RATIONAL DRUG DESIGN:

AN INFORMATION DRIVEN APPROACH TO THE DESIGN OF AN ANTHRACYCLINE ANALOG

by

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A thesis

submitted in partial fulfillment

of the requirements for the degree

Master of Science in Biology

Boise State University

May 2009

BOISE STATE UNIVERSITY GRADUATE COLLEGE

DEFENSE COMMITTEE AND FINAL READING APPROVALS

of the thesis submitted by

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Thesis Title: Rational Drug Design: An Information Driven Approach to the Design of an Anthracycline Analog

Date of Final Oral Examination: 18 March 2009

The following individuals read and discussed the thesis submitted by student Charlene Rebecca Young, and they also evaluated her presentation and response to questions during the final oral examination. They found that the student passed the final oral examination, and that the thesis was satisfactory for a master's degree and ready for any final modifications that they explicitly required.

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The final reading approval of the thesis was granted by Henry A. Charlier, Jr., Ph.D., Chair of the Supervisory Committee. The thesis was approved for the Graduate College by John R. Pelton, Ph.D., Dean of the Graduate College.

DEDICATION

For my son who has been very patient and although has had many goals and aspirations in the last eight years, wants to grow up to be a paleontologist, at least for now.

To his granddad, who before his death was within weeks of completing his own graduate degree, a man who taught his grandson to dream - opening a lot of doors for him.

To my husband who has paid the bills, took care of the most important things, kept everything organized and gave me this opportunity.

ABSTRACT

Over the past fifty years anthracyclines have been used to treat a wide variety of cancers. Combination therapies with anthracyclines have the potential to greatly increase treatment success. Despite the great potential of anthracyclines in the treatment of cancer, their use has been limited due to the risk of chronic cardiotoxicity. The reduction of anthracyclines to an alcohol metabolite has been linked to the development of cardiotoxic side effects. One of the principal enzymes responsible for catalyzing the formation of the anthracycline alcohol metabolite is human carbonyl reductase 1 (HCBR). Controlling the reduction of anthracyclines by HCBR may offer a means to reduce the risk of cardiotoxicity during treatment.

The structure activity relationships responsible for the recognition and binding of the anthracycline substrates were investigated. Molecular modeling studies implicated Met 234 as a possible determinant of anthracycline specificity for HCBR. In order to test this, site directed mutagenesis was used to convert the methionine to a cysteine in a histidine expression system. The histidine tagged HCBR was found to have reduced enzyme activity and coenzyme binding compared to native enzyme. Further, the cysteine 234 mutant enzyme was found to be inactive, although it still appeared to possess coenzyme and anthracycline binding capability. It is clear that the addition of the histidine tag has impaired enzyme function and that such a modification may mask any effects introduced by mutating Met 234 to a cysteine (NIH-INBRE Grant # P20RR16454).

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LIST OF ABBREVATIONS

HCBR 1	Human Carbonyl Reductase 1 Enzyme (EC 1.1.1.184)
HCBR'	Human Carbonyl Reductase 1 Enzyme with pTrcHis2 TOPO Histidine Tag
M234C HCBR	Mutant HCBR 1 Enzyme with Cysteine at Position 234
CBR	Carbonyl Reductase
CBR V88	Human Carbonyl Reductase 1 polymorphism with Valine at position 88
CBR I88	Human carbonyl Reductase 1 polymorphism with isoleucine at position 88
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
MDR	Multi-Drug Resistant
ABC	ATP-binding Cassette Superfamily
SDR	Short-Chain Dehydrogenase Enzyme Family
Tyr	Tyrosine Amino Acid
Lys	Lysine Amino Acid
Met	Methionine Amino Acid
Cys	Cysteine Amino Acid
Ala	Alanine Amino Acid
Ser	Serine Amino Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ATP	Adenosine Triphosphate

AMP	Ampicillin
LB	Luria Bertani
NADPH	β-nicotinamide Adenine Dinucleotide Phosphate, Sodium Salt
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate

CHAPTER 1

CANCER THERAPIES: ANTHRACYCLINE TREATMENTS AND POTENTIAL HEALTH RISKS

Anthracyclines in the Treatment of Cancer

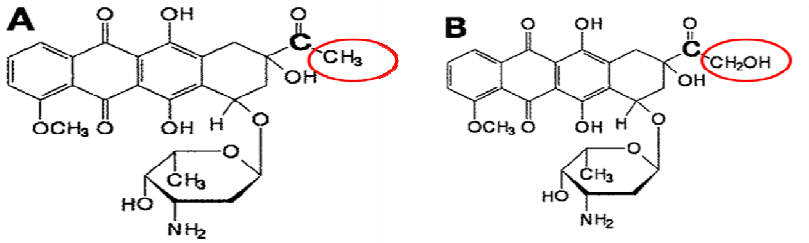
History of Anthracycline Therapies

Anthracyclines are antibiotics that are effective treatments for a variety of aggressive cancers.[1] Among cancer treatment drugs, anthracyclines have activity on the widest range of cancers.[1] Doxorubicin has been considered the most potent neoplastic agent in the anthracycline class of drugs and has the widest spectrum of use. [2] Only a few types of cancers are unresponsive to anthracycline therapy. [2]

Daunorubicin and doxorubicin are two types of anthracyclines that were first isolated from the *Streptomyces peucetius* bacteria in the early 1960's and are still widely used. These two drugs are good models for cancer research because the structures of these drugs differ by only one hydroxyl group; however, they differ in cytotoxic behavior (Figure 1.1).[1]

Doxorubicin is used in the treatment of solid tumors and includes breast, lung, thyroid, ovary carcinomas, childhood solid tumors, soft tissue sarcomas, cancers of the blood and aggressive lymphomas. Daunorubicin is used to treat acute leukemias such as lymphoblastic and myeloblastic leukemias.[1-3] Over 2000 analogs of daunorubicin have been made and the data show that small structural changes induce large changes in chemical behavior. [2]

Figure 1.1: Structure of Daunorubicin and Doxorubicin



A) Daunorubicin, B) Doxorubicin
These two anthracyclines have very similar structures; however, they differ greatly in behavior (Table 1.1). The only difference in structure between daunorubicin and doxorubicin is the addition of one hydroxyl group on C-14.

Even though anthracyclines have been shown to be very effective in the killing of cancer cells the clinical use of anthracyclines is limited by significant side effects for some patients. In 1967 it was discovered that daunorubicin could induce fatal cardiotoxicity. [2] Other analogs have been made that aim to either increase the cytotoxicity or decrease cardiotoxicity of this treatment; however, the cardiotoxicity of these treatments remain high. The clinical use of daunorubicin and doxorubicin covers over fifty years and an analog that is a potent cancer cell killer and has diminished cardiotoxicity properties has yet to be made.

Cytotoxic Mechanisms of Anthracyclines

The cytotoxicity mechanisms are not well understood for anthracyclines. Anthracyclines are passively transported through the membrane in an uncharged form.[4] pH gradients along the membrane influence the accumulation of daunorubicin in cells.[4] Anthracyclines are targeted to the nucleus of the cell where it causes cell damage at the DNA level.[5] Cytotoxic mechanisms are thought to involve DNA intercalation, interruption of the topoisomerase II enzyme and free radical production.[1,6,7]

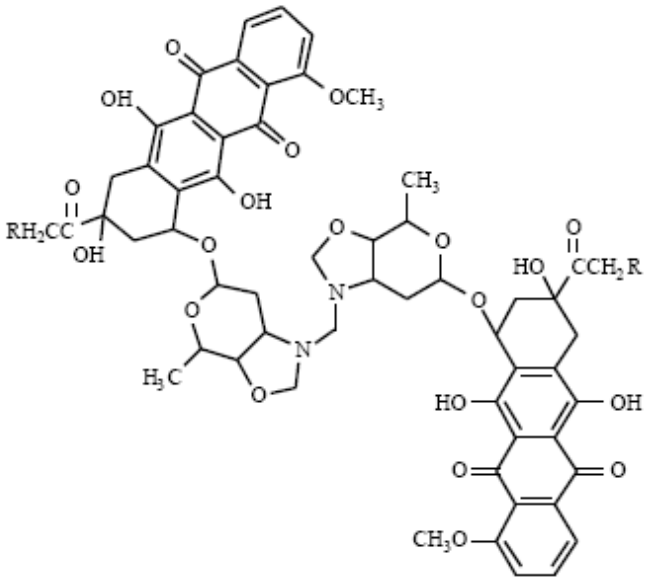
Intercalation into DNA Strands

Intercalation involves the drug inserting directly into the DNA molecule and is essential for drug activity. Doxorubicin and daunorubicin intercalate with B-DNA and show little affinity for A-DNA, left-handed Z-DNA and RNA duplexes.[3] The intercalation is DNA sequence specific where daunorubicin prefers to intercalate at alternating pyrimidine-purine stretches.[8] The anthraquinone intercalates between two base pairs of DNA and prefers to bind at CPG islands.[3]

The amino sugar also intercalates into the DNA but the pattern of intercalation is variable between the different drugs. The amino group determines the specific DNA sequence to which the drug will bind.[9] In both daunorubicin and doxorubicin, the amino sugar of both drugs extends into the minor groove of DNA but the conformation of the anthracycline molecule can vary depending on the surrounding DNA sequence. Daunorubicin's amino sugar has direct hydrogen bonding to the DNA while other anthracyclines make van der Waals contact with base pairs. The hydrogen bond acceptor varies for those drugs that have hydrogen bond contacts with the amino sugar and DNA.[8]

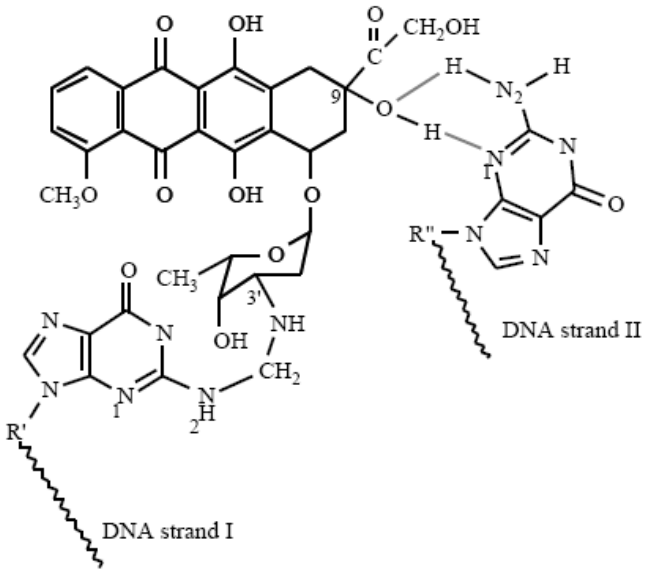
Recent studies have also focused on the role that formaldehyde may play in the formation of DNA-adducts by anthracyclines. Studies have found that the presence of formaldehyde aided the adduct formation when anthracyclines were present. It was found that formaldehyde joins two anthracycline drugs together by a bridge at the 3' amino group of the sugar group (Figure 1.2).[10] The resulting molecule is called doxoform. Doxoform then interacts with the DNA causing DNA-adducts (Figure 1.3).[5]

Figure 1.2: Formaldehyde Conjugated with Doxorubicin



Formaldehyde has been found to interact with anthracycline molecules. Here formaldehyde binds the 3' amino group of the anthracycline sugar joining two anthracycline molecules together. Doxoform(R=OH), Daunoform (R=H). [5]

Figure 1.3: Formaldehyde Aids Doxorubicin Adduct Formation



Structure of the formaldehyde-mediated doxorubicin adduct to DNA molecules. The presence of formaldehyde may increase the ability for doxorubicin to intercalate into DNA and cause cellular damage to cancer cells. [5]

Cancer cells have been found to form formaldehyde. The metabolism of anthracyclines as well as the oxidative stress induced by treatment can lead to formaldehyde formation in the cell as well.[11] The presence of formaldehyde may increase the DNA-adduct formation and lead to increased cancer cell death.

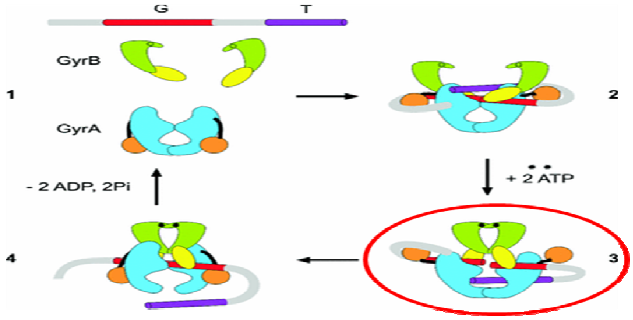
The insertion changes the structure of DNA and inhibits the replication of cancer cells by blocking the synthesis of macromolecules and mRNA transcription. It is believed that intercalation interferes with DNA polymerases, DNA methyltransferases (DNMT1), DNA ligases and helicases. DNA repair enzymes are also inhibited from binding the anthracycline intercalated DNA and inhibits repair to damaged DNA.[12] This interruption of the DNA repair enzymes ability to function is also crucial to the interruption of the topoisomerase II enzyme discussed below. All of these effects can lead to cancer cell death by activating apoptotic response systems.

Interruption of the Topoisomerase II Enzyme

Anthracyclines are well known topoisomerase II poisons and this property is believed to play a significant role in the mechanism of anthracycline cytotoxicity.[1] The role of topoisomerase II enzymes in the cell is to regulate the topology of DNA during DNA replication and transcription activities.[13] Topoisomerase II can catalyze several reactions that include knotting and unknotting DNA, catenation and decatenation of sister chromosomes and relaxation of super helical twists.[14] The role of topoisomerase II is essential for cell viability. Without this enzyme cells face the potential of not being able to separate chromosomes for replication, fail to transcribe genetic information, lack the ability to manage the topology of DNA regions and would fail to replicate.[13]

One role of Topoisomerase II in the cell is to relieve stress that is produced at the replication fork during DNA replication. During the cytotoxic response of anthracyclines this characteristic is exploited. In the normal cell cycle, during DNA replication, DNA helicases unwind segments of DNA for gene transcription and this process introduces topological stress on the DNA strands. Topoisomerase II makes transient double stranded nicks in the DNA strand ahead of the replication fork during DNA synthesis to relieve this stress (Figure 1.4).[15]

Figure 1.4: Regulation of Topoisomerase II by Anthracycline Drugs



Topoisomerase II relieves stress that builds up on a DNA strand during DNA replication. This enzyme regulates the topology of DNA by cutting a double stranded DNA segment and passing another double strand segment through the region. The cut region is re-ligated by topoisomerase II. In this figure the double stranded DNA segment labeled G will undergo cutting. The double stranded segment labeled T will be passed through the G segment.

In stage 1 the two subunits of topoisomerase II come together. In step 2 the double stranded DNA is covalently bound to the enzyme and segment G is cut. In step three the T segment of DNA is passed through the DNA. And in step four the G segment of DNA is re-ligated. This is a reversible reaction that requires the input of ATP.[16]

Anthracyclines are able to prevent the re-ligation of the strands by stabilizing form 3 of topoisomerase II. The topoisomerase enzyme is transformed into a DNA damaging enzyme.[16]

The catalytic reaction of topoisomerase II occurs in discrete steps. To initiate this reaction the enzyme forms a covalent bond between the tyrosine residues and the DNA sugar-phosphate backbone of a double stranded region of DNA – which has been referred to as the gate region. This is followed by topoisomerase II cutting the double stranded DNA at the gate region, and then passing another double stranded DNA region through the cut gate. After the duplex region has been passed through the cut DNA the covalent

bond is reversed leading to the re-ligation of the DNA strands and the release of the bound DNA gate region. The enzyme undergoes a conformational change so that it is ready to start the reaction over again with another region of DNA.[16]

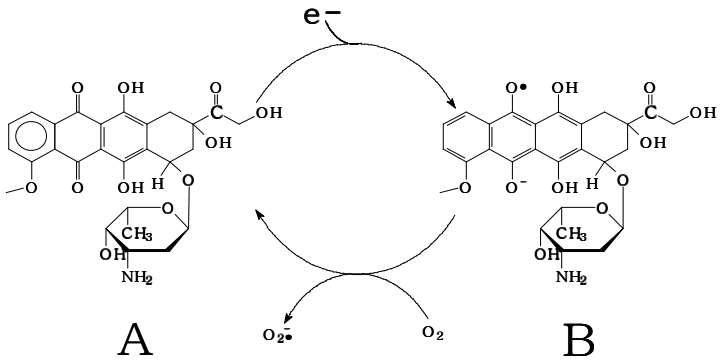
Anthracyclines are able to stabilize the enzyme-DNA covalent adduct, thus preventing the re-ligation of the DNA strands. The anthracyclines bind the enzyme's active site and when the DNA strands are cut, the DNA is irreversibly, covalently bound to the catalytic tyrosine residues in the enzyme (Figure 1.4). In the presence of anthracyclines these topoisomerase II-DNA complexes increase in number. The result is that topoisomerase II is transformed into a DNA damaging agent and the DNA molecules of the cell undergo fragmentation at many different sites.[17] Such DNA damage can overwhelm the cell and will cause growth arrest in the G1 and G2 phase and ultimately the cell will undergo apoptosis.[1]

Topoisomerase II poisons affect the viability of both normal cells and cancer cells; however, the response is more pronounced in the cancer cell. There are several key characteristics that distinguish cancer cell phenotypes from normal cells. Proliferation states between these two types of cells can be substantially different. Cancer cells often proliferate at an increased rate than normal cells leading to an increase in the proteins that are needed for cell replication.[18] Due to the importance of topoisomerase II in the cell replication cycle[13] this enzyme can be up-regulated in the cancer cell. Anthracycline cytotoxicity is hypothesized to rely on the increased topoisomerase II targets to induce the cancer cell deaths.

Oxidative Damage and Free Electron Radical Formation

Reactive oxygen species generated by anthracycline re-dox cycling have also been implicated in the cytotoxic mechanism of anthracyclines (Figure 1.5).[1,6,7,19,20]

Figure 1.5: Reactive Oxygen Generation by Doxorubicin.



Single electron reduction/oxidation cycling between the doxorubicin quinone (**A**) and the semiquinone (**B**) radical. This is a cyclic reaction that regenerates the parent compound and generates oxygen free radicals. The reaction can be catalyzed by many enzymes in the body.

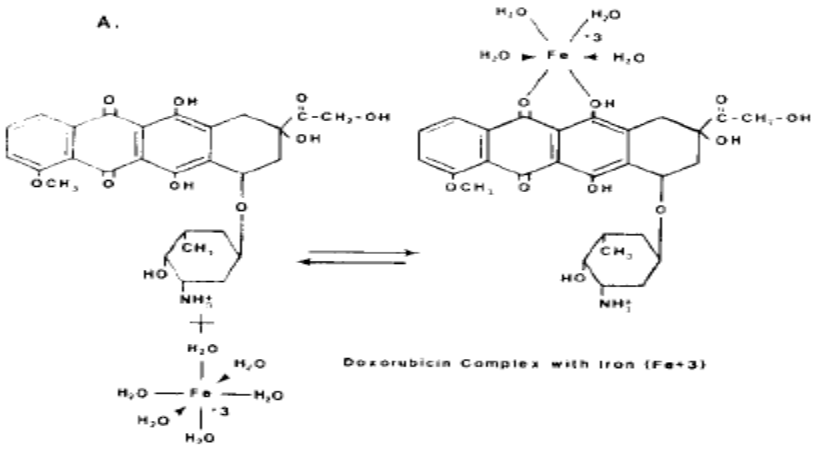
There is evidence that as doxorubicin is added to heart tissue *in vitro* superoxide formation is increased.[6] Other drugs that contained a quinone group, like daunorubicin, were also tested and found that they significantly increased oxygen free radicals. In addition, drugs that lack a quinone group, such as 5-iminodaunorubicin, did not significantly increase oxygen free radicals. This data implies that in the body the quinone group is undergoing a re-dox reaction mediated by the enzyme NADH dehydrogenase.[6]

The role of reactive oxygen species in anthracycline cytotoxic mechanisms is still a matter of debate.[1] Single electron re-dox cycling in anthracyclines has been demonstrated using several different NADPH-dependent oxido-reductase enzymes found in the body.[6,7,19,20] The quinone structure acts as an electron acceptor. This forms a semiquinone species that is highly reactive. In the presence of molecular oxygen the semiquinone reacts to generate radical oxygen species and the parent quinone structure is

regenerated. The parent quinone is now available to undergo another reaction in a cyclic manner. The radical oxygen species that are generated can be superoxide anions or hydrogen peroxide. Both of these species can be damaging to cells and are potentially harmful to tissues.[1]

This re-dox cycling that occurs with the quinone has been associated with the release of intracellular stores of iron. Anthracycline drugs bind to the free iron to form an iron/drug complex (Figure 1.6). This complex can be very damaging to cells by converting the superoxide anions and hydrogen peroxide species generated by the one electron cycling events into a hydroxyl radical. A hydroxyl radical is a highly reactive species that is more damaging than reactive oxygen species.[1]

Figure 1.6: Anthracyclines/Iron Complex



Doxorubicin can form complexes with iron in cells. The interaction can lead to the formation of hydroxyl radicals and may be a potential cytotoxic mechanism. [21]

Damage to lipid membranes may also occur due to these oxidative reactions. The aglycone portion of the anthracyclines has increased lipid solubility and tends to intercalate into the lipid membrane of the cell. The quinone portions of the drug can still

undergo oxidation reactions creating reactive oxygen species near the membrane of the cells. Lipid peroxidation can result and damage cancer cells.[1]

It is still being debated whether these free radical and oxidative reactions occur appreciably at clinically relevant concentrations of anthracyclines. Several investigators have made the point that the effects seen as a result of generating reactive oxygen species are observed at anthracycline concentrations that are higher than would be achieved during treatment in a clinical setting. Further, these free radical species have been implicated in the mechanism of cardiotoxicity often associated with anthracycline treatments and any cytotoxic benefit may be outweighed by the cardiotoxic effects.

Potential Side Effects of Anthracycline Therapies

Acute Side Effects of Anthracycline Treatment

Patients undergoing an anthracycline treatment regime may or may not suffer side effects from their treatment. Some side effects are an acute response that manifest within hours or days of beginning treatment while other side effects may occur years after treatment.[22] The most severe side effect is cardiotoxicity that can manifest itself in an acute or chronic manner and has the potential to manifest twenty years after treatment.[22]

A study conducted by Griffin et.al. asked cancer patients to identify and rank the severity of their chemotherapy side effects.[23] They found that patients experienced twenty different symptoms, thirteen were physical symptoms and seven were psychosocial symptoms. The top five symptoms that were experienced by patients were loss of hair, nausea, fatigue, vomiting and increased urination. The symptoms that patients ranked as the most severe were: nausea, tiredness and loss of hair. These researchers also found that the types of symptoms suffered or the severity of the

symptoms did vary depending on patient factors such as age, gender, and extent of disease.[23]

Nausea, Vomiting and Fatigue Symptoms Impact Patient Health

Mild or moderate nausea and vomiting are very common with anthracycline based treatments; however, some patients suffer severe symptoms.[24] These symptoms can be controlled clinically with the prescription of anti-nausea drugs.[25] Fatigue associated with doxorubicin can affect a significant portion of patients. Studies have found that 99% of chemotherapy patients suffer some degree of fatigue during treatment.[26] Another study reported that two thirds of breast cancer patients reported moderate or severe fatigue during treatment.[25] This symptom seems to be limited to treatment and tends to disappear after treatment is completed;[25] however, these symptoms can persist months up to years after treatment.[26] Fatigue may be due to anemia, treatment induced sleep disturbance and depression symptoms.[25]

Decrease in White Blood Count and Risk of Infection

Within fourteen days patients can suffer from myelosuppression which is a drop in white blood cell count. Most female patients undergoing treatment for breast cancer will see their white cell count return to normal by their next anthracycline treatment. Life threatening infections that result from the suppressed white blood cell count occurs in less than two percent of patients.[25]

Ovarian Failure and Menopausal Symptoms

Doxorubicin treatment may induce ovarian failure which can mimic menopausal symptoms and is a common side effect in women. Patients can be at an increased risk of osteoporosis and cardiovascular disease due to this ovarian failure during treatment. Risk

factors include patient age and total dose administered.[24] Clinicians can offset menopausal symptoms by prescribing intake of calcium and vitamin D, weight bearing exercise and monitoring bone density.[25] Premature menopausal symptoms can include amenorrhea which can be reversible or permanent. If a patient is still amenorrheic one year after treatment they are expected to not regain ovarian function.[24]

Weight Gain and Risk for Secondary Disease States

Several different chemotherapy regimens can cause weight gain. Doxorubicin can cause weight gain in breast cancer patients; however, this symptom is less pronounced in doxorubicin treatment than with other chemotherapy treatments. Weight gain is hypothesized to occur due to decreased physical activity, ovarian failure, increased food consumption, reduced basal metabolic rate and a decrease in lean body mass. Weight gain may be associated with reoccurring cancer in some patients.[25] Women with breast cancer who gained greater than 5.9 kg of weight during treatment were 1.5 times more likely to have a reoccurrence of the cancer and 1.6 times more likely to die of breast cancer compared to woman who gained less.[27] Premenopausal women are at a higher risk for weight gain versus postmenopausal patients. Patients who experience chemotherapy induced menopause are at the highest risk for weight gain.[24] The longer drug administration times may also increase weight gain symptoms. The increase in weight in some patients may further impact their physical health[24] possibly contributing to diabetes, cardiovascular disease and changes in orthopedics.[27]

Chemotherapy-Induced Leukemia

Some researchers have suggested that chemotherapy regimens such as doxorubicin can cause chemotherapy induced acute myeloid leukemia in some patients.[25]

Anthracycline therapies are believed to have a higher incidence of leukemia development following treatment as compared to other chemotherapy treatments. 0.1% to 1.5% of patients that have undergone standard dose anthracycline therapy are expected to develop chemotherapy-associated leukemia;[24] however, other researchers suggest that the risk is no greater than that for the general population.[25] Patients undergoing chemotherapy and radiation; however, may be at increased risk for the development of leukemia after treatment.[25] Doxorubicin-induced leukemia is associated with changes in monocytes and a specific cytogenic abnormality that can develop a few years after treatment. Doxorubicin induced leukemia does not have to involve myelodysplasia which is associated with being a precursor to the development of acute myeloid leukemia.[25]

Cardiotoxic Side Effects of Treatment

Anthracyclines are effective in treating a wide range of cancers; however, clinical use is limited by a dose dependent chronic cardiotoxicity. Some groups of people have a higher incidence of complications including younger children (less than four years), older patients, females, and patients with a history of hypertension or cardiovascular diseases. Cumulative dose and combination therapy have also been implicated in raising the risk of cardiotoxicity.[28] Analogs have been developed in attempts to reduce the incidence of cardiotoxicity and include epirubicin and idarubicin; however, they have not overcome the cardiotoxic side effect. Patients are limited to a lifetime cumulative dose that aims to reduce their risk of developing chronic cardiotoxicity. Patients can suffer acute heart problems or chronic heart failure that may manifest several years after completing therapy.[28]

It has been proposed that doxorubicin accumulates in the heart tissue followed by chronic cardiomyopathy. There is evidence that this problem is correlated to dose, dose schedule and patient age.[1] Also, different patients respond differently to the anthracycline regimens. Older people suffer different cardiomyopathies than children, females respond differently than males, ethnicity puts some races at more risk and Down Syndrome patients also face increased cardiomyopathies after treatment.[28]

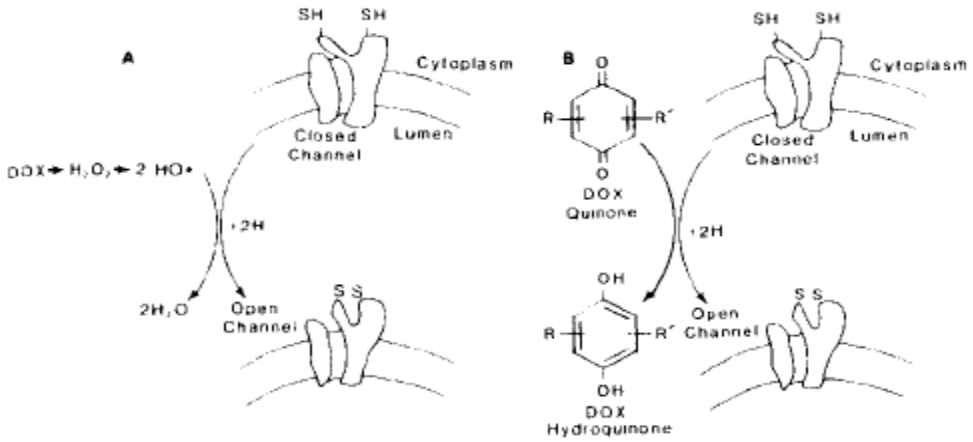
Physical Manifestations of Cardiotoxicity

Hallmarks of the cardiotoxic side effects include both acute and chronic heart failure and lead to systolic and diastolic dysfunction of the heart tissue. Cardiotoxic damage during anthracycline treatment is believed to involve damage to the myofibrils, sarcoplasmic reticulum and mitochondrial damage.[29-33]

Early cardiac dysfunction includes changes in the sarcoplasmic reticulae caused by vacuole formation. Sarcoplasmic reticulae are part of the smooth endoplasmic reticulum organelles found in eukaryotic cells and they are made up of tubules and vesicles. The job of the sarcoplasmic reticulae is to store calcium ions until heart muscle cells are stimulated when they release calcium stores driving the excitation and contraction of heart muscle cells (Figure 1.7).[34]

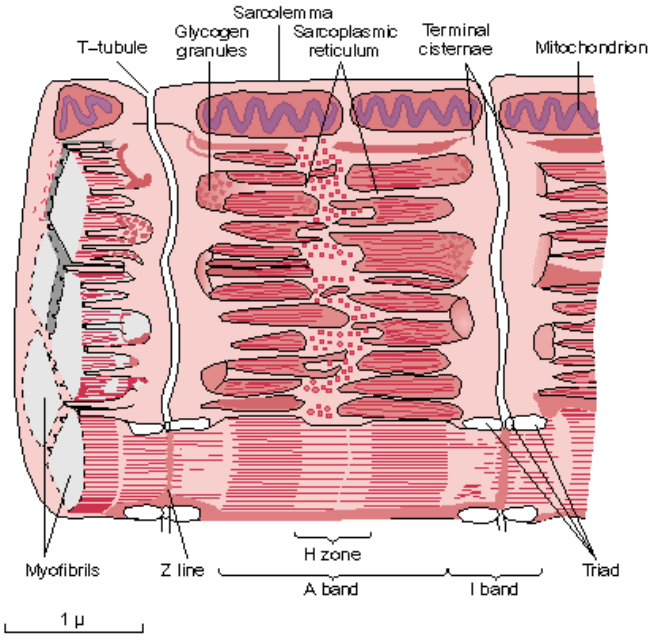
Sarcoplasmic reticulum is composed of two parts: longitudinal tubules and terminal cisterna (Figure 1.8). The terminal cisterna is responsible for the storage of calcium needed to control relaxation and contraction of muscle fibers. Doxorubicin has been found to inhibit calcium uptake and stimulate calcium release by the terminal cisterna.

Figure 1.7: Doxorubicin's Interaction with the Ca^{++} Channels of Sarcoplasmic Reticulum



In the cardiotoxic response of anthracyclines, doxorubicin is believed to prevent the uptake of Ca^{++} into the sarcoplasmic reticulum's terminal cisternia. Doxorubicin is also believed to promote the release of Ca^{++} previously stored. This leads to an imbalance in Ca^{++} in the cell. [21]

Figure 1.8: Structure of the Sarcoplasmic Reticulum

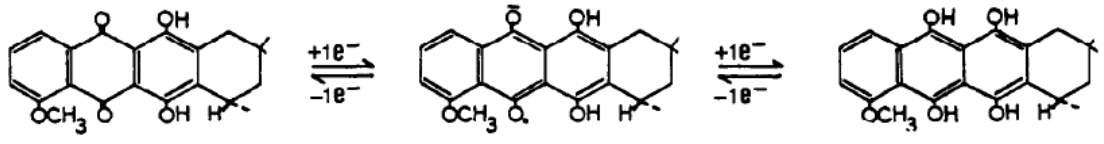


Muscle fibers have a structure called the sarcoplasmic reticulum that helps regulate Ca^{++} stores in the muscle fibers, myofibrils. They contain two important parts: the longitudinal tubules and terminal cisternae. Doxorubicin is hypothesized to interrupt the Ca^{++} channels of the terminal cisternae. Damage from treatment can cause the sarcoplasmic reticulae to expand and displace mitochondria and other elements from the cell. Image from: (http://www.nvo.com/jin/nss-folder/scrapbook9/m3.gif).

The drug was not found to disrupt the function of the longitudinal tubules.[35] It is believed that hydrogen peroxides, oxidizing agents and free radicals can promote the release of calcium in cells. All of these affects can impair contractility and cardiac relaxation. In later stages of cardiac dysfunction, damaged sarcoplasmic reticulae can expand in size and displace contractile elements and mitochondria in the cell.[36]

Mitochondria are membrane bound organelles that are considered the power houses of cells. They are responsible for the ATP generation in cells and in very active tissues such as heart tissues there can be several hundred mitochondria in one cell. Mitochondria also take in calcium ions from the cytosol. During anthracycline treatment, disruption of the mitochondria is observed where the mitochondria swell, are inhibited from calcium uptake and formation of calcium inclusions and electron dense bodies are observed.[37] It has been found that doxorubicin interacts with the electron transport chain found in the mitochondria (Figure 1.9). Doxorubicin can act as a final electron acceptor in the reaction mediated by NADH dehydrogenase found at the complex I. This leads to an oxidation reaction involving the quinone group of the anthracycline resulting in generation of oxygen free radicals.[38]

Figure 1.9: Doxorubicin Interaction with Electron Transport Chain in Mitochondria



Doxorubicin can act as a final electron acceptor in the electron transport chain at complex 1 in the mitochondria. This reaction can generate free electron radicals that are damaging to the mitochondria and to the cell. [39]

Mitochondria have been well established in the apoptosis response of cells. One mechanism that these organelles use to induce apoptosis is to release cytochrome *c* molecules. Cytochrome *c* has been well established to induce a cellular cascade that ends with the activation of caspase-3 which induces apoptosis in cells. It has been found that doxorubicin administration can cause the release of cytochrome *c* from mitochondria, leading to activation of caspase-3, followed by cardiomyocyte apoptosis.[38] This damage to the mitochondria disrupts calcium balance in the cell, disrupts ATP production and is believed to induce apoptosis in cardiac cells.

These early dysfunctions are followed by disorganization and degeneration of myofibrils found in the heart muscle tissue. This damage includes less dense myofibrillar bundles, disruption in Z-disc structures and damage to actin filaments.[40] Formation of vacuoles in the cytoplasm and an increase in the number of lysosomes are also observed.[1]

Combination Therapies Lead to Synergized Cardiotoxic Risk

Another promising treatment for cancer therapy involves the use of Trastuzumab (Herceptin). This drug has been shown to be an especially promising treatment for aggressive breast cancers; which is the second leading cause of cancer in women.[41] Clinical trials have shown that this treatment has decreased the recurrence of cancer by one half and has decreased mortality by one third in patients. Trastuzumab treatment faces the same failings that anthracycline treatment does: cardiotoxicity.[42]

Trastuzumab is a monoclonal antibody that has cytotoxic effects on cells that over express the human epidermal growth factor receptor (Her2/Neu) on cell surfaces. While these receptors are over expressed in certain breast cancers they are also expressed in the heart. It is this expression in the heart cell membranes which is the likely cause of the

cardiotoxic effects seen in Trastuzumab therapy. The Trastuzumab cardiotoxicity is interesting because the toxicity that is observed is reversible in contrast to anthracycline toxicity which is a nonreversible event.[41]

One recent advance in chemotherapy treatment has been to combine Trastuzumab treatment with anthracycline treatment; however, when these two drugs are used together they greatly increase the risk of cardiac dysfunction in patients.[41] When Trastuzumab is used alone 7% of the patients developed heart failure, when anthracyclines are used alone a small percentage of patients developed heart failures; however, when these two drugs are used together 28% of the patients developed heart failure. It is hypothesized that when these heart cells undergo another assault, such as one by anthracyclines, the effect is synergized.[43] This synergy is reflected in the dramatic increase in the percent of patients who develop heart failures. After a combination therapy the cardiotoxicity is also nonreversible favoring the toxicity model of anthracyclines.[42] Understanding the mechanism of cardiotoxicity in anthracycline therapies and being able to address this side effect may considerably improve combination therapies, such as this one, leading to increased ability to treat aggressive cancers.

Drug Resistance Side Effects Limit Effectiveness of Anthracycline Treatment

P-glycoproteins Transport Anthracyclines Out of the Cell

Patients undergoing anthracycline therapy may also face drug resistance problems. One possible mechanism includes active drug transport of the anthracyclines out of the cell. One such mechanism includes the multi-drug resistant (MDR) proteins found in the membranes of cells. MDR proteins are responsible for the excretion of a large range of drug substrates. One such MDR protein is p-glycoprotein which transports

a wide range of substrates. Membrane transport proteins like p-glycoproteins contribute to drug resistance by decreasing intracellular drug concentrations by an active, ATP-dependent transport system.[44] Several anticancer drugs have been found to be substrates of p-glycoprotein, including doxorubicin.[45] In mice undergoing treatment with anthracyclines, it was found that doxorubicin also up-regulated p-glycoprotein expression.[46] P-glycoproteins have been shown to be expressed in the heart tissue and the expression of these transport proteins varies between individuals. Multi-drug resistant knockout mice, which lack p-glycoproteins, show an accumulation of anthracyclines in the heart tissue while mice with a normal complement of p-glycoprotein showed a reduction of intracellular levels of anthracyclines.[47]

The treatment of cells by an acute dose of doxorubicin has shown a sharp increase in the expression of p-glycoproteins located in liver cells. The doxorubicin treatment altered RNA synthesis of the p-glycoproteins as well. The results of this study indicate that exposure to anthracyclines up-regulate the p-glycoproteins and may contribute to drug resistance seen in patients.[45]

Non-MDR Protein Pumps Aid Anthracycline Resistance

There is significant evidence supporting the active transport of anthracyclines by p-glycoproteins; however, there is also evidence that non-MDR proteins also act on the removal of anthracyclines from the cell. One proposed protein is multidrug resistance-associated protein which is a 190 kD membrane bound glycoprotein. It also belongs to the ABC superfamily of proteins[48] although the non-p-glycoprotein transport could involve several different protein candidates.

A study completed by Versantvoort investigated the drug resistance of two multidrug resistant cell lines that did not express the p-glycoprotein.[4] The investigators made the cell membranes permeable, administered daunorubicin and then monitored the amount of daunorubicin accumulation in the cell to indicate the efflux of the drug. Cells that lacked the p-glycoproteins had a five fold efflux of drug compared to the controls that express p-glycoproteins suggesting that other mechanisms of active transport are important to daunorubicin efflux.[4]

Multi-drug resistant phenotypes not only efflux anthracycline molecules but also may inhibit the cytotoxic response of anthracyclines in cancer cells. Multi-drug resistance phenotypes have protein pumps that are located on the cell membrane. These protein pumps respond to a broad spectrum of drugs that initiate a drug efflux response.[18] Prior to anthracycline treatment, exposure to structurally and functionally unrelated drugs can initiate the multi-drug resistance response. This can result in a faster efflux response when the anthracycline treatment is administrated. The active transport of the anthracyclines prevents the accumulation of the drug in the cell.[4] The result is that the number of anthracyclines molecules that make it to the nucleus are significantly reduced preventing the cytotoxic effects on molecular targets. This decrease of anthracyclines in the nucleus may contribute to drug resistance by demanding an increased dosage of drug to see the same effects in patients.[4]

Disruption in Intracellular Distribution of Anthracyclines May Lead to Drug Resistance

Anthracyclines are targeted to the nucleus where there are several cytotoxic targets previously discussed. One mechanism of resistance includes the shifting of

anthracycline location in the cell. When free drug is present in the cell anthracyclines are found in the cytoplasm and can be sequestered in acidic vesicles or bound to membranes of vesicles. The cells may be able to compartmentalize the drug in the cell limiting its ability to kill cells.[48]

Alterations in Gene Expression of Anthracycline Targets Aid Drug Resistance

Drug resistance can also occur due to alterations in the gene expression of anthracycline specific molecular targets. One such target is the topoisomerase II enzyme. To decrease the cytotoxic effect of anthracyclines cancer cells can down regulate the amount of topoisomerase expression in cells, increase the degradation of this enzyme, or introduce mutations in the enzyme.[48]

Drug Metabolism May Affect Anthracycline Drug Resistance

Another mechanism of drug resistance is due to drug metabolism. Daunorubicin treated tumor cells have an 8-fold increase in the IC_{50} value for the drug compared to cells that were not pretreated with the drug. This indicated that the treated cells would need a larger concentration of drug to achieve the same tumor cell killing results. These treated tumor cells also showed a 6-fold increase in carbonyl reductase activity as compared to the non-treated cells. The carbonyl reductase converts the parent drug daunorubicin to daunorubicinol.[44] This metabolite is less efficacious in cell killing compared to the parent anthracycline. Therefore, the conversion of daunorubicin to daunorubicinol reduces the amount of parent drug in the cell and decreases the overall cytotoxicity.

Proposed Mechanisms of Cardiotoxicity

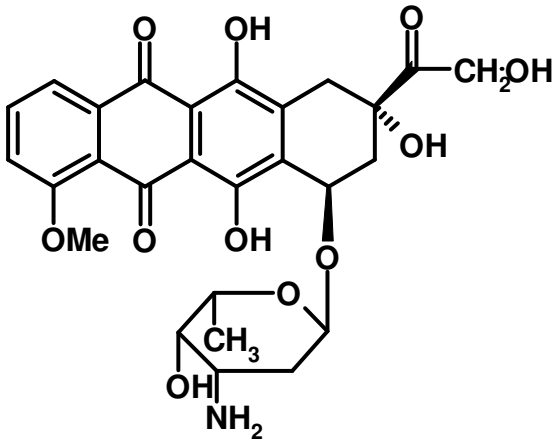
The mechanism of anthracycline cardiotoxicity is not well understood. Two prevailing hypotheses involve metabolite formation and single electron re-dox cycling leading to the generation of reactive oxygen species.[1] Several mechanisms have been proposed that may contribute to the cardiotoxicity and a few of these mechanisms are discussed below.

Reactive Carbonyls

Current research has focused on the metabolism of the carbonyls found in anthracyclines because they have been linked to cardiotoxicity (Figure 1.10). Doxorubicin and daunorubicin have two carbonyls that can be reduced by reductases in the body: the quinone carbonyls and the C-13 carbonyl. HCBR 1 is believed to prefer the C-13 carbonyl as a substrate compared to the quinone carbonyls.[49]

Figure 1.10: Reactive Carbonyls Found in the Anthracyclines

A. Quinone **B. Carbon 13**



It has been established that there are two reactive carbonyls in the anthracycline molecule. A) The quinone structure is a carbonyl that can be reduced by different enzymes in the body. B) Carbon 13 is also a carbonyl that can be reduced. This is an important carbonyl to this research because the reduction of this carbonyl by HCBR 1 is linked to the cardiotoxicity observed in patients undergoing anthracycline therapy.

Quinone Structure and the Formation of Reactive Oxygen Species Impact Cardiotoxicity

It is also hypothesized that the quinone structure of anthracyclines undergoes metabolism by several different enzymes in the body both cytosolic and in the mitochondria. When the quinone undergoes a single electron reduction, reactive oxygen species can be formed that are damaging to the tissue (Figure 1.5). Metabolism of anthracyclines reduces the drug to a semiquinone which can react with molecular oxygen creating superoxide species. This quinone group can undergo many re-dox cycles.[50] These free radical species have been implicated in the development of cardiotoxicity during anthracycline treatments. Plausible mechanisms include free radical peroxidation of membrane lipids leading to the damage seen in sarcoplasmic reticulum observed during anthracycline therapy.[51]

The quinone structure is believed to cause cardiotoxicity by disrupting the calcium balance in the heart tissue. There are two prevailing ideas about the mechanism that causes this: free radical formation or a mechanism independent of quinone derived free radicals. It has been demonstrated that the quinone structure is necessary for daunorubicin induced cardiotoxicity. This cardiotoxicity causes impairment of calcium release in the sarcoplasmic reticulum but does not involve free radical damage. In studies done by Shadle the effects of daunorubicin, a quinone containing species, and 5-iminodaunorubicin, a non-quinone containing analog, were compared.[52] They found that the 5-iminoduanorubicin was less effective at causing cardiac damage than the daunorubicin drug suggesting that the quinone structure is necessary for cardiotoxicity. This study implies that this mechanism of cardiotoxicity involves the quinone moiety but

is independent of free radical formation and that this mechanism disrupted the calcium balance in the heart tissue.[52]

Others have shown that the single electron re-dox cycling known to occur with the quinone in anthracyclines may contribute to anthracycline cardiotoxicity.[53] In a later study, it was established that as doxorubicin exposure in cells increased the superoxide anion formation also increased. This study provides a link for drug exposure to the formation of free radicals that may be toxic to cells.[20]

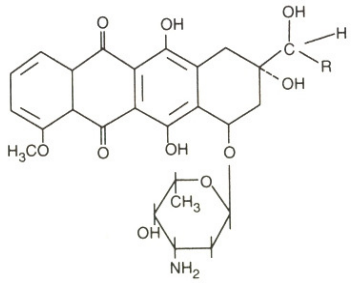
Oxygen free radicals are not the only free radicals that are formed during anthracycline treatments. Tissues are also at risk of the formation of hydroxyl radicals when in the presence of hydrogen peroxide and iron. It is believed that in the mitochondria, at Complex I, the anthracycline initially undergoes reduction to form a semiquinone species. In the mitochondria the anthracycline is an electron acceptor for the electron transport chain (Figure 1.9).[50] This semiquinone goes on to react with molecular oxygen to form a superoxide radical as well as hydrogen peroxide.[53] The hydrogen peroxide will go on to react with iron in the cell to create hydroxyl radicals that are damaging to tissues.[20] This cascade of events may be especially damaging to heart tissue versus other organs in the body because the heart is poorly defended against hydroxyl radicals. The heart uses two main enzymes to detoxify oxygen free radicals: superoxide dismutase and selenium-dependent glutathione peroxidase. Doxorubicin treatment can decrease the concentration of the selenium dependent glutathione peroxidase leaving the heart tissue vulnerable to the buildup of hydrogen peroxide and lipid peroxides.[54] The hydrogen peroxide can further react with iron in the cell creating

hydroxyl radicals that lead to alterations in calcium homeostasis in mitochondria or sarcoplasmic reticulum.[20]

Carbon-13 Metabolism is Linked to Cardiotoxicity

Recent research has focused on the metabolism of the C-13 carbonyl of the anthracycline parent drugs. Anthracyclines are believed to be metabolized by several different enzymes in the body. One important enzyme is human carbonyl reductase 1 (HCBR 1) which is believed to have activity on the quinone and C-13 carbonyls in the drug molecule. Cells that are exposed to anthracyclines show an up-regulation of the enzyme HCBR 1 which has been shown to reduce the parent compound to the metabolite. The metabolite is much less efficient at killing tumor cells and has been shown to be much more cardiotoxic than the parent anthracycline (Figure 1.11).[44] A detailed discussion of the anthracycline metabolism by HCBR 1 is provided later.

Figure 1.11: Structure of the Anthracycline Metabolite



Anthracyclines are believed to be metabolized by the enzyme HCBR 1. The result is a cardiotoxic metabolite that is damaging to tissues and lacks cytotoxic properties. Daunorubicinol: R = CH_3, Doxorubicinol: R= CH_2OH.[3]

Anthracycline Alcohol Metabolites are Linked to Cardiotoxic Side Effects

Other theories about cardiotoxicity rely on the formation of oxygen free radicals as the underlying cause to heart tissue damage. However, studies that have employed free radical scavengers have failed to prevent cardiotoxic damage at the time of

anthracycline administration. This finding inspired a study done by Olson and coworkers to see if another mechanism played a role in cardiotoxicity.[55]

This study found that doxorubicinol is more cardiotoxic to the heart tissue than the parent drug doxorubicin. Doxorubicinol depressed systolic myocardial function in heart muscle cells, inhibited calcium pump activity and sarcoplasmic reticulum lost the ability to store calcium in the heart. Administration of the parent drug doxorubicin did not show any of these acute effects.[55]

They also measured the ability of the heart to convert doxorubicin to doxorubicinol and found that within 45 minutes a substantial amount of doxorubicinol was formed. They believed that this metabolism was important to cardiac accumulation of doxorubicinol. They also measured the ability of the metabolite to kill cancer cells and found that doxorubicin was 5 to 28 times more cytotoxic than the metabolite.[55]

This study lends support to the idea that a mechanism other than free radical formation and damage leads to cardiotoxic side effects. This study provides evidence that the parent drug doxorubicin accumulates in the heart and that the heart has the ability to convert the drug to doxorubicinol. Furthermore, Olson provides evidence that as the metabolite builds in the heart a toxic threshold is meet and damage is done to ion pumps, sarcoplasmic reticulum organelles and systolic action of the heart is inhibited.[55]

Human Carbonyl Reductase 1: A Carbonyl Reducing Enzyme Found in the Body

History of Human Carbonyl Reductase 1

Human carbonyl reductase 1 (HCBR; EC 1.1.1.184) is an enzyme that is a member of the short chain dehydrogenase family (SDR) which contains a conserved amino acid region shared by members of this family.[56] The NADPH binding domain

contains a Rossman fold with a conserved region GlyXXXGlyXGly (where X is any amino acid) in residues at positions 12-18. Another conserved region of SDR families is a TyrXXXLys sequence. The C-terminal portion of the enzyme has the lowest amount of homology and may play a role in determining substrate specificity for the carbonyl reductase enzymes.[56] In the cell there are other carbonyl reducing enzymes that belong to a separate family called the aldo-keto reductase superfamily. At one time HCBR 1 was included in this family but was moved to the SDR family when it was found to have more homology with members of this family.

HCBR 1 catalyzes the NADPH dependent reduction of a wide variety of carbonyl groups. Known substrates include aromatic hydrocarbons, aldehydes, ketones, quinones and pharmaceuticals such as anthracyclines. These substrates range from those endogenous to the body to several xenobiotic molecules that contain carbonyl groups. Menadione is considered an excellent substrate and is often used as a model substrate in studies of carbonyl reductase. Catalytic amino acids include Ser 139, Tyr 193, and Lys 197. These amino acids help to transfer a hydride from the NADPH molecule to the carbonyl carbon of the substrate and a proton to the developing alcohol metabolite.[56]

HCBR 1 is monomeric with a molecular weight of 30.3 kD and is found in the cytosol of the cell. The gene encoding for HCBR 1 is located on chromosome 21 (21q22.12) and codes for a 277 amino acid protein. The enzyme is expressed in a wide range of tissues, including brain, liver, heart, skin, and placenta. Variants of carbonyl reductase are found throughout nature in many different taxa.[57]

Humans have two CBR genes, one that codes for HCBR 1 and one that codes for HCBR 3. HCBR 3 is 62 kb downstream from HCBR 1 on chromosome 21. HCBR 3

function has not been clearly identified as of now but is currently being investigated.[56] Other organisms have a gene encoding for carbonyl reductase 2; however, this gene is lacking in humans.

Possible Functions of HCBR 1

Carbonyl reductase is believed to be involved in a variety of cellular processes such as the metabolism of several endogenous compounds, detoxification of exogenous carbonyl compounds, drug resistance, and signal transduction. However, a definitive role for carbonyl reductase in humans is not well defined.[56]

HCBR Detoxifies Quinone Containing Compounds in the Body

It has been found that *in vitro,* HCBR shows activity on several quinone containing compounds which are known to be a source of oxidative stress and therefore are considered toxic in the body. It is hypothesized that HCBR 1 metabolism protects the body from oxidative stress. Quinone containing compounds that are not reduced can undergo enzyme-catalyzed single electron re-dox cycling leading to the generation of superoxide anion and other free radicals that are damaging to tissues. The role of detoxifying quinones by HCBR 1 has not been directly observed in human tissues; however, HCBR 1 shows a high specificity for quinone containing compounds in *in vitro* studies.[58]

HCBR 1 May Reduce Prostaglandins and Steroids in the Human

Carbonyl reductase has also been shown to reduce prostaglandins and steroids *in vitro* as well.[59] However, it is unclear if this observed activity has physiological relevance because carbonyl reductase has low catalytic efficiencies for these substrates.[60]

A Possible Neuroprotective Role for HCBR 1 – The Reduction of 4-Oxononenal in the Brain

HCBR 1 may have different physiological roles depending in which tissue it is located. In the brain tissue HCBR 1 is hypothesized to play a neuroprotective role. 4-Oxonon-2-enal is a product of lipid peroxidation and is a brain neurotoxin. Carbonyl reductase has been shown to reduce this compound[60] to the less reactive 4-hydroxy-2-nonenal and this metabolism may be protective for the brain tissue.[60]

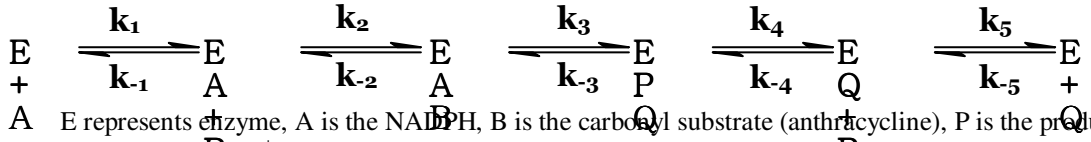
The central nervous system can be vulnerable to oxidative stress because of its high concentration of oxygen and lipids and low antioxidant defenses. A recent study with *Drosophila melanogaster* has found that a CBR gene, called sniffer, may play a role in detoxifying 4-oxonon-2-enal, a product of lipid peroxidation, which reduces neuronal damage in normal brains. This sniffer gene has a large homology with human carbonyl reductase and is only expressed in the brain, specifically in the entire cortex, where all neuronal cell bodies are localized. Flies with mutations in the CBR gene had a shorter life span and showed neurodegeneration while flies that over expressed CBR were protected from neuronal damage caused by oxidative stress. By reducing 4-oxonon-2-enal in the brain tissue, HCBR 1 may contribute to the protection of the brain from diseases that result from oxidative stress such as Alzheimer's, Parkinson's or Huntington's disease.[61]

HCBR 1 has a wide variety of potential physiological substrates indicating that the function of CBR in the body may be complicated. Further study needs to be done to better define its physiological role.

Kinetic Mechanism of HCBR 1

Kinetic analysis done by Bohren et al. establishes that HCBR has a sequential ordered Bi Bi kinetic mechanism which is a common mechanism found in NADPH dependent dehydrogenase families (Figure 1.12).[57] The coenzyme NADPH, binds to the enzyme first and the substrate carbonyl binds to the enzyme second. The resulting product alcohol is released before $NADP^+$. There are three types of HCBR and this study also found that all three forms of enzymes have the same kinetic mechanisms.[57]

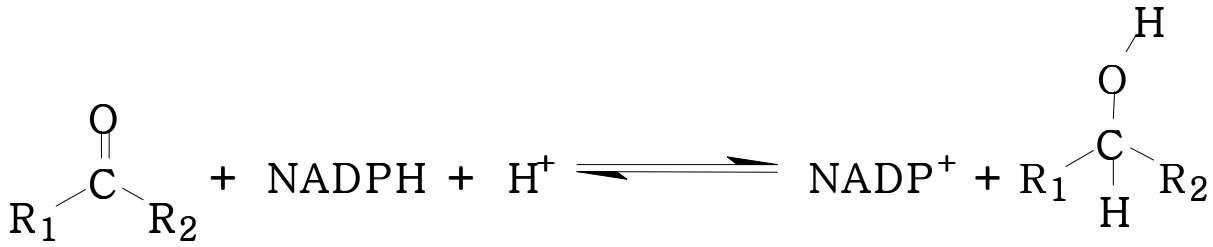
Figure 1.12 Ordered Bi Bi Kinetic Mechanism Proposed for Human Carbonyl Reductase.



E represents enzyme, A is the NADPH, B is the carbonyl substrate (anthracycline), P is the product alcohol, and Q is $NADP^+$. Lower case "k" is the rate constant for the step. In this mechanism, NADPH binds before the carbonyl substrate and $NADP^+$ is release after the product alcohol.

The reaction catalyzed by HCBR 1 is a 2 electron reduction reaction, whereby the carbonyl is converted to an alcohol (Figure 1.13). The hydride on the C4 position of the nicotinamide ring of NADPH is transferred to the carbonyl carbon of the substrate followed by the transfer of a proton via a proton relay system on the enzyme to the developing alkoxide, thus yielding an alcohol metabolite.

Figure 1.13 Re-dox Reaction of HCBR 1, NADPH and Carbonyl Substrate



HCBR 1 mediates the re-dox reaction of carbonyl compounds. A hydrogen is transferred from the NADPH molecule to the carbonyl group. The result is an alcohol metabolite.

Human Carbonyl Reductase 1: Metabolism of Anthracyclines is Linked to Cardiotoxicity

HCBR 1 Metabolizes Anthracyclines

Several studies have implicated HCBR 1 in the metabolism of anthracyclines.[1,49,62] This metabolism of carbon 13 leads to an alcohol product that is damaging to heart tissue.[56,63,64] Single electron re-dox cycling of the quinone carbonyls can lead to the production of free radicals that are hypothesized to be damaging to tissues. Recent studies have found that HCBR 1 can reduce these carbonyls as well and may affect the generation of quinone derived reactive oxygen species.[49] Given that HCBR 1 reduces both the carbonyl at C13 and the quinone carbonyls, the extent to which HCBR 1 participates in the complete metabolism of anthracyclines needs to be better understood.

In a study done by Loveless it was found that all mammalian tissues catalyzed the reduction of daunorubicin.[62] Daunorubicin is a preferred substrate than doxorubicin for the HCBR 1 enzyme reflected by the increased k_{cat}/K_m values for daunorubicin (Table 1.1).[49] All tissues showed activity on the anthracycline drugs; however, their efficiencies did differ depending in which tissue it was found.[62]

Table 1.1: HCBR 1 Kinetic Properties of Two Anthracycline Substrates

	Daunorubicin	Doxorubicin
k_{cat} (s^{-1})	1.27(0.03)	0.27(0.02)
K_m(μM)	108(7)	90(16)
k_{cat}/K_m($\mu M^{-1}s^{-1}$)	$1.18(0.05)x10^{-2}$	$2.90(0.3)x10^{-3}$

Two anthracycline drugs show very different catalytic properties when metabolized by HCBR 1. These differences are apparent even though there is a small difference in structure for these two drugs: they only differ by one hydroxyl group on the Carbon 14 (Figure 1.1).[49]

Carbon 13 Metabolism of Anthracyclines by HCBR 1

Two studies have linked human carbonyl reductase levels to the development of cardiomyopathies in mice models.[64,65] In one study transgenic mice over expressed human carbonyl reductase in the mouse heart.[65] Expressing mice developed severe cardiomyopathies and had enlarged hearts compared to normal non-expressing mice. Immunohistochemical staining of the heart tissue of expressers showed that HCBR 1 expression was uniform, abundant and occurred in myocytes of the heart. More importantly after a single injection of doxorubicin expressers converted 82% of doxorubicin to doxorubicinol compared to only 5% of non-expressers. This result correlated the increased concentration of HCBR 1 with that of the increased doxorubicinol formation in the heart. Doxorubicinol formation was correlated to heart damage in expressers by a serum creatine study. Elevated levels of creatine kinase are linked to muscle damage. Expressers were found to have 60% elevated creatine levels than nonexpressers indicating muscle damage.[63]

A second study involving carbonyl reductase knockout mice demonstrated that lower CBR levels lead to protection against anthracycline cardiotoxicity.[64] They found that CBR1 (+/-) mice had lower levels of CBR and were protected from cardiotoxicity

after exposure to doxorubicin. The knockout mice showed a decrease in circulating metabolite after exposure to doxorubicin compared to normal (+/+) mice. It is believed that the lower amount of CBR enzyme leads to less conversion of parent compound to metabolite.[64]

A recent study provides a partial explanation for the varied response to anthracycline cardiotoxicity observed in patients. Gonzalez-Covarrubias et. al. examined the properties of a natural polymorphism in the human carbonyl reductase one (HCBR 1) gene.[66] They found that between different human populations there is a variation at position 88 whereby some people have a valine and others have an isoleucine. This site is associated with the NADPH binding in the active site of HCBR 1. This study found that the two different forms of HCBR 1 had different K_m and V_{max} values for daunorubicin. They also found that those with a valine at 88 had an increase production of doxorubicinol when exposed to doxorubicin. The results of this study suggests that those with the valine 88 polymorphism may be more at risk of anthracycline-associated cardiotoxicity than those with isoleucine 88 due to the substantial increase in metabolite formation after exposure to doxorubicin.[66] Also this study shows the rational in studying HCBR 1 by mutating key binding amino acids in the active site. Different polymorphisms of HCBR 1 may display different specificities and kinetics for its substrates thus revealing information about substrate binding and catalytic mechanism for carbonyl reduction. This information can be exploited to design a more effective, less cardiotoxic anthracycline analog.

Specific Aims of this Project

Specific Aim 1: Molecular Modeling of HCBR 1 and Known Substrates

Using the crystallized structure of the HCBR 1 enzyme and structures of the anthracyclines I will study the relationship between the enzyme and its substrates. The crystallized structure of HCBR 1 has been solved and can be accessed through the RCSB Protein Data Bank using the accession number, 1wma. This structure shows the enzyme complex with the cofactor $NADP^+$ and an inhibitor bound in the active site. This allows for the structures of various substrates like menadione, daunorubicin and doxorubicin to be aligned in the active site. Analyses can be done to determine which amino acids in the HCBR active site appear to interact with the substrates and possibly play a role in substrate binding and orientation.

Specific Aim 2: Design a New Expression Vector for HCBR 1

In order to facilitate purification of mutated forms of HCBR 1 a poly-histidine tag will be added to the C-terminus of the protein to facilitate purification. Introducing mutations into a protein has the potential to inactivate or alter the protein structure of the enzyme and this purification system should allow for quick purification of the enzyme regardless of its condition after purification. Specific aim 2 will address these problems by designing a new expression vector utilizing a histidine tag added to the protein. The protein will now be purified by a Ni^{2+} resin and visualization of the enzyme can be done easily with a SDS-PAGE gel analysis.

Specific Aim 3: Evaluate the Role of Met 234 in Doxorubicin vs. Daunorubicin Specificity

Due to the information gained in specific aim one, specific amino acids in the HCBR 1 enzyme were chosen to undergo mutation. I targeted position 234 which has a native methionine. It is hypothesized that this amino acid provides steric hindrance with substrates in the active site. It is believed that by mutating the 234 amino acid to amino acids with smaller side chains, such as Ala, Cys, or Ser, that more room will be generated in the active site. I expect to see increased affinity for substrates that will be measured by an increased catalytic efficiency for substrates.

Statement of Hypothesis

Anthracyclines undergo metabolism by HCBR 1 in the body and I believe that there are key amino acids that determine substrate specificity and help guide substrates into the active site. I hypothesize that methionine 234 prevents the binding of different anthracyclines due to a steric hindrance with side chains of anthracyclines. For instance, daunorubicin has a higher specificity for HCBR 1 than doxorubicin. Modeling studies show that the longer side chain of doxorubicin is partially hindered by its interaction with Met 234. Such an adverse interaction is not observed with daunorubicin. If this amino acid is mutated to smaller amino acids than more room will be generated in the active site and an increased catalytic efficiency will be observed for the doxorubicin molecule compared to the daunorubicin molecule.

Summary

Carbonyl reductase plays a significant role in metabolizing anthracyclines, lowering cytotoxic properties and increasing cardiotoxic properties of the drug and should be a pharmacological target for improved drug design. By understanding the relationship between HCBR and its substrates a drug can be designed that resists metabolism and lowers anthracycline cardiotoxicity. Targeting HCBR can increase the drugs lifespan in the cell and improve cytotoxic properties of the drug. In order to design these new anthracycline treatments a better understanding of the kinetic mechanism of substrate recognition and substrate binding is needed. Ultimately a drug will be designed that is a poor substrate for HCBR and resists metabolism and/or novel inhibitors will be identified that can block HCBR activity during anthracycline administration.

In light of the evidence that anthracycline combination therapies can be highly effective at treating certain cancers it is clear that resolving the cardiotoxic problems can be an important improvement to cancer therapies. This study can provide further information about anthracyclines and their metabolism by a key enzyme. This information can be used to enhance the cytotoxic properties and reduce risk of heart tissue injury during therapy. New drug designs are important to providing care for patients that would otherwise be turned down for treatment.

CHAPTER 2

MATERIALS AND METHODS

Materials

Menadione was from Aldrich Chemical Company (Milwaukee, WI), and rutin were obtained from Sigma Chemical Company (St. Louis, MO). Reduced β-nicotinamide adenine dinucleotide phosphate, sodium salt, 99% (NADPH) was purchased from Life Science Resources (Milwaukee, WI). Doxorubicin HCl and daunorubicin HCl were from Pharmacia &Upjohn Co. (Kalamazoo,MI) and Bedford Laboratories (Bedford, OH), respectively.

Molecular Modeling of Crystallized HCBR 1 Enzyme

Crystallized Structure of HCBR 1

The HCBR 1 enzyme has been crystallized and can be accessed at the Protein Data Bank, www.rcsb.org/pdb, with the accession number 1WMA.[67] The structure has a bound inhibitor molecule and a bound co-enzyme molecule (NADPH) present in the model.

Analyzing Active Site of Crystallized HCBR 1

The crystallized structure was viewed with the WinCoot program, version 0.3.3, http://www.ysbl.york.ac.uk/~lohkamp/coot/wincoot.html. The structure of doxorubicin was downloaded from the RCSB Protein Data Bank, accession number 1D12. Using the bound inhibitor as a model for the position of the anthracycline C-13 carbonyl, doxorubicin was aligned in the enzyme active site so that the carbonyl was placed near

the known catalytic amino acids described in chapter one. The structure was analyzed for amino acids that appear to interact with the substrate. Amino acids that were less than 3 Å from the substrate were of interest. This distance is within that expected for hydrogen bond interactions.

Creation of an Expression System for HCBR 1

Amplification and Ligation of Human Carbonyl Reductase 1 Gene into pTrcHis2 TOPO Vector

The HCBR 1 gene, formally isolated from the liver, was PCR amplified from the HCBR 1 pET5a construct previously reported by Slupe et. al.[49] The forward primer was 5'- GAATTCATGTCGTCCGGCATCCATGTAG-3' and the reverse primer was 5'-CCACTGTTCAACTCTCTTCTCTGA-3'.

The amplified PCR product was incubated with the pTrcHis2 TOPO vector at room temperature for 5 minutes. The resulting ligation mixture was transformed into Invitrogen One Shot Top10 *E. coli* cells and grown in SOC media in a shaker set at 37°C and 225 rpm for thirty minutes. The growth was plated onto Luria-Bertani (LB) agar plates supplemented with 100µg/mL of ampicillin and placed in a 37°C incubator overnight. The resulting colonies were counted on the following day (Table 2.1).

Table 2.1: Ligation of HCBR 1 Gene and pTrcHis2 TOPO Vector

Volume of Ligation Mixture	Number of Colonies
25µL	7
50 µL	19
100 µL	37
115 µl	19

Ligation mixtures were inoculated into Top10 *E. coli* cells and were plated onto LB plates supplemented with 100 µg/ml ampicillin and left to grow overnight in 37°C oven.

Colonies were chosen and used to inoculate 3 mL LB-AMP broth, and allowed to shake at 225 rpm overnight at 37°C. Plasmids were isolated from these growths with the Eppendorf Fast Plasmid Mini standard procedure. Plasmids were screened for the correct gene orientation using a restriction enzyme digests with EcoRI. The digests were analyzed by gel electrophoresis using a 1% agarose gel. Correct orientation resulted in two bands: 5 kb and 850 base pair in the lane. From this point forward the HCBR 1 gene with histidine tag will be referred to as HCBR 1'.

Protein Expression of Native HCBR 1' and Expression of the Recombinant Protein

E. coli Growths of Native HCBR 1'

Native HCBR 1' plasmids were transformed into Invitrogen One Shot Top10 *E. coli* cells and plated onto LB-AMP plates. These plates were placed in 37°C incubator overnight. One colony was used to restreak another LB-AMP plate and the resulting colonies were used to inoculate an overnight 3 mL LB-AMP culture. After growing for about 12 hours with shaking at 37°C, the culture was added to a 50 mL LB-AMP culture and allowed to grow with shaking overnight at 37°C. The following morning the overnight culture was used to inoculate 4.8 L of LB-AMP broth. Once the OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM. The induced cells were grown for another 12 h before they were harvested by centrifugation at 5000 x g.

Purification of Native HCBR 1'

The cell pellet was re-suspended in a 1 mM imidazole, 50 mM $NaPO_4$ buffer, pH 8.0 and sonicated in an ice water bath using a Misonix Sonicator 3000. The cell suspension received 10 x 30 second pulses at a power setting of 7. A 3 minute rest period was allowed between pulses. The resulting crude lysate was centrifuged at 50,000 x g

for sixty minutes using a Sorvall Evolution refrigerated centrifuge. The resulting supernatant was retained and was diluted 1:4 with a 1 mM imidazole, 50 mM $NaPO_4$ buffer, pH 8.0. Approximated 1-2 mL of Talon Ni^{2+} resin was poured into a column and equilibrated with the same buffer. The diluted lysate was added to the resin followed by a wash with a liter of 1 mM imidazole, 50 mM $NaPO_4$ buffer, pH 8.0.

The protein was eluted from the resin with increasing imidazole concentrations in a 50mM $NaPO_4$ buffer, pH 8.0. The four elution buffers used have the following imidazole concentrations: 1 mM, 50 mM, 250 mM, and 1 M imidazole.

SDS-PAGE Analysis, Concentration, and Buffer Exchange.

The elution fractions were analyzed for protein using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to locate the HCBR 1' protein. The protein of interest eluted from the column in the 50 mM imidazole, 50 mM $NaPO_4$ buffer, pH 8.0. The fractions containing the HCBR 1' protein were concentrated and buffer exchanged to 100 mM sodium phosphate, pH 7.0.

Creation and Characterization of the M234C HCBR 1´

Creation of Mutated Human Carbonyl Reductase 1 Gene Constructs

Site-Directed Mutagenesis

The QuickChange site-directed mutagenesis kit by Stratagene (Cedar Creek, TX) was utilized to mutate the HCBR 1 gene. The amino acids that were targeted were methionine 234, methionine 141, and tryptophan 229. This method mutates a native gene in a vector by utilizing complimentary primers that contain a mutation. As such the entire plasmid is copied during the process.

Primer Design

The sequence for the native HCBR 1 gene (P16152) was downloaded from the ExPaSy Proteomics Server (http://www.expasy.org). The codons coding for amino acids Met 141, Met 234 and Trp 229 were located. Table 2.2 lists the primer sequences that were designed to mutate these amino acids to alanine, cysteine, or serine.

Table 2.2: Primer Sequences for the QuickChange Mutagenesis Procedure

Intended substitution	A.A. Position	Forward Primer Sequence	Reverse Primer Sequence
Met → Ala	141	5'GTG GTG AAC GTA TCT AGC ATC **GCG** AGC GTC AGA GCC CTT AAA AGC 3'	5' GCT TTT AAG GGC TCT GAC GCT **CGC** GAT GCT AGA TAC GTT CAC CAC 3'
Met → Ser	141	5' GTG GTG AAC GTA TCT AGC ATC **TCG** AGC GTC AGA GCC CTT AAA AGC 3'	5' GCT TTT AAG GGC TCT GAC GCT **CGA** GAT GCT AGA TAC GTT CAC CAC 3'
Met → Cys	141	5' GTG GTG AAC GTA TCT AGC ATC **TGC** AGC GTC AGA GCC CTT AAA AGC 3'	5' GCT TTT AAG GGC TCT GAC GCT **GCA** GAT GCT AGA TAC GTT CAC CAC 3'
Met → Trp	141	5' GTG GTG AAC GTA TCT AGC ATC **TGG** AGC GTC AGA GCC CTT AAA AGC 3'	5' GCT TTT AAG GGC TCT GAC GCT **CCA** GAT GCT AGA TAC GTT CAC CAC 3'
Met → Ala	234	5' GGGTGGGTGAGAACTGAC**GCG**GCGG GACCCAAGGCC 3'	5' GGC CTTGGGTCCCGCCGC**GTC**AGTTCTCA CCCACCC 3'
Met → Cys	234	5' GGGTGGGTGAGAACTGAC**TGC**GCGG GACCCAAGGCC 3'	5' GGCCTTGGGTCCCGCGCA**GTC**AGTTC TCACCCACCC 3'
Met → Ser	234	5' GGGTGGGTGAGAACTGAC**TCG**GCGG GACCCAAGGCC 3'	5' GGCCTTGGGTCCCGCGCA**GTC**AGTTC TCACCCACCC 3'
Met → Trp	234	5' CCA GGG TGG GTG AGA ACT GAC **TGG** GCG GGA CCC AAG GCC ACC 3'	5' GGT GGC CTT GGG TCC CGC **CCA** GTC AGT TCT CAC CCA CCC TGG 3'
Trp → Ala	229	5' CTG AAT GCC TGC TGC CCA GGG **GCG** GTG AGA ACT GAC ATG GCG 3'	5' CGC CAT GTC AGT TCT CAC **CGC** CCC TGG GCA GCA GGC ATT CAG 3'
Trp → Ser	229	5' CTG AAT GCC TGC TGC CCA GGG **AGT** GTG AGA ACT GAC ATG GCG 3'	5' CGC CAT GTC AGT TCT CAC **ACT** CCC TGG GCA GCA GGC ATT CAG 3'

Primers were designed to mutate key amino acids in the native HCBR 1 gene sequence. The sites of interest are Met 234, Met 141 and Trp 229. The letters in the bold underlined font represent the mutated codons in the primer.

Primers were designed according to the recommendations of Stratagene for the QuickChange II site-directed mutagenesis kit:

1) Mutation is designed into the primer and located in the middle of the primer with approximately 15 bases flanking both sides of the mutation. The primer ends in a G or C base. The primers anneal to the same sequence on the opposite DNA strands.

2) Primers were designed with a melting temperature of greater than 78°C. The melting temperature calculation is:

$$T_m = 81.5 + 0.41(\%GC) - \frac{675}{N} - \%mismatch$$

N = primer length in bases

Thermocycling Parameters

Following the protocol provided in the QuickChange II site-directed mutagenesis kit by Stratagene, the following were added to a 1.5mL centrifuge tube: approximately 50 ng of DNA, 100 ng of forward and reverse primer, dNTP's, and 1.25 Units of DNA polymerase. The reactions were placed in a thermocycler. The PCR thermocycling settings are listed in the following table.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	16	95°C	30 seconds
		55°C	1 minute
		68°C	5minutes

After the PCR thermocycling was complete the reactions were placed on ice for two minutes.

Removal of Native HCBR 1' Plasmids

In order to remove the plasmids containing the native HCBR 1 gene 0.5µL of DPN1 restriction enzyme (10 U/µL) were added to each PCR reactions, pipetting thoroughly to mix. The DPN1 restriction enzyme recognizes methylated plasmids. Only the native plasmids have methylated sites and are recognized by the DPN1 restriction enzyme and these plasmids will undergo digestion. This leaves a population of mutated plasmids behind.

The digestion reactions were micro-centrifuged at 7,000 x g and air bubbles were removed. The DPN1/QuickChange II reactions were incubated at 37°C for 1 hour. After a one hour digestion the PCR product was transformed into Stratagene XL1- Blue Supercompetent *E. coli* cells and were plated on LB-AMP plates and incubated at 37°C overnight.

Plasmid Prep Procedure

Five colonies of each mutation were selected to undergo plasmid preparation and these plasmids were sequenced. The Promega PureYield Plasmid Midiprep System was utilized and the standard DNA purification protocol was followed.

Gene Sequencing

The mutated plasmids were sent into Genewiz Laboratory, Genewiz inc, (South Plainfield, NJ) for sequencing. The HCBR 1 gene was sequenced using the following pTrcHis2 TOPO primers: Forward 5' GAGGTATATATTAATGTATCG 3', Reverse 5' GATTTAATCTGTATCAGG 3'. These primers bind the vector in the flanking regions surrounding the HCBR 1 gene. The resulting sequencing information was analyzed using the Bioedit, version 7.0.0, software program.

An HCBR 1 gene sequence used for comparison was created from the sequence available at Expasy.org (accession number P16152), and the bases for the histidine tag were added to the end of the gene according to the manual provided for the pTrcHis2 TOPO vector system. This sequence was then used to align with the plasmid sequences. When the gene was located it was analyzed for the gene mutation coding for the mutated amino acids. The rest of the gene was analyzed to confirm that no other unattended mutations had occurred.

Mutant HCBR 1' Protein Expression and Purification

Protein Production

Sequenced HCBR' plasmids were transformed in Invitrogen One Shot Top10 *E. coli* cells and grown as described above for the modified native protein. The protein was expressed and purified in a manner similar to that described for the native HCBR'.

Kinetic Characterization of Native and Mutant Forms of HCBR' with Menadione Substrate

Extinction Coefficient Determination

The extinction coefficient for the HCBR 1' were estimated using the ProtParam tools at http://ca.expasy.org. The known HCBR 1 protein sequence was downloaded from the ExPaSy Proteomics Server, accession number P16152, and the extra amino acids from the pTrcHis2 TOPO vector were added to the sequence. This sequence was entered into the ProtParam tools and an extinction coefficient of 0.668 $mL \cdot mg^{-1}$ at 280 nm was estimated. The mutation was added to the sequence and was found to have an extinction coefficient of 0.673 $mL \cdot mg^{-1}$ at 280 nm.

Specific Activity Determination and Steady State Kinetic Analysis

Reaction kinetics were measured by monitoring the change in NADPH concentration over time spectrophotometrically ($\varepsilon_{340} = 6220\ M^{-1}cm^{-1}$) using a Varian Cary 100 Bio.[68] Initial slopes of the progress curves were obtained from fitting the curves to either a line or a parabola, and these slopes were used to calculate the initial velocities. Specific activity for the modified native HCBR 1 enzyme in a standard assay comprised of 250 μM menadione and 50 μM NADPH in 100 mM sodium phosphate, pH 8.0 was

measured and compared to that previously published.[49] The extinction coefficient 0.668 $mL \cdot mg^{-1}$ was used to estimate protein concentration at 280 nm in a Cary 100 spectrophotometer. The volume of protein was varied from 3 µl to 10 µl of enzyme and the change in absorbance per minute was recorded. Specific activity values were calculated for each trial.

Menadione saturation kinetics were measured using 50 µM NADPH in 100 mM sodium phosphate and menadione concentrations ranging from 3µM to 200µM. The resulting kinetics data were fit to the Michaelis-Menten equation (eq. 1) using the HYPERW program.[69]

$$v_o = \frac{V_{max} A}{K_m + A} \qquad \text{(eq. 1)}$$

Where ν_o is the initial velocity, V_{max} is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration (in this case it is menadione). From the estimates of these constants the k_{cat} (catalytic rate constant) and the k_{cat}/K_m (catalytic efficiency) was calculated. The M234C mutant protein was found to be inactive under the standard assay conditions, so several other assay conditions were investigated in an effort to detect a measurable enzyme activity. Several concentrations of menadione and NADPH were investigated and the assay was monitored for up to 20 minutes. Also, both daunorubicin and doxorubicin (300 µM concentrations were used for both) were tested as substrates. Also, dithiothreitol (up to 1 mM) was also added to the assays in case the addition of an active-site cysteine led to unintended disulfide bond formation. The total time for each assay measured up to 20 minutes.

Characterization of Coenzyme and Substrate Binding

Binding of coenzyme and substrates were studied by fluorimetry. A 4 μM solution of protein (either the native or mutant HCBR 1') was titrated with either NADPH, $NADP^+$, daunorubicin, or doxorubicin (concentrations ranging from 0.50 μM to 115 μM) and the resulting reduction in protein fluorescence was measured (Ex. 292 nm; Em. 333 nm) using an Olis DM45 spectrofluorimeter (Olis, INC., Bogart, GA). The data was fit to the Stern-Volmer equation (eq. 2) and a K_d value was determined. The program uses a modified Stern-Volmer equation to measure the disassociation of substrate and enzyme.

$$\frac{f}{f_o} = \frac{f_1}{\left(1 + \frac{B}{K_d}\right)} + (1 - f_1) \qquad \text{(eq. 2)}$$

Where $f_1$is the fraction of protein fluorescence that is quenched, *B* is substrate concentration and K_d is the dissociation binding constant for the substrate and enzyme.

Circular Dichroism of Native and M234C HCBR 1'

Circular dichroism was done to assess changes in secondary structure between the native HCBR 1' and the M234C HCBR 1' proteins. A far UV analysis was done on the samples which covered the 275 to 175 nm spectrum. The spectropolarimeter was blanked with a 100 mM sodium phosphate buffer, pH 7.0 and was subtracted from the data. 0.5 mg/mL solutions of both proteins were made and 200μL of solution was added to a 1 mm cell that was placed in a spectropolarimeter (Jasco J810). Data was collected at 1 wavelength per second. Two runs of three scans were collected. Each run was averaged and then both runs were averaged.

The raw data was analyzed by comparing the wavelength to the $\Delta\theta$ (mdeg/M*cm). The data was corrected in order to account for any miscalculations in concentrations of the proteins. The data was also analyzed by Dichroweb, http://www.cryst.bbk.ac.uk/cdweb/html/home.html, this website compares data to a database of known proteins.

CHAPTER 3

RESULTS

CREATION OF AN EXPRESSION SYSTEM FOR HCBR 1

Ligation of HCBR 1 Gene into pTrcHis2 TOPO Vector

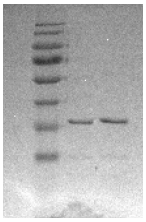
The HCBR 1 gene was successfully amplified and inserted into the pTrcHis2 TOPO vector in the correct orientation. The histidine tag was added to the C-terminus of the gene. Two extra amino acids were added to the N-terminus and twenty-nine amino acids were added to the C-terminus of the enzyme. The results were verified by a restriction enzyme digest with EcoRI and resulted in two bands at 5kB and 850 bp. The mutations were also confirmed by sequencing of the gene in the plasmid.

Protein Expression of Native HCBR 1'

It was found that from a 4.8L culture 11 mgs of protein were obtained. The protein was eluted from the nickel column with the lower than expected concentration of 50 mM imidazole, in 50 mM sodium phosphate, pH 8.0. At such a low concentration of imidazole buffer it seems apparent that the native HCBR 1' protein is weakly bound to the Ni^{2+} resin, perhaps because the C-terminal poly-histidine tag is partially obscured through interactions with the remaining protein. Typically it is expected that elution will occur in the imidazole range of 250 mM to 1 M. SDS-PAGE analysis adds more evidence that the protein of interest was obtained at > 90% purity.

The molecular weight of the modified enzyme is estimated to be 33.80 kD. The major protein band that occurs is slightly above the 33 kD band in the ladder indicating that the major band is in the correct region for the weight that is expected. The gels have one major band occurring but there are two minor bands at 24 kD and 22 kD which comprise < 10% of the total protein. The identity of these bands has yet to be determined, although they may be HCBR 1' proteins that have undergone degradation (Figure 3.1).

Figure 3.1: SDS-PAGE Gel of NHCBR'



Lane 1: Molecular Weight Ladder, EZ-run Prestained REC Protein Ladder (Fischer), Lane 2: 0.757mg/mL NHCBR' sample, Lane 3: 0.894 mg/mL NHCBR' sample. The major band is located just slightly above 33kD band in ladder, in the region that is expected. There is a minor band at about 24kD and 22kD.

Characterization of Native HCBR 1' with Menadione Substrate

Specific Activity

The specific activity of the modified native protein was found to be 0.63 ± 0.08 µmol/mg·min. The reported specific activity in the standard assay for the HCBR 1 pET5a enzyme is 5.1 ± 0.7 µmol/mg·min.[49] The specific activity for the modified native protein is 8 fold less than that of the unmodified protein. It is hypothesized that this lower value is due to the C-terminal peptide comprised of 29 amino acids including the histidine tag interfering with the active site in some way.

Steady State Kinetics Studies with the Modified Native HCBR

Table 3.1 summarizes the steady state kinetic values for both the published unmodified HCBR enzyme[49] and the His-tagged HCBR enzyme. The K_m for the His-tagged enzyme was found to be 3.5 fold lower than the unmodified enzyme which indicates that this modified enzyme is reaching its maximal catalytic efficiency at lower substrate concentrations. The k_{cat}, turnover number, for His-tagged HCBR was seven fold lower than that of the unmodified enzyme, indicating that the modified enzyme produces much less product molecules in the same amount of time than that of the unmodified enzyme. The value of k_{cat}/K_m, or catalytic efficiency, for the His-tagged enzyme is approximately half of that observed for the unmodified enzyme. So this suggests that overall the His-tagged enzyme is two-fold less efficient in catalyzing the reduction of menadione as is the unmodified enzyme. This information also supports the idea that the histidine tag may somehow interfere with enzyme catalysis.

Table 3.1: Steady State Kinetic Constants for NHCBR', M234C HCBR', and Published HCBR 1 pET5a.

Enzyme	**Substrate**	**k_{cat}, s^{-1}**	**K_m, µM**	**k_{cat}/K_m, $\mu M^{-1}s^{-1}$**
NHCBR'	Menadione	0.53 ± 0.02	11±2	0.049 ± 0.007
M234C HCBR'	Menadione	None	None	None
HCBR 1 pET5a[a]	Menadione	3.7 (0.1)	38 (3)	9.8 (0.5) x 10^{-2}

([a]Data from pET5a is from Slupe 2005).[49] The above kinetic constants were established for the three forms of the HCBR 1 enzyme. There is a significant difference between the pET5a enzyme and the histidine tagged enzymes. There is a significant difference between the NHCBR 1' and the M234C HCBR'.

All of the data collected for the steady-state kinetics indicate that when the histidine tag was added to the HCBR enzyme the efficiency of the enzyme to reduce the substrate menadione was diminished. The substrate menadione has been used as a model substrate to study this enzyme's catalytic process by several different researchers. There

are many benefits to using menadione as a substrate as opposed to using the anthracyclines of interest. These benefits include cost, ability to dispose and safety. Although steady state kinetics have yet to be completed using the anthracyclines it is hypothesized that lower catalytic efficiencies will also be observed with other substrates such as doxorubicin or daunorubicin for the His-tagged native enzyme.

Characterization of Coenzyme and Substrate Binding

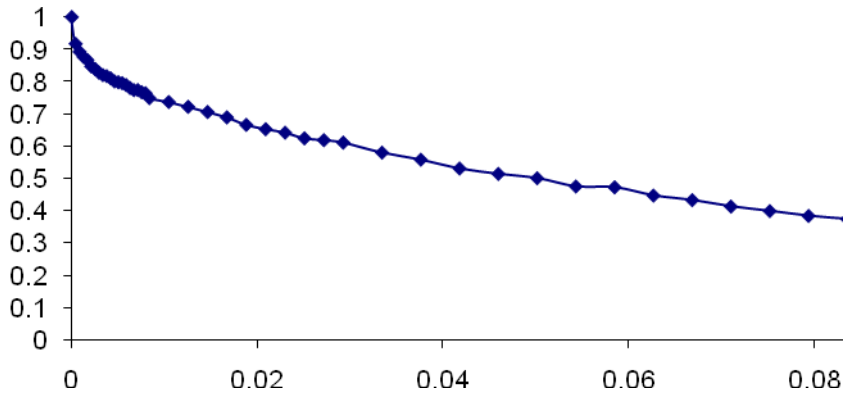
Due to the diminished activity of this modified enzyme, fluorimetry was used to look at independent binding of coenzymes and substrates to the active site. Such a diminished catalytic efficiency and turnover number as seen for the His-tagged native enzyme could arise if either coenzyme binding or carbonyl substrate binding were disrupted. These series of fluorimetry studies are trying to find information about which molecules show perturbations in enzyme binding.

Native HCBR 1 enzyme has a tryptophan (Trp 229) in the active site of the enzyme that reports on binding to the enzyme active site. As compounds bind the active site the fluorescence of the tryptophan is decreased. Several coenzymes/substrates were examined. The binding of NADPH was examined (Figure 3.2) and quenching of the enzyme was observed. Each fluorescence value is divided by the fluorescence of just enzyme to yield the relative fluorescence, f/f_0. This calculation normalizes the data.

NADPH is known to fluoresce in the same region as the HCBR enzyme. Studies with $NADP^+$ were done because this molecule does not fluoresce in the same region. $NADP^+$ is known to bind the active site like NADPH. Figure 3.3 shows the results of the $NADP^+$ fluorimetry study. It was expected that $NADP^+$ quenching would mirror

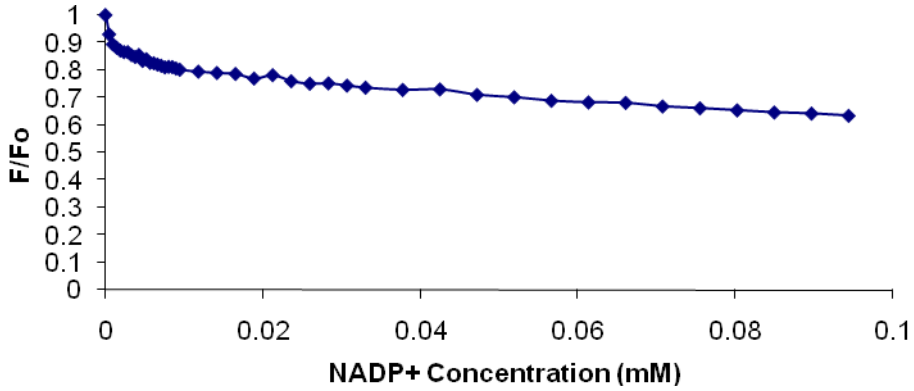
NADPH quenching. The ability to cause quenching for both of these molecules does appear to be different.

Figure 3.2: Fluorescence Quenching of NHCBR 1' and NADPH Coenzyme



Coenzyme binding ability was expolored with fluorescence quenching studies. A 4µM NHCBR' solution was made and a 1mM solution of NADPH was added in 1µL increments. The fluorescence values were recorded and graphed. As the active site Trp 229 is quenched a decrease in fluorescence is observed.

Figure 3.3: Fluorescence Quenching of NHCBR 1' and $NADP^+$

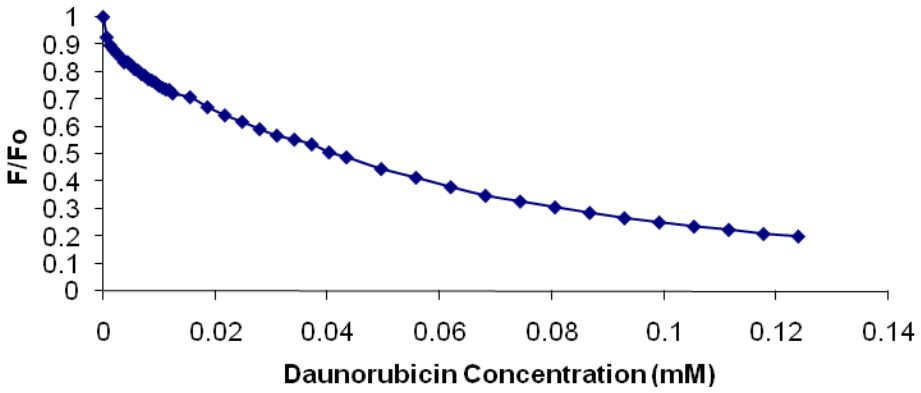


Coenzyme binding ability was expolored with fluorescence quenching studies. A 4µM NHCBR' solution was made and a 1mM solution of $NADP^+$ was added in 1µL increments. The fluorescence values were recorded and graphed. $NADP^+$ is prefered for fuorescence because, unlike NADPH, it does not fluoresce in the same region has the NHCBR'. As the active site Trp 229 is quenched a decrease in fluorescence is seen.

Next the binding of anthracycline substrates were studied by fluorimetry. There was a significant drop in fluorescence when daunorubicin was added to the assay (Figure 3.4). When doxorubicin was added to the assay there was a drop in fluorescence but not to the extent of the daunorubicin (Figure 3.5).

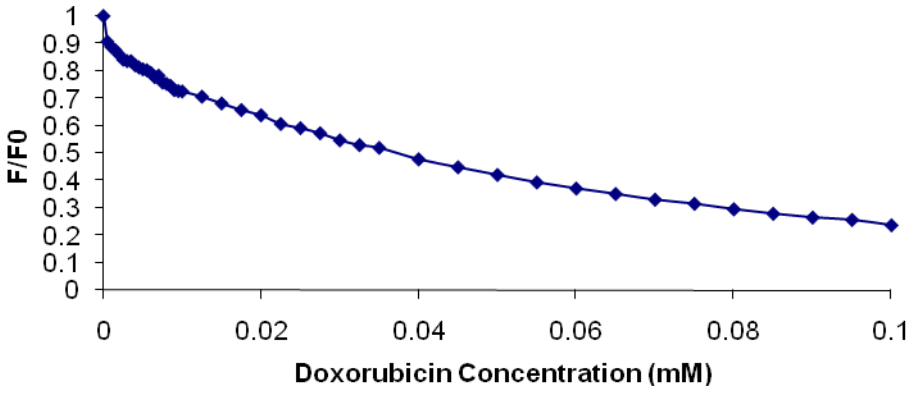
The results of the fluorimetry studies have lead to the hypothesis that the histidine tag may potentially be interfering with the coenzyme binding site of the enzyme, although the details of that mechanism have not been elucidated.

Figure 3.4: Fluorescence Quenching of NHCBR 1' and Daunorubicin



Substrate binding ability was expolored with fluorescence quenching studies. A 4µM NHCBR' solution was made and a 1.24mM solution of daunorubicin was added in 1µL increments. The fluorescence values were recorded and graphed. As the active site Trp 229 is quenched a decrease in fluorescence is seen.

Figure 3.5: Fluorescence Quenching of NHCBR 1' and Doxorubicin



Substrate binding ability was expolored with fluorescence quenching studies. A 4µM NHCBR' solution was made and a 1mM solution of doxorubicin was added in 1µL increments. The fluorescence values were recorded and graphed. As the active site Trp 229 is quenched a decrease in fluorescence is observed.

K_d values were established for the coenzymes and substrates (Table 3.2). These values reflect the disassociation of substrate from enzyme and can be represented by the following equation:

$$C \leftrightarrow E + S$$

$$K_d = \frac{[E][S]}{[C]} \qquad \text{(eq. 3)}$$

C= Enzyme/substrate complex
E= Enzyme
S= Substrate

The K_d value represents the concentration of substrate when half of the binding sites of an enzyme are occupied. A smaller K_d value indicates a more tightly bound substrate. The substrate has a higher affinity for the enzymes binding site than a substrate

with a larger K_d value. The K_d value is not the preferable way to measure binding of substrates because substrates potentially can bind active sites without being in the optimal position for catalysis.

Table 3.2: K_d Values for the Binding of Coenzyme and Anthracyclines to Native and M234C HCBR'

Substrate	Native K_d(μM)	M234C K_d(μM)
Doxorubicin	22.67 (19.62 - 26.16)	35.8 (31.8 - 40.0)
Daunorubicin	25.45 (17.79 - 35.95)	31.5 (27.2 - 36.63)
$NADP^+$	5.49 (4.55 - 6.5)	19.53 (15.83 - 23.96)
NADPH	13.58 (11.32 - 16.16)	26.83 (23.5 - 30.61)

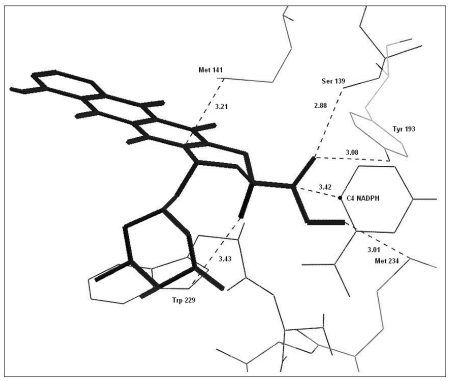
The binding of coenzymes and substrates were assessed by fluorimetry and resulting data were fit to the Stern-Volmer equation (eq. 3). A smaller K_d value indicates higher affinity between active site and molecule. This data suggests that for both coenzyme and substrates the NHCBR' active site may have a stronger affinity than the mutant. The numbers in parentheses represent the 67% confidence interval.

The scientific literature is lacking published K_d values for the unmodified enzyme and anthracycline substrate binding. The data that was collected for the pTrcHis2 TOPO enzyme cannot be directly compared to the pET5a HCBR; however, information can still be gained as far as trying to identify which molecules exhibit impaired binding to the pTrcHis2 TOPO enzyme.

Molecular Modeling of Crystallized HCBR 1 Enzyme

Molecular modeling of the HCBR 1 enzyme and the doxorubicin molecule resulted in the identification of methionine 234 as an amino acid that potentially interacts with bound substrates and limits the size of the substrate that can fit in the active site (Figure 3.6).

Figure 3.6: Models of Anthracycline Binding to the Active Site of Human Carbonyl Reductase



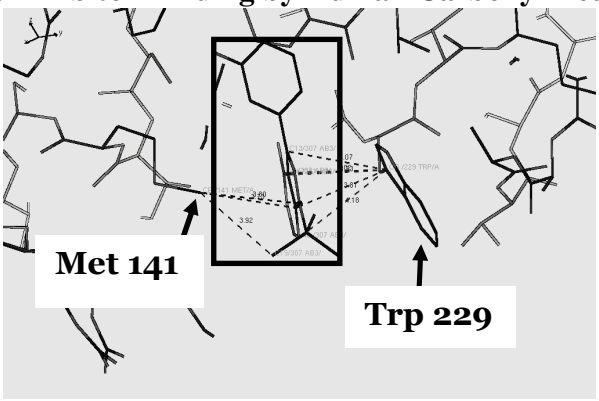
Model of doxorubicin C13 carbonyl reduction by human carbonyl reductase showing the close contact between the side chain of doxorubicin and the methionine 234 amino acid.

It was found that methionine 234 appears to interact with the C-13 side chain of doxorubicin (Figure 3.6) in such a manner as to obstruct proper binding and orientation in the active site required for optimal chemistry. Daunorubicin which has a smaller C-13 side chain does not appear to have such a conflict with Met 234. This may be the reason why daunorubicin was found to be a better substrate for HCBR than was doxorubicin.[49] It is hypothesized that the Met 234 limits the binding of substrates in the active site because it sterically hinders the substrates. The Met 234 is about 3Å away from the doxorubicin molecule which is about the distance for a hydrogen bond.

It was found that mutating methionine 234 to serine, alanine or cysteine in a computer based model increased the space in the active site for the side chain of doxorubicin. This increased distance is hypothesized to allow the doxorubicin more ability to bind in the active site without interference from other amino acids in the active site and thus allow for more optimal orientation in the active site.

Trp 229 and Met 141 were also indentified as potentially important amino acids (Figure 3.7). They appear to surround the aglycone structure of the anthracycline molecules. The anthraquinone has two points that are 3Å away from both the Trp 229 and Met 141. It is hypothesized that these two amino acids help to stabilize the substrates when they are in the active site undergoing catalysis. The mechanism of this stabilization has not been defined to the extent of the hypothesized interaction of Met 234 and substrate side chains discussed in this paper.

Figure 3.7: Inhibitor Binding by Human Carbonyl Reductase



An inhibitor is bound to the active site of HCBR. Met 141 and Trp 229 are designated by arrows and the inhibitor is blocked in. The pyrazolopyrimidine ring of the inhibitor is sandwiched between Met 141 and Trp 229 side chains.

Mutating Trp 229 and Met 141 to Ala, Ser or Cys in the computer model also lead to increased space in the active site. The hypothesis is that this larger space will allow more space for the maneuvering of the substrate molecule leading to increased binding in the active site.

Characterizing the M234C HCBR 1'

Creation of Mutated Human Carbonyl Reductase 1 Gene Constructs

Site-Directed Mutagenesis of HCBR 1'

Several mutated plasmids of the HCBR 1' gene were made (Table 3.3). Several sites were targeted. The methionine 234 site was successfully mutated to cysteine, alanine, serine and tryptophan. The methionine 141 site was successfully made into serine, tryptophan, alanine, and cysteine. The tryptophan 229 site has not been mutated as of now. One double mutant was created – S141/S229. The large numbers of mutated colonies on the mutated plates was a good indication that the procedure was successful. The DPN1 control had the same concentration of native plasmid and underwent the PCR procedure without any primers followed by DPN1 digestion. There were five or less colonies on theses plates indicating that the DPN1 restriction enzyme was able to digest all parental DNA in the PCR reactions. Five colonies were chosen from each mutation and the plasmids were purified and the genes were sequenced.

Table 3.4 summarizes the sequencing results of these plasmids. Most plasmids contained the desired mutation although a small number of plasmids did not. Some plasmids that were sent in did not result in a clear enough sequence for analysis which may be due to improper preparation of plasmid prior to sequencing. At least one colony of each desired mutation was created and had the correct sequence for the rest of the gene. The double mutated plasmid did not have a clear enough sequence to insure that both mutations are present. They will need to undergo further sequencing to confirm the results.

Table 3.3: Results of the Quick Change Mutagenesis Procedure

PCR Reaction	Number of Plates	Number of Colonies	Expectations
S141,E244 HCBR'	2	155	Hundreds
A234,E244 HCBR'	2	195	Hundreds
S 234,E244 HCBR'	2	398	Hundreds
C234,E244 HCBR'	2	274	Hundreds
DPN1 Control	2	less than 5 colonies	None
E. coli only Control	2	None	None

PCR Reaction	Number of Plates	Number of Colonies	Expectations
Trp141,E244 HCBR'	2	18	Hundreds
A141,E244 HBCR'	2	13	Hundreds
S141, E244 HCBR'	2	136	Hundreds
Trp234, HCBR'	2	6	Hundreds
C141, E244, HCBR'	2	43	Hundreds
DPN1 Control	2	None	None
E. coli only control	2	None	None

Under optimal conditions the quick change mutagenesis protocol produces several hundred colonies of mutated plasmids. The large number of plasmids that resulted in the mutated plates was good evidence that the mutations worked. A DPN1 control utilized native template plasmid and underwent the DPN1 digestion. The low recovery of colonies indicates that the DPN1 was able to efficiently digest all native plasmids.

Protein Expression

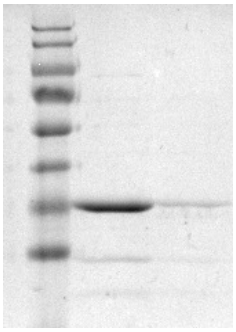
M234C HCBR 1' Protein Production

To better identify mutated enzyme production the HCBR 1 gene was inserted into the pTrcHis2 TOPO vector. The advantage of adding a histidine tag to the gene allowed the enzyme to bind a Ni^{2+} column. It helps in several regards, first it immediately separates bacterial carbonyl reductases from HCBR 1 and second it is easy to sample the fractions on an SDS-PAGE gel allowing the visualization of the mutated proteins regardless of activity.

It was found that a 4.8L of culture yielded about 9.5 mg of mutated protein. As with the native His-tagged construct, the protein eluted from the nickel column with the 50 mM imidazole, 50 mM sodium phosphate, pH 8.0 buffer. It also shows weak binding to the column by the fact that it eluted off the column in a low imidazole buffer.

SDS-PAGE analysis provided more evidence that the protein band observed in the SDS-PAGE gel is the targeted protein, M234C HCBR'. Figure 3.8 shows the results of the SDS-PAGE. Lane 1 is the Fischer EZ Run pre-stained REC protein ladder. Lane 2 is the M234C HCBR 1' sample, Lane 3 is the NHCBR' sample. The banding pattern of the native and the mutant are similar. They both have a major band slightly above the 33 kD ladder band. They both show the same minor bands occurring about 24 kD and 22 kD. The estimated molecular weight of the mutant is 33.64 kD. The gel reflects that the major band for the mutant is in this region.

Figure 3.8: SDS-PAGE Gel of M234C and NHCBR'



Above is an SDS-PAGE gel of the M234C and the native HCBR 1'. Lane 1: Fischer EZ Run Prestained REC protein ladder. Lane 2:M234C HCBR 1' and Lane 3: NHCBR'. The bands appear in the 33kD region as expected.

Table 3.4: Sequencing Results of the Mutated HCBR 1' Plasmids

Plasmid name	Colony Number	Sequence Correct for Mutation
A234,E244 HCBR'	1	Yes
A234,E244 HCBR'	2	Yes
A234,E244 HCBR'	3	Yes
C234, E244 HCBR'	1	Yes
C234, E244 HCBR'	2	Yes
C234, E244 HCBR'	4	Yes
S141, E244 HCBR'	2	Yes
S234, E244 HCBR'	1	Yes
S234, E244 HCBR'	2	Yes
S234, E244 HCBR'	3	Yes
S234, E244 HCBR'	4	Yes
Trp234, E244 HCBR'	1	Yes
Trp234, E244 HCBR'	2	Yes
Trp234, E244 HCBR'	3	Yes
Trp234, E244 HCBR'	4	Yes
A141,E244 HCBR'	1	Yes
A141,E244 HCBR'	3	Yes
A141,E244 HCBR'	4	Yes
C141,E244 HCBR'	1	Yes
C141,E244 HCBR'	2	Yes
C141,E244 HCBR'	3	Yes
C141,E244 HCBR'	4	Yes
Trp141, E244 HCBR'	1	Yes
Trp141, E244 HCBR'	2	Yes
Trp141, E244 HCBR'	3	Yes
Trp141, E244 HCBR'	4	Yes

Plasmids were sequenced to identify that the correct mutation was present in the plasmid. The gene was also analyzed to confirm that no other unintended mutations had occurred.

Characterization of M234C HCBR 1' Protein

Activity Studies

Menadione Activity Study

The M234C HCBR 1' enzyme was first assayed with the standard enzyme assay that has been previously established.[49] The 234 mutant did not display any measurable activity with the standard assay protocols with assay times up to 10 minutes.

The assay was then modified by varying the concentration of menadione from 25 µM to 180 µM. This lower range of menadione concentrations was selected to control for possible substrate inhibition. Even after 20 minutes, no measurable enzyme activity was detected.

NADPH Activity Study

A wide range of NADPH concentrations were also explored in order to test possible problems with the binding of coenzyme NADPH. The NADPH concentration was increased from the standard assay to see if there is any activity with the M234C HCBR 1'. The NADPH concentration ranged from 300 µM to 500 µM. Again, no measurable activity was recorded from the mutant.

Anthracycline Activity Studies

An assay was done with both doxorubicin and daunorubicin. The assays were done under standard assay conditions and 300 µM anthracycline in place of menadione. Also NADPH concentrations were increased from 50 µM to 150 µM. As with all other attempts, no activity was recorded with this mutant.

DTT Activity Study

An assay was done with the compound dithiothreitol (DTT) was used to assay any disulfide bonds that may have been formed due to the addition of the Cys 234 mutation. It is not believed that HCBR has any naturally occurring disulfide bonds and so DTT is not hypothesized to interfere with the activity of the protein. There was no activity observable with pre-incubation of the enzyme with DTT.

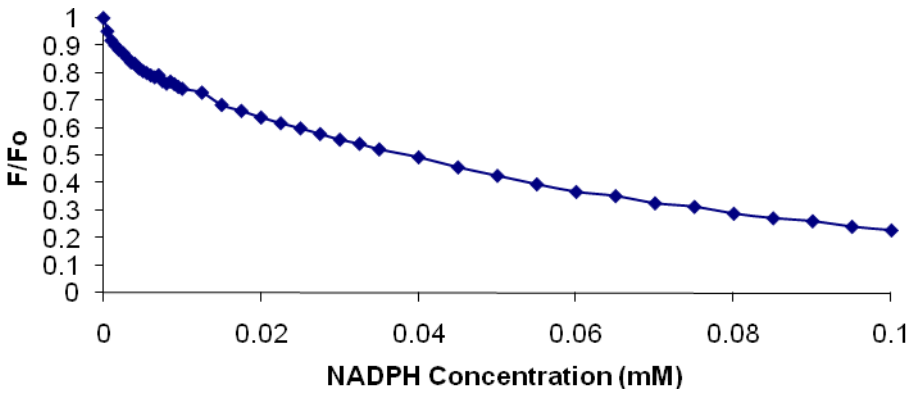
Fluorimetry Studies

NADPH Fluorimetry

As with the native His-tagged construct, the coenzyme and substrate binding properties of the Cys 234 mutant enzyme were investigated using fluorimetry. Figure 3.9 shows the fluorescence quenching as the NADPH is added. From this data a K_d was estimated to be 26.83 µM (Table 3.2).

A previously reported K_d for a variant of HCBR1, also with a histidine tag, was 6.3 ± 0.6µM for CBR1 V88 and 3.8 ± 0.5µM for CBR1 I88 (Covarrubias et. al. 2007). These researchers added a 6x His-tag to the N-terminus of the HCBR1 protein by inserting the gene into a pET28 vector. This vector adds the His-tag to the N-terminus of the enzyme and also adds between 10-24 extra amino acids.

Figure 3.9: Fluorescence of M234C HCBR 1' and NADPH Coenzyme



Coenzyme binding ability was expolored with fluorescence quenching studies. A 4µM M234C HCBR' solution was made and a 1mM solution of NADPH was added in 1µL increments. The fluorescence values were recorded and graphed. As the active site Trp 229 is quenched a decrease in fluorescence is observed.

The K_d values reported in the Gonzalez-Covarrubias are smaller K_d values than the K_d values that were observed in this study (Table 3.2). This smaller K_d value may indicate that the pTricHis2 TOPO His-tag used in this research may have a lower affinity for the binding of the coenzyme reflected by the increased K_d values. Within this research the coenzyme K_d values vary for the native and the M234C indicating that there is a difference in affinity between these two species (Table 3.2). The K_d values indicate that the NADPH coenzyme is binding the M234C HCBR 1' with less affinity than the native HCBR1' enzyme. This may indicate a disruption in the coenzyme binding site and it is hypothesized that this interruption is due to the polyhistidine tag, although the mechanism for this disruption has not been worked out yet. This data about the binding of NADPH to the mutated enzyme further supports the diminished kinetic activity observed in the NHCBR 1' enzyme. There is evidence to support the hypothesis that the

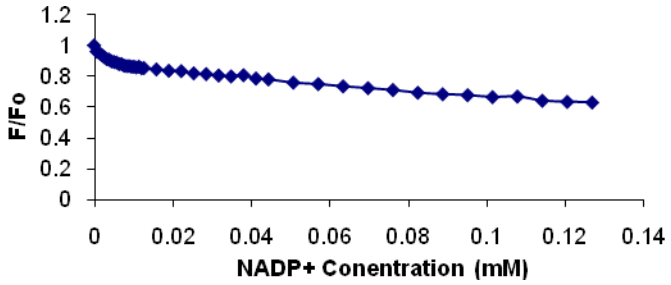
polyhistidine tag added to both of these enzymes is interfering with the binding of the cofactor.

NADP$^+$ Fluorimetry

Fluorimetry studies with the molecule NADP$^+$ and HCBR 1 have been completed in this lab before; however, the information has not been published. NADP$^+$ is a product for the enzyme re-dox reaction and shares close structure with NADPH, varying only by a hydrogen and charge. It is preferred to complete fluorescence quenching studies with NADP$^+$ because it does not fluoresce in the same region as HCBR 1. It is expected that HCBR 1 enzyme will bind the NADP$^+$ with similar affinity as the NADPH molecule. It is expected that binding of the NADP$^+$ will lead to quenching of the enzyme fluorescence (Figure 3.10).

The NADP$^+$ fluorescence quenching data suggests that the HCBR 1' is binding the NADP$^+$ molecule with a higher affinity than it binds the NADPH molecule (Table 3.2).

Figure 3.10: Fluorescence of M234C HCBR 1' and NADP$^+$

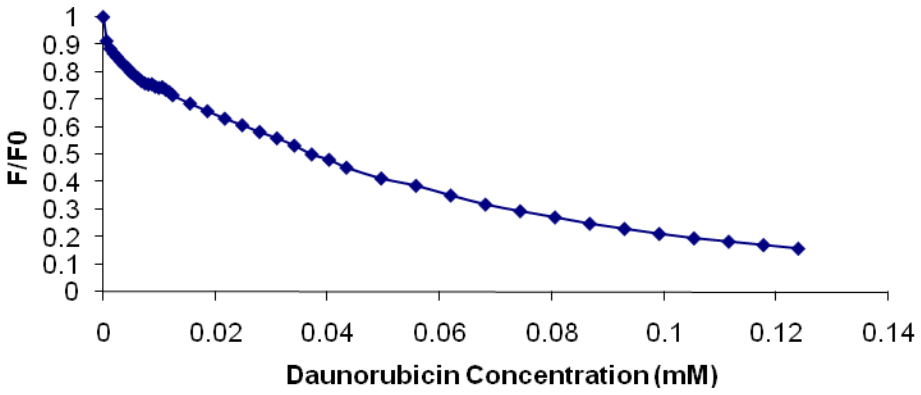


Coenzyme binding ability was expolored with fluorescence quenching studies. A 4µM M234C HCBR' solution was made and a 1mM solution of NADP$^+$ was added in 1µL increments. The fluorescence values were recorded and graphed. NADP$^+$ is preferred in fluorescence studies because it does not fluoresce in the same region as the protein. As the active site Trp 229 is quenched a decrease in fluorescence is seen.

Anthracycline Binding by Mutant HCBR

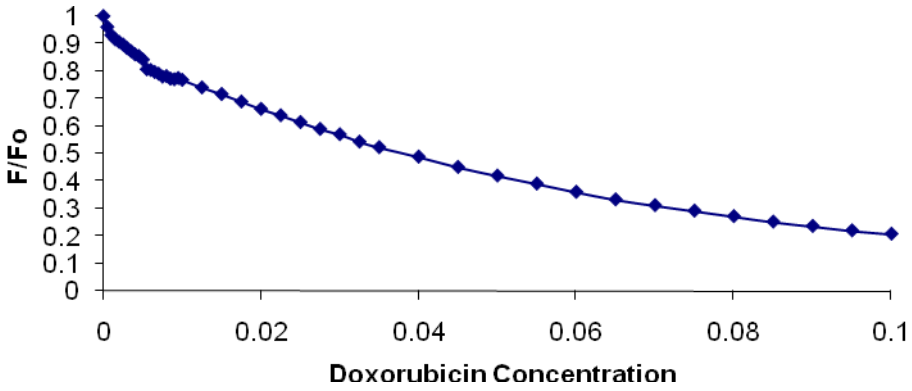
An intrinsic protein fluorescence quenching assay was done with the anthracyclines daunorubicin and doxorubicin. Both effectively quench the protein fluorescence (Figures 3.11 and 3.12). K_d values were found to be 35.8 µM and 31.5 µM for doxorubicin and daunorubicin, respectively (Table 3.2).

Figure 3.11: Fluorescence of M234C HCBR 1' and Daunorubicin



Substrate binding ability was expolored with fluorescence quenching studies. A 4µM M234C HCBR' solution was made and a 1.24mM solution of daunorubicin was added in 1µL increments. The fluorescence values were recorded and graphed. As the active site Trp 229 is quenched a decrease in fluorescence is observed.

Figure 3.12: Fluorescence of M234C HCBR 1' and Doxorubicin



Substrate binding ability was expolored with fluorescence quenching studies. A 4µM M234C HCBR' solution was made and a 1mM solution of doxorubicin was added in 1µL increments. The fluorescence values were recorded and graphed. As the active site Trp 229 is quenched a decrease in fluorescence is observed.

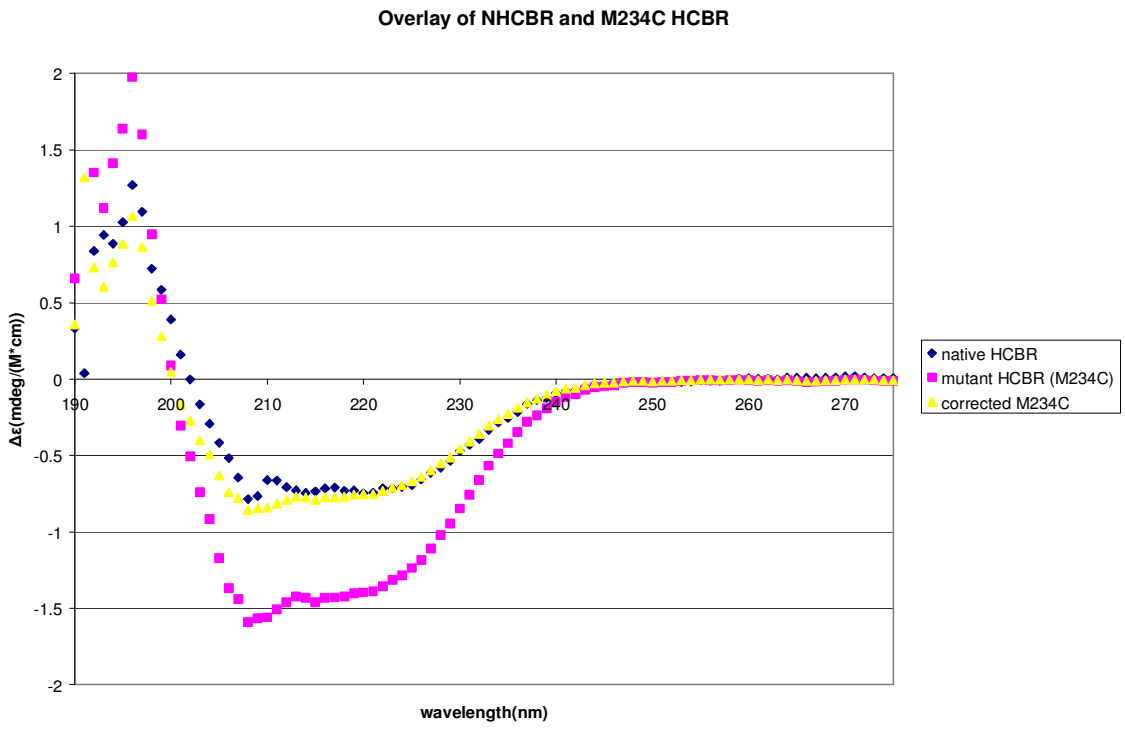
There is no previously published data from which a comparison can be made regarding these K_d values. The decrease in fluorescence as the substrate was added is good evidence that the substrates are able to bind the mutant. The K_d values can be deceiving; however, because this study doesn't insure that optimal binding for catalysis has occurred in the active site. A substrate can bind without optimal position for catalytic reactions to occur but still induce fluorescence. Based on comparison to the anthracycline K_d values obtained for the native HCBR', there may be a modest decrease in anthracycline binding affinity when methionine 234 is mutated to cysteine. The results from these experiments do not support the hypothesis to specific aim 3. It was hypothesized that the M234C HCBR 1' would allow more room in the active site for the

substrate doxorubicin to bind. However, K_d values in this study do not show a significant difference between the two anthracycline substrates.

Circular Dichroism of Native HCBR' and M234C HCBR 1'

The circular dichroism technique was used to assess any difference in secondary structure between the NHCBR' and the M234C HCBR' enzyme. These enzymes are the same with the exception that the mutant has a cysteine amino acid at position 234. This experiment was trying to confirm that the mutated enzyme was folded correctly. It was found that there was no significant difference in secondary structure between the two enzymes (Figure 3.13).

Figure 3.13: Circular Dichrosim of Native and M234C HCBR 1'



It was found that there was no significant difference in secondary structure between native and M234C HCBR 1'.

This may be evidence that the mutated enzyme is folded correctly in reference to the NHCBR' enzyme. This is evidence that the lack of activity that is seen in the mutant compared to the native may be indicative that amino acid 234 has an important role in docking substrates in the active site. It has been observed that when the methionine 234 is mutated to a cysteine that there is no observable activity in the mutant.

Conclusion for Results

The results of these studies indicate that the NCHBR 1' enzyme does not behave the same as the previously published HCBR1 enzyme.[49] This histidine modified enzyme; however, still shows activity with the known substrate menadione and NADPH, even though the binding of the NADPH molecule may be somewhat perturbed. The M234C HCBR' enzyme also has been affected by the addition of the histidine tag; however, when compared to the native enzyme with histidine tag the mutant does not show any observable activity. This difference in kinetic behavior between these two histidine tagged enzymes is important and may be preliminary evidence that suggests that position 234 in the HCBR 1 enzyme is an important amino acid that helps control substrate binding in the enzyme active site.

CHAPTER 4

DISCUSSION

DESIGNING A POOR ANTRHACYCLINE SUBSTRATE FOR HUMAN CARBONYL REDUCTASE 1

Application of the Structure-Based Drug Design for HCBR 1

Structure based drug designs give researchers an information guided approach to the study of their biological target, quite often times a protein. Bioinformatics servers store large amount of data about a protein that many separate researchers are studying. Structural information can be used to categorize the protein into a protein family. Protein families and superfamilies are composed of proteins that have a high degree of similarity due to conserved regions and domains found within the protein. Once a protein has been put into a family insights can many times be gained about the structure/function relationships of the protein based on the highly conserved regions and the corresponding functions in other members of that family.[70]

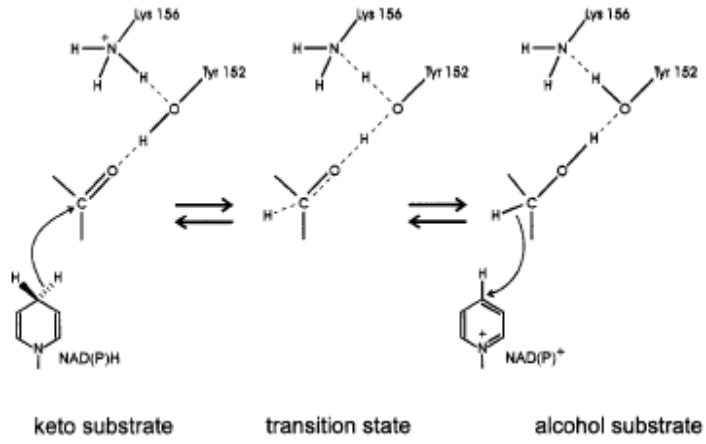
SDR Superfamily Characteristics

HCBR 1 (E.C. 1.1.1.184) is a member of the SDR superfamily of proteins which consists of over 3000 members. The SDR family is ubiquitous, occurring in both prokaryotic and eukaryotic organisms. They have a role in the metabolism of a wide range of biological compounds including alcohols, sugars, aromatic hydrocarbons, steroids, and prostaglandins. All SDR proteins share conserved primary structural

elements that indicate common fold, active site, reaction mechanism, and co-substrate/substrate binding regions. The general structure is a cofactor-binding region in the N-terminus, catalytic active site in the central portion and a substrate binding region of the C-terminus. Those members that are oligimers have a C-terminal extension that allows for binding subunits. The C-terminal region of the SDRs has the greatest variability.[71]

The catalytic residues of SDRs are found in a region comprised of a conserved Tyr-x-x-x-Lys region. The Tyr residue exists in an ionized form which is stabilized by the side chain of the Lys (Figure 4.1). The positive charge of the Lys attracts the oxygen of the carbonyl substrate establishing a partial positive charge on the carbon and a partial negative charge on the oxygen. This allows for a nucleophillic attack from the hydride of the NADPH nicotinamide ring.[72]

Figure 4.1 Nucleophillic Attack of the Substrate



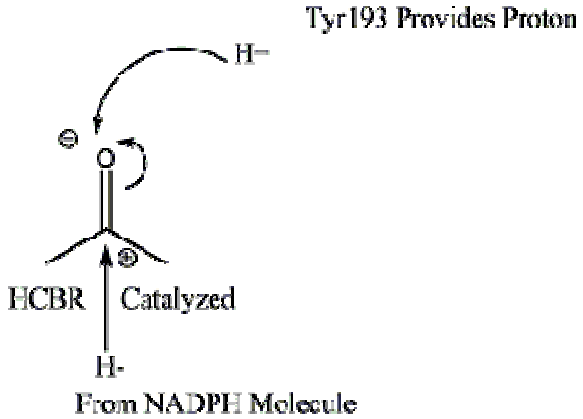
The active site of SDR proteins have a conserved Tyr-x-x-x-Lys residues. The sidechain of the Lys residue stabilizes the ionized form of the Tyr residue. The oxygen of the carbonyl group is attracted to the Tyr residue creating a slightly negative charge on the oxygen. This leaves a partial positive charge on the carbon. The H atom of the NADPH initiates a nucleophillic attack on the carbonyl group and a proton is donated from the Tyr residue. [72]

Analyzing the HCBR 1 Crystallized Structure

The RCSB Protein Databank has a x-ray crystal structure of HCBR 1 submitted by Tanaka et.al.[67] This structure is important to the research discussed in this thesis because it has both a bound inhibitor molecule and the bound coenzyme, NADPH. This information confirms the active site for both the cofactor NADPH and the active site for the substrate. Examination of this active site structure of HCBR 1 has lead to the identification of what are believed to be the catalytic amino acids.

The HCBR 1 enzyme catalyzes the transfer of the pro-4S-hydrogen atom of the nicotinamide ring of NADPH to the carbonyl carbon of the substrate.[59] In the literature a catalytic triad of amino acids have been described, namely Tyr 193, Ser 139, and Lys 197.[56,67,73] This region is a homologous region that has been found throughout the carbonyl reductase families. The role of these enzymes is to facilitate the movement of a proton from the hypothesized proton donor, Tyr 193, to the oxygen of the substrate carbonyl through a hydrogen bonding network (Figure 4.2).

Figure 4.2: Daunorubicin Reduction by HCBR 1



HCBR 1 catalyzes the reduction of anthracyclines at the C-13 carbonyl. A hydride is transferred from the Pro-4S-H atom of the NADPH nicotinamide ring. The hydride acts as a nucleophile that attacks the slightly positive C-13 carbonyl. This pushes electrons from the double bond to the oxygen. The oxygen accepts a proton from the Tyr 193 amino acid in the HCBR 1 active site.

Analysis of the Active Site of HCBR 1

The x-ray crystal structure of HCBR was used in the computer program Wincoot to dock the known substrate doxorubicin and daunorubicin. Using the bound inhibitor as a model the C-13 carbonyl that is known to undergo reduction by HCBR 1 was aligned in the correct position. The research conducted by Slupe et.al.[49] has established that the C-13 carbonyl of anthracyclines is the preferred substrate over that of the quinone carbonyls for HCBR 1. Examinations of the interaction of the anthracyclines and amino acids in the active site led to the identification of Met 234, Met 141 and Trp 229 as potential amino acids that play a significant role in recognizing and docking substrates in the active site. This analysis has also led to the hypothesis that site 234 selects substrates due to steric interference. It was noted that the anthracycline daunorubicin is a better substrate for this enzyme than doxorubicin represented by its higher catalytic efficiency value.[49] The only difference in these molecules is that daunorubicin lacks the alcohol group attached to C-13 side chain observed in the doxorubicin molecule. This difference in structure has led to the postulation that the alcohol group on the C-13 side chain of doxorubicin interferes with Met 234 and disrupts optimal binding for catalysis. It is hypothesized that this interaction leads to the lowered catalytic efficiency as compared to that for daunorubicin. This research project used site-directed mutagenesis procedures to mutate the amino acids to those with a smaller side chain to test the steric hindrance hypothesis. The research examining the changes in enzyme behavior due to the site-directed mutagenesis is still ongoing.

Recently Carlquist and coworkers undertook a computational chemistry experiment where they used the computer program ArgusLab 4.0.1 to dock known

flavonoid inhibitors into the active site of HCBR 1.[74] Their results also suggest that Met 234 and Trp 229 may play a significant role in binding substrates/inhibitors in the active site. Unlike our hypothesis, they suggest that these amino acids participate in chemical interactions with the substrates by anchoring the substrate with hydrogen bonds. They postulate that the Trp 229 residue participates in aromatic-aromatic interactions to increase affinity with the substrates.[74] Their recognition of these two amino acids as being important to substrate-enzyme interactions agrees with our hypothesis that these amino acids play a significant role in substrate binding. We do disagree with the mechanism that these amino acids use to interact with the substrate – especially the Met 234 site.

Receptor-Based Drug Design Cycle

The receptor-based drug design cycle focuses on small, nonprotein ligands. A 3-D structure of a protein-ligand complex is used as the basis for the rational design of a new ligand. The information that is gathered is then used to synthesize a new drug candidate with features that are believed to be beneficial. To test a hypothesis about the new drug design site-directed mutagenesis of the enzyme is often used and data about the enzyme-ligand interaction is collected.[75]

The research of HCBR 1 is also utilizing a receptor-based drug design. The literature states that HCBR 1 is known to be a promiscuous enzyme having a large range of unrelated substrates in the laboratory. Although a lot of substrates have been identified in the laboratory setting the physiological role and the actual physiological substrates have yet to be identified.

In this project we are aiming to learn about the chemical interactions that occur between the anthracyclines and HCBR 1. Concerning the Met 234 site we believe that this amino acid sterically hinders the binding of doxorubicin due to the extra alcohol group on the C-13 side chain leading to a lower catalytic efficiency compared to daunorubicin. The end result is the hope that a new anthracycline drug analog can be designed that changes the C-13 side chain perhaps by adding a bulky residue.

Designing analogs of anthracyclines is not new. Over 2000 anthracyclines have undergone similar modifications.[1] The goal of all of these analogs was to reduce the cardiotoxic side effects that are observed in anthracycline treatment.[2] Researchers have added large chemical groups to different parts of the drug such as the amino group, the sugar moiety or the aglycone group.[2] The hope was that the anthracycline derivatives would have a lower cardiotoxic side effect but have the same or improved cytotoxic properties.[1] Data has shown that these derivatives have not always changed the cardiotoxic properties, on an equivalent drug dosage, and in many cases have significantly decreased the cytoxic properties of the anthracycline.

We hope that our approach will result in a different outcome for our designed anthracycline derivative. The C-13 side chain is one region that has not been altered in the other derivatives. We believe that our rational drug design has given substantial support for the changing of the C-13 side chain. If the future kinetic data supports our hypothesis, we would consider adding a bulky group to the C-13 side chain to prevent the binding of the anthracycline in the active site of HCBR 1. This in turn would prevent the metabolism of anthracyclines by HCBR 1. We believe that this analog would directly

address the issue of cardiotoxicity because data suggest that C-13 metabolism is a major driving force in the cardiotoxic side effects seen in anthracycline treatment.

Design of Ineffective Substrate

The goal of this research project is to collect information about the mechanism of HCBR 1 anthracycline specificity so that a new anthracycline analog can be designed. The goal is that the new analog is an ineffective substrate for HCBR 1. The result will be a new anthracycline analog that no longer fits into the active site and resists metabolism to an alcohol product. The challenge is designing an anthracycline that maintains its cytotoxic behavior and reduces or eliminates its cardiotoxic side effects.

Research shows that there is significant evidence that the HCBR 1 enzyme has a significant role in metabolizing anthracyclines to an alcohol product in the cell. There is significant evidence that this alcohol product is a significant cause of the cardiotoxicity observed in the heart tissue of some patients. This research suggests that the C-13 side chain has an important role in the binding of the anthracycline to the HCBR 1 enzyme and modifications to the anthracyclines C-13 should be explored with the end goal of creating an ineffective substrate for HCBR 1.

Analyses of the Activity of M234C HCBR 1'

Mutating Site 234 to Cysteine Yields No Discernable Activity to Date

To explore the relationship of the Met 234 and its interactions with substrates the HCBR 1 enzyme was successfully mutated to Cys 234, Ala 234, Ser 234 and Trp 234. To date only the activity of M234C has been addressed. Research is ongoing to explore the activity changes of the Ala 234, Ser 234 and Trp 234.

The pTrcHis2 TOPO system was used to add the histidine tag to the end of the HCBR 1 gene. The histidine tag was successfully added to the C-terminus of the protein. This kit also adds two extra amino acids to the N-terminus and 23 extra amino acids before the six histidines on the C-terminus end of the protein. The extra 23 amino acids are epitopes that are used to bind antibodies for western blot procedures. There is more than one way to attach a histidine tag to the end of a protein including vectors like the pTrcHis TOPO as well as conventional PCR reactions. Addition of a histidine tag by PCR utilizes primers designed with a histidine overhang. As the gene is amplified the histidine tag is incorporated into the gene. The pTrcHis TOPO kit was chosen because it has been used in a neighboring lab without significant impacts on the expression of the protein.

Possible Complications Inhibiting M234C Activity

During the course of this research it was found that the histidine tag added by the vector may be interfering with the activity of the NHCBR. It has been found that choosing to use the pTrcHis2 TOPO vector system has proved to have some inherent problems for this enzyme. Our data shows evidence that the histidine tag got in the way of catalysis by interrupting the binding of the NADPH cofactor. This discovery complicates the analysis of results seen for the 234 mutations.

Several of the results suggest that the M234C mutation does not have activity that can be measured by the techniques used in this laboratory. The His-tagged native HCBR protein, even though activity was decreased due to the histidine tag, did show a measurable amount of activity. It remains possible that if other more sensitive techniques such as fluorimetry for measuring enzyme activity were used, perhaps activity for

M234C could be measured. It is worth noting that M234C is the only 234 mutant that was fully characterized. There is preliminary data to suggest that the Ala 234 mutation also appears to lack activity; however, more studies need to be done to support this claim.

If the result that the M234C HCBR' has no activity holds true then the following may be concluded. One possibility is that the Met 234 makes an important interaction in positioning compounds in the active site as previously suggested.[74] If so, our hypothesis that Met 234 acts to restrict the size of substrate that can productively bind in the active site through sterics is not correct. Another possibility is that Met 234 assists in coenzyme binding in some way and its role is essential to the catalytic activity of the enzyme. This option is supported in part by our fluorescence quenching studies. And yet another plausible explanation is that Met 234 is required for the structural integrity of the active site and that changing it to cysteine disrupted this structure, thereby rendering the enzyme inactive. More work needs to be done in order to resolve this problem.

M234C May Display Substrate Binding Ability

After the realization that the histidine tag interfered with the native HCBR 1' expression fluorimetry studies were done to try and assess the ability of the NADPH and $NADP^+$ cofactor binding and substrate binding independent of each other. K_d values, a rate constant that represents the dissociation of substrates from enzymes, were generated from the data collected. A smaller K_d value indicates a greater affinity for that substrate.

Due to the fact that the environment of the coenzyme binding pocket and the substrate binding pocket are different K_d values cannot be directly compared between these two different active sites in the enzyme. However, the experiments are trying to

assess the ability of both the native and M234C HCBR' enzyme to bind these substrates independently.

In the native HCBR' $NADP^+$ has twice the affinity than the NADPH molecule. We were expecting that $NADP^+$ and NADPH would bind the enzyme with similar affinity. We choose to use both the NADPH and $NADP^+$ because NADPH fluoresces in the same region as the HCBR 1 enzyme, whereas $NADP^+$ does not fluoresce in the same region. $NADP^+$ only differs from NADPH by one hydrogen and it possesses a positive charge on the nicotinamide ring. It is known that the $NADP^+$ is able to bind the HCBR 1 enzyme lacking the histidine tag. The fact that we see a difference in affinity between these two coenzymes in the HCBR' is surprising and may indicate that this form of enzyme is not binding the coenzyme as efficiently as HCBR. The results do show that the HCBR' is not completely unable to bind the coenzyme. This information coupled to the fact that the HCBR' has decreased activity may indicate that the coenzyme binding site has been interrupted by the addition of the histidine tag.

The M234C HCBR' was expected to have the same K_d values for the coenzymes. The mutations that are introduced at the 234 site are not hypothesized to affect the binding site of the coenzyme. M234C did not have as large of a difference between the affinities of NADPH and $NADP^+$; however, there was a difference between the K_d values. These may indicate that the M234C has different affinities for the two different coenzymes.

The native enzyme displayed more affinity for both the coenzymes as compared to the M234C. Considering the NADPH data the native HCBR' has twice the affinity than the mutant. For $NADP^+$ the native has four times the affinity than the mutant.

The hypothesis for this series of fluorimetry studies was that the native and M234C HCBR' enzymes would bind the coenzyme in the same or very similar manner. These data results raise the question as to why the native has more affinity for both coenzymes than the mutant. Both of these enzymes are in the same histidine tag system and are hypothesized to be affected by the histidine tag in the same manner; however, they clearly show a difference in the ability to bind coenzymes. This data may suggest that the M234C has some perturbation in the coenzyme active site leading to a decrease in binding ability of the coenzymes. More studies will need to be done to substantiate any changes in the mutant coenzyme site.

The coenzyme site is located near the N-terminus region of the protein and the histidine tag was added to the C-terminus of the protein. It was not assumed that the histidine tag would affect the coenzyme site in any way; however, I believe that there is evidence that the histidine tag is interfering with the active site in some way. Any changes that may have occurred in the coenzyme binding site have not been defined as of yet. The mechanism of the interference that the histidine tag may or may not have with the coenzyme site has not been defined as of now.

The ability of the native and mutant HCBR' enzymes ability to bind anthracycline substrates were also investigated. In the native HCBR' daunorubicin and doxorubicin have about the same affinities for the active site. Although it is important to consider that the doxorubicin error on this data is larger than the $NADP^+$ data. Steady state kinetics concerning the HCBR enzyme and anthracyclines indicate that daunorubicin is a better catalytic substrate than doxorubicin reflected by a larger catalytic efficiency value. Due to this information, I was expecting that daunorubicin would have a higher affinity for the

substrate active site than doxorubicin; however, the fluorimetry data does not support this idea.

The M234C enzyme has a similar trend concerning the affinities of the two anthracycline substrates. Daunorubicin and doxorubicin have essentially the same K_d value; although, daunorubicin has a slightly increased affinity. In this data the error values are very comparable as well. Unlike the native HCBR', activity studies of M234C indicate that the substrates are not undergoing a re-dox reaction. The fluorimetry data indicates that the mutant is able to bind anthracyclines although the binding may occur in a manner that does not facilitate catalysis reactions. These results may indicate that the catalytic machinery has been interrupted in some way in this enzyme. Further study and analysis will need to be done to understand what changes have occurred in the substrate active site of the mutant enzyme.

Comparing between the native and mutant form, the native has slightly more affinity for both daunorubicin and doxorubicin. The difference is not as great as compared to the coenzyme data between the two forms of enzyme. Suggesting that the binding ability of the two forms of enzymes are similar; however, steady state kinetics indicate that activity varies greatly. Investigation of Met 234's role in the catalysis reaction is warranted to understand the difference seen in catalysis but perhaps not in binding ability of substrates between native and mutant.

The doxorubicin data is very important to this study because our hypothesis was that we would see a greater ability for the doxorubicin to bind the M234C mutant and thus see an increased catalytic efficiency. The fluorimetry data does not support this hypothesis. The native has increased affinity for the doxorubicin molecule compared to

the M234C mutant. Both the steady state kinetic data and the fluorimetry data disagree with the stated hypothesis for specific aim 3. These results raise questions as to the role of Met 234 in the binding of substrates. Perhaps this interaction involves more than just a steric hindrance in the active site.

Overall, concerning the fluorimetry data the native HCBR' enzyme has more affinity for all four molecules used compared to the M234C form. All of the results were surprising and unexpected. The coenzyme data indicate that the histidine tag is interfering with the binding of substrates and the substrate data indicate that the M234C is not able to bind doxorubicin with greater affinity.

Circular dichroism studies were done to try and assess the folding ability of both the NCHBR' and M234C HCBR' enzymes. The folding of the enzyme was also trying to get at the question as to why the catalysis machinery has been interrupted. If the mutant enzyme is not folded correctly this could interrupt the catalysis ability of the enzyme. These experiments suggest that there is no difference in secondary structure between the native and the M234C HCBR' enzymes. Providing preliminary evidence that M234C HCBR' may be folded correctly but lacking catalytic activity with the menadione substrate.

Conclusion

It is difficult to assess the activity of the M234C due to the interruption posed by the histidine tag. The ideal situation would be to compare the data collected on the M234C HCBR' mutation to the published literature for the pET5a HCBR enzyme; however, due to the histidine tag complications it is not possible to do this comparison. Even though the chosen expression system is not an ideal system it is possible to compare

the data from the NCHBR' to the M234C HCBR' within reason. It is clear that the histidine tag is interfering with the binding of the NADPH cofactor; however, it is still possible to measure activity for the NHCBR'. The M234C mutation can be assumed to have the similar problems with the histidine tag interfering with NADPH binding. Unlike the NHCBR' the M234C does not have any measurable activity. This data lends some support and evidence that the 234 mutations have interrupted the catalysis ability of the HCBR 1' enzyme. Further study and analysis will need to be done to determine what roles the 234 position plays in the catalysis reaction.

CHAPTER 5

FUTURE WORK

IMPROVING THE HIS-TAGGED EXPRESSION SYSTEM FOR HCBR

Reducing the Histidine Tag

Several lines of evidence suggest that the pTrcHis2 TOPO system is not a good expression system for this protein. The result of this expression vector was a native protein that did not have the comparable kinetic behavior as that previously published.[49] The fact that the histidine tagged protein eluted off in a low imidazole concentration buffer may also suggest a weak binding of this histidine tag to the nickel resin, perhaps because of interaction with the remaining protein, thus obscuring the availability of the histidines to bind to the nickel resin. Overall the character of the protein is compromised and a new expression system must be explored.

Current research is ongoing to delete the extra DNA bases that code for the extra 23 amino acids that were added with the pTrcHis2 TOPO vector. There are two techniques that are being used to try and reverse this condition.

One technique utilizes the Phusion site-directed mutagenesis kit. With this kit primers are designed that flank the region that is to be deleted. One primer starts on the leading strand at the first histidine and extends the plasmid. The second primer binds the lagging DNA strand at the end of the HCBR 1 gene and the primer extends the plasmid. The result is that the region in the middle of the HCBR 1 gene and the His-tag (the aforementioned 23 added amino acid residues) is removed.

Another procedure that is being explored is to design a primer with a histidine overhang that is directly attached to the C-terminus of the HCBR gene. The overhang will be incorporated into the HCBR 1 gene and the result is that the gene is amplified and the histidine tag is added to the end of the gene. The amplified gene will then be inserted back into the pTrcHis2 TOPO expression vector.

If either one of these techniques work the six histidine tag will be added directly to the end of the gene. Our computer modeling techniques show that the last amino acid in the protein is on the surface of the HCBR 1 enzyme which is the desired position when adding a histidine tag. We believe that if the histidine tag can be successfully added to the last amino acid that the histidine tag will be on the surface of the protein and will be available to bind to the nickel resin. It seems likely that we will see an increased affinity for the histidine tagged HCBR 1 to the Ni^{++} resin and we will observe that an increased concentration of imidazole will be needed to elute off the protein.

When these experiments are complete we will be looking for several key changes in the native HCBR 1 protein with histidine tag. First we will be looking to see if the histidine tag binds to the nickel resin with a higher affinity. We expect the protein to require higher concentrations of imidazole for elution from the nickel resin because to date these proteins have been eluting off in a very weak concentration of imidazole. If the concentration of imidazole needed to elute the protein increases this will indicate a better binding of the protein to the nickel resin.

Next, kinetic assays will need to be done to fully assess the behavior of the newly constructed variant of the His-tagged HCBR 1 protein. In order to move forward, the kinetic behavior of the native must be the same or very similar to the published values for

HCBR kinetics. If this expression system results in restoring the kinetic behavior than this expression system will be a better expression system than the current pTrcHis2 TOPO system.

Assess the Activity of Mutated 234 HCBR 1

Once a protocol is established that will remove the extra amino acid residues added by the initial histidine tag then this procedure will be used to remove the extra bases from the Cys 234, Ala 234, Ser 234 and Trp 234 genes.

The proteins will be expressed in the established purification protocol. Steady state kinetics studies will be done to establish if there is or is not activity with these mutations. If there is observable activity than a k_{cat}, K_m and k_{cat}/K_m values will be determined for menadione, daunorubicin, and doxorubicin.

In the event that these mutations continue to lack activity than further experiments will need to be done to establish the condition of the proteins. One such experiment could include crystallization of the enzyme with a known substrate. This would give the researcher an ability to look at a 3-D representation of the enzyme and see if there are changes in the position of the substrate and the new interaction of the 234 side chain.

Met 141 and Trp 229 Mutated HCBR 1

It is hypothesized that three amino acids work in tandem to guide and dock substrates in the active site. These three amino acids are Met 234, Met 141 and Trp 229. Met 234 has been discussed extensively in this paper; however, it is important to mention the hypothesized role of Met 141 and Trp 229. They are also believed to restrict substrate binding in the binding pocket via steric hindrance although this mechanism has not been as clearly defined as the proposed mechanism of Met 234. The Met 141 and Trp

229 amino acids are observed surrounding the aglycone ring structure of both daunorubicin and doxorubicin in our computer based models. It is hypothesized that mutation of these amino acids, individually and in tandem, to amino acids with smaller side chains such as Cys, Ser or Ala that an effect on the catalytic efficiencies with known substrates will be observed.

Ongoing research indicates that the Met 141 mutants have activity with the known substrate menadione, unlike the M234C HCBR' enzyme. The 141 mutant enzymes are currently in the same histidine tag system as previously mentioned. This limits the ability to define steady state kinetic values as for 141 mutations as of now. Future experiments will involve making these mutations in an enzyme construct whereby a histidine tag does not interfere with enzyme activity. This system may include adding the histidine tag directly to the end of the enzyme in the same manner as the M234C. Another option is to remove the histidine tag completely from these specific mutations. Due to the fact that these mutations currently show activity it may be possible to purify these mutants using the standard anion exchange purification procedure outlined by Slupe et. al.[49] In this system the data could be directly compared to the published HCBR pET5a steady state kinetic values leading to the ability to define the changes that have occurred in these mutations.

Conclusion: Information is the Beginning of Drug Design

The results that are generated from the analyses of 234, 141 and 229 mutations will guide our understanding of the substrate-enzyme interactions in the active site. This information is crucial to the rational drug design approach that this laboratory has undertaken to design a new anthracycline analog. We believe that the information will

allow a unique design to the anthracycline that will lead to lowered cardiotoxic risk because the anthracycline analog will resist metabolism by HCBR 1.

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