A Thesis entitled

Metal Binding Characteristics of Heterocyclic and Carbocyclic Anticancer Drugs

by

Brittany Bezoski

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Pharmacology and Toxicology

________________________________________

Dr. Miles Hacker (deceased), Major Research Advisor

________________________________________

Dr. Ezdihar Hassoun, Committee Chair

________________________________________

Dr. Marcia McInerney, Committee Member

________________________________________

Hermann von Grafenstein, Committee Member

________________________________________

Dr. Amanda Bryant-Friedrich, Dean
College of Graduate Studies

The University of Toledo
December 2016
Note

Research in this thesis was conducted by Brittany Bezoski under the supervision of Dr. Miles Hacker, who also provided all of the material used for the research. Unfortunately, Dr. Hacker passed away before the thesis was completed and defended. Dr. Ezdihar Hassoun (Committee Chair), and committee members, Dr. Marcia McInerney and Dr. Hermann Grafenstein acted as reviewers and approvers of the thesis in the context of satisfying Bezoski’s degree requirement, but had no role in the research.
For decades, the anthracyclines such as doxorubicin, have been among the most common and effective group of antineoplastic drugs. Unfortunately, their use has been limited by their serious and irreparable cardiotoxicity, as a side effect. This damage to the heart was believed to be due to the drugs’ interactions with Fe$^{2+}$. Investigators began to develop analogs in hope to reduce the cardiotoxic effects but retain the anticancer activity, and were successful in developing mitoxantrone and pixantrone. Pixantrone was developed to primarily replace the anthracylines, as it displayed broader spectrum of activity and lower toxicity. Further drug development resulted in another analog, BBR-3378, in the hope of further increasing the anticancer activity and reducing the cardiotoxicity. To investigate whether the harmful side effects of the drugs are specifically caused by the interaction with iron, interactions of doxorubicin, mitoxantrone, pixantrone, and BBR-3378 with iron were tested and compared with the drugs’ interactions with other metals, including copper and zinc, using spectroscopy as a readout. Doxorubicin and mitoxantrone appeared to interact with iron, whereas pixantrone and BBR-3378 did not. However, all
of the four drugs interacted with zinc and copper, but copper interactions with the drugs were interrupted by the addition of albumin. The finding that doxorubicin and mitoxantrone interact with iron, but pixantrone does not, supports the role of iron in the contribution to the anthracycline-induced cardiotoxicity. And the fact that BBR-3387, does not seem to interact with iron, makes it a promising candidate for further studies to test its anticancer activity. Also, the finding that there is no distinction in the interactions of all of the drugs whether they are cardiotoxic or not, with other metals like copper and zinc may indicate that those metals may not contribute to the drugs’ toxicity. This might be further supported by the finding that the interaction of copper with drugs in the presence of albumin was interrupted, which may normally happen in natural bodily functions.
Acknowledgements

First of all, I would like to express my deepest gratitude to Dr. Ezdihar Hassoun. She has gone above and beyond to help me with completing my Thesis, more than I can ever repay. I would have not have been able to do this without her. I also would like to thank my committee members, Dr. Marcia McInerney, and Hermann von Grafenstein for their support.

I am extremely thankful to Dr. Miles Hacker, who took me on as his student. He was a great teacher and an even better person, and I learned a lot from him. I also would like to thank Luis Mullins, who worked with me in Dr. Hacker’s lab. He was very helpful with assisting me with my project.

Lastly, I would like to thank my parents and sister who encouraged me to complete my thesis. This would not be possible without all of their support.
# Table of Contents

Abstract iii
Acknowledgements v
Table of Contents vi
List of Figures vii

1 Introduction
  1.1 Cancer 1
  1.2 Chemotherapy 3
  1.3 Anthracyclines 7
  1.4 Cardiotoxicity 9
  1.5 Doxorubicin 16
  1.6 Mitoxantrone 19
  1.7 Pixantrone 21
  1.8 BBR-3378 23
  1.9 Aims and Objectives 24

2 Materials and Methods
  2.1 Drug and Metal Preparation 25
  2.2 Metal Binding Assay 26
  2.3 Interactions with ferrous – iron 26
  2.4 Interactions with cupric – copper and BSA 27
  2.5 Interactions with zinc 27
3 Results

3.1 Interactions with Iron

3.1.1 Doxorubicin and iron 28

3.1.2 Mitoxantrone and iron 29

3.1.3 Pixantrone and iron 30

3.1.4 BBR-3378 and iron 31

3.2 Interactions with Copper / Albumin

3.2.1 Doxorubicin and copper / albumin 32

3.2.2 Mitoxantrone and copper / albumin 35

3.2.3 Pixantrone and copper / albumin 37

3.2.4 BBR-3378 and copper / albumin 39

3.3 Interactions with Zinc

3.3.1 Doxorubicin and zinc 41

3.3.2 Mitoxantrone and zinc 43

3.3.3 Pixantrone and zinc 44

3.3.4 BBR-3378 and zinc 45

4 Discussion 46

References 50
# List of Figures

| Figure 1.1: Chemical structure of an anthracycline | 8 |
| Figure 1.2: Haber-Weiss Reaction | 12 |
| Figure 1.3: Chemical structure of daunorubicin and doxorubicin | 16 |
| Figure 1.4: Chemical structure of idarubicin and epirubicin | 19 |
| Figure 1.5: Chemical structure of Mitoxantrone | 19 |
| Figure 1.6: Chemical structure of Pixantrone | 21 |
| Figure 1.7: Chemical structure of BBR-3378 | 23 |
| Figure 3.1: Interaction between doxorubicin and iron | 29 |
| Figure 3.2: Interaction between mitoxantrone and iron | 30 |
| Figure 3.3: Interaction between pixantrone and iron | 31 |
| Figure 3.4: Interaction between BBR-3378 and iron | 32 |
| Figure 3.5: Interaction between doxorubicin and copper | 34 |
| Figure 3.6: Interaction between doxorubicin/copper and albumin | 34 |
| Figure 3.7: Interaction between mitoxantrone and copper | 36 |
| Figure 3.8: Interaction between mitoxantrone/copper and albumin | 36 |
| Figure 3.9: Interaction between pixantrone and copper | 38 |
| Figure 3.10: Interaction between pixantrone/copper and albumin | 38 |
| Figure 3.11: Interaction between BBR-3378 and copper | 40 |
| Figure 3.12: Interaction between BBR-3378/copper and albumin | 41 |
Figure 3.13: Interaction between doxorubicin and zinc 42
Figure 3.14: Interaction between mitoxantrone and zinc 43
Figure 3.15: Interaction between pixantrone and zinc 44
Figure 3.16: Interaction between BBR-3378 and zinc 44
Chapter 1

Introduction

1.1 Cancer

When cell division becomes uncontrollable, it results in a tumor, which may be either benign or malignant. While benign tumors are non-cancerous and non-life-threatening because they are confined in a small area of the body and do not tend to spread, malignant tumors are considered cancer, tend to grow faster than benign tumors, and spread throughout the body by a process known as metastasis. Their harm is also facilitated by angiogenesis, a process of creating new blood vessels that feed them. Cancer types are classified according to the tissue origin and include the following:

**Carcinomas**: they originate from epithelial cells, such as lung, breast and colon.

**Sarcomas**: they originate from connective tissues, such as bone, cartilage, fat, and muscle.

**Lymphomas**: they originate in the lymph nodes and other parts of the immune system.
**Leukemias:** they are non-solid tumors developing in the bone marrow, and are also found in the vasculature.

**Germ cell tumors:** they originate from ovarian and testicular cells.

**Blastomas:** they originate from immature cells or embryonic tissue.

While there are several causes for cancer, several risk factors may contribute to them. The most common risk factors besides genetic predisposition, are environmental, including tobacco, alcohol, diet, and pollutants (Anand et al., 2008).

Currently, there are several options for the treatment of cancer, which are basically dependent on the type, location, and the stage; with the latter determined by the tumor growth and its metastasis. The treatments include chemotherapy or hormonal therapy, surgery, and radiation.

Surgery is usually used to remove tumors before they metastasize. It can also be used to diagnose whether tumors are malignant or benign by examining biopsied cancer tissues, and to prevent cancer development in specific organs or tissues of pre-identified high-risk people expected to develop cancer in those organs or tissues.

Radiation therapy is also used for the treatment by using high-energy radiation that damages the DNA of the cells, preventing them from dividing, and leading to their death. Healthy cells are also expected to be killed, but that is usually kept minimal. This practice is aimed to cure cancer completely, and/or to prevent its reoccurrence.

Surgery and radiation are very efficient cancer treatments, but only when the cancer is localized. Metastasis is characterized by moving of the tumor cells from one site to
surrounding body parts forming “daughter” tumors that are close to the primary site. Cancer cells may invade the blood and lymphatic vessels, keep circulating in these fluids and transported to other sites. When the cancer cells invade a new site and begin to multiply to form another tumor, the tumor will become difficult to treat, since it cannot be detected until it grows significantly. At this point chemotherapy may be useful. (National Cancer Institute at: https://www.cancer.gov/about-cancer/understanding/what-is-cancer).

1.2 Chemotherapy

Chemotherapy is an approach to kill cancer cells, using drugs. Based on the fact that cancer cells are rapidly dividing, chemotherapeutic drugs have been designed to interfere with cell division. This is especially effective when tumor cells are in the process of metastasis, or in subclinical metastasis, where there are not enough cancer cells to be detected. There are many different classes of chemotherapeutic agents used for that purpose with each having a different strategy to either slow or halt the growth of cancer cells. Unfortunately, several of these drugs can also be toxic to healthy cells, especially those undergoing rapid cell division, such as bone marrow cells, and cells of the digestive system, and hair follicles. Therefore, the resulting side-effects of chemotherapy include: hair loss, immunosuppression, and inflammation of the lining of the digestive tract.
Alkylation Agents are the earliest drugs used for cancer chemotherapy. While nitrogen mustard was first tested during World War II for its use as a chemical warfare agent, it was also investigated for its potential use as a therapeutic agent for the treatment of lymphoma and leukemia. The chemical was first tested in a human patient in 1942 (Gilman, 1963), but its anticancer activity was unexpectedly discovered following an accidental spill of sulfur mustards on troops, in WWII, as men exposed to the mustard gas were observed to have significant reductions of illnesses in the bone marrow and lymph nodes (Marshall, 1964). The chemical was therefore the first alkylating agent utilized clinically and it was the start of modern cancer chemotherapy.

Alkylation agents act by damaging the DNA through an addition of an alkyl group to the DNA nucleotides. The agents in this class are commonly bifunctional, i.e., they form covalent bonds with two different nucleophilic sites of the DNA bases, resulting in interstrand or intrastrand cross-links. Such alterations on the DNA molecule potentially affects cell division, leading to cell death. However, other agents are monofunctional because they transfer a single alkyl group to the DNA strands, i.e., and that results in DNA miscoding and strand damage.

Antimetabolites are drugs that interfere with a cell’s metabolic processes by acting as false compounds to those normally needed for some biological functions, and will therefore stop those functions, leading to cell death. This class of drugs currently includes the pyrimidine antagonists, purine antagonists, and folate antagonists. It had been observed that a diet deficient in folic acid could provide improvement in leukemia patients, and that led to the development of folic acid antagonists that were first described
as effective in cancer treatment in 1948. Farber et al. (1948) found a folic acid analog that was effective in the reduction of childhood leukemia, which then led to the development of the antifolate, methotrexate, that is still used today to treat childhood leukemia. Folate antagonists, or antifolates act by inhibiting dihydrofolate reductase that catalyzes the conversion of folate to 5,6,7,8-tetrahydrofolate, required for nucleotide synthesis. Once nucleotide synthesis is blocked, the processes of DNA replication and cell division will be also stopped.

Purine antagonists act by inhibiting production of the purine nucleotides, adenine and guanine, while pyrimidine antagonists act by blocking synthesis of the pyrimidine nucleotides, cytosine and thymine in the DNA molecule, and cytosine and uracil in RNA. Purines and pyrimidines are essential molecules for nucleic acid synthesis, therefore blockage of these molecules will result in prevention of DNA synthesis and cellular division.

**Plant alkaloids** are compounds that are derived from plants and are used as anticancer agents. There are several classes of plant alkaloids, including the vinca alkaloids, taxanes, and the podophyllotoxins. The vinca alkaloids act by inhibiting the microtubular synthesis, while the taxanes act by preventing depolymerization of the preformed microtubules. Since microtubules are essential for cell division, interference with their function and/or formation will lead to death of dividing cells.

Epipodophyllotoxins, semi-synthetic derivatives of podophyllins, and the derivatives of the podophyllotoxins, act by inhibiting DNA topoisomerase II. This enzyme is essential for DNA replication and therefore many other chemotherapeutic agents had been
designed to target its function, and are known collectively as topoisomerase inhibitors. The drugs act by inhibiting either type I or type II topoisomerase, which are essential for DNA replication. The process of DNA replication involves unwinding of the DNA double strands, which creates a twisting tension that builds up in the rest of the coiled portion. Topoisomerase I and II act to modify the topology of the DNA without altering the structure and sequence (Minotti et al., 2000). While topoisomerase I causes breaks in one strand of a DNA double helix to release this tension, topoisomerase II causes breaks in both strands, reseals them after changing the twisting status of the double helix, and preventing the DNA from being supercoiled. Prevention of tension in the DNA strands is essential, as such tension can block further transcription and repair of the broken strand, resulting in cell apoptosis. Since inhibitors of the topoisomerase enzymes can kill all cells undergoing DNA replication, and that the rate of cancer cells division is much higher than that of the normal cells, topoisomerase inhibitors can potentially target cancer cells.

**Targeted therapy** is a different approach to cancer chemotherapy, where small molecules and monoclonal antibodies are used to target malignant, rather than the normal cells. This therapy is different from the traditional chemotherapy because it interferes specifically with essential molecules for tumor growth and progression, rather than killing rapidly dividing cells. The first molecules used in targeted cancer therapy were the estrogen/estrogen receptor complex, that is required for the growth of many types of breast cancer. In the 1970s, tamoxifen was first used as targeted cancer therapy, and was then followed by the development and approval of several other drugs that interfere with estrogen’s ability to promote the growth of Estrogen Receptor-Positive breast cancers.
This observation stimulated the interests of investigators to explore other targets that may be useful in the treatment of cancer. Tyrosine kinase receptors and associated signaling pathways were among those targets. Tyrosine kinase is an important enzyme for the activation of signal transduction cascades through phosphorylation. Many of the signaling cascades are growth factors associated with cell growth and development. However, mutations in this class of enzymes can result in their continuous activity, leading to unregulated cell growth and division, and eventually to the development of cancer. Tyrosine kinase inhibitors act by disrupting the enzyme activity; leading to the inhibition of growth signaling. (National Cancer Institute at: https://www.cancer.gov/about-cancer/understanding/what-is-cancer).

1.3 Anthracyclines

The anthracyclines are among the most common and effective drugs used for the treatment of cancer. They include daunorubicin, doxorubicin, epirubicin, and idarubicin that are used for the treatment of different types of cancer including leukemias and lymphomas, and breast, uterine, ovarian, and lung cancers. Most patients are commonly treated at some point with an anthracycline drug during their systemic cancer chemotherapy (Hortobagyi, 1997). The molecules contain a tetracyclic ring that has
adjacent quinone and hydroquinone moieties, a short-side chain with a carbonyl group at C-13, and an aminosugar (daunosamine) moiety attached by a to the C-7 of the ring by a glycosidic bond (Menna et al., 2007).

Figure 1.1: Chemical structure of an anthracycline

Because of the planar structure of the molecules, they can cause DNA intercalation, a process where the compounds are inserted between the DNA base pairs. While intercalation was initially believed to be the mechanism for the drug’s cytotoxicity, other cytotoxic mechanisms have been identified, such as generation of free radicals that attack the DNA causing damage, formation of DNA crosslinking, interference with DNA unwinding, direct effects on cellular membranes, and inhibition of topoisomerase II enzyme (Gewirtz, 1999). Of all the proposed mechanisms, topoisomerase II inhibition is believed to be the primary mechanism for the induction of cytotoxicity by the anthracylines (Hande, 1998). Studies by Minotti (2007), have indicated that the anthracycline’s anticancer activity is attributed to the stabilization of a reaction
intermediate, where DNA strands are cut and covalently linked to the tyrosine residues of topoisomerase II, leading to the blockade of DNA reclosing. The anthracyclines also described as “topoisomerase II poisons” because they stabilize the cleavable topoisomerase-DNA complex, converting it into a DNA damaging agent. As a result of that, double and single-strand DNA breaks accumulate and will eventually lead to DNA damage and cytotoxicity (Zunino, 1990). The anthracyclines’ effect on topoisomerase II is mainly attributed to the unique structure of the molecules, where rings B, C, and D are important for the intercalation with the DNA, and that the external moieties, such as the sugar group and the cyclohexane ring A of the molecule are important for the complex formation and stabilization (Dal Ben et al., 2007).

Derivatives of the anthracylines, including the anthracenediones and aza-anthracenediones believed to act by a similar mechanism, because they also contain a planar chromophore used as an intercalating agent, and that their antitumor activity is based upon the stabilization of the topoisomerase-DNA cleavable complex, leading to prevention of the strand breaks resealing.

1.4 Cardiotoxicity

As with any antineoplastic drug, the anthracylines use for the treatment of cancer is limited because of their adverse effects. Cardiac toxicity has been identified as the main
toxicity of the anthracyclines, and also found to be dose-dependent and irreversible (Elliot, 2006). The toxicity was identified with the drugs by their first use during the 1960’s (Elliot, 2006). Hrdina et al., (2000) have listed the following cardiotoxic effects associated with the use of the drugs:

“Acute toxicity” that is produced immediately after administration of one or two doses, and it involves production of vasodilation, hypotension, and cardiac dysrrhythmias. However, these toxic effects are reversible and do not require discontinuation of an anthracycline-based treatment (Menna et al., 2007).

“Subchronic toxicity”, which involves production of myocarditis and pericarditis early (1-3 days) into treatment. However, this kind of toxicity is uncommon.

“Chronic toxicity”, which is the most common and serious toxicity that develops late in the course of the treatment with the drugs, or shortly after completion of the therapy course. The effects involve production two life-threatening conditions: dilated cardiomyopathy and congestive heart failure (CHF). These effects develop in the majority of the patients within a year from the completion of a cumulative anthracycline treatment (Menna et al., 2007).

Delayed or late-onset chronic toxicity, which was recognized during studies conducted in the early 1990’s on childhood cancer survivors, and indicated that this toxicity may develop 10 to 15 years after the conclusion of treatment.
Several mechanisms have been suggested for the anthracycline-induced damage to the heart including disruption of calcium homeostasis, effects on histamine release, interference with the autonomic control of the heart, and alteration of cardiac protein expression. In addition to these mechanisms, free radicals generated by the anthracyclines were found to play a major role in the anthracylines-induced cardiotoxicity, both, *in vitro* (Link et al, 1996) and *in vivo* (Luo et al., 1997). There are two main pathways for the generation of free radicals from the anthracyclines: the redox-cycling of the anthracycline molecule and the formation of anthracycline-ferric ion complexes (Hrdina et al., 2000). Iron-mediated formation of reactive oxygen species (ROS) that lead to the production of myocardial oxidative stress remain to be the most commonly proposed mechanism of toxicity (Simunek et al., 2009).

The quinone moiety of ring C in the anthracycline tetracycle allows the compound to act as an electron acceptor, and when accepting the electron, it forms the semiquione free radical. Flavoproteins act as catalysts by accepting electrons from NADH or NADPH and subsequently donating them to the compounds to form the semiquinone radical. While the semiquinone is stable under anoxic conditions, it rapidly converts back to the parental quinone under normoxic conditions by donating its unpaired electron to an oxygen molecule, resulting in the generation of superoxide radicals (O$_2$•-). This is known as “redox-cycling” and it is well demonstrated in Figure 1.2. Redox-cycling can be very damaging because it can lead to overproduction of superoxide radicals from only a small amount of the anthracycline (Kiezer et al., 1990).
The $O_2^\cdot$- can undergo dismutation to $H_2O_2$, either spontaneously or by the action of superoxide dismutase (SOD). While $H_2O_2$ is a relatively stable molecule, but together with $O_2^\cdot$– they can generate the highly toxic hydroxyl radicals ($OH^\cdot$) (Hrdina et al., 2000). This reaction is known as the Haber-Weiss reaction (eq. 3), which is a very slow reaction, unless catalyzed by transition metals, such as Fe. The Fe-catalyzed Haber-Weiss reaction involves two steps: in the first step, ferric ion ($Fe^{3+}$) is reduced to ferrous ion ($Fe^{2+}$) by $O2^\cdot$– (eq. 1); and the second step is the Fenton reaction that involves the reaction $Fe^{2+}$ with $H_2O_2$ (eq. 2) (Simunek et al., 2009).

In summary:

$$Fe^{3+} + O_2^\cdot - \rightarrow Fe^{2+} + O_2 \quad (eq. \ 1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\cdot \quad (eq. \ 2) \quad (Fenton \ reaction)$$

$$O_2^\cdot - + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot \quad (eq. \ 3) \quad (Haber-Weiss \ reaction)$$

**Figure 1.2: Haber-Weiss Reaction**
Unlike O$_2$ •– or H$_2$O$_2$, both of which can be readily detoxified by enzymatic systems, OH• cannot be detoxified. Further OH• has a very short half-life and is extremely reactive towards DNA causing DNA damage, towards the lipids of the cellular membrane leading to lipid peroxidation, and towards the proteins leading to protein damage (Hrdina et al., 2000). Overproduction of free radicals including ROS that can attack different cellular macromolecules is known as oxidative stress that can lead to cellular damage and death. Mitoxantrone does not undergo redox cycling, but it has been shown to be cardiotoxic as well. This may indicate that there is a second mechanism or pathway for the generation of free radicals from the anthracyclines.

One suggestion for this mechanism may involve the formation of anthracycline-ferric ion (ANT-Fe$^{3+}$) complex through two different pathways. One is dependent on the presence of a reducing system, and the other one occurs in the absence of that system through radicals formation from the complex itself. The reducing system in the first pathway that involves the reduction of the ANT-Fe$^{3+}$ to ANT-Fe$^{2+}$ by flavoproteins such as, NADH cytochrome P450 reductase and the thiols of cystein or glutathione. Low-molecular-weight reducing agents can be as effective as the endogenous reducing system. The reduced complex can react with O$_2$ to form O$_2$•–, which can subsequently dismutate to H$_2$O$_2$ and enter into the Haber-Weiss reaction to generate the harmful hydroxyl radicals. The ANT-Fe$^{3+}$ can also reduce its chelated Fe through an intramolecular redox reaction in the absence of a reducing system, either by the oxidation of the C9 side chain, or the hydroquinone moiety at ring C, forming an ANT free radical chelate with Fe$^{2+}$ (ANT•-
The complex can be oxidized to yield O$_2$ •–. In the presence of O$_2$, and O$_2$ •– can react with H$_2$O$_2$ to generate OH• (Simunek et al., 2009).

Compared to the other tissues, the cardiomyocytes seem to be especially susceptible to anthracyclines-induced oxidative stress, which can be attributed to several reasons, including their ability to accumulate more of the drugs, as compared to with the other tissues. This has been well demonstrated by Showalter et al. studies in 1986 observing higher accumulation of anthracycline in chick embryo heart cells, as compared with the liver cells and the murine L5178Y lymphoblasts. A second reason is that cardiac cells are rich in mitochondria. It is generally accepted that these organelles are important target for the anthracycline molecular effects and that cardioselective mitochondrial dysfunction has been shown to be implicated in the chronic anthracycline cardiototoxicity (Link et al., 1996). The third reason is the weak antioxidant activity of the cardiac tissues. The tissues lack catalase (Doroshow et al., 1980), and catalase is an antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide into water molecules. Investigators have also shown that doxorubicin selectively down-regulates cardiac glutathione peroxidase, another antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide, and that suggests exposure of the cardiomyocytes to high levels of hydrogen peroxide.

The association of Fe in ANT-induced oxidative stress and cardiototoxicity is supported by several studies. Myers et al. (1986) have indicated formation of anthracycline-Fe complexes and the potential of oxidative damage of the biomembranes, *in vitro*. Hershco
et al (1993) have demonstrated an increase in Fe-mediated anthracycline cardiotoxicity in the isolated rat cardiomyocytes. The toxicity of doxorubicin was increased by prior Fe loading, indicated by higher release of lactate dehydrogenase (LDH) and more distinct reduction of cell contractility. The combination of Fe and doxorubicin apparently induced an additive effect, because at the concentrations used, Fe alone had only a minimal effect on LDH release and no effect on contractility, and doxorubicin alone had only a minor effect on contractility. A few years later, Link et al. (1996) showed that Fe loading to the rats treated with doxorubicin resulted in severe weight loss and a significant increase in mortality. In both of those studies, the unfavorable effects of Fe on doxorubicin cardiotoxicity could be eliminated by the use of the Fe chelator deferoxamine. Panjrath et al. (2007) have shown that dietary Fe supplementation increased doxorubicin cardiotoxicity significantly. The investigators concluded that the Fe body stores and its bioavailability in the tissues may be important independent predictors of susceptibility to doxorubicin-induced cardiotoxicity in humans.

Hemochromatosis results from defects in the genes coding for the hemochromatosis protein (Hfe). The disorder is associated with excessive levels of Fe in the body. Miranda et al. (2003) have assessed the contribution of Hfe to the sensitivity of the cardiomyocytes to doxorubicin-induced cardiotoxicity by using Hfe−/− and Hfe+/− mice, and they found that the rate of mortality from doxorubicin cardiotoxicity was significantly greater in both Hfe−/− and Hfe+/− mice than in wild-type animals. Also, doxorubicin treated Hfe−/− mice were found to have higher mitochondrial damage and Fe deposits in the heart than those of the wild-type mice.
1.5 Doxorubicin

In the early 1960s, the first anthracycline was isolated from the actinobacterium *Streptomyces peucetius* and was given the name daunorubicin, which is a combination of the name Dauni, referring to a pre-Roman tribe that lived in an area of Italy where the compound was isolated, and rubis, which refers to the word ruby, a French word that describes the color (Di Marco et al, 1981). Daunorubicin was the first compound that was found to be effective in reducing tumor size murine models and was later tested clinically (Di Marco, 1981). The compound was found to be very effective in the treatment of acute leukemia, but unfortunately it also produced fatal cardiotoxicity (Weiss, 1992). In an effort to eliminate the cardiotoxicity of the compound but retaining its antitumor activity, investigators started to develop analogs of daunorubicin. This led to the isolation of doxorubicin, also known as adriamycin, which is referring to the Adriatic Sea.

Figure 1.3: Chemical structure of daunorubicin (left) and doxorubicin (right)
Doxorubicin was isolated from a mutated form of *Streptomyces* (Arcomone et al. 1987), and it only differs from daunorubicin by the presence of a single hydroxyl group. Di Marco et al (1977) have demonstrated better activity of the compound against murine tumors than that of doxorubicin and a better therapeutic index than that of daunorubicin when tested in the same model. Doxorubicin was marketed in the United States in 1974, and as of today, it remains to be used as an anticancer agent because of its wide spectrum of antitumor activity. While the drug is used to treat different types of cancer, including Hodgkin’s disease, leukemia, lymphomas, breast cancer, sarcomas, childhood solid tumors, and non-Hodgkins lymphomas (Simunek, 2009), its use is still limited by its dose-related cardiotoxicity. The recommended cumulative lifetime dose of the drug is 450 to 550 mg/m² (Hortobagyi, 1997). In order to reduce / prevent the problem of the drugs-associated cardiotoxicity, investigators started to explore different strategies. Some strategies were directed towards limiting the cumulative dose by the drug to target only the cancer site, or by decreasing the amount of drug reaching the heart. This could be achieved by the use of the liposomal formulations of daunorubicin and doxorubicin, that were found to deliver lower amount of the drug to the heart than the non-liposomal forms and were therefore less cardiotoxic (Forssen, 1979).

Another approach involved cardioprotection through iron chelation. Dexrazoxane, is a clinically approved drug that is believed to act by undergoing hydrolysis, yielding the Fe-chelating metabolite ADR-925 that structurally resembles the chelating agent, ethylenediaminetetraacetic acid (EDTA). This metabolite binds to free and loosely bounded Fe, including Fe that is complexed with the anthracyclines, thus preventing
production of ROS that damage cellular components. The compound has been repeatedly demonstrated to be effective in protecting the myocardium of animals and humans against the anthracyclines (Jones, 2008). Data analyses of the clinical trials that involved the use of Dexrazoxane, has indicated that the antitumor action of the anthracyclines was not compromised (Jones, 2007). However, later clinical trials for the use of the compound in combination with other drugs for the treatment of acute myeloid leukemia and myelodysplastic syndrome in children found the use to be associated with secondary leukemias (Tebbi, 2007). Accordingly, in July of 2011 the US Food and Drug Administration released a statement restricting the use of dexrazoxane, to only in adult patients with breast cancer who are receiving > 300 mg/m2 doxorubicin or > 540 mg/m2 epirubicin, and that the general approval for the use of dexrazoxane, for cardio-protection was withdrawn (Tebbi, 2007). Efforts by investigators continued for decreasing the cardiotoxicity of the anthracyclines through searching for new analogues with better antineoplastic activity and less toxicity (Weiss, 1992). However, only a small number of those analogs, including idarubicin and epirubicin made it past clinical trials. Idarubicin is a daunorubicin analogue that is only used for the treatment of acute myelogenous leukemia. Epirubicin is another analogue that is derived from doxorubicin through compounds epimerization. While both idarubicin and epirubicin are used clinically, none of them was proved to have better efficacy or lower toxicity (Cortes-Funes, 2007), and therefore the search for better alternatives continued.
In the continued search for more effective and less toxic anthracycline analogs, a number of multi-ringed planar compounds were synthesized. Among these compounds were the
anthracenediones, that lack the amino sugar moiety and the tetracyclic A ring of
doxorubicin, but retain the planar polycyclic aromatic ring structure (Faulds, 1991). From
this class mitoxantrone, was identified as the most active and the only compound of the
first generation anthracenediones class to be approved for clinical use (Hande, 1998). The
drug was approved by the FDA in 1987 for use in the treatment of adult acute myeloid
leukemia, and in 1996 it was approved for use in the treatment of symptomatic hormone-
refractory prostate cancer. Mitoxantrone was also used for the treatment of breast cancer
and lymphomas (Hande, 1998). Aside from its antitumor activity the drug was found to
be useful in the treatment of multiple sclerosis (MS) (Fox, 2006) and was approved in
2000 for the use in worsening cases of relapsing–remitting MS, secondary progressive
MS, and progressive-relapsing MS.

Similar to the anthracyclines, the mitoxantrone’s mechanism of action involves DNA
intercalation and inhibition of topoisomerase II. The drug is known to have slightly less
antitumor activity and less cardiotoxicity than that of doxorubicin, and can be better
tolerated than doxorubicin. Approximately twice as much of the mitoxantrone can be
administered before heart failure is produced (Hande, 1998). However, the better
tolerance and less cardiotoxicity of mitoxantrone doesn’t mean that the drug is
completely free of cardiotoxicity. The drug’s toxicity is believed to be due to the
presence of the 5,8-dihydroxy substitution groups on the molecule that favor the drug
binding to iron, similar to the ANT-Fe$^{3+}$ complex pathway and its associated pathway for
free radical generation, discussed under the anthracyclines. The search for better
analogues continued and resulted in, the development of a second generation of anthracenedione compounds, which lack the 5,8-dihydroxy substitution groups.

### 1.7 Pixantrone

![Chemical structure of Pixantrone](image)

Figure 1.6: Chemical structure of Pixantrone

Synthesis and screening of new second-generation anthracenedione analogues that have wider spectrum of action and lower toxicity than mitoxantrone or doxorubicin continued. Among those analogues was pixantrone. Pixantrone is a novel aza-anthracenedione compound similar to mitoxantrone, but is different from it in the way it contains a pyridine ring instead of the 5,8-dihydroxyphenyl ring that is believed to be associated with the generation of free radicals from the molecules (Krapcho et al., 1994). A nitrogen is also present in the anthraquinone ring of the molecule, which provides additional hydrogen-bonding sites for the interaction with topoisomerase II and also for DNA
intercalation (Jamal-Hanjani et al., 2011), that are known to be the basic mechanisms of action of the compounds predecessors, the anthracycline. This has been confirmed by studies of Krapcho et al, (1994) indicating that presence of the nitrogen at the 2-position of the ring is associated with significant effect on the biological activity of the compound. Pixantrone is first activated by formaldehyde, in the form of a drug/formaldehyde conjugate or the use of formaldehyde-releasing drugs, then it intercalates selectively at CG and CA dinucleotides of the DNA molecule, impairing topoisomerase II, and causing DNA cleavage (Evison et al., 2007).

Pixantrone proved to have a broader therapeutic range and a greater anticancer activity than the other anthracyclines, in clinical trials for the treatment of a number forms of leukemia (Cavalletti et al, 2007). The drug was also found to have activity when tested in vitro, against murine and human tumor cells, including leukemia and non-small lung cancer cells (Faive et al., 2001). Pixantrone was also tested in mice and found to be equally effective as mitoxantrone, but it induced no cardiotoxic effects, even after repeated administration. (Cavalletti, 2007). The study also showed that pixantrone did not worsen a pre-existed heart muscle damage, which suggested a further advantage of the drug if used by patients pretreated with anthracyclines (Cavalletti, 2007). Human clinical trials revealed the effectiveness of pixantrone in treating patients with Non-Hodgkins lymphoma, and in 2012, the drug received a conditional marketing authorization in the European Union as monotherapy to treat adult patients with Multiply Relapsed or Refractory Aggressive Non-Hodgkin B-Cell Lymphomas.
1.8 BBR-3378

Another approach to reduce the potential of the compounds to generate free radicals through redox cycling was the development of anthrapyrazoles and aza-anthrapyrazoles (Sissi et al., 2004). The compounds have a pyrazole ring that is fused to the anthraquinone moiety. Similar to their anthracenedione predecessors, the anthrapyrazoles are intercalating agents that act by inhibiting topoisomerase II enzyme. Structural modification of the B ring appeared to reduce the molecule's tendency to form semiquinone free radicals and the subsequent generation of superoxide anion. Studies on the anthrapyrazoles antitumor effects in model tumors and in doxorubicin-resistant cells have indicated a large array of antitumor activity (Begleiter, 2006).

Figure 1.7: Chemical structure of BBR-3378
1.9 Aims and Objectives

The study was intended to answer the following:

1. Previous studies suggested that the cardiotoxic effects of the anthracyclines is due to binding of the drugs to the body iron. Does pixantrone display little to no cardiotoxicity because it does not interact with iron?

2. Interaction of some of the anthracyclines and their analogues with iron was suggested to contribute to the compounds’ cardiotoxicity through generation of free radicals and/or ROS. Do these drugs interact with body metals other than iron?

3. If the drugs interact with other metals, is there a distinction in the interaction characteristics of the different classes with those metals?

To answer the above questions, we studied the interactions of those drugs with iron and also with copper and zinc and assessed that by recording changes in the optical density/absorption spectra of the drug solutions with, and without metals.
Chapter 2

Materials and Methods

2.1 Drug and Metal Preparation

0.02M Tris-HCl buffer was prepared and pH was adjusted to 7.0. Doxorubicin, mitoxantrone, pixantrone, and BBR-3378 were dissolved in the Tris-HCl buffer to obtain concentrations of 400μM. The stock solutions of the drugs were diluted down to 25μM for use in the metal binding assays. Solutions of 10mM ferrous sulfate dissolved in Tris-HCl buffer were prepared daily. Cupric sulfate was dissolved in Tris-HCl buffer to obtain a concentration of 1mM. A solution of 200 mg/ml albumin was made by dissolving bovine serum albumin (BSA) in water. Zinc sulfate was dissolved in Tris-HCl buffer to obtain a concentration of 50μM.
2.2 Metal Binding Assay

Absorption spectra were recorded on a Molecular Devices SpectraMax M5 UV/Vis. For all experiments, 800 µl Tris-HCl was added to a cuvette and scanned via spectrophotometer for a reference. Then 800 µl of the drugs’ solutions were added to new cuvettes and scanned for blanks. The metal solutions were added to the cuvettes containing the drugs and were covered with parafilm and inverted by hand several times to ensure proper mixing. The solutions were then scanned at wavelengths set for each specific drug, and were ranging from 350nm to 750nm. Each experiment was done in triplicate to ensure accurate results.

2.3 Interactions with ferrous – iron

Iron binding assays were carried out with all four drugs. Ten µl of 50mM ferrous sulfate solution was added to each drug and scanned approximately every 2 minutes. This addition took place 5-8 times for a total of 50-80 µl of ferrous solution added to the drugs’ cuvette.
2.4 Interactions with cupric – copper and BSA

Copper binding assays were carried out with all four drugs. In the first experiment, 5 µl of 1mM cupric sulfate solutions were added to each drug and mixtures were scanned approximately every 2 minutes. This addition took place 8 times for a total of 40 µl cupric sulfate solution added to the drugs’ cuvette. In a second experiment, BSA was also added to the mixtures of drug and cupric sulfate solutions. Increments of 10 µl of albumin were added and, mixtures were scanned approximately every 2 minutes. BSA solution addition was done 8 times for a total of 80 µl added to the drug/cupric sulfate mixtures.

2.5 Interactions with zinc

Zinc binding assays were carried out with all four drugs. Ten µl of 50mM zinc sulfate solutions were added to each drug and scanned approximately every 2 minutes. This addition took place 8 times for a total of 80 µl zinc sulfate solution added to the drugs’ cuvette.
Chapter 3

Results

3.1 Interactions with iron

3.1.1 Doxorubicin and iron

Figure 3.1 represents the interaction between doxorubicin and iron. The optical density changes with iron additions to the sample. The primary peak (450 to 510 nm) shows the highest optical density of the blank at approximately 2.9, and the lowest optical density of the solution with the highest iron concentration at approximately 2.1. At about 525 nm, an isosbestic point was formed and it switched so that the blank had the lowest optical density and solution with the highest iron concentration had the highest optical density.
3.1.2 Mitoxantrone and iron

Figure 3.2 represents the interaction between mitoxantrone and iron. The optical density seemed to change as more iron was added to the drug. Unlike doxorubicin that had just one peak, there was a distinctive “M” shape formation of two separate peaks, in response to changes in wavelength. At both of those peaks, the blank had the highest optical density at approximately 4.1 and 3.9, and solution of the highest iron concentration had the lowest optical density at approximately 3.0 and 3.1. Similar to the observed formation of isosbestic point with doxorubicin-iron optical density at 525 nm, an isosbestic point was also observed with mitoxantrone-iron, but at about 690 nm. At that point, shifts were
observed so that the blank had the lowest optical density and solution of the highest iron concentration had the highest optical density.

**Figure 3.2** Interaction between mitoxantrone and iron. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 10µl of 50µM ferrous sulfate were added to 25µM mitoxantrone to produce a final concentration of 2.9µM of ferrous sulfate. Each scan was approximately 2 minutes apart.

3.1.3 Pixantrone and iron

Figure 3.3 represents the interaction between pixantrone and iron. The optical density in response to changes in wavelength exhibited a similar “M” shape as that observed with mitoxantrone-iron. There were 2 distinctive peaks at approximately 600nm and 640nm with an optical density of 3.1 and 3.2. At both those peaks, the optical density did not differ as more iron was introduced. There was no distinct isosbestic point observed.
3.1.4 BBR-3378 and iron

Figure 3.4 represents the interaction between BBR-3378 and iron. BBR-3378 also had the characteristic “M” shape in regard to the optical density, with peaks occurring at approximately 370nm and 480nm. The optical density stayed fairly consistent as iron solution was added. There were only slight changes at each peak, and there was no distinct isosbestic point formation.
3.2 Interactions with Copper / Albumin

3.2.1 Doxorubicin and Copper / Albumin

Figure 3.5 represents the interaction between doxorubicin and copper. The optical density changes with addition of copper solutions to the sample. The major peaks for the
absorbance observed between 430 and 520 nm, and the highest and lowest optical densities were observed with the blank and the highest iron concentration at approximately 2.5, and 1.1, respectively. At about 525nm, an isosbestic point was formed, where there was a shift so that the blank displayed the lowest optical density and, while solution of the highest iron concentration displayed the highest one.

Figure 3.6 represents the interaction between the doxorubicin and copper after the addition of albumin (BSA). The figure shows that the peak of absorbance occurred between 420nm and 525nm, and the highest optical density (of approximately 2.0) was observed with addition of the highest BSA concentration. However, the blank displayed the lowest optical density at approximately 0.13, and an isosbestic point was also formed at 525nm.
Figure 3.5: Interaction between doxorubicin and copper. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 5 µl of 50mM cupric sulfate were added to 25 µM doxorubicin to produce a final concentration of 2.4mM of cupric sulfate. Each scan was approximately 2 minutes apart.

Figure 3.6: Interaction between doxorubicin/copper and bovine serum albumin (BSA). The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 10 µl of bovine serum albumin were added to the 25 µM doxorubicin plus 40 µl copper combination to produce a final concentration of 90.9mg/l. Each scan was approximately 2 minutes apart.
3.2.2 Mitoxantrone and Copper / Albumin

Figure 3.7 displays the interaction between mitoxantrone and copper. The optical density changes as more of the copper solution was added to the sample. There are 2 distinctive peaks occurring at roughly 610 nm and 660 nm. At the two peaks, the blank displayed the highest optical densities of approximately 4.1 and 3.9, while the samples with the highest iron concentration displayed the lowest optical densities of approximately 2.0 and 2.1. Also, as the copper concentration was increased, the spectrum lost its distinctive “M” shape. An isosbestic point was formed at about 685nm where there were shifts in absorbances with the blank displaying the lowest optical density and samples with the highest iron concentration displaying the highest one.

Figure 3.8 shows the interaction between the mitoxantrone and copper after the addition of albumin. The figure displays 2 main peaks of absorbances occurring at around 610 nm and 660 nm, and an isosbestic point at 685nm. At the first peak (610nm) addition of the highest concentration of albumin was associated with the observation of the highest optical density of approximately 3.1, and addition of the lowest albumin concentration was associated with the observation of the lowest one at 1.4. However, at the second peak that occurred at 660nm, the highest optical density (2.9) was observed with addition of the third highest concentration of albumin, while the lowest optical density was observed with addition of the lowest albumin concentration. Notably, the wavelength regained its “M” shape as more albumin solution was added.
Figure 3.7: Interaction between mitoxantrone and copper. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 5μl of 50mM cupric sulfate were added to 25μM mitoxantrone to produce a final concentration of 2.4mM of cupric sulfate. Each scan was approximately 2 minutes apart.

Figure 3.8: Interaction between mitoxantrone/copper and bovine serum albumin (BSA). The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 10μl of bovine serum albumin were added to the 25μM mitoxantrone plus 40μl copper combination to produce a final concentration of 9.9mg/l. Each scan was approximately 2 minutes apart.
3.2.3 Pixantrone and Copper / Albumin

Figure 3.9 shows the interaction between pixantrone and copper. Unlike iron, the optical density changed as more of the copper solution was added to pixantrone. Two separate peaks of absorbance were displayed at 600nm and 640nm. At both peaks, the highest optical densities (3.4 and 3.6) were displayed by the blank, and the lowest optical densities (2.5 and 2.1) were displayed by samples with the highest copper concentrations. An isosbestic point at 680nm was also displayed, but the optical densities at which became fairly similar. Also as seen in mitoxantrone, the characteristic “M” shape display of optical densities associated with changes in the wavelength was lost, as the copper concentration was increased.

Figure 3.10 shows the effects of albumin addition to the pixantrone/copper combination. Once again, changes in optical densities that were associated with changes in the wavelength showed 2 main peaks at 600nm and 640nm and an isosbestic point at 680nm. At each peak, addition of the highest albumin concentration was associated with the highest optical densities (2.6 and 2.7), while the blank displayed the lowest ones (1.8 and 1.4). Also, changes in optical densities associated with changes in the wavelength regained the “M” shape with the repeated addition of albumin solution.
Figure 3.9: Interaction between pixintrone and copper. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increment of 5μl of 50mM cupric sulfate were added to 25μM pixintrone to produce a final concentration of 2.4mM of cupric sulfate. Each scan was approximately 2 minutes apart.

Figure 3.10: Interaction between pixintrone/copper and bovine serum albumin (BSA). The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increment of 10μl of bovine serum albumin were added to the 25μM pixintrone plus 40μl copper combination to produce a final concentration of 90.9mg/l. Each scan was approximately 2 minutes apart.
3.2.4 BBR-3378 and Copper/Albumin

Figure 3.11 shows the results of interaction between BBR-3378 and copper. Changes in the optical density associated with changes in the wave length resulted in the occurrence of a wider “M” shape peaks than those seen with pixantrone or mitoxantrone. The two peaks appeared at 370nm and 480nm. At both peaks, the lower concentrations of copper were associated with the higher optical densities (3.0 and 3.4), and the highest concentrations of copper were associated with the lowest optical densities (2.4 and 2.8). An isosbestic point also appeared right after the appearance of the second peak at 510nm, where there was a slight switch, so that the lower and the higher copper concentrations were associated with the lower and higher optical densities, respectively. The figure also shows that the absorption spectrum did not lose the characteristic “M” shape with the addition of more copper.
Figure 3.12 shows the results of the interaction between the BBR-3378/copper combination and albumin. The optical density was only little altered as more albumin was added. There spectrum displayed two main peaks, one at 370nm, and one at 480nm. It appeared as if the optical densities were fairly equal among all the scans during the first peak, but they slightly changed at the second peak so that the highest optical density (3.0), and the lowest optical density (2.4) were associated with the highest and the lowest albumin concentrations, respectively. An isosbestic point was also formed, where the lower albumin concentrations albumin was associated with the highest optical densities.
3.3 Interactions with Zinc

3.3.1 Doxorubicin and Zinc

Figure 3.13 represents the results of interaction between doxorubicin and zinc. Zinc addition to the drug resulted in changes the drug’s optical density. The figure shows that the optical density increased as more zinc was added. At the primary peak (490nm), the
higher concentrations of zinc were associated with the highest optical densities, while the blank and the lower concentrations of zinc were associated with the lower optical densities.

Figure 3.13: Interaction between doxorubicin and zinc. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 10µl of 50mM zinc sulfate were added to 25µM doxorubicin to produce a final concentration of 4.5mM of zinc sulfate. Each scan was approximately 2 minutes apart.
3.3.2 Mitoxantrone and Zinc

Figure 3.14 demonstrates the results of interaction between mitoxantrone and zinc. Two peaks were displayed, one at 610nm and another at 660nm, giving the spectrum a characteristic “M” shape. The optical density increased as more zinc was added. At the two main peaks, the highest optical densities of 3.6 and 3.2 were associated with the higher concentrations of zinc, and the lowest optical densities of 3.1 and 2.7, were associated with the blank and the lower concentrations of zinc.

![Figure 3.14](image)

*Figure 3.14: Interaction between mitoxantrone and zinc. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 10μl of 50mM zinc sulfate were added to 25μM mitoxantrone to produce a final concentration of 4.5mM of zinc sulfate. Each scan was approximately 2 minutes apart.*
3.3.3 Pixantrone and Zinc

Figure 3.15 demonstrates the results of interaction between pixantrone and zinc. The figure displays two separate peaks at 600nm and 640nm, giving it the “M” shaped similar to the observations with the other drugs/metals. The optical densities appeared to be fairly similar during the beginning and the end of the scan, but it only changes at the two peaks where the higher concentration of zinc was associated with the highest optical densities, and vice versa.

Figure 3.15: Interaction between pixantrone and zinc. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 10μl of 50mM zinc sulfate were added to 25μM pixantrone to produce a final concentration of 4.5mM of zinc sulfate. Each scan was approximately 2 minutes apart.
3.3.4 BBR-3378 and Zinc

Figure 3.16 demonstrates the results of the interaction between BBR-3378 and Zinc. The figure displays the typical “M” shape of the spectrum that was associated with changes in the wavelength, and was observed with all of the BBR-3378/metal interactions. The two peaks appeared at 360nm and 470nm, and there appeared to be only a slight change in optical densities with the addition of more zinc. The figure also shows that the higher concentrations of zinc associated with highest optical densities throughout the scan.

Figure 3.16: Interaction between BBR-3378 and zinc. The x-axis is the wavelength (nanometers) and the y-axis is the optical density. Increments of 10μl of 50mM zinc sulfate were added to 25μM BBR-3378 to produce a final concentration of 4.5mM of zinc sulfate. Each scan was approximately 2 minutes apart.
Chapter 4

Discussion

Studies on the cardiotoxicity of the anthracylines have indicated the role of production of free radicals and ROS from the drugs when they interact with the body iron (Menna et al, 2007). We used the principles of spectrophotometry where compounds interactions can result in changes in optical densities to investigate the interactions between certain anticancer drugs and important metals found naturally in the body. The tested drugs were therefore expected to have a specific optical density over a definitive wavelength, but that optical density changes if metals are added to, and interact with the drugs. The study also identified the occurrence of any isosbestic points at a specific drug/ metal interaction. By definition, the isosbestic point is a specific wavelength at which the total absorbance of a sample does not change during a chemical reaction or a physical change of the sample. At this point, drugs are expected to be completely saturated with the metal.

The results of interaction of doxorubicin with iron showed a change in optical density with repeated addition of iron, where the drug-iron mixture resulted in a change of about 0.8 from the doxorubicin’s primary peak. This means that the compound did interact with
iron. This is also true with mitoxantrone, where the optical density changed by the addition of ferrous to the drug. The first and the second peaks that were primarily observed with the drug alone shifted by about 1.1 and 0.8, respectively upon iron addition. Contrary to the observed results with doxorubicin and mitoxantrone, pixantrone results indicated little to no interaction with iron, because the optical densities of the two pixantrone’s peaks remained the same after the addition of iron. This may confirm previous studies suggesting the potential interactions of doxorubicin and mitoxantrone with iron (Cavalletti et al, 2007). Since previous studies indicated the potential contribution of iron interactions with the drugs to their observed cardiotoxic effects (Cavalletti et al, 2007), the no observed interaction of pixantrone with iron may suggest a non-potential for the compound to produce cardiotoxicity. Similarly, BBR-3378 showed only a slight interaction with iron at its first and second peaks that differ by 0.1 or less, and 0.3, respectively, suggesting non-potential for the drug to induce cardiotoxicity. The reason for the observed non-interaction of pixantrone and BBR-3378 with iron is the absence of the 5,8-dihydroxyphenyl ring in the drug molecules that is believed to be associated with free radical generation by doxorubicin and mitoxantrone (Cavalletti et al, 2007).

Since specific drugs are known to interact with iron, and their interactions with the metal was found to be associated with their cardiotoxicity, other body metals may have similar effects to those produced by iron and may also contribute to the observed effects. We
tested the effects of copper and zinc, since they are the most abundant body metals, besides iron.

The results suggest that all four drugs interact with copper, as indicated by changes in the optical density when more cupric solution was added to the samples. Since there was no apparent distinction in the interaction of the metal with doxorubicin and mitoxantrone, in comparison with pixantrone and BBR-3378 that lack the iron binding site, the copper binding site to the drugs is suggested to be different from that of iron.

Albumin is one of the most common proteins of the blood. Therefore, BSA was used in the studies to have a preliminary idea of how the observed in vitro interactions of copper with the drugs can be influenced in vivo, in the presence of blood proteins, such as albumin. The results show that addition of albumin to drugs mixed with copper solution resulted in shifting the peaks back to those observed with the drugs alone. The best conclusion that can be derived at this point that copper may not interact with the compounds in vivo because of the presence of albumin, and therefore the role of this body metal in the drugs’ cardiotoxicity may be excluded. Although this may be true because there is no current evidence of the involvement of this metal in that effect, further in vivo studies are required to confirm this suggestion.

Similar to the effects of copper, zinc appeared to interact with all four drugs as indicated by the small changes in the absorption spectra of the drugs when zinc was added. Again,
since there was no selectivity of the drugs in their interaction with the metal, compared to their interactions with iron, it is suggested that the zinc binding site on the drugs is different from that of iron.

The non-selectivity of the binding characteristics of the four drugs to copper and zinc as compared with that of iron, together with the fact that iron interaction with doxorubicin and mitoxantrone may contribute to the cardiotoxicity of the two drugs suggest that interactions with copper and zinc may not contribute to the cardiotoxicity of the drugs.

In conclusion, we studied the metal binding effects on some of the anthracyclines and their analogues. Our results have demonstrated the interaction of iron with doxorubicin and mitoxantrone, but not with pixantrone and BBR-3378. We also concluded that all four drugs have weak interactions with copper, which can be interrupted with the addition of bovine serum albumin. The results also demonstrated the interactions between zinc and the four drugs. Since copper and zinc interacted with the four drugs, unlike iron that interacted only with doxorubicin and mitoxantrone, we suggest that the iron binding sites on the drugs are different from those of the copper and zinc binding sites.
References


