Determining the intracellular localization and efficacy of novel anticancer agents in human breast cancer cell lines through the use of fluorescent microscopy

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A Thesis

Entitled

Determining the Intracellular Localization and Efficacy of Novel Anticancer Agents in Human Breast Cancer Cell Lines Through the Use of Fluorescent Microscopy.

By

Eric Richard Koegle

Submitted as partial fulfillment of the requirements for

The Master of Science in Pharmaceutical Sciences in Pharmacology and Toxicology

Advisor: Dr. Miles Hacker

College of Graduate Studies

The University of Toledo

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Breast cancer is the second most frequently diagnosed cancer and it ranks second among cancer deaths in women. The anthracycline antibiotic doxorubicin, more commonly known as Adriamycin, is a drug widely used in breast cancer chemotherapy. The mechanism of action of this particular drug is still somewhat unclear, although it is thought to interrupt the progression of a known DNA unwinding enzyme, Topoisomerase II. Another drug used in breast cancer is the anthracenedione, Mitoxantrone. This drug is used in the treatment of metastatic breast cancer. Mitoxantrone is also a Topoisomerase II inhibitor. In some cases, patients become resistant to chemotherapies or have malignancies that recur. These recurrences are sometimes due to cells that are resistant to the current chemotherapeutic tactic being applied. These cells that resistant express P-glycoprotein. P-glycoprotein is an ATP-dependent efflux pump that has
evolved to remove harmful substances from cells. In the case of chemotherapy failure, it is a good chance that patients exhibit an overproduction of P-glycoprotein in their tumor cells. This leads to a population of cells that has the unique ability to rid itself of drug and express multi-drug resistance.

Although these chemotherapeutic agents are effective anticancer drugs their efficacy is limited by drug-induced cardiotoxicity and therapy must be stopped once a patient has received a cumulative dose of 550mg/m\(^2\). Researchers have sought other equally effective Topoisomerase II inhibitors that lack the cardiotoxicity. The development of 9-aza-anthracyrazoles has led to drugs that are not only active against breast cancer, but are non-cardiotoxic. Furthermore, an interesting observation has been made in which compounds containing two terminal tertiary amine side chains, as seen in the compounds BBR3378 and BBR3409, are active in PGP expressing MCF-7 cells.

The purpose of this thesis is to determine the potency of these novel cancer drugs and also to determine intracellular drug distribution in cells. The two colorimetric assays that were used during this project were the MTT cell proliferation assay and a trypan blue exclusion assay.
Dedication

This Master’s Thesis is dedicated to the people who made this possible. To my parents, Karen and Wilbur, and my grandparents Nancy and Richard, who gave constant support, encouragement, and motivation especially during rough times which allowed this project to happen. I also would like to mention my brothers, David and Michael, and my friends who have helped me keep the stress obtained by a master’s student to a minimum.
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Introduction

Breast Cancer

Worldwide, breast cancer is the second most common type of cancer after lung cancer. In the United States in 2007, there were 180,510 estimated new cases and 40,910 deaths attributed to breast cancer with approximately 99% of the patients being female. It is estimated that in 2008, 182,460 women will be diagnosed with and 40,480 will die of cancer of the breast (1). Breast cancer is the most frequently diagnosed cancer in women. This diagnosis frequency is attributed to breast self-examinations (BSE) which was widely discussed in the 1990s as a useful tool for detecting breast cancer at an early stage. Today mammograms have been slowly replacing the BSE as the main method of breast cancer detection. Mammographies are highly accurate and can detect breast cancer at an early stage when treatment may be more effective (2). Women older than 40 are encouraged to have a mammogram every one to two years. Another screen for breast cancer is magnetic resonance imaging (MRI) which is even more sensitive in detecting abnormalities but is less specific to breast cancer than a mammogram. This decrease in specificity may lead to more false positives, which may have undesirable effects on the patient such as financial and psychological costs (3). MRIs may be necessary for patients who have a strong family history of breast cancer, have very dense or scarred breast tissue or have BRCA-1 and BRCA-2 mutations.
As cells age, they die and are replaced by new ones. In some cases, this orderly process goes wrong, as new cells are being produced but old cells do not die. A collection of these extra cells may form a mass of tissue called a growth or tumor. There are two types of tumors, benign and malignant. Benign tumors are non-cancerous and are rarely life threatening. Cells from benign tumors do not invade surrounding tissue and do not spread throughout the body. These tumors can be easily removed and usually do not grow back. The other type of tumor is a malignant tumor. These tumors are cancerous and are generally more serious than benign tumors. Malignant tumors can be surgically removed but often recur. Malignant tumors have the potential to be metastatic and spread to other parts of the body. Metastatic spread is the primary cause of tumor deaths.

In most cases when breast cancer cells metastasize, they utilize the lymph duct system and locate to nearby lymph nodes. Lymph nodes are part of the lymphatic system which serves to produce, store and carry white blood cells that fight infections and other diseases. The spread of breast cancer is often determined by the presence of cancerous cells inside the lymph nodes. The farther away from the breast cancer that the cells are found, the more advanced the disease is.

There are many risks factors that may increase the chance of developing breast cancer, although the exact mechanism of these factors is unknown. Certain genetic changes can cause normal breast cells to become cancerous. The two major genes that affect breast cancer cells are oncogenes and tumor suppressor genes.

**Oncogene**

An oncogene is a gene that participates in the onset and development of cancer when deregulated. Genetic mutations resulting in the activation of oncogenes may
increase the chance that a normal cell will develop into a tumor cell. Proto-oncogenes encode proteins that have normal function, but when these genes are altered or expressed abnormally they contribute to the pathogenesis of cancer. One such proto-oncogene is known as ErbB-2. Once this ErbB-2 proto-oncogene is activated it becomes an oncogene. The most important oncogene related in breast cancer is the HER2/neu gene. Oncogenes are known to speed up the rate of cellular division in cells. Approximately 15 to 20% of breast cancers have an amplification of the HER2 gene or over expression of its protein product. Over expression of this receptor in breast cancer is associated with increased disease recurrence and a less favorable prognosis (4). HER2 is a normal cell surface protein involved in cell development. In normal cells, HER2 controls the cell growth and division aspects of the cell cycle. When the gene HER2 is activated in cancer cells, tumor formation is accelerated. If this gene is not expressed correctly, cell cycle progression would be halted, thus effectively inhibiting cell division.

**Tumor Suppressor Gene**

Conversely, tumor suppressor genes are figuratively thought to be in a battle with oncogenes to prevent DNA damage and keep normal regulation of a cell under control. Tumor suppressor genes have a dampening effect on the regulation of the cell cycle to promote apoptosis. Tumor suppressor genes repress select genes essential for continuation of the cell cycle. These suppressor genes also prevent cells with DNA damage from going through cellular division. These cells undergo apoptosis if the DNA damage cannot be repaired (5). The BRCA-1 gene is a tumor suppressor gene that is involved with producing the BRCA-1 protein. This particular protein is directly involved in repairing DNA double strand breaks inside the nucleus of cells. Mutations in the
BRCA-1 gene have the ability to cause an increase in the risk of breast cancer. If this particular gene becomes mutated, the protein made from the gene may not function properly. Researchers believe that a dysfunctional BRCA-1 gene may be unable to help fix mutations that occur in other genes. These defects can accumulate and may allow cells to grow uncontrollably to form a tumor (6).

The function of the BRCA-2 gene is very similar to that of the BRCA-1 gene. The proteins made by both genes are essential for DNA repair. BRCA-2 mutations are usually insertions or deletions of a small number of DNA base pairs in the gene. As a result of these mutations, the protein product of BRCA-2 is abnormally short and does not function properly. As with the BRCA-1 gene, mutations in this gene also lead to an increase in the risk of cancer. Mutations in the BRCA-1 and BRCA-2 genes accounts for approximately 5 to 10% of all breast cancer cases (2, 7, 8).

**Signs and Symptoms of Breast Cancer**

The earliest sign of breast cancer is usually an abnormality detected on a mammogram. Less common symptoms include persistent changes to the breast, such as thickening, swelling, distortion, tenderness, skin irritation, scaliness, or nipple abnormalities such as ulceration, retraction, or spontaneous discharge. Typically, breast pain results from benign conditions and is not an early symptom of malignant breast cancer (2).

**Risk Factors**

Aside from being female, age is the single most important factor affecting breast cancer risk. This risk is also increased by the mutations in the tumor suppressor genes
(BRCA-1 and BRCA-2), a family history, high breast tissue density and high dose radiation to the chest as a result of other medical procedures. The use of oral contraceptives and being overweight adversely affects survival for postmenopausal women with breast cancer. For women with high risk of breast cancer, drugs such as tamoxifen can be used which helps decrease the risk. Tamoxifen is approved by the FDA for the prevention of breast cancer in women. Tamoxifen inhibits tumor growth in the estrogen receptor positive breast cancer. Tamoxifen must be metabolized by the liver to produce active metabolites which results in the cells entering cytostasis (9, 10).

**Reactive Oxygen Species**

Oxidative phosphorylation is a metabolic pathway that utilizes the energy released by the oxidation of nutrients to produce adenosine triphosphate, or ATP. During oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors, such as oxygen. These electrons get transported across the inner mitochondrial membrane in a process called chemiosmosis. This accumulation of electrons on the outside of the membrane forms potential energy in the form of a pH gradient. This energy is then tapped by ATP synthase to create ATP when a subsequent electron flows back inside the inner mitochondrial membrane and down the gradient.

Although oxidative phosphorylation is essential to cellular metabolism, it creates reactive oxygen species (ROS) such as hydroxyl radical and superoxide which can damage DNA and cell membranes. ROS are also generated by the interaction between the cycling between the quinone and semiquinone forms of the anthracyclines with iron as shown in Figure 1 (11). These ROS have the ability to damage the heart because they lack the cardioprotectants that other organs possess. The heart requires an endless supply
of energy. Reactive oxygen species can slow production of ATP. ROS can then damage the myocytes resulting in myofibrillar loss, cytoplasmic vacuolization and cardiac arrhythmias.

These reactive oxygen species include hydroxyl radicals (OH•) superoxide anion (O2•−). Hydrogen peroxide (H2O2) production leads to the formation of more free radicals. The tri-electron reduction product of molecular oxygen is the hydroxyl radical. This is an extremely reactive species that has a very short half life and can react with all biological molecules (12).

\[
\text{H}_2\text{O}_2 + e^{-} \rightarrow \cdot\text{OH} + \cdot\text{OH}
\]

Hydroxyl Radical Formation

Superoxide anion is formed by the addition of one electron to the ground state dioxygen. It is very reactive and unstable in aqueous solutions due to its ability to react spontaneously with itself producing hydrogen peroxide and molecular oxygen (12).

\[
\text{O}_2 + e^{-} \rightarrow \text{O}_2\cdot\cdot^{-}
\]

Superoxide Radical Formation

Hydrogen peroxide, formed from the reduction by two electrons of molecular oxygen, is not a free radical but is very reactive towards cellular macromolecules and is the source for the most dangerous species, the hydroxyl radical (12). Hydrogen peroxide is also formed from superoxide dismutase. Under normal conditions there is a balance between prooxidant (ROS) formation and antioxidants of the cells. However, when this balance is disturbed and shifted towards formation of ROS, the biological system is said to undergo oxidative stress.

\[
2 \text{O}_2\cdot^{-} + 2 \text{H}^{+} \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]

Hydrogen Peroxide Formation
Figure 1. Anthracycline and Radical Production

Adriamycin (Doxorubicin)

Daunorubicin was the first of these anthracycline compounds discovered. This particular drug was isolated from a soil sample and contained a bacterium that produced a red colored antibiotic. This antibiotic was found to be extremely effective against tumors in mice and rats. The drawback to daunorubicin was that it produced fatal cardiac toxicity. Scientists decided to investigate this bacterium and chemically modified it to produce another anthracycline derivative, adriamycin. Adriamycin exhibited better activity against solid murine tumors than daunorubicin, relatively higher therapeutic index and less of a threat of cardiotoxicity.

There are currently seven anthracyclines on the market. The class of anthracycline compounds has a general mechanism of action of inhibiting DNA and RNA synthesis. Anthracyclines intercalate between the base pairs of the DNA strands which effectively prevents the replication of that cell. It is said that anthracycline antibiotics intercalate into the guanine-cytosine regions of the B strand of DNA double stranded
helix. The base pairs located above and below the drug break to prevent excessive van der Waal’s interactions. This intercalation causes the base pairs to separate which induces structural changes to the DNA strand, such as lengthening of the DNA strand or twisting of the base pairs. These structural changes can cause inhibition of the replication process and death. The main characteristic of cancerous cells is that they grow uncontrollably. These particular cells would be more affected by interrupting DNA/RNA synthesis (13). These compounds also create iron-mediated free oxygen radicals that damage DNA and cellular membranes, however, this is not the main mechanism of action. These anthracycline compounds also have the capability of also inhibiting Topoisomerase II enzymes. This enzyme relieves the double helix of torsional strain created by the positive super coils during DNA replication. The Topoisomerase II enzyme effectively cuts both strands of the DNA helix simultaneously in order to change the linking number of the molecule (14). This is accomplished by Topoisomerase II cutting twice on one single strand of DNA, this gap in the DNA allows the other helix to pass through twice. Changing of the linking number effectively promotes chromosome disentanglement because it relaxes the helix by effectively unwinding the DNA molecule by two turns (15). The planar aromatic chromophore portion of doxorubicin as seen on the left handed side of the molecule in Figure 2 allows this compound to be an effective intercalator. It also has a six-membered daunosamine sugar that sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures (16).
Figure 2. Adriamycin (Doxorubicin)

The side effects of doxorubicin, however, are severe enough to give itself the nickname, “Red Devil.” Doxorubicin can cause acute side effects such as nausea and vomiting as well as cardiac arrhythmias. These arrhythmias are not necessarily related to doxorubicin induced cardiotoxicity. Doxorubicin is known to cause neutropenia as well as complete alopecia. There is a potential for occurrence of secondary acute myelogenous leukemia and myelodysplastic syndrome in patients when treated with anthracyclines including doxorubicin. The occurrence of refractory secondary AML or MDS is more common when anthracyclines are given in combination with DNA-damaging anti-neoplastic agents or radiotherapy, when patients have been heavily pretreated with cytotoxic drugs, or when doses of anthracyclines have been escalated. Peripheral neurotoxicity in the form of sensory and/or motor disturbances have been reported in patients treated with doxorubicin, mostly in combination with cisplatin.
Seizures and comas also have been reported in patients treated with doxorubicin in combination with cisplatin or vincristine.

When cumulative doses of doxorubicin reach $550\text{mg/m}^2$, the risks of developing cardiac side effects, including congestive heart failure, dilated cardiomyopathy, and death dramatically increase. The cardiotoxicity has been attributed to a dose dependent decline in mitochondrial oxidative phosphorylation which results in a decline in oxidative phosphorylation and a fall in overall ATP production (11).

**Mitoxantrone**

![Mitoxantrone structure](image)

**Figure 3. Mitoxantrone**

Mitoxantrone is an anthracenedione, characterized by an aromatic tricyclic system with polar side chains (Figure 3). Similar to doxorubicin, mitoxantrone is a Topoisomerase II inhibitor where it disrupts DNA synthesis and repair in cells (17). Since these two drugs are very similar in structure and identical in mechanism of action, they have the same side effects. Nausea, vomiting, hair loss, and immunosuppression are acute symptoms of mitoxantrone. The main concern in patients undergoing either doxorubicin or mitoxantrone treatment is irreversible cardiomyopathy.
Patients undergoing these therapies undergo regular monitoring by echocardiograms or multiple uptake gated acquisition scans. In echocardiograms, ultrasounds are used to produce an accurate assessment of the velocity of blood and cardiac tissue. This allows assessment of cardiac valve areas and function, any abnormal communications between the left and right side of the heart, any leaking of blood through the valves and calculation of the cardiac output as well as the ejection fraction. In the case of the multiple uptake acquisition scan, a small amount of stannous chloride is injected into the patient’s bloodstream to prepare the red blood cells for tagging with the radioactive dye. This injection is followed by a subsequent injection of a radioactive substance, Technetium-99m-pertechnetate that labels the cells in vivo. The patient is then placed under a gamma camera that detects the low level radiation given off by the Technetium-99m. Images are then analyzed on a computer to give detailed results of the heart and also more reproducible means of measuring and monitoring the ejection fraction of the left ventricle of the heart, which is most important in measuring heart performance. The advantage of this type of scan compared to an echocardiogram is that it is more accurate and less invasive.

**Search for Non-cardiotoxic Compounds**

The cardiotoxicity associated with doxorubicin and mitoxantrone, encouraged researchers to look into the structure activity relationship in order to eliminate the risk. Doxorubicin undergoes redox cycling through the aromatic ring, giving rise to the accumulation of free radical species. There are other anthracycline derivatives in the market, such as epirubicin, which is mechanistically similar to doxorubicin. Epirubicin has been shown to be less effective at higher doses than doxorubicin is at standard doses,
this is why doxorubicin continues to be in use more often. Epirubicin however, is favored over doxorubicin in some chemotherapeutic regimens because it causes fewer side effects. The reduced toxicity attributed to epirubicin may be because of its hydroxyl group attached at the 4’ carbon on the sugar causing the drug to be eliminated faster than other anthracyclines.

There are also liposomal anthracyclines available. These liposomal drugs are encapsulated within a hydrophobic membrane. This makes an ideal delivery system for cancer chemotherapy because the drug locates to the tumor sites. In healthy blood vessels, there are endothelial cells that line the blood vessels that are held together by tight junctions. These tight junctions are less existent in tumor blood vessels. These liposomes can then penetrate the tumor blood vessels and enter the cancerous areas easily. This phenomenon is referred to as the enhanced permeability and retention effect (18).

There are also drugs that are considered to be cardiac protectants. One such drug is dexrazoxane which is an iron chelator. Iron works to convert the semiquinone to a semiquinone radical. This anthracycline radical formation aids in the production of damaging ROS that can be made from hydrogen peroxide. The mechanism of action for dexrazoxane is to chelate free iron thus limiting the formation of free radical generating anthracycline-iron complexes. This may minimize the anthracycline-iron complex mediated oxidative damage to cardiac and soft tissues.

Redox cycling in the anthracycline compounds is a problem that can give way to free radicals. To combat this, a nitrogen atom has been introduced into the carbocyclic aromatic planar system (19). Extensive synthetic work has been performed and led to the discovery of the aza-anthrapyrazoles, where C-N substitutions were introduced at
different positions of the anthrapyrazoles ring system, giving different regioisomers. The cytotoxicity of these derivatives was substantially affected by the location of the bioisosteric nitrogen. In fact, only 9-aza derivatives showed activity \textit{in vitro} and \textit{in vivo} (20).

The drugs used throughout this thesis are those that are in the class of 9-aza anthrapyrazoles. This class of compounds has been proven to be greatly effective against breast cancer. The effectiveness of mitoxantrone, doxorubicin and pixantrone have been proven through clinical trials. Pixantrone is a novel 2-aza anthracenedione that was produced to combat the cardiotoxicity associated with these compounds. This drug is a new chemical compound used in the treatment of Non-Hodgkin’s lymphoma and other various hematological malignancies, solid tumors and immunological disorders. Pixantrone has been designed to reduce the potential for severe cardiotoxicities, as well as to potentially increase the activity and simplify administration compared to the currently marketed anthracyclines. Pixantrone could allow repeat therapy in relapsed patients and combination therapy with a broader range of chemotherapies. This drug is less likely to cause tissue damage and can be administered through a peripheral vein, eliminating the need for a central line. The discovery of novel 9-aza anthrapyrazoles, such as those used throughout these experiments, led to compounds that are less cardiotoxic yet still have the activity. BBR3378 and BBR3404 were the two experimental compounds used in this study.
BBR3378 is a 9-aza anthrapyrazoles compound that has tertiary amine side chains. BBR3378 is an intercalating agent that inhibits Topoisomerase II. It has previously shown that intracellular distribution of the 9-aza anthrapyrazoles compounds is regulated by the side chain terminal amines (21). BBR3378 contains tertiary terminal amine side arms which cause the drug to localize into distinct cytoplasmic vesicles in both wild type and P-gp expressing cells. Primary amine derivatives showed a higher DNA binding affinity than tertiary amine derivatives, however, the increased lipophilicity of the tertiary amine derivatives facilitated their cellular uptake (Figure 4) (22). It has been determined that overcoming MDR resistance is critical in the development of new antineoplastic compounds.

Multi drug resistance (MDR) is defined as the ability of a cell to exhibit resistance against a wide variety of compounds that are structurally and functionally unrelated. There are many mechanisms that a cell can obtain to become resistant to cytotoxic agents. Some of the mechanisms used by tumor cells to protect themselves against chemotherapeutic agents are 1) multi drug resistance associated proteins (MRPs), which
are also drug transporters, 2) alterations in drug targets, 3) alteration in drug metabolism, 4) repair of drug induced DNA damage and 5) the inability to perform apoptosis, programmed cell death. However, one of the most common mechanisms by which cells become multi drug resistant is through the increased expression of a 170-kDa membrane glycoprotein called P-glycoprotein. This glycoprotein is encoded by the MDR1 gene and is an ATP-dependent extrusion pump for drugs and physiological substrates. This p-glycoprotein is over expressed in the MCF-7 A10 breast cancer cells that are used throughout the cell death assays along with the observational studies.

**Figure 5: Structure of BBR3409**

BBR3409 is another novel 9-aza anthrapyrazoles derivative (Figure 5). This also bears the dual tertiary amine side arms resembling the BBR3378 compound. This intercalating agent locates predominantly inside the cytoplasmic vesicles. This chemical along with BBR3378 does not lack the tertiary amine side arms, therefore, it is not cross resistant in P-glycoprotein expressing cells (23). These terminal tertiary amines are critical in overcoming multi-drug resistance.
Objectives

With the discovery of the 9-aza anthrapyrazoles, researchers have found a drug that appears to be useful in the treatment of breast cancer and no longer poses a grave cardiotoxic threat. The compound BBR3378 is a drug that has two side arms containing tertiary amines. It has been proven that tertiary amine side arms are essential for the activity of this compound in cells over expressing P-glycoprotein. The compound BBR3409 is structurally similar to BBR3378, however it contains an additional carbon in one of it’s terminal amine chains. Previous studies have demonstrated that both BBR3378 and BBR3409 are active in cells expressing multi-drug resistance. Previous studies suggest that side arms of these drugs dictate how it distributes intracellularly. One unique property of aza-anthrapyrazoles is that they fluoresce naturally. Using this natural property, we will study in a kinetic fashion the intracellular distribution of these two drugs in both the wild-type MCF-7 and the P-glycoprotein over expressing MCF-7 A10 breast cancer cells in vitro.
Materials and Methods

Materials

Two human breast cancer cells lines were used throughout these studies. The MCF-7 wild type cell line was epithelial cells collected from the mammary gland tissue of the breast in the adenocarcinoma disease state obtained from ATCC (Manassas, VA). The other breast cancer cell line that was used was the MCF-7 A10 a MCF-7 cell line that over expresses MDR1 (p-glycoprotein) was obtained from ATCC (Manassas, VA).

Both drugs, BBR3378 and BBR3409 were provided as a gift from NOVIS Pharmaceuticals (Milan, Italy).

Powdered RPMI-1640 medium with L-glutamine without sodium bicarbonate and Sodium pyruvate (C$_3$H$_5$NaO$_3$) was purchased from Sigma-Aldrich (St. Louis, MO).

Fetal Bovine Serum, MEM amino acids, 0.05% Trypsin - 0.53 mM EDTA without calcium and magnesium 1x and 5,000 I.U. Penicillin/mL and 5,000 μg/mL Streptomycin solution 50x was obtained from Cellgro (Lawrence, KS).

Chloroquine Diphosphate salt was obtained from ICN Biomedicals Inc (Costa Mesa, CA).

Sodium bicarbonate (NaHCO$_3$) and PBS: phosphate buffered saline 10X solution with a pH of 7.4 ± 0.1 (1x) was purchased from Fisher Scientific (Pittsburgh, PA).

Non-drying Ultraviolet Immersion oil for microscopy Type B was obtained from Cargille Laboratories (Cedar Grove, NJ).
Olympus CX41 microscope with a U-LH50HG fluorescence attachment with a 460-490 nm excitation and 500 nm emission filter was connected to an Olympus U-RFLT50 mercury bulb burner purchased from B&B Microscopes was used throughout all the photographic data obtained.

A Nikon D70S SLR camera with Nikon Capture was used for all photography of the cells.

A MTT cell proliferation assay kit was purchased from ATCC (Manassas, VA).

A LysoTracker Red kit was purchased from Invitrogen (Carlsbad, CA).

**Storage Conditions**

Cell cultures were stored in liquid nitrogen (-160ºC). Media were stored at 4ºC in the dark. Test compounds and dilutions were stored under appropriate conditions in the 4ºC cooler in the dark.

**Media Preparation**

RPMI-1640 medium was prepared according to the manufacturer’s instructions and was supplemented with NaHCO₃ (0.075%), 10% fetal bovine serum, MEM amino acids and C₃H₃NaO₃ (1.0 mM.) The mixture was sterile filtered and stored at 4ºC.

**Cell Thawing**

The cell cultures were kept in liquid nitrogen until ready for use. The cells were carefully removed from the liquid nitrogen storage container. Cells were then rapidly thawed by placing the vials inside a 37ºC hot water bath for roughly 90 seconds. The cryoprotective media (10% glycerol) was quickly diluted with media.
**Incubation Conditions**

Incubations were carried out in a Stericult 200 Incubator set to 37°C, 5% CO₂, at 95% humidity.

**Passaging of Cells**

RPMI-1640 medium, 1x sterile PBS and trypsin EDTA were placed in a warm water bath set at 37°C and warmed prior to use. Next, T-75 flasks (Falcon) (Franklin Lakes, IL) of cells are placed inside the sterile hood to be passaged. Old medium is aspirated off the plate through the use of a sterile Pasteur pipette this will not remove the cells as they are adherent cell lines. Following aspiration of the PBS 1x trypsin EDTA was added and the flasks were then transferred back inside the sterile incubator for about four minutes to allow the protease to work. This cell suspension was centrifuged for about five to ten minutes at 800 rpm. The media in the tube was then aspirated leaving a cell pellet and 10 mL of fresh media was added. Cell concentrations were determined using a Bright Line hemocytometer. Approximately 5 x 10⁵ cells are seeded onto a new T-75 flask to which 15mL of fresh media is added.

**Setting up Observation Slides**

Six well plates are used in the observation experiments. Microscope cover slips are first sterilized with 70% ethanol over night then in each of the wells. The plate is then covered and set aside inside the hood overnight to allow any remaining ethanol to evaporate. Cells were prepared at a concentration of 3 x 10⁵ cells per mL and 1 mL of the cell suspension was added to each well containing a dried microscope cover slip. The plate was incubated overnight to allow the cells to adhere to the cover slip. The
appropriate drug concentration was then added to each well and the plate was then incubated for the desired time. The cover slip was removed and placed face down onto a microscope slide. A few drops of UV immersion oil was placed on top of each microscope cover slip for proper observance with ultraviolet light.

**MTT Cytotoxicity Assay**

Once an accurate cell count has been determined, dilute or concentrate to $5 \times 10^3$ cells per 100 μL. According to the scheme made up for the experiment, pipet out 100 μL to each well of a 96 well plate. The plate was returned to the incubator overnight. The next day cells were dosed with 5 μL of the appropriate drug concentration. After 72-96 hours, 10 μL of MTT reagent was added to each well and the plates were then retuned to the incubator for two to four hours. Finally, 100 μL of detergent was added to each well and the plate was incubated in the dark at room temperature overnight to lyse the cells and solubilized any purple precipitate formed. The absorbance at 570 nm was measured for each well using a KC Junior spectrophotometer. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.
Cell Count Assay

Once an accurate cell count has been determined, dilute or concentrate to around 2 x 10^4 cells per mL. Place 1 mL into each well of a 24 well plate according to the specific scheme made up for that day. Once finished seeding out the plate, it was placed inside the incubator overnight to allow the cells to adhere. The next morning, I cells were dosed with 50 μL of the appropriate drug. The plate was then placed back inside the incubator for a period of three days, until the controls were confluent. Once the controls reached confluency, each well in the plate was aspirated and after and 0.25 mL of Trypsin EDTA was added to each well. After five minutes, the plates were then removed from the incubator and placed back inside the hood where 0.75 mL of fresh media was added to each well in order to inactivate the trypsin. The cells were triturred and resuspended in a total of 1mL. Each well of the plate was placed inside a microfuge tube for temporary storage before they were counted. Tubes were all numbered and the drug and concentration were marked on a separate sheet. Cell counts were then determined using a hemocytometer for each microfuge tube by the trypan blue exclusion method.
Results

BBR3378 in MCF-7 Data

Figure 6. Concentration of BBR3378 vs. Absorbance in MCF-7

The data obtained from the experiment performed in Figure 6 shows that the cell is responsive to increasing concentrations of BBR3378. As concentrations of drug increase, there is a decrease in absorbance, indicating more cell death and a decrease in the number of cells reducing the yellow tetrazolium MTT to purple formazan precipitate. There is a great decrease in absorbance at 570 nm to the concentration of 5.84 μM of drug. This is the reference used in indicating the LC50 concentration of this particular
drug. At a dilution factor of 1:250 and a concentration of 5.84 μM, BBR3378 appears to be a potent drug for breast cancer chemotherapy.

Figure 7. MCF-7 cells after instant exposure to BBR3378

Figure 7 is a picture of MCF-7 breast cancer cells exposed to 5.84 μM BBR3378. This picture was taken instantly after the drug was introduced to the microscope slide. As seen in Figure 7, the drug is entirely dispersed throughout the cell. It does not localize particularly into any region of the cell.
Figure 8. MCF-7 cells exposed to BBR3378 for 15 minutes

After about 15 minutes of exposure, the drug appears to group into the nucleus and also into the more acidic regions of the cell, more specifically cytoplasmic vesicles (Figure 8).

Figure 9. MCF-7 cells exposed to BBR3378 for 50 minutes
After 50 minutes of exposure, the drug appears to efflux from the nucleus and concentrates in intracellular vesicles. These vesicles are shown as small spheres in Figure 9.

![Image](image1.jpg)

**Figure 10. MCF-7 cells exposed to BBR3378 for 120 minutes**

Figure 10 represents MCF-7 cells that have been exposed to BBR3378 for 120 minutes then washed with 1x PBS. It is clear that the drug moves from the nucleus of the cell into the cytoplasm and then into the vesicles.

![Image](image2.jpg)

**Figure 11. MCF-7 cells exposed to BBR3378 for 180 minutes**
After 180 minutes of exposure, the cells still fluoresce and the characteristic drug present in the cytoplasmic vesicles remains.

**Figure 12. MCF-7 cells exposed to BBR3378 for 300 minutes**

As seen in Figure 12, after being exposed to BBR3378 for 300 minutes then washed away, the drug still remains inside the cell. There are many bright spherical vesicles present indicating that the drug’s main route of elimination in the MCF-7 breast cancer cells are through the lysosomes.

**Figure 13. MCF-7 cells exposed to BBR3378 for 330 minutes**
A very small amount still remains inside the lysosomes. Figure 13 was taken 90 minutes post wash after the 300 minute exposure and still had traces of drug present inside the intracellular vesicles.

**BBR3378 in MCF-7 A10 (ADR) Data**

Figure 14 shows the effects of BBR3378 in another breast cancer cell line, the MCF-7 A10 (ADR) strain. This particular strain was selected because it exhibited a multi-drug resistance because it over-expresses p-glycoprotein. As seen from the data above, BBR3378 has an LC50 concentration of 14.6 μM. There is also a slight upward shift in this dose response curve possibly indicating that the concentration of the drug was so high that the color of BBR3378 may have played a role in the spectrophotometric analysis. Also, the cell line expressing MDR does not have as high of absorbance
readings as the wild-type strain. This is probably due to the fact that the doubling time of these cells are much longer than that of its wild-type sister.

Figure 15. MCF-7 A10 cells after instant exposure to BBR3378

Figure 15 is demonstrating the effects of instant exposure of MDR cells to BBR3378. As seen from the picture, the drug instantly goes through the cell membrane and directly into the cytoplasmic vesicles.

Figure 16. MCF-7 A10 cells exposed to BBR3378 for 30 minutes
Figure 16 shows the effects of the drug thirty minutes after washing off the microscope slide of cells exposed to BBR3378. This drug disperses throughout the whole cell.

Figure 17. MCF-7 A10 cells exposed to BBR3378 for 180 minutes

Figure 17 represents the effects seen 180 minutes after washing of the microscope slide of cells exposed to BBR3378. The drug is rapidly being forced out of the cell, but there is still a very small amount of drug left inside the intracellular vesicles.

Figure 18. MCF-7 A10 cells exposed to BBR3378 for 240 minutes
Figure 18 shows the effects of BBR3378 240 minutes after washing the microscope slide of cells exposed to the drug. There still is drug present inside the lysosomes of these cells, but the cell is actively working to rid itself of drug.

**BBR3409 in MCF-7 Data**

![Concentration of BBR3409 vs. Absorbance in MCF-7](image)

Figure 19. **Graph of Concentration of BBR3409 vs. Absorbance in MCF-7**

Figure 19 represents BBR3409, another drug containing terminal tertiary amine side arms, in a MTT assay with MCF-7 wild type breast cancer cells. The above figure shows the effects of varying concentrations of BBR3409 on cell survival. It was determined that the LC50 concentration of BBR3409 is 9.24μM. Comparing Figure 19 to Figure 14, there is a decreased absorbance in BBR3409 versus BBR3378. This decrease in the absorbance means that there were fewer cells that were living indicating more cell death.
Figure 20. MCF-7 cells after instant exposure to BBR3409

Figure 20 shows MCF-7 cells that have been exposed to BBR3409 instantly. As seen in the picture above, the drug enters quickly and is distributed throughout the cell.

Figure 21. MCF-7 cells exposed to BBR3409 for 30 minutes

Figure 21 shows MCF-7 cells 30 minutes after exposure to BBR3409. The drug penetrates right into the nuclear envelope with great ease. As seen in this figure, the nucleus fluoresces brightly along with the cytoplasmic lysosomes.

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Figure 22. MCF-7 cells exposed to BBR3409 for 60 minutes

Figure 22 shows 60 minutes exposure of BBR3409 in MCF-7 cells. The drug is still present inside the nucleus but is concentrating in the cytoplasmic vesicles.

Figure 23. MCF-7 cells exposed to BBR3409 for 120 minutes
MCF-7 cells that have been exposed to BBR3409 for 120 minutes are shown in Figure 23. The cell is still fluorescing brightly and there is still drug present inside the nucleus of the cell. The drug stays inside the nucleus more than twice as long as BBR3378.

Figure 24. MCF-7 cells exposed to BBR3409 for 180 minutes

Figure 24 is showing cells exposed to BBR3409 for 180 minutes. As can be seen, there is no drug in the nucleus and drug remains in the lysosomes.
Figure 25. Concentration of BBR3409 vs. Absorbance in MCF-7 A10

The MCF-7 A10 cells were exposed to varying concentrations of BBR3409 is shown in Figure 25. The LC50 of BBR3409 in this p-glycoprotein expressing cell line appears to be around 11.823 μM. This gives more insight to the aza-anthrapyrazoles bearing tertiary terminal amine side arms being not cross resistant in cells over expressing P-glycoprotein because of this dose response curve.
Figure 26. MCF-7 A10 cells after instant exposure to BBR3409

Figure 26 is that of MCF-7 cells over expressing P-glycoprotein exposed to 11.823 μM of BBR3409. The cytoplasmic vesicles fluoresce when exposed to BBR3409.

Figure 27. MCF-7 A10 cells exposed to BBR3409 for 30 minutes
After 30 minutes of exposure to BBR3409, the drug distinctively clusters inside the lysosomes.

Figure 28. MCF-7 A10 cells exposed to BBR3409 60 minutes

After 60 minutes of exposure to BBR3409, the pattern of drug distribution is identical to that seen after 30 minutes of exposure.

Figure 29. MCF-7 A10 cells exposed to BBR3409 for 120 minutes
Again, 120 minutes post exposure to BBR3409 the drug distribution pattern remains unchanged.

**Figure 30. MCF-7 A10 cells exposed to BBR3409 for 180 minutes**

At 180 minutes post exposure, the cells begin to lose fluorescence (Figure 30). The cytoplasmic vesicles still have drug in them, but the intensity of the fluorescence is diminishing.
Figure 31. Effect of Various Concentrations of BBR3378 and BBR3409 on MCF-7 Cell Numbers

Figure 31 shows the cell count assay results. This data compares the relative concentrations of both BBR3378 and BBR3409 and shows their effectiveness when compared to a control.

Figure 32. Effect of Various Concentrations of BBR3378 and BBR3409 on MCF-7 A10 (ADR) Cell Numbers
Figure 32 shows the results of the cell count assay that was performed on MCF-7 A10 cells. This was a quantitative method to measure the effectiveness of the BBR compounds.

Figure 33. MCF-7 cells exposed to LysoTracker Red

MCF-7 cells were stained with LysoTracker Red in Figure 33. This dye stains the lysosomes. This is due to the weakly basic amines that selectively accumulate into low pH regions.
MCF-7 A10 cells were stained with LysoTracker Red. This dye stains the lysosomes. This is due to the weakly basic amines that selectively accumulate into low pH regions more specifically the lysosomes (Figure 34).
Discussion

The two human breast cancer cell lines used throughout this study were the MCF-7 wild type and the MCF-7 A10 P-glycoprotein over expressing cells. The MCF-7 A10 cells over express P-glycoprotein represent breast cancer that exhibits multi-drug resistance and this is ideal in showing if these aza-anthrapyrazole derivatives are active against this form of breast cancer. These cell lines were also used to study the intracellular drug distribution of another aza-anthrapyrazole derivative BBR3422, a drug that lacks the terminal tertiary amine side arms (25).

Studies have shown that anthrapyrazoles having primary or secondary amine side arms were clustered in the cell nucleus, whereas compounds having tertiary amines on both side arms localized in distinct cytoplasmic vesicles (24). In this study, both BBR3378 and BBR3409 distributed throughout the breast cancer cells in a time dependant fashion. BBR3378 exhibited distribution into the nucleus within minutes of exposure. Previous studies have not shown that anthrapyrazoles bearing terminal tertiary amines on both side arms distribute into the nucleus (21). BBR3378 begins to enter the nucleus about fifteen minutes post exposure in wild type MCF-7 breast cancer cells. Within thirty minutes after drug first is noticed in the nucleus, the drug moves into the cytoplasmic vesicles. BBR3378 was determined to distribute and localize to the lysosomes in the cytoplasm which was confirmed through the use of LysoTracker Red which was purchased from Invitrogen (Carlsbad, CA). This LysoTracker sensor is a red fluorescent dye that stains the acidic compartments in live cells. Cells were stained with
LysoTracker Red and pictures taken from these cells were then compared to pictures of cells dosed with either BBR3378 or BBR3409. In the case of BBR3378, drug remains inside the wild type cell for more than six hours. There is a noticeable time dependant removal of drug from the cell which is indicated by diminished fluorescence. This diminishing fluorescence may be a direct result of cellular autophagy. Cellular autophagy is a catabolic process by which the drug or parts of the cell that contain the drug is bottled up in a vesicle which then subsequently fuses with a lysosome. This lysosome then degrades the contents of the vesicle and can then prepare the contents for excretion. These drugs may accumulate in the lysosomes due to a pH trapping effect. There is such a low pH in the lysosomes that it attracts these quinone like structures which can be rapidly reduced within the lysosome.

In the multi-drug resistant breast cancer cells, BBR3378 does not gain entry into the nucleus. At instant exposure to drug, it appears as if there is a mechanism that prohibits this compound from entering the nucleus. Nuclear uptake of drug appears to be affected from the over expression of P-glycoprotein. From the MTT assay, once a concentration of drug is reached, the absorbance at 570 nm fluctuates. As suggested earlier, absorbance of drug itself could have played a role in the spectrophotometric analysis. Once the drug gains entry into the cell, it localizes in the lysosomes. In this P-glycoprotein over expressing cell, drug excretion is greatly increased. When comparing the MCF-7 wild type to the MDR strain, there is a great difference in time at which fluorescence diminishes from the cells. The MDR strain rids itself of drug very quickly. This indicates that BBR3378 is being actively transported out of the cell by P-glycoprotein. Cellular autophagy facilitates removal of drug from these cells. This is indicated by the large fluorescent vesicles that appear in the pictures. The process of
cellular autophagy bundles and collects drug deposits throughout the cell and prepares it for excretion.

BBR3409 has a very similar distribution pattern when compared to BBR3378. BBR3409 gains entry into the nuclear envelope very quickly in the MCF-7 wild type cancer cells. It appears as if this particular drug concentrates more in the nucleus when compared with BBR3378. The nuclei of these cells fluoresce more brightly indicating that more drug localizes there. After about one hour of exposure to drug, the intensity of fluorescence shifts from the nucleus to the cytoplasmic vesicles. This unique drug remains inside the nuclear envelope for just over two hours. While BBR3409 has an LC50 that is higher in wild type breast cancer cells than BBR3378, there appears to be a threshold concentration of drug that makes the cell more sensitive. When comparing the dose response curves for the two drugs, BBR3409 has a very distinctive steep drop in both, the absorbance at 570 nm and the cell number. This alludes to the threshold concentration of BBR3409 that may be effective. This suggests that only specific concentrations of drug are effecting in killing these cells. All concentrations of drug used prior to the fall in absorbance may not be effective in cellular lethality. In the case of BBR3378, there is a gradual response when comparing the doses of the drug. This drug appears to be highly susceptible to autophagy. The cell may be attempting to protect itself from this foreign drug that it will bundle cytoplasmic vesicles and organelles into these larger clusters. This drug has nearly twice the LC50 concentration of BBR3378, which could be a result from this clustering that occurs during cellular autophagy.

BBR3409 in the A10 resistant breast cancer cells does not penetrate into the nucleus of these cells. Just like in the case of BBR3378, distribution across the nuclear envelope is nonexistent. Although this membrane is very porous this study concludes
that drug does not enter it. This may be because of the over expression of P-glycoprotein. This protein works to remove drug that may have accumulated within the nucleus very quickly. Localization of drug in the P-glycoprotein over expressing cells is clearly lysosomal. The intensity of fluorescence of the lysosomes is extremely high which confirms, the similar distribution of both drugs. Excretion of both drugs in the MCF-7 A10 cell line is nearly identical in time. There is not a threshold concentration of drug that is effective in these multi drug resistant cells. The concentration curve (Figure 32) shows that BBR3409 has a higher specific activity in treating MCF-7 A10 breast cancer cells. The LC50 of BBR3409 in the MCF-7 A10 line is 11.823 μM whereas BBR3378 is 14.6 μM.

BBR3378 and BBR3409 were nearly equipotent in their ability to induce cell death and it has been shown that these drugs accumulate inside the lysosomes of these two cell lines. Both drugs exhibit an identical distribution pattern. In MCF-7 wild type cells, both drugs penetrate into the nucleus and through time dependence then distribute into the intracellular vesicles that were determined to be the lysosomes. Using Analysis of Variance (ANOVA), in the wild type MCF-7 cancer cells, the difference between the two drugs, BBR3378 and BBR3409, was found to be statistically significant, p < 0.05. In the wild type cells, BBR3378 had an LC50 concentration of 5.84 μM and BBR3409 was 9.24 μM. This is nearly a two fold increase in concentration of drug for BBR3409 to become as effective as BBR3378. Using Analysis of Variance (ANOVA), the difference between the two drugs in MCF-7 A10 cells was found to be statistically non significant, p > 0.05. Both drugs exhibited nearly the same IC50 values. Both drugs were unable to gain access to the nucleus in these P-gp over expressing cells. Both BBR3378 and BBR3409 do collect inside the lysosomes and become excreted over time.
Conclusions

In this study, the drugs, BBR3378 and BBR3409 were tested for its intracellular distribution and efficacy inside MCF-7 wild type and MCF-7 A10 multi-drug resistance human breast cancer cell lines. These compounds were found to be effective against both human breast cancer cells lines exhibiting approximately similar LC50s. Results obtained by MTT were duplicated in cell count assays where viable cells were counted utilizing the trypan blue exclusion method. These compounds are 9-aza substituted which combats the effects of redox cycling making these compounds less cardiotoxic than its derivatives. Both drugs distributed throughout the wild type cell in a kinetic fashion, slightly fluorescing in the nucleus at first post exposure then effluxed rapidly into the intracellular vesicles in the wild type breast cancer cells. These vesicles were determined to be acidic through the Lysotracker dye, where it appears that the drug sequesters itself into these acidic regions, more specifically the lysosomes for removal from the cell. In the P-glycoprotein over expressing cells, the drug does not gain access to the nucleus and the drug collects inside the lysosomes. The distribution patterns of both drugs are similar with the only difference being in the intensity of fluorescence.
Recommendations for Future Studies

1. DNA intercalation is the main mechanism of action for these aza-anthrapyrazole compounds. Future studies should include DNA binding studies. This would give an accurate measurement of how efficient these BBR compounds intercalate DNA. A DNA retardation assay can be performed to show the degree of intercalation and inhibition of DNA replication.

2. Since these drugs are said to inhibit Topoisomerase II activity, it is important to show the degree of inhibition. This can be shown using the methods as described in *A Novel Homogenous Assay for Topoisomerase II Action and Inhibition*, 2005. This assay analyzes the effects of topoisomerase II inhibitors through the concept of dual-color fluorescence cross-correlation spectroscopy. This spectroscopy technique can detect double stranded DNA breaks because the two strands carry spectrally different fluorophores at the opposite 5’ ends.

3. As efficacy of the drug is determined through the amount of drug present in cells, it is important to quantify the amount of intracellular drug. This can be done through an assay of intracellular drug accumulation which can be measured through the use of flow cytometry using the drug’s fluorescent properties.
References


