THE ANTICANCER MODE OF ACTION OF THE QUINONE NATURAL PRODUCT
CRIBROSTATIN 6

BY

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DISSERTATION

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ABSTRACT

Cribrostatin 6 is a quinone-containing natural product that induces the death of cancer cell lines in culture, but its mechanism of action and scope of activity are unknown. Quinone based drugs have shown potential in the clinic, making cribrostatin 6 an interesting compound to study. The mode of action of cribrostatin 6 is investigated herein. Through a variety of experiments including cell cycle analysis, transcript profiling, and analysis of reactive oxygen species (ROS) production, a hypothesis for the mode of action of cribrostatin 6 is developed. ROS generation is likely the primary mechanism by which cribrostatin 6 induces apoptosis in cancer cells. The synthesis of cribrostatin 6 derivatives was undertaken using a previously published synthesis as inspiration. The cytotoxicity of the derivatives in cancer cell lines was determined, and several derivatives are equally as potent as cribrostatin 6. Given the success of certain ROS producers as anti-cancer agents, cribrostatin 6 and its derivatives have potential as novel chemotherapeutic agents.
**Table of Contents**

1 Reactive oxygen species as a cancer therapy................................................................. 1

1.1 Reactive oxygen species ............................................................................................. 1

1.2 Creation of ROS in the cell ....................................................................................... 1

1.3 ROS modulation in the cell ....................................................................................... 2

1.4 ROS and cancer cells ................................................................................................. 3

1.5 Determining ROS levels ......................................................................................... 5

1.6 Mechanisms of cell death related to ROS ............................................................... 7

1.7 ROS-producing compounds in clinical use............................................................... 8

1.7.1 *Compounds with unknown mechanisms of ROS creation* .................................. 9

1.8 Reductive activation of quinones by enzymes and environment ................................ 13

1.8.1 *Activation by 1-electron reduction* ................................................................. 13

1.8.2 *Activation by 2-electron reduction* ................................................................. 14

1.8.3 *Activation in hypoxic environments* ............................................................... 15

1.9 Bio-reductively activated clinical anticancer agents .................................................. 17

1.10 Compounds that are ROS scavengers and other mechanisms ................................ 21

1.11 The cribrostatin family of heterocyclic quinones .................................................... 22

1.11.1 *Known activities of the cribrostatin family* .................................................... 23

1.11.2 *Cribrostatin 6* .................................................................................................. 24

1.12 Conclusions ............................................................................................................. 25
1.13 References ................................................................................................................................. 27

2 The Anti-Cancer Mode of Action of Cribrostatin 6 ........................................................................ 33

2.1 Cytotoxicity evaluation of cribrostatin 6 in mammalian cancer cell lines ................................. 33

2.1.1 Treatment of cancer cell lines with cribrostatin 6: IC_{50} values ........................................ 33

2.1.2 Cytotoxicity of rapidly dividing and quiescent 3T3 cells treated with cribrostatin 6 .......... 35

2.2 Assessment of cribrostatin 6 in assays reporting on cell death ................................................. 36

2.2.1 Cribrostatin 6 induces apoptosis in cancer cells ..................................................................... 37

2.2.2 Cribrostatin 6 does not inhibit topoisomerases ................................................................. 38

2.2.3 Cribrostatin 6 does not intercalate DNA .......................................................................... 41

2.2.4 Cribrostatin 6 does not induce cell cycle arrest ............................................................... 42

2.2.5 ROS is produced rapidly in cribrostatin 6 treated cells ..................................................... 43

2.2.6 HMOX-1 is upregulated in cribrostatin 6 treated cells ....................................................... 44

2.2.7 Incubation of cribrostatin 6 treated cells in hypoxic environments .................................. 49

2.2.8 Effect of anti-oxidant treatment on the anti-cancer potency of cribrostatin 6 ................ 50

2.3 Other assays attempted ............................................................................................................. 51

2.3.1 Hemolysis ............................................................................................................................. 51

2.3.2 The effect of cribrostatin 6 on DNA synthesis in E. faecalis ............................................. 52

2.3.3 The Effect of Cribrostatin 6 on DNA Synthesis in U-937 Cells ........................................... 54

2.3.4 The effect of cribrostatin 6 against testicular cancer cell lines ....................................... 55

2.4 Conclusion: the anti-cancer mode of action of cribrostatin 6 ................................................. 57
3 Synthesis of Cribrostatin 6 and Its Analogs ................................................................. 68
  3.1 Previous syntheses of cribrostatin 6 ........................................................................ 68
    3.1.1 Nakahara and Kubo-- Linear synthesis of cribrostatin 6 ..................................... 69
    3.1.2 Markey and Kelly-- Aromatic cross coupling route to cribrostatin 6 .................... 69
    3.1.3 Knueppel and Martin-- Electrocyclic ring opening .............................................. 70
  3.2 Modified Nakahara and Kubo synthesis of cribrostatin 6 ..................................... 71
  3.3 Synthesis and evaluation of cribrostatin 6 analogs .................................................. 74
    3.3.1 Choice of derivatives ......................................................................................... 74
    3.3.2 Synthesis of unsubstituted quinones ................................................................. 76
    3.3.3 Non-polar side chain analogs ............................................................................. 77
    3.3.4 Polar side chain analogs ................................................................................... 78
    3.3.5 Reduced and bicyclic analogs .......................................................................... 80
  3.4 Biological evaluation of cribrostatin 6 analogs ...................................................... 81
  3.5 Further testing of derivatives ................................................................................ 83
  3.6 Future directions .................................................................................................. 86
  3.7 Conclusions ....................................................................................................... 87
  3.8 Acknowledgements ............................................................................................. 87
  3.9 References .......................................................................................................... 88
1 Reactive oxygen species as a cancer therapy

1.1 Reactive oxygen species

Reactive oxygen species (ROS) refer to a group of oxygen-containing molecules that include superoxide radical anions (O$_2^-$), peroxides, and hydroxyl radical (OH·) (Figure 1.1). Superoxide radical anions are the product of a 1-electron reduction and have short lifetimes in the cell as they react quickly with antioxidants or transform to another ROS, hydrogen peroxide.$^1$ Peroxides are the least reactive of the common ROS species, therefore, they can persist in the cell longer than superoxide anions and hydroxyl radicals.$^1$ Hydroxide radicals are uncharged oxygen radicals that also have short lifetimes but are very damaging, as they react readily with a variety of cellular macromolecules.$^1$ As will be discussed later, all these ROS can damage cells in multiple ways.

$$\cdot\text{O}-\text{O}^- \quad \text{H-\text{O}-\text{O}-H} \quad \cdot\text{O}-\text{H}$$

Figure 1.1 Lewis structures of ROS.

1.2 Creation of ROS in the cell

ROS production is a natural process of normal cells. Most ROS produced by normal cells are byproducts of the oxidative phosphorylation process in the mitochondria. In a normal cell, the mitochondria is the largest source of ROS because electrons can escape and react with molecular oxygen during electron transfer through the electron transport chain. As much as 2% of the oxygen consumed by the mitochondria for ATP synthesis is transformed into superoxide anions.$^2$ As noted in section 1.1, superoxide anions can react to form peroxides, which can then produce more highly toxic hydroxide radicals. In addition to the electron transport chain, ROS can be produced from detoxifying enzymes such as cytochrome P450s. ROS are normal
byproducts of vital cellular processes; however, as ROS can cause damage to the cell, endogenous cellular systems for scavenging ROS have evolved.

1.3 ROS modulation in the cell

Cells maintain redox balance and limit the amount of ROS in a cell by two mechanisms. The first way is through ROS scavengers glutathione and thioredoxin. Both molecules neutralize ROS by oxidation of dithiols, although glutathione is a small molecule and thioredoxin is a protein (Figure 1.2). The cell maintains a pool of both of these molecules to neutralize ROS, and there are different enzymes that recycle the glutathione and thioredoxin for use again through reduction of the disulfide bond.

Figure 1.2 Glutathione forms dithiols in the neutralization of ROS. Oxidized glutathione is reduced by the enzyme glutathione reductase.

The second method used to maintain cellular redox balance is through the ROS neutralizing enzymes superoxide dismutases (SOD) and catalase (Figure 1.3). SOD catalyzes the formation of hydrogen peroxide from superoxide. Although not as reactive as superoxide, hydrogen peroxide still is damaging to the cell because it can travel to the nucleus and oxidize nuclear DNA. Thus, another enzyme, catalase, transforms hydrogen peroxide into molecular
oxygen and water. Not immediately involved in cellular maintenance, although an integral part of this process, is NADPH which provides the reducing power for many of the enzymes involved in ROS neutralization.

\[ \cdot\cdot\cdot\cdot O - O - \xrightarrow{\text{SOD}} H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2 \]

**Figure 1.3** Two enzymes that neutralize ROS. Superoxide dismutase reduces superoxide radical anions to peroxide. Peroxide is then transformed by catalase to molecular oxygen and water.

Through the mechanisms of ROS scavenging and ROS neutralizing, normal cells are able to maintain a balance of ROS produced and ROS destroyed so that the cell can maintain homeostasis. If this balance is interrupted by reduction of the cell’s ROS buffering capacity or an increase in ROS, the cell could die from damage caused by ROS. Therefore, it is of the utmost importance to maintain the redox balance for cells to live.

### 1.4 ROS and cancer cells

It has been well established that cancer cells have a greater concentration of endogenous ROS than normal cells.\(^4^,^5\) There have been several theories to explain this phenomenon. One is that cancer cells are more metabolically active than normal cells, thus requiring a greater supply of ATP. Additional metabolic burden stresses respiration and the electron transport chain, creating more superoxide anion.\(^1\) In addition, in a damaging cycle, ROS can damage mitochondrial DNA, which causes mutations to members of the oxidative phosphorylation process and in turn produce more ROS.
As with all cells, ROS can damage lipids, proteins, and DNA; however, ROS are implicated in a much more insidious mechanism in the cancer cell. It is believed that ROS increase cancer cell proliferation, which encourages uncontrolled tumor growth. One mechanism proposed for this effect is that ROS interfere with the MAPK signaling pathway disrupting normal metabolic regulation thereby allowing uncontrolled metabolism and growth. A second way ROS promote cancer cell survival is DNA damage (Figure 1.4). ROS damage DNA beyond the repair capabilities of the cell so that mutations occur during replication that may be advantageous to cancer cell growth. As previously discussed, the cycle of mitochondrial DNA damage then codes for faulty respiration proteins, allowing greater ROS leakage. In addition to damage on a genetic level, proteins can be oxidized by ROS, damaging the mitochondrial membrane and the proteins involved in ATP synthesis which, in turn, promote further production of ROS.

Due to the sustained imbalance in oxidative stress in cancer cells, it is believed that this feature of cancer cells could be exploited for chemotherapeutic selectivity. As illustrated in Figure 1.5, increasing ROS in a cancer cell could tip the already strained redox balance, overpowering the ROS buffering capacity of the cancer cell and cause cell death. In contrast, in normal cells, the endogenous ROS buffering capacity can protect cells from some elevation in ROS levels.
Figure 1.5 A hypothesized mechanism for the selectivity of ROS for cancer cells. In normal cells, the redox buffering capacity can accommodate exogenous oxidative stress from a ROS producer. In cancer cells, the load of endogenous ROS is already straining the buffering capacity of the cell, causing redox imbalance and cell death when an exogenous source of ROS is added.

1.5 Determining ROS levels

ROS can be observed in living cells with dyes such as reduced acetylated dichlorofluoroscein (H$_2$DCFDA). This dye can diffuse into cells in its nonfluorescent diacylated form. Once inside the cell, enzymes remove the acyl groups trapping the dye inside the cellular
membrane, and peroxides oxidize the dye to its fluorescent form (Figure 1.6). The fluorescent product can be observed in live or fixed cells using either microscopy or cell flow cytometry.

![Chemical structures](image)

**Figure 1.6 Reduced diacyl dichlorofluorescein can be used as a ROS indicator.**

Alternatively, ROS can be indirectly sensed through use of N-acetyl cysteine (NAC). NAC is a precursor to glutathione, a major ROS scavenger. In addition, NAC is a radical scavenger in its own right. If cells in culture are co-treated with NAC and compound, levels of ROS (as seen by a dye like DCF) and transcription upregulation are generally reduced if the compound works through a ROS regulated mechanism.

A very powerful new biological method is transcript profiling. In this experiment, cDNA created from sample mRNA is hybridized to a microarray chip that contains thousands of oligonucleotides representing many genes. The transcripts that are upregulated in a cell can then be identified by fluorescence. Transcript profiling has recently been applied as a method to evaluate the effect of compound on thousands of transcripts, providing a unique signature to each compound.\(^{10,11}\) Frequently in ROS-producing compounds, the transcription of genes involved in oxidative stress, like heat shock proteins and thioredoxins, are upregulated.\(^{12}\) Commonly, ROS induction of genes is then confirmed by co-treating with NAC and observing reduction in the upregulation of these transcripts.\(^{13,14}\)
1.6 Mechanisms of cell death related to ROS

Reactive oxygen species induction as a direct method of inducing cancer cell death is emerging as an anti-cancer strategy.\textsuperscript{1,3,9,15,16} There are three basic ways ROS can affect a cell (Figure 1.7).\textsuperscript{1,9,17} In the first, cells exhibit ROS damage is as a byproduct from cytotoxins with another primary mechanism (e.g. cisplatin). Although ROS are produced during cisplatin treatment, the ROS are created after DNA crosslinking by cisplatin. These ROS probably contribute to cell death, but are not primarily responsible for the induction of cell death. The second, and most direct, way ROS induces cell death occurs when macromolecules in the cell are damaged by ROS created directly by reduction and oxidation of a compound. If sufficient ROS are generated, cells cannot recover from ROS-induced damage, and intracellular stores of antioxidant molecules are exhausted. The third major way cells die from ROS exposure is from compounds that induce a small amount of ROS that can cause downstream signaling for the actual molecules that induce cell death. In this chapter, and in the thesis in general, we will primarily be discussing compounds that act through the second mechanism, direct ROS production.
Figure 1.7 Cellular ROS induces death in at least 3 ways. ROS can be the downstream effect of common cancer drugs like camptothecin and paclitaxel after damage is done to DNA or microtubules. Alternatively, ROS can be produced directly by the anticancer drug. Finally ROS can be part of a signaling pathway that ultimately causes cell death.

1.7 ROS-producing compounds in clinical use

There are currently a handful of anti-cancer compounds in clinical trials or FDA approved that use ROS generation as a primary mechanism of cell death (Figure 1.8). The next two sections describe compounds currently in clinical trials as anti-cancer chemotherapeutics in which ROS is believed to have a primary cell death effect, but for which the mechanism of ROS production is unknown. The following section describes experimental therapeutics that produce ROS through redox cycling. The final section discusses ROS-producing molecules activated with enzymes. These sections set the stage for Chapter 2, where the mode of action of cribrostatin 6 will be investigated.
1.7.1 Compounds with unknown mechanisms of ROS creation

Elesclomol. The thiohydrazide elesclomol is a potent proapoptotic compound.\(^{14}\) In cancer cell lines, elesclomol treatment caused a 2.4 fold increase in ROS-related fluorescence of DCF-DA after 24 hours. Antioxidant treatment prevented the accumulation of DCF signal in cells, thus suggesting ROS production in cells.\(^{14}\) Transcription profiling of elesclomol-treated Hs294T melanoma cells shows upregulation of transcripts that are involved in redox stress, including HSP70 and metallothioneins.\(^{14}\) Hsp70 transcripts are increased when treated with elesclomol, but co-treatment with N-acetyl cysteine attenuates Hsp70 RNA induction.\(^{14,18}\)

Elesclomol has been evaluated in several clinical trials. A phase I clinical trial in patients with metastatic melanoma established a weekly tolerated dose of elesclomol at 213 mg/m\(^2\) when co-administered with 80 mg/m\(^2\) paclitaxel.\(^{19}\) In phase II clinical trials, elesclomol in conjunction
with paclitaxel treatment was evaluated in patients with stage IV metastatic melanoma. Progression-free survival was increased from 56 days for patients given monotherapy with paclitaxel to 112 days for the elesclomol/paclitaxel co-administration group. Elesclomol continued to stage III trials, however, these were closed due to safety concerns. The final analyses of this stage III trial are expected in 2010.

Arsenic trioxide. Arsenic trioxide is the most common therapeutic arsenical compound used today. This drug is most potent in acute promyelocytic leukemia for two reasons, both of which reflect the proposed modes of action of arsenic trioxide: APL is highly sensitive to ROS, and ROS cause differentiation of APL cells. APL cells have low levels of glutathione, thus less antioxidant buffering capacity, enabling ROS produced by arsenic trioxide to damage and kill the cell. It is believed that NADPH oxidase activity is induced by arsenic trioxide to produce ROS. Knockdown of P47PHOX, a component of NADPH oxidase, with siRNA indicated that NADPH oxidase is in fact the main source of ROS in arsenic trioxide treated cells, an observation supported by upregulation of NADPH components in transcription analysis. The second path arsenic trioxide takes as an anti-cancer agent is to differentiate APL cells through indirectly activating PML-RARα fusion protein. This causes the APL cells to mature to myeloid cells.

Arsenic trioxide has been found to be useful as a single entity agent for treatment of APL, with low incidence of drug resistance and high remission rates (>80%). Arsenic trioxide was approved in 2000 by the FDA for treatment of APL as a single agent, and many clinical trials are on-going to explore its use in conjunction with other chemotherapeutics and for treatment of other forms of cancer.
Motexafin gadolinium. Motexafin gadolinium is a porphyrin-like experimental therapeutic for the treatment of cancer. This compound is MRI active and accumulates in tumors over extended periods of time, although the serum half-life is quite short. Motexafin gadolinium is thought to induce cell death by producing ROS over the normal buffering capacity of cells. The gadolinium metal does not change oxidation state, but the organic macrocyclic ring produces \( \pi \)-radical anions that are stabilized by the electrophilic gadolinium. Reduction of redox sensitive molecules in the cell produces superoxide anions and depletes the cells of important antioxidants and redox sensitive metabolites, thereby causing cell death. In addition, motexafin gadolinium causes release of intracellular zinc from thioredoxin enzymes, which are zinc dependent enzymes, thereby inhibiting thioredoxin reductase and reducing the buffering capacity of cells.

Motexafin gadolinium is being actively evaluated in the clinic. There are have been several phase I and II studies on its effect on brain metastases in combination with whole brain radiation therapy (WBRT). Interestingly, motexafin gadolinium shows limited effectiveness in metastases that come from cancers other than non-small cell lung cancers. In the case of non-small cell lung cancers, there was a 5.4 month improvement in median time to neurologic progression with patients treated with WBRT and motexafin gadolinium rather than just WBRT. However, there was no significant increase in progression-free survival time.

Menadione. Menadione is a prototypical redox cycling molecule. In cells treated with menadione, an increase in the amount of ROS observed by DCF is attenuated by addition of NAC. Menadione can create ROS through at least two reduction pathways. The first is an unusual series of reactions as the quinone is directly modified by ascorbate as shown in Figure 1. Ascorbate can reduce menadione to the semiquinone. In turn, the semiquinone reduces...
molecular oxygen to produce superoxide anions with concomitant reoxidation of the semiquinone back to the quinone menadione.\textsuperscript{35} This process can continue until either the cellular stores of molecular oxygen or ascorbate is depleted. As ascorbate is relatively non-toxic and bioavailable, this redox cycling is a promising strategy. Indeed, the one study published so far using a combination of sodium ascorbate and menadione sodium bisulfite orally in radiotherapy and chemotherapy refractory advanced prostate cancer shows prostate serum antigen doubling times increase significantly in 13 of 17 patients.\textsuperscript{37} The drug combination has orphan drug status for treatment of advanced bladder cancer but has not been approved yet.

Also responsible for menadione’s toxicity is bioreductive activation. Cytochrome p450 reductase reduces the menadione quinone to a semiquinone that reverts with molecular oxygen to produce toxic superoxide anions (Figure 1.10).\textsuperscript{38} In the absence of exogenous ascorbate, this is how menadione exerts its anti-cancer action.\textsuperscript{34} Menadione can also be modified by the NQO1 enzyme, producing a non-toxic hydroquinone (Figure 1.10).\textsuperscript{39} An in depth discussion of the manner by which compounds are bio-reduced, and the effects of such products \textit{in vivo}, is provided in the next section.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.9.png}
\caption{Menadione can be chemical reduced by ascorbate in a cell to give superoxide radical anions. Figure adapted from Verrax et al.\textsuperscript{35}}
\end{figure}
1.8 Reductive activation of quinones by enzymes and environment

Quinones and many other types of molecules are affected by enzymes and their oxygen environment to modulate their biological activity (Figure 1.10). Following is a brief discussion of the two major enzymes involved in bioreductive activation as well as the effect of hypoxic environments on some anticancer agents.

![Figure 1.10 General quinone reduction scheme. A quinone can be reduced by 2 electrons to give the corresponding hydroquinone. Alternatively, reduction by 1 electron yields a semiquinone. Under normal oxygen levels, the semiquinone can oxidize to the quinone, in the process reducing O₂ and producing superoxide radical anions. Similarly, an equilibrium exists with the hydroquinone and quinone.](image)

1.8.1 Activation by 1-electron reduction

The predominant enzymes responsible for 1-electron reduction of anticancer drugs are the cytochrome P450 reductases. In an electron transfer pathway, the electron is transferred from NADPH to FADH to FMNH before being transferred by the enzyme to the quinone as illustrated in Figure 1.11. If the substrate is a quinone, the product from this reaction is a semiquinone. As described previously, the semiquinone can be oxidized to the parent quinone in the presence of oxygen, generating superoxide anions.
Figure 1.11  P450 reductase uses cofactors FADH and FMNH to transfer electrons from NADP to reduce a quinone by 1 electron. ⁴⁰

Cytochrome P450 enzymes are found in nearly all tissues in the body. These enzymes have many different functions and accept a varied collection of substrates that can be part of biosynthesis, degradation, and activation of both xenobiotics and endogenous compounds. ⁴⁰⁻⁴² Cytochrome P450s activate some investigational drugs to active molecules. It is important to note that cytochrome P450 reductases are different from cytochrome P450s. Using NADPH, the enzymatic heme core of cytochrome P450s reduces molecules by 2 electrons to form active species of some drugs. ⁴³ In contrast, cytochrome P450 reductase does not have a metal center and reduces molecules by 1 electron.

1.8.2 Activation by 2-electron reduction

NAD(P)H: quinone oxidoreductase 1 (NQO1, DT-diaphorase) is an enzyme that will typically reduce a quinone by 2 electrons, yielding a hydroquinone (Figure 1.12). ⁴⁴ This process is the major pathway for 2-electron quinone bioreduction enzyme for quinones. Unlike P450, NQO1 does not contain a metal center. Instead, the enzyme exerts its catalytic action through the cofactor FAD, NAD(P)H, and the enzyme pocket. ⁴⁵ NQO1 is thought to cause detoxification of many quinones, resulting in excretion of the typically less reactive hydroquinone; however, there are some compounds that are made biologically active by this 2-electron reduction process.
1.8.3 Activation in hypoxic environments

Due to a reduced number (or incorrectly formed) blood vessels, a rapidly growing tumor contains regions of hypoxia. Hypoxia is commonly defined at 0.1%-2% oxygen in the local environment, compared to ~20% oxygen in normoxic tissues. Often, hypoxia is of a transient nature and it is difficult to predict the location or duration of this condition. Hypoxia presents several challenges for treating solid tumors: reduced availability of drug because of reduced number and integrity of blood vessels, resistance to drugs due to slower cellular metabolism, and ineffectiveness of radiation treatments as oxygen is not present to fix DNA damage. However, as will be discussed later, the differences between normoxic and hypoxic cells offer a method of selective cancer treatment.
Semiquinones created via 1-electron reduction cannot be oxidized to quinones in the absence of oxygen. The net result is that the semiquinone accumulates, thus there is less hydroquinone. By testing compounds in cell culture in both hypoxic and normoxic environments, information about 1-electron vs. 2-electron bioreduction can be gathered. If the compound is less toxic in hypoxia, this indicates superoxides created by semiquinone reoxidation are the more active species in cell death. If the compound is more toxic in hypoxia, the product of the 1-electron reduction (semiquinone) or the 2-electron reduction product (hydroquinone) is the more potent species (Figure 1.14).
Testing compounds in hypoxia can yield information about the mode of action. Comparing the IC$_{50}$ values in hypoxia and normoxia can indicate if the superoxide anion is an important participant in cell death. Comparing the IC$_{50}$ of cells co-treated with dicumarol (an inhibitor of NQO1) in addition to compound can indicate if the hydroquinone or semiquinone is the more relevant species involved in cell death.

1.9 Bio-reductively activated clinical anticancer agents

To illustrate the above concepts of bioreduction, examples of anti-cancer compounds mitomycin C, tirapazamine, and AQ4N (Figure 1.15) will be discussed.

Mitomycin C. Mitomycin C (MMC) is an FDA approved drug for treatment of solid tumors. MMC can be activated by at least two enzymes to give the active hydroquinone that
alkylates DNA, causing cell death (Figure 1.16).\textsuperscript{49} The first pathway is thought to be predominantly catalyzed by cytochrome P450 reductase.\textsuperscript{50} This results in a semiquinone that then rearranges and reacts further to give the active alkylating species. In normoxia, the enzyme NQO1 converts MMC to the hydroquinone, which then goes on to form the alkylating species.\textsuperscript{51} MMC is a poor substrate for P450 reductase and NQO1, but the high concentration of these enzymes in the cell result in the rapid reaction of MMC with these enzymes.\textsuperscript{49} In hypoxic situations, the semiquinone followed by rearrangement in the preferred pathway; however, because both pathways lead to the active alkylating compound, MMC has nearly no selectivity for hypoxic cells over normoxic cells.\textsuperscript{52} Lack of hypoxic vs. normoxic cell selectivity is believed to contribute to the maximum tolerated toxicity of this agent.

Figure 1.16 MMC can be bio-reduced by two different enzymes to give the same alkylating agent. This phenomenon explains the poor hypoxic selectivity of MMC.
**Tirapazamine.** Tirapazamine is the prototypical bio-reductively activated, hypoxia specific anti-cancer drug. The structure contains two \( N \)-oxide moieties that are reduced by cytochrome P450s in hypoxia to amines.\(^{53}\) The \( N \)-oxides are enzymatically reduced to a highly toxic radical species after enzymatic processing, and these species damage DNA through both double and single strand breaks (Figure 1.17).\(^ {53}\) In normoxia, the tirapazamine radical reacts with molecular oxygen and reforms the parent compound and superoxide anions that are much less toxic than the tirapazamine radical.\(^ {53}\) In addition, molecular oxygen outcompetes tirapazamine \( N \)-oxides for the P450’s heme iron centers, thus reducing the amount of tirapazamine radical formed.\(^ {54}\) Both of these facets leads tirapazamine to be selectively toxic to hypoxic cells. Because of its specificity for hypoxic tissues, tirapazamine is being evaluated in clinical trials in conjunction with ionizing radiation therapy. In addition to killing hypoxic cells, tirapazamine serves an oxygenating agent to sensitive hypoxic cells to radiation therapy.\(^ {54}\) Currently in several clinical trials, there are mixed results in tirapazamine cotreatment with radiation and cisplatin.\(^ {54}\)

![Figure 1.17](image)

Figure 1.17  Tirapazamine is activated in hypoxia to a radical intermediate. The radical intermediate then can abstract \( H \) from a biomolecule which will induce cell death. In normoxia, molecular oxygen will react with the radical intermediate first causing superoxide anion formation and less cellular damage. Adapted from Brown.\(^ {53}\)
AQ4N. AQ4N (bis-N-oxide banoxantrone) (Figure 1.15) is a bio-reductively activated quinone prodrug whose reduction product AQ4 is more toxic in hypoxic environments. Thus, as for tirapazamine, AQ4N has been the subject of several clinical trials for the treatment of solid tumors. The reduced form of AQ4N, AQ4, is a stable molecule that both binds DNA and acts as a topoisomerase II inhibitor. It is important to note that AQ4N does not share DNA binding and topoisomerase II inhibition properties with its reduced form, AQ4. Unlike MMC, the quinone moiety does not undergo reduction, instead, the N-oxide groups on AQ4N get reduced to their amine form in two steps by 2-electron reductases, commonly cytochrome P450s (Figure 1.18). The mechanism of this reaction is proposed to involve iron (II) in cytochrome P450 that catalyzed the cleavage of the nitrogen-oxygen bond in the N-oxide. This molecule is more potent in hypoxia because environmental oxygen outcompetes the N-oxide for iron binding, reducing the rate of AQ4N reduction by P450s. It is important that the targeted cancerous tissue express high levels of P450s relative to normal tissue to produce selectivity, and this has been shown to be the case.

Figure 1.18  AQ4N is reduced by cytochrome P450 to its active reduced form AQ4. AQ4 is a potent topoisomerase II inhibitor, but AQ4N has no topoisomerase II inhibitory activity. Adapted from Patterson.
In clinical trials AQ4N has been shown to be tolerated in doses up to 20 mg/kg; 18 hours after dosing AQ4 is found localized in tumor tissues. Not unsurprisingly, due to its requirement for hypoxic tissue, AQ4N is not highly effective by itself. Currently a clinical trial is ongoing to investigate radiation and temozolomide treatment in conjunction with AQ4N.

1.10 Compounds that are ROS scavengers and other mechanisms

*Imexon.* Although originally investigated in the 1970’s, imexon has been recently re-enrolled into anti-cancer clinical trials (see Figure 1.8 for structure). Currently the compound is in phase II trials for the treatment of metastatic non-small cell lung cancer in combination with doxetaxel. Imexon-treated cells show an increase in superoxide radical and other ROS (as seen by microscopy), followed by apoptosis. This intracellular ROS enhancement is most likely due to imexon reacting with the pool of glutathione, thus, reducing the antioxidant capacity of the cell. In patients with various advanced metastatic cancers treated with imexon, peripheral blood mononuclear cells exhibit an upregulation of thioredoxin reductase-1, glutaredoxin-2, and peroxiredoxin-3. All of these genes are involved in the antioxidant function of the cell. Although imexon is an electrophile, it does not appear to alkylate DNA, but does react *in vitro* with cysteine residues and reduces the cellular levels of glutathione. The compound is not highly potent - the IC\textsubscript{50} in 8226 myeloma cells at 24 hours is 284 µM and 48 hours is 41 µM. The MTD was calculated to be 875 mg/m\textsuperscript{2} (~23 mg/kg in an average human) every 5 days to give a serum level of 53 ug/mL (477 µM).

*Fenretinide.* Fenretinide (Figure 1.8) is a synthetic retinoic acid-type molecule, however, its mechanism of action is different from all trans-retinoic acid (ATRA). First of all, fenretinide does not bind retinoic acid receptors in the way retinoic acids do since it lacks an acid moiety. However, fenretinide does affect transcription of these receptors. Unlike ATRA,
fenretinide produces ROS within 15 minutes of its addition to cells in culture, as measured with DCF.\textsuperscript{63} But while NAC reduces apoptosis caused by fenretinide, the actual role of ROS in cell death is unknown. Although fenretinide is less potent in hypoxia, which indicates a superoxide radical mechanism, this effect is subtle in a majority of cell lines tested and cell death can probably be explained by a biochemical pathway other than ROS damage.\textsuperscript{64} Ascorbic acid can completely rescue fenretinide-induced cell death, further suggests an indirect ROS-induced death.\textsuperscript{63} Ultimately, fenretinide causes build up of ceramide in cells, which induces apoptosis.\textsuperscript{65} Despite the uncertainty of its mechanism, fenretinide is in phase III trials for cervical and bladder cancer.\textsuperscript{66}

1.11 The cribrostatin family of heterocyclic quinones

There are several quinone-containing compound families found in nature. One cytotoxic quinone family is the cribrostatins, isoquinoline quinones isolated from the sea sponge \textit{Cribrochalina} (Figure 1.19).\textsuperscript{67-69} In addition to the original six cribrostatins, cribrostatin 7 has been isolated from \textit{Petrosia} sponge.\textsuperscript{70} Although grouped into the same family, the cribrostatins have a wide range of molecular complexity. Cribrostatin 4 contains three stereogenic centers, whereas all the other cribrostatins contain no stereochemistry. Interestingly, increasing molecular complexity does not correspond with increased anti-cancer potency. All members of this family have anti-neoplastic activity with \textit{IC}_{50} values in the range of 0.045-25 \textmu M against murine leukemia cell line P-388. These compounds are also anti-bacterial and anti-fungal. Of this family of compounds, we have chosen to investigate cribrostatin 6 due to its high potency and novel tricyclic imidazole isoquinoline quinone system.
Figure 1.19 The Cribrostatin family of toxic isoquinoline quinones.

1.11.1 Known activities of the cribrostatin family

There have been various reports in the literature regarding the potency of the cribrostatins in inducing death in cancer cell lines. Much of the data has been summarized in Table 1.1. Overall, cribrostatin 5 is the most potent compound in a variety of cell lines, with cribrostatin 6 also fairly potent.\textsuperscript{68} Cribrostatin 4 has a wide range of mediocre potencies ranging from 3.8 \(\mu\text{M}\)-42.5 \(\mu\text{M}\),\textsuperscript{68} and cribrostatin 7 seems to be the least potent with an \(\text{IC}_{50}\) in HCT-116 greater than 100 \(\mu\text{M}\).\textsuperscript{70}
Table 1.1  Anti-cancer activity of the cribrostatin family in cell culture compiled from the literature.  "-" indicates no report of data.

1.1.2 Cribrostatin 6

Cribrostatin 6 is a blue isoquinoline quinone isolated from the sea sponge *Cribrochalina* and originally reported by Pettit *et al* in 2003. From a dichloromethane extraction of the sponge, Pettit *et al* used P-388 murine leukemia cell death, as well as the blue color, to guide fractionation/purification of the sponge extract to obtain anti-cancer molecule cribrostatin 6. From 350 kg of wet sponge, 195 g of dichloromethane extract was obtained. From the extract, 88 mg of cribrostatin 6 was purified and characterized. Pettit *et al* took 10 years to deduce the structure of cribrostatin 6, owing to the difficulty in obtaining x-ray quality crystals.

Cribrostatin 6 has a broad range of anti-neoplastic activities. In addition to inducing death in cancer cell lines in culture (Table 1.1), cribrostatin 6 also has anti-bacterial and anti-fungal properties. As shown in Table 1.2, cribrostatin 6 can inhibit growth in a variety of pathogens, both sensitive and drug resistant bacteria and fungus. For larger lists of bacteria and fungus cribrostatin 6 has been tested against, see the referenced literature. In the course of these bacterial studies, the MTD of cribrostatin 6 in mice was determined to be between 750-1000 µg/kg when given two times per day in methanol diluted in PBS by I.P. injection for 5 days.
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>MIC in µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>16&lt;sup&gt;69&lt;/sup&gt;, 10.4&lt;sup&gt;72&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRSA</td>
<td>16&lt;sup&gt;69&lt;/sup&gt;, 2-16&lt;sup&gt;71&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>32&lt;sup&gt;69&lt;/sup&gt;</td>
</tr>
<tr>
<td>VRE</td>
<td>32&lt;sup&gt;69,71&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>2&lt;sup&gt;69&lt;/sup&gt;, 1-8&lt;sup&gt;71&lt;/sup&gt;, 10.4&lt;sup&gt;69&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>0.5&lt;sup&gt;69&lt;/sup&gt;, 0.5-16&lt;sup&gt;71&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td>2.6&lt;sup&gt;69&lt;/sup&gt;, 2&lt;sup&gt;69&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 1.2 MIC of cribrostatin 6 in selected bacterial and fungal strains.

### 1.12 Conclusions

The utility of quinones as anti-cancer *drugs* is unquestioned, as demonstrated by FDA-approved compounds such as doxorubicin, mitomycin C, and mitoxantrone. However, despite decades of study, the mechanism by which many cytotoxic quinone-containing compounds induce cell death remains unclear. The obvious electrophilic and redox-cycling properties of quinones has dominated many of these mechanistic investigations, and indeed several of these compounds appear to induce death via covalent alkylation of DNA,<sup>73-75</sup> whereas others generate cellular reactive oxygen species (ROS), leading to cell death.<sup>1,76,77</sup> In many cases evidence points to more than one of the above mechanisms of death being operational in quinone-treated cells. For example, doxorubicin both inhibits topoisomerase II and generates ROS through a 1-electron reduction pathway.<sup>78,79</sup> There is also evidence that doxorubicin forms covalent adducts with DNA in cell culture as well as in carcinomas of human patients.<sup>80,81</sup> MMC can be activated to a cytotoxic species through both 1- and 2-electron reduction pathways to give the MMC semiquinone and hydroquinone, respectively.<sup>82</sup>

Cribrostatin 6 is a quinone that is potent against cancer cells in cell culture, and it is the subject of this thesis. Specifically, my goals were to determine the mode of action of cribrostatin 6 in cancer cells and synthesize derivatives to find more potent compounds. Presented in the
following chapters are the results of these experiments. Chapter 2 outlines the battery of biological assays used to elucidate the mode of action of cribrostatin 6. The data indicated a ROS-mediated cell death mechanism, likely through a 1-electron reduction pathway. Chapter 3 discusses the synthesis of twelve compounds to explore the SAR of cribrostatin 6 and their evaluation against lymphoma and leukemia cell lines. The clinical successes of anti-cancer molecules with a ROS-generating mechanism suggests that analogous compounds could also be effective anti-cancer drugs.
1.13 References


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NIH; clinicaltrials.gov; Vol. 2009.


2 The Anti-Cancer Mode of Action of Cribrostatin 6

Sections from Chapter 2 have been taken from Hoyt, M.T.; Palchaudhuri, R.; Hergenröther, P.J. “Cribrostatin 6 induces death in cancer cells through a reactive oxygen species (ROS)-mediated mechanism” Investigational New Drugs 2010. Copyright Springer Science+Business Media, LLC

Cribrostatin 6 was synthesized then evaluated in a battery of biological assays with the goal of defining the mechanism by which it induces death in cancer cells. As described in this chapter, these experiments indicate that cribrostatin 6 has broad cytotoxic activity (including against both quiescent and rapidly-dividing cells), does not appreciably inhibit topoisomerase, and appears to induce cell death through the generation of reactive oxygen species.

2.1 Cytotoxicity evaluation of cribrostatin 6 in mammalian cancer cell lines

As described in section 1.11, cribrostatin 6 was identified as an anti-cancer agent in the P-388 mouse leukemia cell line.¹⁻³ To characterize its spectrum of anti-cancer activity, the toxicity of cribrostatin 6 was evaluated in a variety of cancer cell lines including HeLa (human cervical cancer), MCF-7 (human breast cancer), SK-MEL-5 (human melanoma), U-937 (human lymphoma) and HL-60 (human leukemia). Cribrostatin 6 used for all experiments described herein was synthesized as described in chapter 3.

2.1.1 Treatment of cancer cell lines with cribrostatin 6: IC₅₀ values

To determine the IC₅₀ values for cribrostatin 6, cells were incubated with compound over a range of concentrations (0-100 µM). After 24 hours, the cellular toxicity was determined by either a sulforhodamine B assay⁴ for adherent cell lines (HeLa, MCF-7, and SK-MEL-5) or MTS assay for suspension cell lines (U-937 and HL-60), and logistical dose-response curves were used to calculate IC₅₀ values. In general, cribrostatin 6 exhibited reasonably potent (≤10 µM) toxicity across cell lines from various origins, with U-937 cells being the most susceptible of the
cell lines tested (Table 2.1). The potency of cribrostatin 6 was also tested in HL-60 VCR, a HL-60 cell line that is resistant to vincristine and colchicine by virtue of enhanced expression of P-glycoprotein (Pgp).\(^5\) Pgp actively pumps many compounds out of the cancer cell, thus enabling the cell to resist the effect of many cytotoxins.\(^5\) Cancers with enhanced expression of multi-drug resistant markers like Pgp generally have a poor prognosis. Cell lines expressing Pgp are resistant to the effect of many cytotoxins; likewise, tumors expressing Pgp respond poorly to chemotherapy.\(^6\) The HL-60 VCR cell line used was created by treating HL-60 cells with increasing amounts of vincristine.\(^7,8\)

The IC\(_{50}\) value for cribrostatin 6 in the HL-60 VCR cell line was determined to be 4.8-fold higher than wild type HL-60; however, as shown in Table 2.1, the HL-60 VCR cell line was extremely (>50-fold) resistant to colchicine. Overall, cribrostatin 6 induces cell death across a variety of cancer cell types as assessed by its efficacy against cancer cell lines in culture.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cribrostatin 6 IC(_{50}) (µM)</th>
<th>colchicine IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa (cervical)</td>
<td>10 ± 0.7</td>
<td>-</td>
</tr>
<tr>
<td>MCF-7 (breast)</td>
<td>8 ± 2</td>
<td>-</td>
</tr>
<tr>
<td>SK-MEL-5 (melanoma)</td>
<td>9 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>U-937 (lymphoma)</td>
<td>0.6 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>HL-60 (leukemia)</td>
<td>6 ± 5</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>HL-60 VCR (multi-drug resistant leukemia)</td>
<td>29 ± 5</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Table 2.1 IC\(_{50}\) values for cribrostatin 6 against various human cancer cell lines, and the effect of colchicine versus HL-60 and HL-60 VCR. Cells were treated with compound for 24 hours then analyzed as described in the text. A dash indicates colchicine was not tested in that cell line. Error is standard error of the mean, n=3.
2.1.2 Cytotoxicity of rapidly dividing and quiescent 3T3 cells treated with cribrostatin 6

Chemotherapeutics typically target rapidly dividing cells, and these compounds are frequently inactive against quiescent cells, cells that are resting outside of the cell cycle. Due to this disparity in toxicity, quiescent cancer cells may be responsible for some tumor resistance to common therapeutics. Cancer stem cells are an example of a quiescent cell type that is able to survive chemotherapeutic treatment. In fact, the very trait of quiescence is what is believed to allow these cancer stem cells to resist the effects of chemotherapeutics. There is now a recognition that compounds that kill quiescent cells, such as cancer stem cells, could be of use. One potential application could be in combination therapies, where standard cytotoxins kill the rapidly dividing portion of a tumor and a compound toxic to quiescent cells kills the resistant cancer stem cells.

BALB/3T3 cells were utilized to evaluate cribrostatin 6 versus isogenic quiescent and rapidly dividing cells. These 3T3 cells rapidly divide until the cells contact each other, at which point they become quiescent. Thus, both low density and confluent 3T3 cells were treated with cribrostatin 6 to assess its activity against both actively dividing and quiescent cell types. As a comparison, several other chemotherapeutic agents (representing a variety of anti-cancer mechanisms) were also evaluated. For these experiments cells were incubated with compound for 72 hours, at which point cytotoxicity was determined by the sulforhodamine B assay and IC\textsubscript{50} values were calculated from logistical dose-response curves.

All anti-cancer compounds tested inhibited growth and caused death in rapidly dividing 3T3 cells with nanomolar to low micromolar IC\textsubscript{50} values (Table 2.2). However, when quiescent 3T3 cells were treated with the same compounds for 72 hours, paclitaxel, cisplatin, and etoposide induced no cell death or growth inhibition (IC\textsubscript{50} > 100 µM). Doxorubicin and MMC retained
cytotoxic activity against the quiescent 3T3 cells, although their potencies were reduced 60- and 16-fold, respectively, relative to the non-contact inhibited cells. In contrast, cribrostatin 6 had only minimally reduced (~2.6-fold) potency against quiescent 3T3 cells. Thus, cribrostatin 6 is able to kill 3T3 cells that are not actively dividing.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; vs. quiescent 3T3 (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; vs. dividing 3T3 (µM)</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisplatin</td>
<td>&gt;100</td>
<td>3 ± 0.8</td>
<td>&gt;33</td>
</tr>
<tr>
<td>cribrostatin 6</td>
<td>13 ± 2</td>
<td>5 ± 2</td>
<td>2.6</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>0.6 ± 0.03</td>
<td>0.01 ± 0.003</td>
<td>60</td>
</tr>
<tr>
<td>etoposide</td>
<td>&gt;100</td>
<td>0.05 ± 0.003</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>MMC</td>
<td>16 ± 2</td>
<td>1 ± 0.4</td>
<td>16</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>&gt;100</td>
<td>0.02 ± 0.005</td>
<td>5000</td>
</tr>
</tbody>
</table>

Table 2.2 Assessment of cell death induction in quiescent and actively dividing 3T3 fibroblasts. Quiescent 3T3 cells were grown in media until contact inhibited. Both quiescent and rapidly dividing cells were treated with compound for 72 hours followed by analysis of cell growth by with the sulforhodamine B assay. Error is standard error of the mean, n=3.

2.2 Assessment of cribrostatin 6 in assays reporting on cell death

Most investigations into the mode of action for anti-cancer quinones focus on some combination of DNA intercalation, topoisomerase inhibition, DNA alkylation, and reduction/oxidation-cycling properties. Indeed, several quinones appear to induce death via topoisomerase inhibition, others through covalent alkylation of DNA (sometimes proceeded by bioreductive activation), and others generate cellular reactive oxygen species (ROS), leading to cell death, as discussed in section 1.4.

In many cases, the data suggests more than one of the above mechanisms of death being operational in cells treated with anti-cancer quinones. For example, doxorubicin both inhibits topoisomerase II and generates ROS through a 1-electron reduction pathway. There is also evidence that doxorubicin forms covalent adducts with DNA in cell culture as well as in
carcinomas of human patients.\textsuperscript{24,25} As shown in Figure 1.16, MMC can be activated to a cytotoxic species through both 1- and 2-electron reduction pathways to give the MMC semi-quinone and hydroquinone, respectively.\textsuperscript{26}

2.2.1 *Cribrostatin 6 induces apoptosis in cancer cells*

To determine the mode of cell death induced by cribrostatin 6, annexin V/propidium iodide (PI) co-staining assay was utilized, enabling discrimination between apoptotic and necrotic cell death. U-937 cells were treated with cribrostatin 6 (10 µM) for 12 and 16 hours, then double stained with FITC-conjugated annexin V and PI and analyzed by flow cytometry (Figure 2.1). After 12 hours of cribrostatin 6 treatment a small population of cells (~9%) had either died or was in late stage apoptosis (Annexin V positive, PI positive), and ~24% of cells were in early stage apoptosis (Annexin V positive, PI negative). After a 16 hour exposure to cribrostatin 6, ~56% of cells were in the dead/late apoptotic quadrant, ~36% of cells were in early stage apoptosis, and only ~8% of the cells were viable. Thus, a significant population of cribrostatin 6 treated cells progress through the annexin V positive/PI-negative quadrant, strongly suggesting that these cells are dying through apoptosis.
Figure 2.1 Cribrostatin 6 induces apoptosis in U-937 cells. Cells were treated with 10 µM cribrostatin 6 for the indicated time, then stained with PI and annexin V-FITC and analyzed by flow cytometry. Population movement through the lower right-hand quadrant indicates apoptotic death. Data shown is representative of 3 experiments.

2.2.2 Cribrostatin 6 does not inhibit topoisomerases

Inhibition of topoisomerase enzymes is a known mechanism of approved cancer drugs doxorubicin, camptothecin, mitoxantrone, and etoposide;27-29 many topoisomerase inhibitors contain quinones.30 To determine if cribrostatin 6 inhibits these enzymes, in vitro inhibition assays with purified topoisomerases were performed. To assess the inhibition of topoisomerase I, purified topoisomerase I and supercoiled pBR322 plasmid DNA were incubated together for 30 minutes at 37°C. Topoisomerase I catalyzes the conversion of supercoiled plasmid DNA to relaxed circular plasmid DNA, and the supercoiled and relaxed forms of DNA can be resolved and readily visualized by agarose gel electrophoresis.28,31-33 As shown by the data in Figure 2.2a, cribrostatin 6 inhibits the topoisomerase I-induced relaxation of supercoiled pBR322 only at a concentration of 50 µM. In contrast, camptothecin, a known topoisomerase I inhibitor, inhibits the relaxation of pBR322 at a concentration of 10 µM. Cribrostatin 6 does not appear to be a strong inhibitor of topoisomerase I in vitro.

The inhibition of topoisomerase II can be similarly investigated using a gel electrophoresis DNA shift assay. In this assay, purified topoisomerase II was incubated with
catenated plasmid DNA (kDNA). Topoisomerase II decatenates the kDNA into single plasmids, and agarose gel electrophoresis resolves these two forms of DNA.\textsuperscript{34-36} As shown in Figure 2.2b, a 10 µM concentration of doxorubicin completely inhibited decatenation by topoisomerase II. In contrast, only at 50 µM did cribrostatin 6 inhibit decatenation of kDNA by topoisomerase II, indicating that cribrostatin 6 is not a strong inhibitor of topoisomerase II \textit{in vitro}. In total, the high cribrostatin 6 concentrations needed to inhibit the topoisomerase enzymes \textit{in vitro} suggest that topoisomerase inhibition is not a primary mechanism of cell death induced by this compound.
Figure 2.2 (a) Camptothecin is more potent than cribrostatin 6 in an *in vitro* topoisomerase I inhibition assay. Topoisomerase I and pBR322 supercoiled plasmid DNA were incubated in the presence of vehicle or compound for 30 minutes and then analyzed by gel electrophoresis. Lane 1 contains 1 µg DNA ladder. (b) Doxorubicin is more potent than cribrostatin 6 in an *in vitro* topoisomerase II inhibition assay. Topoisomerase II and kDNA were incubated for 30 minutes in the presence of vehicle or compounds. Lane 1 contains 1 µg DNA ladder.
2.2.3 Cribrostatin 6 does not intercalate DNA

To test if cribrostatin 6 interacts with DNA in a manner similar to known DNA binders doxorubicin and 9-aminoacridine, an *in vitro* ethidium displacement assay was performed based on the known increase in ethidium bromide fluorescence upon intercalation into DNA. DNA binding compounds will displace ethidium bromide and cause an overall decrease in fluorescence. As shown in Figure 2.3, addition of cribrostatin 6 to DNA induces only a small change (~20%) in ethidium bromide fluorescence; in contrast, doxorubicin and 9-aminoacridine show significantly more ethidium bromide displacement. Thus, cribrostatin 6 does not appear to intercalate DNA to the same degree as 9-aminoacridine or doxorubicin.

![Figure 2.3](image_url)

Figure 2.3 Interaction of known intercalators and cribrostatin 6 with DNA, as monitored by an ethidium bromide displacement assay. Error bars are standard error of the mean, n=3.
2.2.4  *Cribrostatin 6 does not induce cell cycle arrest*

Prior to death, cells treated with many cytotoxins arrest in a certain phase of the cell cycle;\cite{38,39} the phase of arrest can be a clue to the mechanism of action of a compound. To determine if cribrostatin 6 arrests cells in a certain phase of the cell cycle, four different cell lines were treated with increasing concentrations of cribrostatin 6 for 16 hours. Cells were then fixed and stained for the amount of DNA present using propidium iodide (PI), and analyzed by flow cytometry. Treatment of U-937, SK-MEL-5, MCF-7, and HeLa cells with cribrostatin 6 caused no significant changes in the levels of cells in each phase of the cell cycle within 16 hours relative to controls (Figure 2.4). This data indicates that cribrostatin 6 does not induce cell cycle arrest in these cell lines. The inability of cribrostatin 6 to induce cell cycle arrest corroborates the data suggesting that topoisomerase inhibition may not be the primary mechanism of action for cribrostatin 6, as small molecules topoisomerase inhibitors are known to arrest cells in distinct phases of the cell cycle. For example, camptothecin arrests cells in S and G2 phase due to DNA replication fork damage.\cite{40,41} Similarly, doxorubicin causes G2/M phase arrest through its inhibition of topoisomerase II.\cite{42} Mitomycin C is known to cause S phase arrest in HeLa cells, which is consistent with its DNA alkylating mode of action.\cite{43} Although most cytotoxins induce some form of cell cycle arrest, there are a handful of compounds known to effect cell death in a cell cycle independent manner.\cite{44-46}
Figure 2.4 Cribrostatin 6 does not cause cell cycle arrest prior to its induction of death. Cells were treated with varying concentrations of cribrostatin 6 for 16 hours after which time the cells were trypsinized, fixed/permeabilized, and analyzed via propidium iodide staining. No significant change in the distribution of cells in growth phases was observed. Error bars represent standard error of the mean, n≥3.

2.2.5 ROS is produced rapidly in cribrostatin 6 treated cells

Reactive oxygen species (ROS) can be a cause or an effect of cancer cell death. To determine if ROS production is an early effect of cribrostatin 6 treatment, and therefore more likely a cause of death of treated cells, U-937 cells were treated with the compound and the levels of ROS produced monitored with dichlorofluorescein diacetate (DCF), a non-fluorescent dye that reacts with peroxides to produce fluorescent dichlorofluorescein (Figure 1.6). The level of intracellular fluorescence produced from this dye was quantified in live cells by flow cytometry. As a positive control for these experiments, t-butyl peroxide was used, as it is known to create peroxides in cells. As shown in Figure 2.5, cells treated with cribrostatin 6 produced significant concentrations of peroxides in one hour, as indicated by DCF staining. Compounds
that do not induce ROS through their primary mechanism, such as etoposide, show no response in this assay (Figure 2.5). Mitomycin C shows a small, but significant response in DCF assays reported in the literature, however, this is over the course of 24 hours. The rapid formation of ROS suggests that it is directly produced by cribrostatin 6 and is not a byproduct of cell death.

Figure 2.5 Cribrostatin 6 produces peroxides in 10 minutes as observed with the dye DCF. U-937 cells (n = 250,000) were treated with compound for the time indicated and ROS was quantified with DCF and flow cytometry. t-Butyl peroxide is known to produce peroxides in cells, whereas etoposide does not. Error bars represent standard error, n=3.

2.2.6 HMOX-1 is upregulated in cribrostatin 6 treated cells

The analysis of global transcriptional changes in response to compound treatment can be used to identify cellular pathways affected by a small molecule, and in some cases can suggest a macromolecular target. Whole genome transcriptional profiling of human lymphoma U-937 cells treated with cribrostatin 6 (15 µM) for 6 hours was performed using the Illumina human HT-12 array. This concentration and time point were selected so that the data gathered could be compared to the Connectivity Map (CMAP), a database of small molecule transcript profiling
The CMAP has been used to aide mode of action studies for several compounds.

Analysis of the data from this experiment indicates that cribrostatin 6 treated cells had elevated levels of several antioxidant transcripts, including heme oxygenase 1 (HMOX1) (28.7 fold change) and various ferritins. These ferritins are under the transcriptional control of NRF2, a transcription factor that is activated under oxidative stress. The transcripts that were elevated and reduced (top 20 for both) in response to cribrostatin 6 treatment of U-937 cells are shown in Table 2.3. Other oxidative stress associated transcripts affected included the upregulation of sulfiredoxin 1 (SRXN1), oxidative stress induced growth inhibitor (OKL38) and the glutamate-cysteine ligase modifier subunit (GCLM) involved in glutathione synthesis. The down regulation of the transferrin receptor (TFRC) involved in iron uptake and the leucine zipper protein NF-E2 involved in hemoglobin production was also observed (Table 3).

HO-1 is a 32 kDa heat-shock protein that prevents cell death by converting heme to the powerful antioxidant biliverdin. Biliverdin production from heme produces carbon monoxide, a potential neurotransmitter, and free iron, which can serve as an oxidative stress signal. Biliverdin is also rapidly degraded into bilirubin, another potent antioxidant. Ferritins, which were also upregulated in cribrostatin 6 treated cells, are responsible for the storage of free iron in a soluble and non-toxic form in the cell.

In order to confirm that treatment of cells with cribrostatin 6 elevates HMOX1 at the protein level, a Western blot for HO-1 was performed on cells treated with cribrostatin 6 for six hours. Hemin, a compound that induces the transcription of NRF2 and HMOX-1, was used as a positive control for elevation of HO-1 protein. As shown in Figure 2.6, cribrostatin 6 treatment (at 15 and 30 µM) elevated HO-1 protein levels in U-937 cells. This elevation could be
prevented by co-treatment of cells with the antioxidant \( N \)-acyl cysteine (NAC), strongly suggesting that HMOX1 upregulation by cribrostatin 6 is ROS-mediated.

![Western blot for HO-1](image)

**Figure 2.6** Western blot for HO-1. Treatment of U-937 human lymphoma cells with cribrostatin 6 induces heme oxygenase 1 (HO-1) protein expression that can be prevented by the antioxidant NAC. Hemin is a known up-regulator of HO-1 protein levels. Cells were treated with hemin at 10 \( \mu \)M and NAC at 10 mM.

The gene expression signature of cribrostatin 6 treated U-937 cells was compared to analogous signatures for over 1300 biologically active small molecules; the macromolecular target is known for most of these compounds. Thus, use of this Connectivity Map database can allow the identification of the mechanism of action of bioactive molecules by comparison to the gene expression signature of molecules with known mode of actions.\(^{51}\) In the case of cribrostatin 6, the connectivity map did not yield strong matches (best score < 0.6) to the compounds in the database. Importantly, this database contains several quinones and multiple topoisomerase inhibitors, including doxorubicin, daunorubicin, mitoxantrone, etoposide, camptothecin, and irinotecan.

In the literature, menadione is known to upregulate p53 regulated genes, and antioxidant response element regulated genes.\(^{58}\) Doxorubicin has been shown to upregulate cystathionine B-synthase which is involved in homo-cysteine synthesis. Other doxorubicin
upregulated transcripts are lipoxigenase involved in inflammation, and DNA repair proteins. MMC treated tissue increases expression of leukocyte antigen related protein and NK4 both involved in immune response, and HSP70. Interestingly, cribrostatin 6 does not increase level of any of these transcripts upregulated by other quinone, ROS producing drugs.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Protein</th>
<th>Function</th>
<th>( p ) value</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMOX1</td>
<td>heme oxygenase 1</td>
<td>antioxidant</td>
<td>0</td>
<td>28.7</td>
</tr>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
<td>inflammation</td>
<td>0</td>
<td>4.7</td>
</tr>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
<td>inflammation</td>
<td>0</td>
<td>4.1</td>
</tr>
<tr>
<td>SRXN1</td>
<td>sulfiredoxin 1 homolog</td>
<td>oxidative stress</td>
<td>0</td>
<td>3.5</td>
</tr>
<tr>
<td>OKL38</td>
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<td>oxidative stress</td>
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Table 2.3 Top 20 up- and down-regulated genome-wide transcripts in human lymphoma U-937 cells treated with cribrostatin 6 (15 µM) for six hours. The gene symbol identification, protein associated with that gene, function of the protein, \( p \)-values corrected for multiple hypothesis testing using the False Discovery Rate method and fold changes in transcript versus DMSO control are shown in the table.
2.2.7 Incubation of cribrostatin 6 treated cells in hypoxic environments

Hypoxic environments can affect the cellular activity of a compound. In chapter 1, tirapazamine was introduced as an example of a molecule activated by hypoxic environments into a potent anticancer agent. Similarly, if a compound causes cell death by producing superoxides from molecular oxygen, then the potency of a compound should decrease in hypoxia. The mechanism of cell death attenuation is explained by a lack of oxygen to propagate the quinone redox cycle. To investigate if cribrostatin 6 displays differential toxicity in hypoxic and normoxic cells, HeLa cells were treated with cribrostatin 6 and incubated in a hypoxic (1% O\textsubscript{2}) or normoxic (ambient O\textsubscript{2} concentration, ~20%) incubator in 96 wells plates. After 48 hours, the plates were analyzed using SRB staining to determine IC\textsubscript{50} values. The toxicities of cribrostatin 6 in each environment were then compared. Tirapazamine was used as a hypoxia active control, and etoposide was used as a non-hypoxic sensitive control. As shown in Figure 2.7, tirapazamine is significantly more potent in hypoxia (by over 35-fold) and the potency of etoposide is not altered in hypoxic environments significantly. But, cribrostatin 6 is less potent to cells grown in hypoxia by more than 10-fold. The reduction in toxicity suggests that cribrostatin 6 produces superoxide anions through redox cycling that cause cell death.
Figure 2.7  Cribrostatin 6 and tirapazamine exhibit differential toxicity in hypoxic and normoxic environments in HeLa cells. Etoposide displays a very small difference in toxicity in hypoxic and normoxic environments. Graphs are representative data from three different experiments.

2.2.8 **Effect of anti-oxidant treatment on the anti-cancer potency of cribrostatin 6**

ROS can be indirectly sensed through use of N-acetyl cysteine (NAC). NAC is a precursor to glutathione, a major ROS scavenger, and NAC is a radical scavenger in its own right. If cells in culture are co-treated with NAC and a ROS producing compound, levels of ROS (as detected by a dye like DCF) and transcription upregulation are generally reduced if the compound induces ROS production. In addition, NAC can rescue cells in culture from ROS-mediated cell death including death caused by mitomycin C, doxorubicin, and menadione. To determine if NAC can rescue cells from cribrostatin 6 treatment, HeLa cells were pretreated with 5 mM NAC for 1 hour prior to treatment with cribrostatin or control compounds in 96 well plates. Cell death was determined by SRB staining after 48 hours.
incubation with compound. Cribrostatin 6 has the most dramatic rescue from cell death by NAC (Figure 2.8). Doxorubicin and menadione show a less potent effect that may reflect their secondary modes of action.

![Graph showing cell death vs. compound concentration with and without NAC](image)

**Figure 2.8** Cribrostatin 6 cell death is rescued by 5 mM N-acetyl cysteine. HeLa cells were preincubated with NAC (5 mM), and then treated with compound at the given concentration. Cell death was assessed 48 hours later by the SRB assay. Data shown is representative of 3 experiments.

### 2.3 Other assays attempted

#### 2.3.1 Hemolysis

Some small molecules will induce the lysis of red blood cells.\(^6^7\) This is a non-desirable trait in an experimental therapeutic, as drug-induced anemia can occur due to insufficient iron carrying blood cells.\(^6^8\) Common anti-cancer agents such as cisplatin, tamoxifen,\(^6^9\) and mitomycin C\(^6^8\) have been reported as causing anemia in cancer patients due to hemolysis. To determine if cribrostatin 6 causes lysis of red blood cells (RBC), cribrostatin 6 was incubated
with isolated human RBC for 2 hours. After this time, the samples were centrifuged for 2 minutes at 10,000 x g. If no lysis occurred, the RBC form a pellet in the bottom of the tube with a colorless supernatant, but if the RBC were lysed, the supernatant turned red due to the heme in the RBC. This color difference can be measured spectrophotometrically.

Cribrostatin 6 was found not to lyse RBC at 10 µM or 50 µM; complete lysis is induced in these experiments by using a hypotonic buffer (Figure 2.9). Interestingly, as a qualitative observation, the RBC pellets at the end of the experiment were very dark colored, a deep brown or black. This effect was not seen with any of the many compounds previously assayed in the Hergenrother laboratory. This might suggest that cribrostatin 6 is oxidizing the iron heme center of the RBC.

![Figure 2.9: Cribrostatin 6 does not cause hemolysis in human red blood cells in vitro. Error bars are standard error, n=4 separate experiments.](image)

**2.3.2 The effect of cribrostatin 6 on DNA synthesis in E. faecalis**

To further elucidate the mode of action of cribrostatin 6, its effect on DNA synthesis was examined using a \(^3\)H-thymidine incorporation assay. The first attempts at this assay were performed in *E. faecalis* strain BM4105 as cribrostatin 6 is known to have antibacterial activity
in *E. faecalis*. E. faecalis in exponential growth phase were treated with $^3$H-thymidine and antibacterials with different modes of action. The antibacterial controls were chosen based on their modes of action: ciprofloxacin inhibits DNA synthesis, tetracycline and rifampicin inhibit protein synthesis. As the bacteria grow, they incorporate the tritiated thymidine into their newly synthesized DNA. At the end of the experiment, the DNA from the bacteria was collected and the incorporation of tritium was quantified with a scintillation counter. Ciprofloxacin will cause a dose dependent reduction of $^3$H-incorporation levels in this experiment, as DNA synthesis has been inhibited. Conversely, tetracycline and rifampacin have little to no effect since they do not inhibit DNA synthesis.

When *E. faecalis* are treated with cribrostatin 6 in this assay, the effect is clearly different from the ciprofloxacin response (Figure 2.10). This indicates that cribrostatin 6 does not interfere directly with DNA synthesis in bacteria.

![Figure 2.10 $^3$H-thymidine incorporation into *E. faecalis* bacteria over a 20 minutes incubation with compounds.](image-url)
### 2.3.3 The Effect of Cribrostatin 6 on DNA Synthesis in U-937 Cells

In an analogous assay to that performed in *E. faecalis*, DNA synthesis inhibition was studied in U-937, a human lymphoma cell line. In this case, the cells were treated with $^3$H-thymidine and control compounds as follows: camptothecin as a topoisomerase I inhibitor, doxorubicin as a topoisomerase II inhibitor, cycloheximide as a protein synthesis inhibitor, actinomycin D as an RNA synthesis inhibitor. In this assay, camptothecin inhibits DNA synthesis; however, doxorubicin shows no effect in this assay which may be a reflection of the fact that it causes G2/M arrest in cells, not S phase arrest (Figure 2.11). Cycloheximide inhibits $^3$H-thymidine synthesis most likely because it is a potent inhibitor of cell growth. Cribrostatin 6 does not display a dose dependent effect in this assay, which may indicate that inhibition of DNA synthesis is not its primary mode of action.

![Figure 2.11](image)

**Figure 2.11** $^3$H-thymidine incorporation in U-937 human lymphoma over a 2 hour treatment with drugs. Error is standard deviation, n=3.
2.3.4 The effect of cribrostatin 6 against testicular cancer cell lines

In collaboration with Dr. Maroun Beyrouthy in the laboratory of Prof. Michael Spinella at Dartmouth University, cribrostatin 6 was tested against several testicular cancer stem cell lines. Testicular cancer is the most common solid tumor found in young men. Although testicular cancer is currently one of the most curable cancers, there is a subpopulation of patients with cisplatin-resistant malignancies who relapse from remission and thus have few therapeutic options.

To determine the efficacy of cribrostatin 6 at causing death of testicular cancer cell lines, cells were plated into a 96 well plate and incubated for 72 hours with cribrostatin 6 at the indicated concentrations (Figure 2.12). Cell lines used were testicular cancer stem cell lines NT2/D1, 577M, N2102Ep, cisplatin resistant testicular cancer stem cell line NT2/D1-R1, and multi-drug resistant lung carcinoma A549. Following incubation with compound, cell viability was assessed using a MTT assay. Cribrostatin 6 was a potent cytotoxin in testicular cancer stem cells, including the cisplatin resistant cell line NT2/D1-R1. In multi-drug resistant A549, cribrostatin 6 had no effect at these concentrations.
Figure 2.12 Cribrostatin 6 is potent against testicular cancer stem cell lines NT2/D1, 577M, N2102Ep, and cisplatin resistant testicular cancer stem cell line NT2/D1-R1. Cribrostatin does not have a cytotoxic effect on A549 multi-drug resistant lung carcinoma. Cells were incubated with cribrostatin 6 (indicated as Hc) at indicated concentrations for 72 hours then cell viability was determined by MTT assay. Data points are average of 3 biological replicates.

To further investigate cytotoxicity cribrostatin 6, testicular cancer stem cell line NT2/D1 was treated with 10 µM retinoic acid for 48 hours. Retinoic acid causes this cancer cell line to differentiate into cells with a neuronal phenotype. Treatment with retinoic acid slowed the growth of the cells, thus the differentiated and control cells were replated at the same cell number before treatment with cribrostatin 6. After 72 hours, cell viability was quantified using a CellTiterGlo assay. The data obtained indicate that cells with neuronal phenotypes are more sensitive to cribrostatin 6 treatment than testicular cancer stem cells (Figure 2.13).
Figure 2.13 Cribrostatin 6 is more toxic to differentiated testicular cancer stem cells. NT2/D1 cells were treated with either retinoic acid (10 µM) or vehicle for 48 hours. Cells were replated to provide an equal number of cells in all wells, and cells were treated with cribrostatin 6 at the indicated concentrations. Incubated for 72 hours with compounds before quantifying cell viability using CellTiterGlo (Promega). Data is average triplicate data.

2.4 Conclusion: the anti-cancer mode of action of cribrostatin 6

Cytotoxic quinones induce cell death through a spectrum of mechanisms, including topoisomerase inhibition and direct DNA damage. The data described herein suggest that the mode of action of cribrostatin 6 does not map onto any of these known cytotoxins. Cribrostatin 6 treated cancer cells die through apoptosis, as detected by positive annexin V/PI staining. In vitro, cribrostatin 6 has only weak intercalative and topoisomerase inhibitory properties, suggesting that DNA binding and topoisomerase inhibition are not primary modes of cell death induction. Indeed, these observations are consistent with data from cell cycle arrest experiments: cribrostatin 6 does not induce cell cycle arrest, unlike quinones such as doxorubicin, menadione, or mitomycin C. In addition, unlike most cytotoxins cribrostatin 6 is able to
induce death in both actively dividing and quiescent cells, suggesting a mechanism of cell death not related to, or dependent on, the cell cycle. Finally transcript profiling and subsequent CMap analysis reveals no match to compounds with known mechanisms of action.

The weight of the available evidence suggests that cribrostatin 6 induces cell death through a ROS-mediated mechanism. As measured with DCF, cribrostatin 6 induces cellular ROS within minutes of treatment. Consistent with this, global transcript profiling shows that HMOX1, an antioxidant gene, is the most elevated transcript in cribrostatin 6 treated cells, with a >28-fold upregulation. Western blots confirm that the HO-1 protein is elevated upon cribrostatin 6 treatment, and that this effect is abrogated upon treatment with the ROS scavenger NAC. Finally, cribrostatin 6 induces death in growing cells without arresting them in a particular phase of the cell cycle, and it is able to kill cells that are not actively cycling; both of these features are consistent with direct ROS generation.

As indicated by CMap results, cribrostatin 6 may share certain features of other quinone anti-cancer agents, but its mode of action is likely different. Similar to menadione cribrostatin 6 loses potency in hypoxia, whereas doxorubicin and MMC that have DNA damage as their primary mode of action retain potency to cells grown in hypoxia. Similar to both doxorubicin and MMC, cribrostatin 6 can kill quiescent cells, albeit at a reduced toxicity. None of the four quinones share transcripts that are upregulated, which is likely reflective of their different modes of action.

Based on the available evidence, the following mechanism is proposed for the anti-cancer activity of cribrostatin 6 (Figure 2.14). The proposed mechanism of action of cribrostatin 6 begins with 1-electron reduction to the cribrostatin 6 semiquinone. The semiquinone is oxidized to the quinone by molecular oxygen producing superoxide radical anions. The superoxide
radical anions are transformed into peroxides and other ROS, causing damage to the cell. Eventually, the damage is too great for the cell to repair, and it undergoes apoptosis. As discussed in chapter 1, such ROS generation could overload the buffering capacity of the cancer cell, while leaving healthy cells alive.

The compound with a mode of action most similar to cribrostatin 6 is menadione. Menadione exerts its cytotoxicity after bioreductive activation by cytochrome p450 reductases. Following reduction to the menadione semiquinone, toxic superoxide anions are created as the semiquinone reverts to the quinone consuming molecular oxygen. The damaging species is the superoxide anions as these can damage the cells and be converted into more reactive molecules. Menadione can also be modified by the NQO1 enzyme, producing a non-toxic hydroquinone. As discussed in chapter 1, menadione is currently in clinical trials in combination with ascorbate. Clinical results for patients with advanced, refractory prostate cancer and advanced bladder cancer have been positive, and the compound is moving forward in trials.

Remaining questions on the mechanism of cribrostatin 6 induced cell death include the identity of the enzyme that causes the one electron bioreduction and the possibility of alkylation of biomolecules after bioreduction. While it appears that cribrostatin 6 does not alkylate DNA in the quinone form (seen in experiments with DNA in topoisomerase assays) some enzyme could bio-reductively activate it. In this case, it would be necessary to determine which enzymes activate cribrostatin 6. Other important questions to answer are: What is damaged in the cell? Experiments to reveal what part of the cell is damaged could explain cribrostatin 6’s mode of action. Likely candidates are the nuclear DNA and mitochondria. What is the role of iron metabolism in cribrostatin 6 toxicity? Cribrostatin 6 can bind metals (an observation made
during synthetic work, although not quantified) and changes the color of red blood cells. This, combined with data indicating that many proteins involved in heme metabolism are upregulated on cribrostatin 6 treatment, could point toward a mechanism of action involving iron mediated cell death.

**Figure 2.14 Proposed mechanism of action of cribrostatin 6.**

The ability of cribrostatin 6 to induce death in quiescent cells could be an advantage in treating solid tumors. In solid tumors, there are quiescent cells called cancer stem cells. Cancer stem cells are resistant to many common chemotherapeutics, and cause regrowth of the tumor after withdrawing chemotherapy or radiation therapy.\textsuperscript{10,11,13} Destroying the population of cancer stem cells by treating with a compound that kills quiescent cells could improve remission rates for cancer patients. Although more animal studies need to be completed, \textit{in vivo} studies have shown that mice can tolerate cribrostatin 6 injected i.p. at 1 mg/kg twice daily for 5 days.\textsuperscript{71} It could be possible to decrease the dose limiting toxicity with a cribrostatin 6 conjugated cancer targeting agent.

Quinone anti-cancer drugs with a variety of modes of action such as doxorubicin, mitomycin C, and mitoxantrone, have shown utility in the clinic. Several of these quinones induce death through ROS-mediated pathways,\textsuperscript{20-22} and others exert their cytotoxic activity via covalent alkylation of DNA.\textsuperscript{17-19} Frequently, these are not the only modes of action. As
discussed in chapter 1, doxorubicin both inhibits topoisomerase II and generates ROS through a 1-electron reduction pathway,\textsuperscript{16,23} as well as potentially forming DNA adducts in human patients.\textsuperscript{24,25} Of the previously discussed anti-cancer quinone modes of action, cribrostatin 6 has only exhibited some form of bioreductive activation and production of ROS. Although this is the only known mechanism of menadione-mediated cancer cell death, and is thus sufficient for cell death, there is a possibility for additional cribrostatin 6 modes of action that should be explored.

In conclusion, cribrostatin 6 appears to induce death in cancer cells through a ROS-mediated mechanism. When compared to similar quinone drugs, cribrostatin 6 has certain attractive features, including its ability to induce death in non-dividing cells and in cells that are resistant to standard anti-cancer agents. Further investigations with cribrostatin 6 will help to shed light on the potential of ROS-generating compounds as anti-cancer agents.

2.5 Acknowledgements

I would like to thank Rahul Palchaudhuri for his help with completing transcriptional profile and western blot experiments.
2.6 References


(7) Ogretmen, B.; Safa, A. R. "Identification and characterization of the MDR1 promoter-enhancing factor 1 (MEF1) in the multidrug resistant HL60/VCR human acute myeloid leukemia cell line". Biochemistry 2000, 39, 194-204.


3 Synthesis of Cribrostatin 6 and Its Analogs

To obtain cribrostatin 6 for biological study, milligram quantities needed to be synthesized in the laboratory. The synthetic pathway used would also need to be versatile to enable the creation of cribrostatin 6 derivatives. These derivatives could be useful for further mechanistic experiments and to find a more potent cytotoxin or a compound with fewer side effects in vivo.

To explore the structure-activity relationship of cribrostatin 6, three families of derivatives were envisioned. The first contained substitutions off the methyl group on the 2 position of the imidazole ring, represented by compound 3.1 (Figure 3.1). The second is substitution of the methyl and ethoxy groups on the 8 and 9 positions on the quinone to provide molecules with both hydrogen and larger groups as substituents, represented by 3.2. The third is reduction or substitution of the double bond at positions 12 and 13 in the isoquinoline, represented by 3.3.

![Figure 3.1 Desired cribrostatin 6 derivatives.](image)

3.1 Previous syntheses of cribrostatin 6

There are currently three very different syntheses of cribrostatin 6 reported in the literature. The advantages and disadvantages of each from the point of view of the synthesis of derivatives is discussed below.
3.1.1 Nakahara and Kubo-- Linear synthesis of cribrostatin 6

Nakahara and Kubo were the first to report a synthesis of cribrostatin 6 and published a series of papers outlining their route.\textsuperscript{1-3} Their synthesis is linear over 10 steps, beginning from synthesized advanced starting material \textbf{3.4} (Scheme 3.1). At the center of their synthesis is a Pomeranz-Fritsch isoquinoline condensation to form isoquinoline \textbf{3.6}. The imidazole ring is formed by cyclizing amide \textbf{3.7} to give the penultimate product \textbf{3.8}. Synthesis of derivatives is difficult with this method as the synthesis is not convergent. Any change in substituents on the quinone must be made in the first step; however, substitution on the imidazole can be determined later in the synthesis.

![Scheme 3.1 Nakahara and Kubo synthesis of cribrostatin 6.](image)

3.1.2 Markey and Kelly-- Aromatic cross coupling route to cribrostatin 6

Markey and Kelly reported a cribrostatin 6 synthesis in 2009 utilizing a palladium catalyzed biaryl cross coupling to attach the imidazole ring to protected hydroquinone \textbf{3.10} (Scheme 3.2).\textsuperscript{4} After addition of a pendent allyl group, product \textbf{3.12} can form the framework of the isoquinoline in alcohol \textbf{3.13} by dihydroxylation followed by oxidative cleavage with concomitant aminal formation. Although this is a vastly different approach from Nakahara’s
synthesis, it contains nearly as many steps (13 from the commercially available material) and is hardly less linear. Any substitution on the quinone must be installed at the first steps of the synthetic pathway and the choice of imidazole substitution must be made early.

Scheme 3.2 Markey and Kelly synthesis of cribrostatin 6.

3.1.3 Knueppel and Martin—Electrocyclic ring opening

Knueppel and Martin published the most recent synthesis of cribrostatin 6 in 3 steps from three simple starting materials: alkyne 3.14, imidazole 3.15, and squarate 3.17 (Scheme 3.3). The key step in the synthesis is an electrocyclic ring opening of a squarate 3.18 which recondenses in a radical mechanism to form the complete skeleton of cribrostatin 6. This synthesis has the advantage of being convergent, allowing for high yields and many positions of substitution. The only drawback of using this synthesis is the limited commercial availability and synthetic accessibility of different squarates and imidazoles, minimizing the possible substitution on the quinone and imidazole rings.
Scheme 3.3  Knueppel and Martin synthesis of cribrostatin 6.

3.2  Modified Nakahara and Kubo synthesis of cribrostatin 6

When the studies described in this dissertation were started, the Martin route had yet to be published. Thus, to obtain cribrostatin 6 for our mechanistic experiments, it was synthesized in a route that closely follows the Nakahara route, although with a slightly different series of transformations. Our aim was to access cribrostatin 6 and derivatives as quickly as possible, not develop a novel natural product synthesis.

Scheme 3.4  Synthesis of aryl aldehyde starting material

Nakahara and Kubo had not sufficiently described the synthesis of their aryl aldehyde starting material, thus, we had to create a synthetic pathway to produce grams of aryl aldehyde 3.4 (Scheme 3.4). To achieve this goal, methyl resorcinol 3.9 was protected with ethyl bromide
to afford diethoxy toluene $3.19$ in 85% yield. This product could be produced on at least a 50 gram scale. Formylating the phenyl ring with tin and dichloromethyl methyl ether produced aldehyde $3.20$. Although not an environmentally friendly process, this reaction gave a pure product with no need to purify before the next step. Additionally, the reaction could be run on a 50 gram scale; however, the addition of dichloromethyl methyl ether produced an exotherm requiring slow addition and continual cooling. A phenol was then installed using the Dakin reaction: oxidation of the aldehyde to the formate ester with mCPBA followed by cleavage of the ester with NaOH to give phenol $3.21$. This procedure could be done on a 25 gram scale, although appropriate precautions need to be taken when using mCPBA in large quantities. The phenol product produced was quite impure, requiring large scale column chromatography to separate mCPBA side products from the phenol. Regioselective formylation of $3.21$ with dry magnesium chloride and paraformaldehyde yielded benzaldehyde $3.22$. Frequently, the benzaldehyde product was pure immediately after aqueous workup requiring no further purification. Interestingly, and as was noted previously,$^6$ this reaction will only work with beads of magnesium chloride, and not powder. Benzyl protection of the phenol afforded Nakahara’s advanced intermediate $3.4$.

The synthetic route to cribrostatin 6 from this point closely followed the Nakahara and Kubo synthesis (Scheme 3.2). A protected alkyl aldehyde was installed by reductive amination of the aryl aldehyde with aminoacetaldehyde dimethyl acetal followed by protection of the free amine with tosyl chloride to produce $3.5$. Once tosylation, the product could be purified by column chromatography to produce 20 grams of the product. The reductive amination product was pure after aqueous extraction. Protected aldehyde $3.5$ was then revealed and underwent nucleophilic attack by the open position of the phenyl ring followed by dehydration to give
isoquinoline 3.6 in a Pomeranz-Fritsch reaction. This step was the limiting step in terms of producing large quantities of intermediate materials. The maximum reliable scale for the conversion of 3.5 to 3.6 was 10 grams. Larger reactions would not give the desired product. The isoquinoline was purified by silica gel column chromatography. A nitrile was added to the isoquinoline by oxidizing the isoquinoline to the N-oxide followed by treatment with trimethylsilyl cyanide. Nitrile 3.24 was easily reduced to the primary amine dihydrochloride salt by palladium catalyzed hydrogenation to give advanced intermediate amine 3.25.

Scheme 3.5 Synthesis of amine 3.25, the key intermediate in the synthesis of several cribrostatin 6 derivatives.

To finish the synthesis of cribrostatin 6, treatment of 3.25 with acetyl chloride and triethylamine gave amide 3.26, which was readily converted to tricycle 3.27 with phosphorous oxychloride. Amide 3.26 was actually produced in a mixture that included tricycle 3.27. The compounds coelute by silica gel chromatography, so the mixture was subjected to phosphorous
oxychloride treatment to resolve the mixture. The acetyl group of 3.27 was saponified to afford phenol 3.8, which was then oxidized with concentrated nitric acid to provide milligram quantities of cribrostatin 6 whose spectra matched that from the original isolation paper.  

![Scheme 3.6: Synthesis of cribrostatin 6 from advanced intermediate 3.25.]

3.3 Synthesis and evaluation of cribrostatin 6 analogs

To explore the structure-activity relationship of cribrostatin 6, derivatives were synthesized by primarily changing the substituent at the 2-position of the imidazole ring. In addition, the derivatives were constructed to test the relationship between reduction potential and cytotoxicity, as described below.

3.3.1 Choice of derivatives

In quinone drugs, it is known that reduction potential is a factor in the toxicity of a compound (Figure 3.2). This relationship can be dependent on the mechanism of the quinone drug. For example, in mitomycin C type compounds that undergo 2-electron bio-reduction, there has been data both supporting and contraindicating a positive correlation between reduction potentials of the compounds and cancer cell potencies. This data is not surprising. As was discussed in chapter 1, MMC undergoes a 2-electron bio-reduction activating the compound to...
alkylate DNA, thus, ROS is not a major contributor to cancer cell death. However, in cardiomyocytes, there is a clear correlation between less negative reduction potential of MMC derivatives and increased cardiotoxicity indicating that toxicity to cardiomyocytes is primarily caused by ROS from a 1-electron bio-reduction. The same pattern may hold true for doxorubicin as it causes cardiotoxicity through a 1-electron bio-reduction ROS mechanism. In the case of napthoquinones that predominantly induce death through a 1-electron bio-reduction with concomitant ROS generation, there is a stronger relationship between less negative reduction potential and more cancer cytotoxicity.

![Diagram showing reduction potentials and bio-reduction processes](image)

**Figure 3.2** Reduction potentials are correlated with 1-electron bio-reduction, but not 2-electron bio-reduction.

One critical assumption is that compounds that have a higher chemical reduction potentials (more easily reduced) are also more easily reduced by bio-reductive enzymes. This has been found to be the case for quinones with 1-electron reduction potentials between -400 and -165 mV. In a separate study MMC analogues with less negative (higher) reduction potentials
were reduced in less time by enzymes xanthine oxidase and NADPH-cytochrome P450 reductase.\textsuperscript{10}

Given the previous assumptions, it follows that for compounds activated by a 1-electron reduction mechanism, the design of compounds with less negative reduction potentials could lead to more potent cytotoxins. State-of-the-art computational methods can be used to determine reduction potentials of quinones; however, these methods not simplistic enough for a synthetic chemist or cancer biologist to use.\textsuperscript{15,16} There is currently no convenient computational method to reliably calculate reduction potentials for molecules, thus, they must be empirically measured. All empirical data must be taken under the same conditions; we are in the process of measuring reduction potentials of all of the cribrostatin 6 derivatives and related compounds.

From data presented in chapter 2, there is good reason to believe that cribrostatin 6 is inducing cell death through a 1-electron bio-reduction pathway. Thus, if cribrostatin 6 is working as a pure 1-electron redox cycling compound, alterations in reduction potential could yield a predictable toxicity pattern for this set of derivatives. To test this hypothesis, a set of varied yet related compounds needed to be synthesized, tested in cytotoxic assays, and the redox potentials measured.

\subsection{3.3.2 Synthesis of unsubstituted quinones}

To begin synthesis of derivatives, the creation of a compound with hydrogens replacing the quinone substituents was first attempted. The Nakahara and Kubo synthesis was the guide during this synthesis which mirrors the previous cribrostatin 6 synthesis (Scheme 3.7).
Scheme 3.7  Synthesis of an unsubstituted quinone derivative.

The starting benzaldehyde was subjected to reductive amination to give amine 3.29. Prior to condensing to the isoquinoline ring 3.31, the amine was tosylated to give 3.30. A nitrile was added to give 3.32 which was reduced to give amine salt 3.33. Acetyl chloride could be coupled to the amine followed by refluxing in POCl₃ to give the penultimate imidazole product, compound 3.35. Unfortunately, oxidation of this product with CAN gave a decomposition product. Attempted oxidation with NaClO₃, H₂O₂, AgO, Jones Reagent, DDQ, TMSI, and BBr₃ did not give the desired quinone, thus, this route was abandoned.

3.3.3 Non-polar side chain analogs

Derivatives with non-polar groups appended to the imidazole were easily accessed by substituting the appropriate acid chlorides for acetyl chloride in the acylation of 3.25 (Scheme 3.8). Acid chlorides corresponding to a tert-butyl, phenyl, and propyl substituent were coupled to 3.25 to give amides 3.37a, 3.37b, and 3.37c. The resulting amides were uneventfully
cyclized, deprotected, and oxidized to quinones 3.40a, 3.40b, and 3.40c in a manner analogous to the synthesis of cribrostatin 6.

Scheme 3.8 Synthesis of non-polar side chain derivatives.

3.3.4 Polar side chain analogs

To deliver derivatives with polar side chains, a common intermediate derived from acylation with 2-benzyloxy acetyl chloride was advanced to the final quinone (Scheme 3.9). Thus, acylation of 3.25 afforded amide 3.41 which could be cyclized with POCl₃ as previously described for cribrostatin 6. Saponification of ester 3.42 with NaOH produced phenol 3.43, oxidation of which gave benzyl ether derivative 3.46. Returning to the primary route, debenzylation of the imidazole side chain by hydrogenation gave primary alcohol 3.44, which was oxidized to the final carbinol derivative 3.45. Interestingly, no oxidation of the alcohol was observed during nitric acid treatment.
Scheme 3.9 Both aldehyde 3.47 and azido 3.48 can be made from common precursor alcohol 3.45. Alcohol 3.45 can be synthesized from amine salt 3.25 through an acid chloride coupling followed by two deprotection steps and the final oxidation.

Derivative 3.45 was transformed into both an azide and an aldehyde. The azide was synthesized by a standard method utilizing diaza(1,3)bicyclo[5.4.0]undecane (DBU) and diphenylphosphoryl azide. This method gave very good yields of azide modified quinone 3.48. Treating 3.45 with Dess-Martin periodinane gave the desired aldehyde derivative 3.47 in 90% yield. Unfortunately, all attempts to transform the alcohol or aldehyde derivatives to a carboxylic acid were not successful resulting in either decomposition of the quinone or no reaction (Table 3.1).
was converted to phenol be obtained after treatment with HNO

derivative was synthesized using high pressure hydrogenation (Scheme 3.10). Cribrostatin 6 precursor 3.27 was subjected to 1000 psi hydrogen at elevated temperatures for 2 hrs in the University of Illinois high pressure hydrogenation facility. Resulting dihydroisoquinoline 3.49 was converted to phenol 3.50 by saponification. A small amount of oxidized product 3.51 could be obtained after treatment with HNO₃, however, this product is unstable in solution and as a solid and begins to revert to cribrostatin 6 in a matter of hours.

Table 3.1 Summary of oxidation reagents used to attempt to synthesize a carboxylic acid derivative.

<table>
<thead>
<tr>
<th>Starting Material</th>
<th>Reagents</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.45, 3.47</td>
<td>Jones</td>
<td>Decomposition</td>
</tr>
<tr>
<td>3.47</td>
<td>NaClO₂</td>
<td>Decomposition</td>
</tr>
<tr>
<td>3.47</td>
<td>oxone</td>
<td>No Reaction</td>
</tr>
<tr>
<td>3.47</td>
<td>AgNO₃</td>
<td>Decomposition</td>
</tr>
</tbody>
</table>

3.3.5 Reduced and bicyclic analogs

To examine the role of the carbon-carbon double bond in the isoquinoline ring, a reduced derivative was synthesized using high pressure hydrogenation (Scheme 3.10). Cribrostatin 6 precursor 3.27 was subjected to 1000 psi hydrogen at elevated temperatures for 2 hrs in the University of Illinois high pressure hydrogenation facility. Resulting dihydroisoquinoline 3.49 was converted to phenol 3.50 by saponification. A small amount of oxidized product 3.51 could be obtained after treatment with HNO₃, however, this product is unstable in solution and as a solid and begins to revert to cribrostatin 6 in a matter of hours.

Scheme 3.10 Synthesis of reduced cribrostatin 6 derivative.

To explore the contribution of the imidazole ring to toxicity, amide 3.37c was saponified to phenol 3.52 (Scheme 3.11). Subsequent oxidation with HNO₃ gave the final quinone 3.53, although concomitant removal of the ethyl protecting group on the remaining phenol was observed.
Scheme 3.11 Synthesis of bicyclic derivative 3.53

3.4 Biological evaluation of cribrostatin 6 analogs

With cribrostatin 6 and eleven of its derivatives in hand, experiments were conducted to assess their ability to induce death in cancer cell lines in culture. In addition to the derivatives synthesized as described herein, seven derivatives were synthesized in Prof. Stephen Martin’s laboratory (University of Texas, Austin) by Daniel Knueppel using their synthetic pathway (Figure 3.3). For these initial experiments, U-937 (human lymphoma) and HL-60 (human leukemia) cell lines were utilized. To determine the IC₅₀ values for cribrostatin 6 and derivatives, cells were incubated with compound over a range of concentrations (0-100 µM). After 24 hours, the cellular toxicity was determined by MTS assay, and logistical dose-response curves were used to calculate IC₅₀ values, as described in chapter 2. The results are displayed in Figure 3.2.
Figure 3.3  

a) IC$_{50}$ values of cribrostatin 6 derivatives in µM.  

b) IC$_{50}$ values in µM of cribrostatin 6 derivatives synthesized by Daniel Knueppel. Error is standard error, n=3.
Analysis of the derivative reveals several structural features to be essential for cribrostatin 6 activity (Figure 3.4). Removing the imidazole significantly attenuates the potency of the compound as observed in 3.60, 3.53, and 3.52. The quinone ring also is an important moiety as indicated by compound 3.8. Substitution on the imidazole ring was tolerated to varying degrees. Substitution of the methyl group with an aldehyde, propyl, or t-butyl group had minimal effects on toxicity. In terms of substituting the groups on the quinone ring, activity was most negatively affected by substituting the ethoxy group to a hydroxyl group on the quinone as in compound 3.57.

![Figure 3.4 Structure-activity relationship of cribrostatin 6 derivatives.](image)

**3.5 Further testing of derivatives**

The four most potent cribrostatin 6 derivatives were chosen for further testing. Because cribrostatin 6 had an attenuated potency in hypoxia, the chosen derivatives were first tested in hypoxia. As discussed in chapter 2, hypoxia is relevant to tumor models as solid tumors have area of hypoxic tissue. Testing of these derivatives was performed as before with HeLa cells that had been kept in 1% oxygen environment for at least 12 hours were treated with varying concentrations of compounds and returned to incubate in 1% oxygen environment for 48 hours. Cell death was then determined by sulforhodamine B assay.
Of the four derivatives tested, two were significantly less potent in hypoxia, increasing the IC50 7 fold. In comparison, cribrostatin 6 has a 5 fold difference between normoxic and hypoxic IC50s. The other two compounds were only 2 fold less potent in hypoxia, thus considered more desirable for treated solid tumors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (µM) in Normoxia</th>
<th>IC50 (µM) in Hypoxia</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cribrostatin 6</td>
<td>10 ± 0.7</td>
<td>&gt;49</td>
<td>5</td>
</tr>
<tr>
<td>3.54</td>
<td>9 ± 1</td>
<td>22 ± 14</td>
<td>2</td>
</tr>
<tr>
<td>3.56</td>
<td>7.1 ± 0.7</td>
<td>14 ± 5</td>
<td>2</td>
</tr>
<tr>
<td>3.47</td>
<td>12.7 ± 0.7</td>
<td>&gt;100</td>
<td>&gt;7</td>
</tr>
<tr>
<td>3.40b</td>
<td>8 ± 2</td>
<td>57 ± 7</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3.2 Cribrostatin 6 derivatives are less potent in hypoxic environments. HeLa cells were incubated with compounds in the appropriate oxygen environment for 48 hours.

The two most hypoxia potent cribrostatin 6 derivatives were tested for their ability and time course to cause apoptosis in cancer cells. U-937 cells were treated with compounds at 10 uM for the indicated amount of time. Cells were then stained with annexin V and propidium iodide and analyzed by cell flow cytometry. For both compounds cell death primarily occurred between 8 and 16 hours of treatment. This is a similar time frame to cribrostatin 6 as illustrated in chapter 2.
To determine if there is a relationship between reduction potential and cytotoxicity, the 5 most potent compounds plus 3 others with a variety of functionality were subjected to cyclic voltametry. From the resulting C.V. the $E_{1/2}$ reduction potentials could be measured. This is the energy needed to reduce the quinone to the semi quinone. In the series tested, there is no correlation between reduction potential and toxicity; however, this relationship is limited by error in both cytotoxicity testing and reduction potential testing. Further investigation with more precise techniques and a larger series of derivatives could give more information about the proposed relationship.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$E_{1/2}$ reduction potential (mV)</th>
<th>U-937 IC$_{50}$ (µM)</th>
<th>HL-60 IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.47</td>
<td>-259</td>
<td>7 ± 2</td>
<td>0.90 ± 0.05</td>
</tr>
<tr>
<td>3.54</td>
<td>-384</td>
<td>4 ± 1</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>3.45</td>
<td>-385</td>
<td>17 ± 7</td>
<td>3 ± 0.8</td>
</tr>
<tr>
<td>3.56</td>
<td>-392</td>
<td>6 ± 2</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>Cribrostatin 6</td>
<td>-396</td>
<td>5 ± 1</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>3.40b</td>
<td>-396</td>
<td>6 ± 3</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>3.60</td>
<td>-507</td>
<td>48 ± 1</td>
<td>14 ± 3</td>
</tr>
</tbody>
</table>

Table 3.3 The reduction potentials of cribrostatin 6 and selected derivatives were calculated using cyclic voltametry spectra. Under these experimental conditions there is no correlation between toxicity in cell culture and reduction potential.

3.6 Future directions

There are several experiments that can be completed in the near future. First, the reduction potentials of the current derivatives should be measured to see if there is a correlation between the cytotoxicity and electrochemistry of these molecules. If there is, this could be a major step forward in determining which further derivatives should be synthesized. Without this data, derivatives to be synthesized include molecules with substituents on the nitrogen imidazoles and further substitution of the double bond of the isoquinoline.

Cell culture studies with these derivatives will yield further information. Annexin V/propidium iodide staining of treated cancer cells will confirm the occurrence of apoptotic death and also the time frame of cell death. Also, testing derivatives in hypoxia may help determine which compounds will go forward to animal testing. Derivatives with the smallest decrease in potency in hypoxic environments are desired.

A major factor in the future of these compounds is how they are tolerated in mice and their effectiveness in in vivo models. The first set of mouse studies with these derivatives will be
toxicity studies in mice. Cribrostatin 6 toxicity to mice is known as described in section 1.11.2, and a derivative that is less toxic could have a higher therapeutic window for \textit{in vivo} cancer studies. If these studies give encouraging results, further studies in mouse cancer models will occur.

### 3.7 Conclusions

A previously published synthesis of cribrostatin 6 was used as the basis for the synthesis of derivatives. In this synthesis, use of a variety of acid chlorides altered the substitution on the 2-position of cribrostatin 6’s imidazole ring. Eleven derivatives were synthesized using our modified synthetic pathway. Although the chosen pathway was linear, it did provide quick access to a variety of derivatives. The synthesized derivatives were tested against U-937 and HL-60 cell lines. While the derivatives of cribrostatin 6 were not more potent than the natural product, they did reveal that the imidazole and quinone rings are both important for cytotoxicity. In future experiments, the derivatives of cribrostatin 6 should be further evaluated for their activity in hypoxic cells and toxicity in mouse models.

### 3.8 Acknowledgements

I would like to thank Daniel Knueppel for synthesizing cribrostatin 6 derivatives and his discussions on the synthesis of cribrostatin 6.
3.9 References

(14) Butler, J.; Hoey, B. M. "The one-electron reduction potential of several substrates can be related to their reduction rates by cytochrome P-450 reductase". *Biochim Biophys Acta* **1993**, *1161*, 73-8.
4 Materials and Methods

4.1 General Procedures

Doxorubicin was obtained from Ochem Incorporation (Des Plains, IL). Tirapazamine was synthesized in the Hergenrother Laboratory following literature procedure \(^1\). The MTS assay reagent was obtained from Promega (Madison, WI). Annexin V-FITC conjugate was purchased from Southern Biotech (Birmingham, AL). All other compounds are from Sigma Aldrich (St. Louis, MO). Plasmid DNA pBR322 was bought from New England Biolabs (Ipswich, MA). Topoisomerases I and II and kDNA were obtained from Topogen (Port Orange, FL). For Western blot and transcriptional profiling experiments, the following materials were purchased from the indicated vendor: protease inhibitors, Cell Signaling Technology (Beverly, MA); mouse monoclonal anti-actin antibody sc-8432, Santa Cruz Biotechnology (Santa Cruz, CA); RNeasy kit, Qiagen (Valencia, CA); rabbit polyclonal antibody SPA-896, Stressgen (Ann Arbor, MI); Illumina HumanHT-12 beadarray, Illumina Inc. (San Diego, CA).

All reactions were conducted in oven-dried glassware when appropriate and air- or water-sensitive liquids were transferred via dry syringe. The reagent used for drying organic extraction layers was magnesium chloride. Flash column chromatography was performed using 230-400 mesh silica gel. Thin-layer chromatography (TLC) was performed on precoated silica glass plates with fluorescent indicator (254 nm) and spots were visualized by UV light (254 or 365 nm). \(^1\)H NMR spectra and \(^13\)C NMR spectra were recorded on a 500 MHz, \(^1\)H (125.7 MHz, \(^13\)C) spectrometer, and spectra are referenced to residual solvent peak. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad peak). Coupling constants are reported in hertz.
4.2 Biological Evaluation

Cell lines and Cytotoxicity

U-937, MCF-7, SK-MEL-5, HeLa, HL-60, and HL-60 VCR cancer cell lines were propagated in RPMI 1640 media supplemented with 10% FBS and 1% Penicillin/Streptomycin at 37°C in 95% air/5% CO₂ atmosphere. In preparation for cytotoxicity assessment, suspension (30,000 cells/well) and adherent (10,000 cells/well) cells were grown in 96-well plates overnight. Compound (0-100 µM), diluted in DMSO, was added using 100X stock solutions (1% DMSO final concentration in wells). Cytotoxicity was determined after 24 hours incubation. For suspension cells, the colorimetric MTS viability assay was utilized according to manufacturer’s protocol. For adherent cells, plates were stained with the protein dye sulforhodamine B to assess overall biomass. In short, cells in media were fixed by addition of 100 µL 10% trichloroacetic acid and incubated at least one hour at 4 °C. Plates were gently washed by submerging in a vessel of tap water 3 times and stained with 100 µL 0.057% sulforhodamine B in 1% acetic acid. Plates incubated at room temperature for 30 minutes and then were washed of unbound dye by submerging plates into a vessel of 1% acetic acid. Plates were dried, dye was resuspended in 200 µL 10 mM Tris buffer pH = 10.5, and after 30 minutes room temperature incubation, the absorbance of wells was read at 510 nm in a microplate reader. DMSO was used as a negative death control (100% viable) and a known cytotoxin as a positive death control (0% viable) to determine percentage cell death. IC₅₀ values were calculated from logistical dose response data using TableCurve 2D (San Jose, CA).
ROS determination with DCF

Untreated U-937 cells were aliquoted (1.5 million cells/tube) from an exponentially growing culture and washed once with 1 mL PBS containing 5 mM glucose. The cell pellet was resuspended in 1 mL PBS with 5 mM glucose and treated with 1 µL of 2.5 mM DCF in DMSO to give a final concentration of 2.5 µM DCF. Cells were protected from light with aluminum foil from this point forward. Cells were incubated at 37°C for 30 minutes then washed with 1 mL PBS with 5 mM glucose. The cell pellet was resuspended in 0.5 mL PBS with 5 mM glucose. Cribrostatin 6 and t-BuOOH were added in the indicated concentrations by adding 1 µL of 100X stock solutions. Samples incubated for the indicated time (0-60 min) and were immediately analyzed using a BD Biosciences LSR II flow cytometer. The geometric mean of the live population was calculated to determine amount of intracellular fluorescence.

Annexin V Apoptosis Assay

Immediately prior to treatment, U-937 cells were plated in 1 mL aliquots in a 24 well plate at 500,000 cells/well. Cells were then treated with 1 µL of a 10 mM cribrostatin 6 DMSO stock solution (10 µM final cribrostatin 6 concentration). The cells were incubated for specified amount of time (4-16 hours), and were then stained according to manufacturer’s protocol with annexin V-FITC and propidium iodide (PI). Briefly, cells were washed with PBS and resuspended in annexin V binding buffer (10 mM HEPES pH = 7.4, 140 mM NaCl, 2.5 mM CaCl₂, 0.1% BSA). Cells were incubated 40 minutes with 5 µL FITC-annexin V conjugate, and then were treated with 10 µL 50 µg/mL PI and analyzed for cellular FITC and PI fluorescence by flow cytometry immediately.
Topoisomerase I and II Inhibition

The Topogen protocol was followed with minor modifications. In this protocol, 2 units of topoisomerase I were incubated at room temperature with compound in buffer for 15 minutes prior to adding pBR322 DNA prediluted in buffer. Likewise, 2 units of topoisomerase II were preincubated with compound and combined with kDNA in ATP buffer. Both solutions were then incubated at 37°C for 30 minutes. A 5 µL volume of 5x stop buffer was then added, and reactions were analyzed by agarose gel electrophoresis on 1% agarose gels followed by staining with 10 µg/mL ethidium bromide and visualization by UV.

Cell Cycle Analysis

One million U-937 cells were seeded in 24 well plates at 1 million cells/mL. Adherent cell lines (SK-MEL-5, MCF-7, HeLa) were grown in 10 cm plates until they were 70% confluent. Cells were treated with cribrostatin 6 (1-20 µM) using 2 µL of the appropriate DMSO stock solution and incubated for 16 hours. Cells were transferred to tubes either by pipetting U-937 or trypsinizing adherent cell lines then all cells were washed with 1 mL PBS. The cell pellet was resuspended and fixed in 1 mL cold 70% ethanol, which was added dropwise while vortexing. Samples were stored at 4°C. For analysis, cells were washed with 1 mL PBS and resuspended in 50 µL 100 µg/mL RNase A. Cells were incubated at 4 °C for at least 4 hours. PI (350 µL at 50 µg/mL) was then added to samples, and DNA content was analyzed by flow cytometry.
**3T3 Quiescent and Dividing Cells Data**

3T3 murine fibroblasts were propagated in high glucose DMEM with 10% calf serum and 1% Penicillin/Streptomycin, taking care to never allow cells to become confluent. Cells were plated in 96 well plates at 10,000 cells/well. For the experiments with rapidly dividing cells, cells were treated the day following plating. Contact inhibited cells grew for 5 days before treatment. Cells were treated with compounds as described for cytotoxicity assays, incubated 72 hours, and analyzed with sulfurhodamine B as described previously.

**Western Blotting**

U-937 cells were pre-treated with N-acetyl cysteine (NAC) (10 mM in H$_2$O) for 18 hours, and then resuspended in NAC-free media for 3 hours prior to treatment with compound. The cells (8 mL of 500,000 cells/mL) were treated with compound from appropriate stock solution for 6 hours (1% final DMSO). The cells were centrifuged, washed with PBS, and lysed with RIPA lysis buffer (Cell Signaling Technology) containing protease inhibitor. Cell debris was removed by centrifugation (16000xg for 5 min) and protein concentration was determined by Bradford assay. Cell lysate containing 60 µg total protein was loaded onto a 12% SDS-PAGE gel and electrophoresed at 120 V for 90 min. The proteins were transferred onto a nitrocellulose membrane at 50 V for 2 hours. The membrane was blocked with 5% BSA in TBST for 2 hours at room temperature, incubated with primary anti-HOMX-1 rabbit polyclonal antibody at 1:5000 dilution in 5% BSA TBST overnight at 4 °C, incubated with secondary HRP conjugated antibody at 1:20,000 dilution for 1 hour in 0.5% BSA TBST at room temperature and visualized by ECL autoradiography after incubation with luminol substrate in the presence of peroxide. The
membrane was stripped in acidic methanol and probed for β-actin using a mouse monoclonal anti-actin antibody at 1:5,000 dilution in 5% BSA TBST overnight at 4 ºC.

**Protection with NAC**

HeLa cells were propagated as previously described. In preparation for cytotoxicity assessment, 10,000 cells/well were grown in 96-well plates overnight. One hour prior to treatment with compounds, cells were pretreated with 5 mM N-acetyl cysteine by adding 1 µL of 500 mM N-acetyl cysteine aqueous solution to each well. After 1 hour incubation, cells were treated with compounds as previously described. Cytotoxicity was determined after 48 hours incubation by using the SRB assay.

**Incubation in Hypoxic Environments**

HeLa cells were propagated as previously described. In preparation for cytotoxicity assessment, 10,000 cells/well were grown in 96-well plates overnight in a 1% oxygen environment in a hypoxic cell culture incubator from New Brunswick Scientific. Cells were treated with compound as previously described and returned to the hypoxic incubator. Cytotoxicity was determined after 48 hours incubation by using the SRB assay.

**Gene Expression Analysis**

U-937 cells (3,000,000 in 6 mL RPMI 1640 with 10% FBS) were incubated in the presence of 15 µM cribrostatin 6 or DMSO vehicle (0.2% final DMSO v/v) for 6 hours in a 12-well plate.
Aliquots (500 µL) were taken at 6 and 24 hours to assess viability (by annexin V/PI staining and flow cytometry) in order to ensure no toxicity occurred at 6 hours but toxicity was observed at 24 hours. After 6 hours of incubation, 5 mL of cells were pelleted (500xg 5 min) and RNA isolated with on column DNase digestion. RNA integrity was assessed using an Agilent Bioanalyzer. Whole genome transcript profiling was performed on the Illumina HumanHT-12 bread array for seven compounds and DMSO control from three independent experiments. The raw bead-level files were processed with Illumina® BeadStudio 3.1, Gene Expression Module v3.2.6 3, without background correction or normalization, to get one value per beadtype for each array. These 48,803 beadtype values were then input into R 4 using the beadarray package 5 from the Bioconductor Project 6. The vsn algorithm 7 was used for background correction, normalization and transformation of the beadtype values to the glog2 scale. Differential expression was assessed using a mixed linear model using the Limma package 8, which uses an empirical Bayes correction 9 that helps to improve power by borrowing information across beadtypes. All the desired pair-wise comparisons between the 8 treatment groups (control + cribrostatin 6 + 6 unrelated compounds) were pulled as contrasts from the model and the p-values were corrected for multiple hypothesis testing using the False Discovery Rate method 10 separately for each contrast. In order to facilitate comparisons of our results with those of Connectivity Map database we used Illumina’s probe mapping file that matched probes from Illumina’s HumanWG-6v3 array (same probes as the HumanHT-12 array) to Affymetrix’s U133Plus2.0 array 11. The top 50 up- and down-regulated probe set IDs ordered according to fold change were used to create the seed for the connectivity map (CMap) database matching. Analysis of the canonical pathways was performed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems) software using a p-value cutoff of 0.05 to define the network eligible genes.
4.3 Chemical Characterization

1-(aminomethyl)-5,7-diethoxy-6-methylisoquinolin-8-ol dihydrochloride (3.25).

Nitrile 1 (3.4 g, 9.4 mmol), in a Fischer Porter bottle, was suspended in 50 mL of 10% HCl/MeOH (v/v) with 400 mg of 5% Pd/C. The flask was charged with H₂ (60 psi) and stirred at room temperature for 6 hours. The reaction was filtered through celite and concentrated to give amine dihydrochloride 2 (3.1 g, 98% yield) as a yellow solid without further purification.

¹H NMR (D₂O) δ 8.39 (d, 1H, J = 6.7 Hz), 8.27 (d, 1H, J = 6.7 Hz), 5.01 (s, 2H), 4.11-4.03 (4H, m, J = 7 Hz), 2.48(3H, s), 1.44 (3H, t, J = 7 Hz), 1.39 (3H, t, J = 7 Hz)

¹³C NMR (D₂O) δ 147.7, 145.1, 144.8, 144.6, 138.0, 130.8, 130.4, 120.7, 117.0, 71.5, 70.3, 41.9, 14.7, 14.5, 11.1.

HRMS: (ESI) calcd for C₁₅H₂₁N₂O₃ (M-2HCl+H)⁺, 277.1552; found: 277.1544

m.p. = decomposition 192-194 °C
General protocol for amidation:

1-(acetamidomethyl)-5,7-diethoxy-6-methylisoquinolin-8-yl acetate (3.26).

Amine 3.25 (500 mg, 1.5 mmol) and a stir bar were loaded in a 100 mL 3-neck-round bottom under nitrogen atmosphere and placed in an ice bath. Compound 3.25 was dissolved with 20 mL of dry CH₂Cl₂. While stirring, 3.25 was treated with dry triethylamine (1.3 mL, 9.03 mmol) using an oven dried syringe and acetyl chloride (1.2 mL, 7.5 mmol). The reaction was stirred overnight at room temperature and was quenched with 50 mL of saturated sodium bicarbonate and extracted with CH₂Cl₂ (3 x 50 mL). The organic material was dried and concentrated. The crude material was purified via silica gel column chromatography eluting with 1:1 ethyl acetate:hexanes to produce amide 3.26 (350 mg, 65% yield) as an off-white solid.

¹H NMR (CDCl₃) δ 8.34 (d, 1H, J = 5.8 Hz), 7.81 (s, 1H) 7.77 (d, 1H, J = 5.8 Hz), 4.99 (br, 2H), 4.00 (q, 4H, J = 7.0 Hz), 2.57 (s, 3H), 2.41 (s, 3H), 2.14 (s, 3H), 1.52 (t, 3H, J = 7.0 Hz), 1.41 (t, 3H, J = 7.0 Hz).

¹³C NMR (CDCl₃) δ 170.4, 169.3, 152.0, 151.5, 149.7, 139.9, 135.8, 129.8, 129.3, 120.3, 114.8, 70.4, 69.8, 45.3, 23.6, 21.5, 16.1, 16.0, 11.1.

HRMS: (ESI) calcd for C₁₉H₂₅N₂O₅ (M+H)⁺, 361.1763; found: 361.1765

m.p = 73-76 °C
1-(benzamidomethyl)-5,7-diethoxy-6-methylisoquinolin-8-yl benzoate (3.37c)

\[
\begin{align*}
\text{Me} & \\
\text{OEt} & \\
\text{O} & \\
\text{N} & \\
\text{N} & \\
\text{Ph} & \\
\end{align*}
\]

H NMR (CDCl\textsubscript{3}) \(\delta\) 8.62-7.40 (m, 10 H), 5.63 (d, 1H, \(J = 7.4\) Hz), 4.85 (d, 1H, \(J = 7.4\) Hz), 4.02 (m, 4H), 2.44 (s, 3H), 1.53 (t, 3H), 1.20 (t, 3H).

C NMR (CDCl\textsubscript{3}) \(\delta\) 167.5, 165.2, 152.6, 151.7, 149.9, 140.3, 136.4, 135.2, 134.7, 131.6, 130.9, 130.1, 129.5, 129.4, 129.1, 128.8, 127.5, 120.8, 114.9, 70.6, 70.3, 45.8, 16.2, 16.0, 113 HRMS: (ESI) calcd for C\textsubscript{29}H\textsubscript{28}N\textsubscript{2}O\textsubscript{5} (M+H)+ 485.2076; found: 485.2093

1-(butyramidomethyl)-5,7-diethoxy-6-methylisoquinolin-8-yl butyrate (3.37.a)

\[
\begin{align*}
\text{Me} & \\
\text{OEt} & \\
\text{O} & \\
\text{N} & \\
\text{N} & \\
\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 & \\
\end{align*}
\]

H NMR (CDCl\textsubscript{3}) \(\delta\) 8.31 (d, 1H), 7.86 (s, 1H), 7.75 (d, 1H, \(J = 7.4\) Hz), 4.97 (s, 2H), 4.00-3.97 (m, 4H), 2.85 (t, 2H), 2.39 (s, 3H), 2.32 (t, 2H), 1.91 (m, 2H), 1.73(m, 2H), 1.50 (t, 3H), 1.38 (t, 3H), 1.10 (t, 3H), 0.98 (t, 3H).

C NMR (CDCl\textsubscript{3}) \(\delta\) 173.2, 172.0, 152.2, 151.3, 149.6, 139.8, 129.8, 129.22, 120.4, 114.7, 70.3, 69.6, 45.1, 38.9, 36.6, 19.4, 18.3, 15.9, 14.1, 14.0, 11.0
HRMS: (ESI) calcd for C_{23}H_{32}N_{2}O_{5} (M+H)^+, 417.2389; found: 417.2388

**5,7-diethoxy-6-methyl-1-(pivalamidomethyl)isoquinolin-8-yl pivalate (3.37b)**

![Chemical structure of 5,7-diethoxy-6-methyl-1-(pivalamidomethyl)isoquinolin-8-yl pivalate (3.37b)]

$^1$H NMR (CDCl$_3$) $\delta$ 8.33 (d, 1H), 7.77 (s, 1H), 5.2 (d, 1H), 4.57 (d, 1H), 4.06-3.80 (m, 4), 2.39 (s, 3H), 1.57 (s, 9H), 1.51 (t, 3H), 1.41 (t, 3H), 1.29 (s, 9H)

$^{13}$C NMR (CDCl$_3$) $\delta$ 178.6, 177.6, 152.7, 151.4, 149.3, 139.8, 136.9, 129.8, 120.7, 114.6, 70.3, 69.8, 45.6, 39.8, 39.0, 28.0, 27.8, 15.9, 15.6, 11.0

HRMS: (ESI) calcd for C$_{25}$H$_{36}$N$_{2}$O$_{5}$ (M+H)$^+$, 445.2702; found: 445.2707

**1-((2-(benzyloxy)acetamido)methyl)-5,7-diethoxy-6-methylisoquinolin-8-yl 2-(benzyloxy)acetate (3.41)**

![Chemical structure of 1-((2-(benzyloxy)acetamido)methyl)-5,7-diethoxy-6-methylisoquinolin-8-yl 2-(benzyloxy)acetate (3.41)]

$^1$H NMR (CDCl$_3$) $\delta$ 8.79 (s, 1H), 8.37 (d, 1H), 7.79 (d, 1H), 7.39-7.29 (m, 10H), 5.03, 4.82, 4.69, 4.02, 3.99-3.93 m, 2.41, 1.52, 1.36
$^{13}$C NMR (CDCl$_3$) $\delta$ 169.8, 168.8, 151.64, 151.61, 149.4, 140.3, 137.3, 137.2, 135.1, 129.8, 129.0, 128.77, 128.71, 128.6, 128.5, 128.3, 128.2, 128.19, 128.16, 128.12, 128.0, 120.0, 114.6, 73.8, 73.7, 70.3, 69.9, 69.7, 67.8, 44.5, 15.9, 15.8, 11.0

HRMS: (ESI) calcd for C$_{33}$H$_{36}$N$_2$O$_7$ (M+H)$^+$, 573.2601; found: 573.2597

General protocol for cyclization:

7,9-diethoxy-3,8-dimethylimidazo[5,1-a]isoquinolin-10-yl acetate (3.27).

Amide 3.26 (500 mg, 1.4 mmol) was dissolved in 15 mL of toluene in a 100 mL round bottom flask and treated with phosphorous oxychloride (POCl$_3$) (405 µL, 4.35 mmol). The reaction was refluxed for an hour and cooled to room temperature. The reaction mixture was quenched with saturated sodium bicarbonate and extracted with CH$_2$Cl$_2$ (3 x 50 mL). The organic layers were dried and concentrated. The crude material was purified via silica gel column chromatography eluting with 0%-10% methanol in dichloromethane to provide compound 3.27 (385 mg, 80% yield) as a white solid.

$^1$H NMR (CDCl$_3$) $\delta$ 7.87 (s, 1H), 7.62 (d, 1H, $J = 7.4$ Hz), 7.5 (d, 1H, $J = 7.4$ Hz), 4.04 (q, 2H, $J = 7$ Hz), 3.98 (q, 2H $J = 7$ Hz), 3.06 (s, 3H), 2.54 (s, 3H). 2.37 (s, 3H), 1.5 (t, 3H, $J = 7$ Hz), 1.4 (t, 3H, $J = 7$ Hz)
$^{13}$C NMR (CDCl$_3$) δ 168.6, 152.1, 150.5, 137.9, 135.5, 124.5, 124.4, 122.1, 119.2, 118.3, 118.2, 108.0, 70.3, 69.7, 21.4, 16.1, 15.9, 12.9, 10.6

HRMS: (ESI) calcd for C$_{19}$H$_{23}$N$_2$O$_4$ (M+H)$^+$, 343.1658; found: 343.1647

m.p. = 191-194 °C

3-((benzyloxy)methyl)-7,9-diethoxy-8-methylimidazo[5,1-a]isoquinolin-10-yl 2-(benzyloxy)acetate (3.42)

$^1$H NMR (CDCl$_3$) δ 7.87 (d, 1H), 7.72 (s, 1H), 7.46-7.28 (m, 10H), 7.08 (d, 1H), 4.97 (s, 2H), 4.81 (s, 2H), 4.59 (s, 2H), 4.50 (s, 2H), 4.00-3.96 (m, 4H), 2.36 (s, 3H), 1.51 (t, 3H), 1.38 (t, 3H)

$^{13}$C NMR (CDCl$_3$) δ 168.3, 152.3, 150.3, 145.3, 137.8, 137.5, 137.0, 135.1, 128.83, 128.8, 128.7, 128.4, 128.39, 128.35, 128.1, 125.8, 125.0, 122.6, 120.2, 118.9, 117.9, 108.3, 73.8, 72.2, 70.3, 69.8, 67.5, 64.4, 16.0, 15.9, 10.6

HRMS: (ESI) calcd for C$_{33}$H$_{34}$N$_2$O$_6$ (M+H)$^+$, 555.2495; found: 555.2485
**7,9-diethoxy-8-methyl-3-phenylimidazo[5,1-a]isoquinolin-10-yl benzoate (3.38c)**

![Chemical Structure](image)

$^1$H NMR (CDCl$_3$) $\delta$ 8.40-8.38 (m, 2H), 7.98 (d, 1H, $J$ = 7.7 Hz), 7.81-7.46 (m, 9H), 7.09 (d, 1H, $J$=7.7 Hz), 4.11 (q, 2H, $J$ = 7.0), 4.02 (q, 2H, $J$=7.0Hz), 2.39 (s, 3H), 1.52 (t, 3H, $J$= 7.0 Hz), 1.29 (t, 3H, $J$=7.0)

$^{13}$C NMR (CDCl$_3$) $\delta$ 164.5, 152.1, 150.8, 141.0, 135.7, 134.3, 130.6, 130.1, 129.5, 129.25, 129.22, 129.1, 128.6, 125.7, 124.9, 124.2, 120.1, 118.8, 118.5, 108.6, 70.3, 70.0, 15.98, 15.92, 10.6

HRMS: (ESI) calcd for C$_{29}$H$_{26}$N$_2$O$_4$ (M+H)$^+$, 467.1971; found: 467.1959

**7,9-diethoxy-8-methyl-3-propylimidazo[5,1-a]isoquinolin-10-yl butyrate (3.38a)**

![Chemical Structure](image)

$^1$H NMR (CDCl$_3$) $\delta$ 7.64 (s, 1H), 7.52 (d, 1H, $J$=7.7), 7.01 (d, 1H, $J$=7.7), 4.00 (q, 2H, $J$=7.0), 3.95 (q, 2H, $J$=7.0Hz), 2.93 (t, 2H, $J$ = 7.4 Hz), 2.78 (t, 2H, $J$ = 7.4 Hz), 2.32 (s, 3H), 1.91-1.83 (m, 4H), 1.48 (t, 3H, $J$ = 7.0), 1.39 (t, 3H, $J$ = 7.0), 1.10 (t, 3H, $J$ = 7.4), 1.02 (t, 3H, $J$= 7.4)
$^{13}$C NMR (CDCl$_3$) $\delta$ 170.8, 152.9, 152.1, 139.6, 136.4, 128.4, 124.8, 118.3, 117.2, 116.1, 113.8, 112.5, 71.0, 70.0, 36.3, 26.2, 21.1, 18.4, 15.9, 15.8, 13.89, 13.84, 10.9

HRMS: (ESI) calcd for C$_{23}$H$_{30}$N$_2$O$_4$ (M+H)$^+$, 399.2284; found: 399.2274

3-(tert-butyl)-7,9-diethoxy-8-methylimidazo[5,1-a]isoquinolin-10-yl pivalate (3.38b)

![Chemical Structure](image-url)

$^1$H NMR (CDCl$_3$) $\delta$

$^{13}$C NMR (CDCl$_3$) $\delta$ 176.1, 152.5, 151.6, 144.3, 136.8, 128.3, 126.0, 118.8, 117.6, 116.0, 113.7, 112.8, 70.9, 69.9, 39.3, 33.6, 28.0, 27.4, 15.7, 15.3, 10.6

HRMS: (ESI) calcd for C$_{25}$H$_{34}$N$_2$O$_4$ (M+H)$^+$, 427.2597; found: 427.2590

General protocol for saponification:

7,9-diethoxy-3,8-dimethylimidazo[5,1-a]isoquinolin-10-ol (3.8).

![Chemical Structure](image-url)

Tricycle 3.27 (200 mg, 0.6 mmol) was dissolved with 15 mL of methanol and treated with sodium hydroxide (70 mg, 1.75 mmol). The reaction stirred overnight at room temperature, then
was acidified with 30 mL of 2N HCl and extracted with CH₂Cl₂ (3 x 50 mL). The organic layers were dried and concentrated. The crude material was purified via silica gel column chromatography eluting with 0% - 5% methanol in dichloromethane to give compound 3.8 (152 mg, 87% yield) as an ivory solid.

¹H NMR (CDCl₃) δ 7.95 (s, 1H), 7.49 (d, 1H, J = 7.6Hz), 7.04 (d, 1H, J = 7.7Hz), 4.04 (q, 2H, J = 7Hz), 3.92 (q, 2H, J = 7Hz), 2.7 (s, 3H), 2.35 (s, 3H), 1.48 (t, 6H, J = 7.0Hz)

¹³C NMR (CD₃OD) δ 146.8, 145.7, 143.2, 135.8, 126.9, 126.1, 117.9, 117.6, 113.1, 112.3, 109.9, 70.4, 69.2, 14.7, 14.5, 9.5, 9.3

HRMS: (ESI) calcd for C₁₇H₂₁N₂O₃ (M+H)⁺, 301.1552; found: 301.1542

m.p. = decomposition 149 °C

3-((benzyloxy)methyl)-7,9-diethoxy-8-methylimidazo[5,1-a]isoquinolin-10-ol (3.43)

```
Me
OEt

EtO
OH

\[ \text{N} \]

O
Ph
```

¹H NMR (CDCl₃) δ 8.13 (s, 1H), 7.85 (d, 1H), 7.31-7.25 (m, 5), 7.18 (d, 1H), 5.16 (s, 2H), 4.57 (s, 2H), 3.92 (q, 2H), 3.49 (q, 2H), 2.36 (s, 3H), 1.50-1.47 (m, 6H)

¹³C NMR (CDCl₃) δ 147.0, 144.8, 142.1, 137.2, 135.6, 128.6, 128.3, 128.1, 126.8, 124.7, 119.3, 118.3, 111.2, 110.7, 72.9, 70.5, 69.9, 63.0, 16.0, 15.89, 10.7

HRMS: (ESI) calcd for C₂₄H₂₆N₂O₄ (M+H)⁺, 407.1971; found: 407.1987
7,9-diethoxy-8-methyl-3-propylimidazo[5,1-a]isoquinolin-10-ol (3.39a)

![Chemical structure of 3.39a]

$^1$H NMR (CDCl$_3$) $\delta$ 7.97 (s, 1H), 7.54 (d, 1H, J= 7.7), 6.99 (d, 1H, J=7.7Hz), 4.03 (, 2H, J=7.0), 3.92 (q, 2H, J=7.0 Hz), 2.98 (t, 2H, J=7.4), 2.35 (s, 3H), 1.89 (sextet, 2H, J=7.4), 1.48 (t, 3H, J=7.0Hz), 1.47 (t, 3H, J=7.0Hz), 1.04 (t, 3H, J=7.4)

$^{13}$C NMR (CDCl$_3$) $\delta$ 146.6, 143.9, 141.2, 140.8, 125.1, 123.1, 122.2, 119.1, 117.9, 112.3, 107.7, 70.1, 69.6, 28.6, 21.0, 15.9, 15.7, 14.0, 10.4

HRMS: (ESI) calcd for C$_{19}$H$_{24}$N$_2$O$_3$ (M+H)$^+$, 329.1865; found: 329.1855

7,9-diethoxy-8-methyl-3-phenylimidazo[5,1-a]isoquinolin-10-ol (3.39c)

![Chemical structure of 3.39c]

$^1$H NMR (CDCl$_3$) $\delta$ 8.21 (s, 1H), 7.94 (d, 1H, J=7.7Hz), 7.81 (m, 2H), 7.53-7.43 (m, 3H), 7.01 (d, 1H, J=7.7Hz), 4.04 (q, 2H, J=7.0 Hz), 3.92 (q, 2H, J=7.0Hz), 2.34 (s, 3H), 1.47 (t, 3H, J=7.0Hz), 1.46 (t, 3H, J=7.0 Hz)

$^{13}$C NMR (CDCl$_3$) $\delta$ 146.7, 144.2, 141.7, 140.2, 132.6, 130.1, 129.2, 129.1, 128.8, 128.3, 126.6, 124.8, 123.0, 120.0, 118.4, 112.3, 108.8, 70.3, 69.8, 16.0, 15.8, 10.5

HRMS: (ESI) calcd for C$_{22}$H$_{22}$N$_2$O$_3$ (M+H)$^+$, 363.1709; found: 363.1708
3-(tert-butyl)-7,9-diethoxy-8-methylimidazo[5,1-a]isoquinolin-10-ol (3.39b)

\[ \text{H NMR (CDCl}_3 \text{) } \delta \text{ 7.99 (s, 1H), 7.87 (d, 1H, } J=7.8 \text{ Hz), 6.94 (d, 1H, } J= 7.8 \text{ Hz), 3.99 (q, 2H, } J= 7.0 \text{ Hz), 3.90 (q, 2H, } J= 7.0 \text{ Hz), 2.32 (s, 3H), 1.56 (s, 9H), 1.47-1.41 (m, 6H)} \]

\[ \text{C NMR (CDCl}_3 \text{) } \delta \text{ 147.1, 146.5, 143.9, 141.5, 126.6, 122.5, 122.4, 121.3, 117.6, 112.5, 107.3, 70.2, 69.7, 33.6, 28.9, 27.5, 15.9, 15.8, 10.4} \]

HRMS: (ESI) calcd for C\textsubscript{20}H\textsubscript{26}N\textsubscript{2}O\textsubscript{3} (M+H\textsuperscript{+})\textsuperscript{+}, 343.2022; found: 343.2014

7,9-diethoxy-3-(hydroxymethyl)-8-methylimidazo[5,1-a]isoquinolin-10-ol

HRMS: (ESI) calcd for C\textsubscript{20}H\textsubscript{16}N\textsubscript{2}O\textsubscript{3} (M+H\textsuperscript{+})\textsuperscript{+}; found:

7,9-diethoxy-3,8-dimethyl-5,6-dihydroimidazo[5,1-a]isoquinolin-10-yl acetate

HRMS: (ESI) calcd for C\textsubscript{19}H\textsubscript{24}N\textsubscript{2}O\textsubscript{4} (M+H\textsuperscript{+})\textsuperscript{+}; found:
7,9-diethoxy-3,8-dimethyl-5,6-dihydroimidazo[5,1-a]isoquinolin-10-ol

\[
\text{Me} \quad \quad \quad \quad \text{OEt} \\
\text{EtO} \quad \quad \quad \quad \text{EtO} \\
\text{OH} \quad \quad \quad \quad \text{N} \quad \quad \quad \quad \text{Me}
\]

HRMS: (ESI) calcd for C_{17}H_{22}N_{2}O_{3} (M+H)^{+}, 301.1552; found: 301.1560

N-((5,7-diethoxy-8-hydroxy-6-methylisoquinolin-1-yl)methyl)benzamide

\[
\text{Me} \quad \quad \quad \quad \text{OEt} \\
\text{EtO} \quad \quad \quad \quad \text{EtO} \\
\text{OH} \quad \quad \quad \quad \text{N} \quad \quad \quad \quad \text{O} \\
\text{NH} \quad \quad \quad \quad \text{Ph}
\]

HRMS: (ESI) calcd for C_{22}H_{24}N_{2}O_{4} (M+H)^{+}, 381.1814; found: 381.1819

General oxidation protocol:

Cribrostatin 6.
Compound 3.8 (100 mg, 0.3 mmol) was treated with 1 mL of nitric acid (60%) in a 50 mL round bottom flask at 0°C. The walls of the flask were rinsed with 5 mL CH₂Cl₂ to insure all material was in contact with the nitric acid. Reaction stirred for 30 minutes and then neutralized with saturated sodium bicarbonate. The aqueous solution was extracted with CH₂Cl₂ (3 x 25 mL) and the organic layers were dried, concentrated, and purified by silica gel column chromatography eluting with 25% ethyl acetate/hexanes to yield cribrostatin 6 (28 mg, 31% yield) as a dark blue/grey solid. The spectra match previously recorded cribrostatin 6 data.¹²

¹H NMR (CDCl₃) δ 8.21 (s, 1H), 7.83 (d, 1H, J = 7.3 Hz), 7.82 (d, 1H, J = 7.3 Hz), 4.37 (q, 2H, J = 7 Hz), 2.67 (s, 3H), 2.03 (s, 3H), 1.41 (t, 3H, J = 7 Hz).

¹³C NMR (CDCl₃) δ 184.7, 180.4, 155.9, 137.4, 129.9, 125.7, 124.7, 124.5, 123.7, 123.3, 107.4, 69.4, 15.8, 12.4, 9.0.

HRMS: (ESI) calcd for C₁₅H₁₅N₂O₃ (M+H)⁺, 271.1083; found: 271.1075

m.p. = 170-172 °C

9-ethoxy-8-methyl-3-propylimidazo[5,1-a]isoquinoline-7,10-dione

¹H NMR (CDCl₃) δ 8.29 (s, 1H), 7.92 (d, 1H, 7.3), 7.18 (d, 1H, 7.3), 4.39 (q, 2H, J = 7.0), 2.98 (t, 2H, J = 7.4), 2.06 (s, 3H), 1.90 (sext, 2H, J = 7.5), 1.41 (t, 3H, J = 7.0), 1.04 (t, 3H, J = 7.4)
$^{13}$C NMR (CDCl$_3$) δ 184.9, 180.6, 156.1, 141.5, 130.0, 125.9, 124.9, 124.6, 123.6, 123.5, 107.4, 69.6, 28.4, 20.3, 15.9, 13.9, 9.1

HRMS: (ESI) calcd for C$_{17}$H$_{18}$N$_2$O$_3$ (M+H)$^+$, 299.1396; found: 299.1388

9-ethoxy-8-methyl-7,10-dioxo-7,10-dihydroimidazo[5,1-a]isoquinoline-3-carbaldehyde

![Chemical Structure](image)

$^1$H NMR (CDCl$_3$) δ 10.09 (s, 1H), 9.66 (d, 1H, J = 7.2), 8.65 (s, 1H), 7.64 (d, 1H, J = 7.2 Hz), 4.47 (q, 2H, J = 7.0Hz), 2.12 (s, 3H), 1.44 (t, 3H, J = 0 Hz)

$^{13}$C NMR (CDCl$_3$) δ 184.1, 181.3, 180.1, 156.9, 136.5, 131.1, 130.1, 129.7, 129.6, 129.0, 122.5, 112, 70.2, 16.2, 9.5

HRMS: (ESI) calcd for C$_{15}$H$_{12}$N$_2$O$_4$ (M+H)$^+$, 285.0875; found: 285.0867

9-ethoxy-3-(hydroxymethyl)-8-methylimidazo[5,1-a]isoquinoline-7,10-dione

![Chemical Structure](image)
$^1$H NMR (CD$_3$OD) $\delta$ 8.51 (d, 1H, J = 7.4 Hz), 8.12 (s, 1H), 7.22 (d, 1H, J=7.4 Hz), 4.97 (s, 2H), 4.35 (q, 2H, J = 7.0), 2.00 (s, 3H), 1.36 (t, 3H, J = 7.0)

$^{13}$C NMR (CD$_3$OD) $\delta$

HRMS: (ESI) calcd for C$_{15}$H$_{14}$N$_2$O$_4$ (M+H)$^+$, 287.1032; found: 287.1022

3-(azidomethyl)-9-ethoxy-8-methylimidazo[5,1-a]isoquinoline-7,10-dione

![Chemical Structure](image)

$^1$H NMR (CDCl$_3$) $\delta$ 8.36 (s, 1H), 8.14 (d, 1H, J = 7.3 Hz), 7.33(d, 1H, J = 7.3 Hz), 4.83 (s, 2H), 4.42 (q, 2H, J = 7.0 Hz), 2.09 (s, 3H), 1.43 (t, 3H, 7.0 =Hz)

$^{13}$C NMR (CDCl$_3$) $\delta$ 184.9, 180.6, 156.5, 135.3, 130.5, 126.6, 126.4, 125.7, 125.6, 123.7, 108.9, 70.0, 46.8, 16.2, 9.4

HRMS: (ESI) calcd for C$_{25}$H$_{36}$N$_2$O$_5$ (M+H)$^+$, 312.3; found: 312.2

3-(tert-butyl)-9-ethoxy-8-methylimidazo[5,1-a]isoquinoline-7,10-dione

![Chemical Structure](image)
$^1$H NMR (CDCl$_3$) $\delta$ 8.32 (s, 1H), 8.26 (d, 1H, J = 7.5), 7.17 (d, 1H, J=7.5), 4.39 (q, 2H, J=7.0Hz), 2.07 (s, 3H), 1.57 (s, 9H), 1.42 (t, 3H, J=7.0Hz)

$^{13}$C NMR (CDCl$_3$) $\delta$ 185.2, 180.9, 156.5, 148.0, 130.3, 127.0, 125.6, 125.4, 124.8, 123.9, 107.2, 69.8, 33.7, 28.5, 16.2, 9.4

HRMS: (ESI) calcd for C$_{18}$H$_{20}$N$_2$O$_3$ (M+H)$^+$, 313.1552; found: 313.1550

9-ethoxy-8-methyl-3-phenylimidazo[5,1-a]isoquinoline-7,10-dione

![9-ethoxy-8-methyl-3-phenylimidazo[5,1-a]isoquinoline-7,10-dione](image)

$^1$H NMR (CDCl$_3$) $\delta$ 8.51 (s, 1H), 8.41 (d, 1H, J = 7.4 Hz), 7.78-7.49 (m, 5H), 7.22 (d, 1H, J = 7.4Hz), 4.43 (q, 2H, J=7.0 Hz), 2.09 (s, 3H), 1.44 (t, 3H, J=7.0Hz)

$^{13}$C NMR (CDCl$_3$) $\delta$ 185.0, 180.8, 156.5, 140.9, 130.4, 129.8, 129.5, 129.4, 128.6, 127.8, 126.0, 125.8, 125.3, 123.9, 108.6, 69.9, 16.2, 9.4

HRMS: (ESI) calcd for C$_{20}$H$_{16}$N$_2$O$_3$ (M+H)$^+$, 333.1239; found: 333.1235

3-((benzyloxy)methyl)-9-ethoxy-8-methylimidazo[5,1-a]isoquinoline-7,10-dione

![3-((benzyloxy)methyl)-9-ethoxy-8-methylimidazo[5,1-a]isoquinoline-7,10-dione](image)
$^1$H NMR (CD$_2$Cl$_2$) δ 8.26 (d, 1H, J=7.3Hz), 8.24 (s, 1H), 7.35-7.28 (m, 5H), 7.22 (d, 1H, J=7.3Hz), 4.99 (s, 2H), 4.52 (s, 2H), 4.38 (q, 2H, J=7.0), 2.06 (s, 3H), 1.40 (t, 3H, J=7.0Hz)

$^{13}$C NMR (CD$_2$Cl$_2$) δ 185.2, 181.0, 156.6, 137.9, 137.7, 130.7, 128.7, 128.3, 128.2, 126.8, 126.5, 125.8, 125.6, 123.6, 108.0, 72.7, 70.0, 64.5, 16.1, 9.3

HRMS: (ESI) calcd for C$_{22}$H$_{20}$N$_2$O$_4$ (M+H)$^+$, 377.1501; found: 377.1492

9-ethoxy-3,8-dimethyl-5,6-dihydroimidazo[5,1-a]isoquinoline-7,10-dione

![Structural diagram](image)

HRMS: (ESI) calcd for C$_{15}$H$_{16}$N$_2$O$_3$ (M+H)$^+$, 273.1239; found: 273.1230

N-((7-hydroxy-6-methyl-5,8-dioxo-5,8-dihydroisoquinolin-1-yl)methyl)benzamide

![Structural diagram](image)

HRMS: (ESI) calcd for C$_{18}$H$_{14}$N$_2$O$_4$ (M+H)$^+$, 323.1032; found: 323.1019
4.4 References

(1) Fuchs, T.; Chowdhury, G.; Barnes, C. L.; Gates, K. S. "3-amino-1,2,4-benzotriazine 4-oxide: characterization of a new metabolite arising from bioreductive processing of the antitumor agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine)". *J Org Chem* 2001, 66, 107-14.


(11) Illumina.

A VPARP Inhibition as a Chemical Biology Tool

A.1 Introduction

Poly(ADP-ribose) polymerases (PARPs) have roles in maladies as varied as neurodegenerative diseases, cancers, inflammatory response, and myocardial infarction. Although the PARP family is a group of mammalian enzymes with 18 putative members, only 8 have been cloned and isolated.1 Little is known about most of the PARPs, except that the enzymes link monomers of ADP-ribose, generated by cleavage of nicotinamid from NAD+, to a wide variety of protein substrates forming a branched polymer. In the last 15 years, there has been much research effort towards understanding the founding member of the family, PARP-1.

A.1.1 PARP-1

PARP-1 is part of the DNA repair machinery. It responds to double- and single-strand DNA breaks in three ways: in cases of minor damage PARP-1 signals for repair by polymerizing ADP-ribose, which is recognized by p53 and XRCC-1; in cases of extreme damage, PARP-1 ADP-ribose polymerization dramatically increases, thus, depleting the cell of NAD+ and leading to necrotic death;2 in cases moderate of damage, the cell undergoes apoptosis during which PARP-1 is cleaved and inactivated by a caspase.3 This caspase-mediated inactivation of PARP-1 prevents futile cycles of DNA damage and repair and preserves cellular energy for the apoptotic program. When activated by damaged DNA, PARP-1 is responsible for 90 % of PARP activity in cells and its kcat is increased by 500 fold.4

PARP-1 inhibition has been an intense area of medicinal chemistry research over the last 20 years, and many highly potent PARP-1 inhibitors have been developed.5,6 PARP-1 inhibitors are effective in animal models of cancer and neuro cardioprotective models.5 The PARP
inhibitor DR2313, when administered shortly after an induced stroke, was shown to protect the brains of rats from oxidative damage by inhibiting the necrotic death that would result from NAD$^+$ depletion.\textsuperscript{7} Similarly, PARP inhibitors protect against heart damage during heart attack models in rats.\textsuperscript{8} In cancer, PARP inhibitors potentiate the effects of DNA damaging agents temozolomide, camptothecin, and radiation.\textsuperscript{9-11} In addition to animal models, there are several PARP inhibitors in Phase I and II clinical trials as chemopotentiators for cancers, and one PARP inhibitor in trials as a single-entity agent for cardiovascular indications.\textsuperscript{12} While in most anti-cancer studies PARP inhibitors are used as potentiators, one PARP inhibitor is in Phase I studies as a single entity anti-cancer agent.\textsuperscript{13}

PARP-1 inhibition has received intense scrutiny, and multiple compounds are now available as inhibitors of PARP-1. However, almost no work has been done to develop small molecule inhibitors of the other PARP isozymes. This is likely due to the fact that the cellular role of these other PARP isozymes is still unclear, and thus their value as a therapeutic target is unknown. We believe that inhibition of other members of the PARP family also has potential therapeutic value. As discussed further below, we believe that inhibition of vault PARP (VPARP) could be useful in multidrug resistant cancers. The development of \textit{isozyme-specific} inhibitors of VPARP would allow this enzyme to be further studied as targets of cancer therapies without interference from other PARPs, namely PARP-1.

As single entity agents, PARP-1 inhibitors do not typically induce death in cancer cells, but rather can potentiate DNA damaging drugs by inhibiting the DNA damage repair process. PARP-1\textsuperscript{(+/−)} mice are still viable, and loss of PARP-1 activity through RNAi does not have an effect in the cell cycle.\textsuperscript{14,15} VPARP\textsuperscript{(+/−)} mice are more sensitive to the effect of certain toxins. Thus, my research goals were the development of \textit{isozyme-specific} inhibitors of VPARP. Of
course, specific targeting of isozymes of the PARP family is a challenge because the poly(ADP-ribose) polymerization site is highly conserved across family members. In addition, all catalytic residues are conserved, and the NAD\(^+\) binding pocket is 90\% homologous among PARP family members.\(^1\)

### A.1.2 PARP inhibitors

There are several different classes of PARP inhibitors, many of which are based on the nicotinamide binding site. The classic inhibitor, 3-aminobenzamide, is a nicotinamide mimic, but isoquinolines, phenanthridinones, and isoindolinones are known to be more potent PARP-1 inhibitors than the simple benzamide/3-aminobenzamide compounds (Figure A.1).\(^6\) There is little information about the isozyme-specificity of the known PARP inhibitors; a few PARP inhibitors have lower IC\(_{50}\)s for PARP-1 than PARP-2, but rarely have other isozymes been evaluated. To date, there are no selective VPARP inhibitors. Isozyme-specific small molecule inhibitors of VPARP could be superior to siRNA in research as they could provide a dose dependent effect, are inexpensive, and are simple to use. Inhibitors developed also have potential applications in anti-cancer research. My goal was to identify isozyme-specific inhibitors of VPARP, and to use these compounds to validate these PARP isozymes as viable targets for anti-cancer therapy.
Figure A.1 Representative PARP inhibitors of four scaffold classes, from left, the benzamides, isoquinolines, phenanthridinones, and isoindolinones. The IC₅₀ values of the actual inhibitors, from left, 22 μM, 0.39 μM, 40 nM, and 2nM.

A.1.3 VPARP

VPARP was initially discovered in a yeast two-hybrid screen designed to identify proteins that interact with the major vault protein (MVP) component of vaults. Vaults are one of the largest of particles in a cell at 13 MDa; they occur as hollow oblong shapes with a cinched “waist” and protein caps on either end. Cryoelectron microscopy of vaults shows that they open to eight petal-like segments per half vault. Although their volume is large enough to contain two ribosomes, only a portion of isolated vaults have density in their cavities; it is not known what accounts for the observed mass. Little is known about the function of vaults. They are found in all human cells, but cells exposed to toxins, such as epithelial intestine, lung, esophagus, and liver cells have higher numbers of vault particles. Upregulation and increased protein levels of MVP are found in multidrug resistant lung cancers, and with increased levels of MVP, VPARP expression and protein levels are also increased. Vaults are composed of 4 different molecules: 96 copies of MVP, 8 copies of VPARP, 2 molecules of telomerase-associated protein 1 (TEP1), and 6 vRNA. VPARP itself contains many different protein domains: a BRCT domain, a MVP interacting domain, an inter-α-trypsin inhibitor heavy chain-related domain, and a PARP domain that shares 28% sequence holomology to PARP-1.
VPARP has been shown to poly(ADP-ribose)ylate itself and MVP. VPARP is found both in the cytoplasm and in the nucleus of human cells with a portion sublocalized in mitotic cells to the mitotic spindle. VPARP associates with telomerase, but it is not necessary for telomerase function. No stimulus has been found that increases VPARP activity in the cell, and the length and branching of the PAR created is unknown.

VPARP deficient mice are viable and show no obvious phenotype. Their vaults are stable with normal morphology, and there is no effect on telomeres. When exposed to different chemical carcinogens, the mice have a slightly increased incidence of tumorigenesis in both lung and colon. The few papers reporting RNAi-mediated knockdown of VPARP show no obvious phenotype upon examination of spindle poles in mitotic cells. The function of vaults and VPARP remains a mystery.

A.2 The search for VPARP inhibitors

A three pronged approach was taken to discovering an isozyme-specific VPARP inhibitor: a small library was built using click chemistry, a homology model based on PARP-1 crystal structure was used to screen a small molecule library in silico, and a 24,000 member small molecule library was screened in an in vitro VPARP enzyme inhibition assay.

A.2.1 Synthesis of a focused library

A focused library based on known PARP inhibitors was synthesized using Huisgen [3+2] cycloaddition chemistry (Figure A.2). Four known PARP inhibitors were chosen as scaffolds because these compounds have relatively high IC$_{50}$s against PARP-1, thus, our system will not be biased toward selective inhibition of PARP-1. A second consideration was that having a scaffold with an aryl amine would simplify synthesis of the library as these are quite easily transformed into azides. The alkynes were all commercially available and include diverse
structural features with regard to rings, straight chains, hydrophobicity, and polarity. The chosen PARP inhibitors had aryl amines that were transformed to azides using sodium azide and sodium nitrite. These were “clicked” with 18 alkynes by heating in a microwave with a copper catalyst. After simple purification, usually by filtration, purity was 80-90 % with yields from 10-90 %, variable primarily based on solubility in methanol. The compounds were tested against recombinantly expressed catalytic fragment of VPARP using a colorimetric NAD$^+$ based PARP substrate developed in this lab.$^{30}$ The catalytic fragment of VPARP was expressed from pET28b vector, purified using Ni NTA resin, and immediately used in testing. Two compounds inhibited VPARP with IC$_{50}$s that could be estimated at 20 µM, however, they inhibited PARP-1 equally well at lower concentrations (Figure. A.3).
Figure A.2  A. Basic reaction used to synthesize aryl azides. B. 3+2 Huisgen cycloaddition to make libraries  C. Commercially available alkynes used to make library
Figure A.3 Focused library VPARP hits. Unfortunately, this inhibited PARP-1 as well.

A.2.2 *In silico* screen of ChemBridge library

The second approach to finding a specific VPARP inhibitor was *in silico* screening using a catalytic fragment VPARP homology model developed by Dr. Jerome Baudry. As our initial experiments suggest it would be difficult to obtain quantities of VPARP for an entire library screen, this virtually screening method was utilized in order to limit the number of compounds that would need to be physically screened in an *in vitro* VPARP inhibition assay. The Hergenrother laboratory possesses a collection of 14,000 compounds that were purchased from ChemBridge Corporation; this library was subjected to the *in silico* screen with VPARP and PARP-1, in the hopes of identifying a VPARP selective inhibitor. The compounds were docked in a rigid model, and the energy minimized compounds were allowed to sample all possible interactions by keeping all bonds freely rotatable. Screening 14,000 compounds took about 24 hours.

380 compounds were chosen from this virtual screening data as promising hits, and small portions (<0.5 µL) of these compounds were taken from the library and tested in singlet against recombinantly expressed catalytic domain of VPARP using a colorimetric assay. From this assay, 26 out of the 380 compounds appeared to be hits (Figure A.4). These compounds were then tested against PARP-1 in singlet; 20 of the compounds also inhibited PARP-1, and thus 6 compounds were potential VPARP inhibitors. These 6 were then purchased from ChemBridge
and tested as VPARP inhibitors over a range of concentrations. Importantly, controls were also performed to determine the absorbance of the compound alone and the compound in combination with the substrate. None of the 6 compounds gave dose dependent behavior independent of their own absorbance. This result was further confirmed by evaluating the compounds in a different PARP-1 assay, one in which NAD+ is converted to a fluorescent product.\textsuperscript{31} \textit{In silico} screening gave no VPARP specific inhibitors.
Figure A.4  PARP-1 inhibition of *in silico* screen ChemBridge hits. Compounds 6138830, 6241008, 6169128, 6171674, 5852203, and 5251828 were ordered from ChemBridge for further testing, but did not show dose dependent inhibition of VPARP.
Figure A.4 Con't
A.2.3 Screening a 24,000 member library

The next step in the effort to identify an isozyme-specific VPARP inhibitor was to screen our entire library of small molecules. This was partially made possible by the development of a chromogenic PARP substrate in our laboratory, ADP-ribose-pNA (Figure A.5). Because recombinantly expressed VPARP does not remain active longer than one day, it was necessary to express, purify, and use VPARP all in one ~12 hour time period. To screen the 24,000 compounds, 8 liters of *E. coli* harboring the VPARP expression vector were grown 10 times over 30 days. In addition, several plates needed to be rescreened because the controls did not respond appropriately.

![Figure A.5 Chromogenic PARP substrate ADP-ribose-pNA.](image)

For the screen, VPARP (5 µM in 40 µL buffer) was added to the wells of 384 well plates. Compounds were added through the use of a 384-well pin transfer apparatus, which transfers ~50 nL at a time for a final compound concentration of ~10 µM. The ADP-ribose-pNA substrate was added, and the plates were allowed to incubate for 12 hours at room temperature. After 12 hours, the absorbance of the wells was read at 405 nm. Both the 14,000 member ChemBridge library and 10,000 member Marvel library were tested in this manner. From this screen 147 primary hits were identified; these compounds were rescreened in duplicate at compound concentrations of 10 and 100 µM. Twenty compounds appeared to be possible hits and were ordered from ChemBridge Corporation and tested for their ability to inhibit VPARP in a dose
dependent manner. Appropriate controls were conducted monitoring the absorbance of the actual compound during the course of the assay. From these experiments, the 20 compounds were reduced to 2 (Figure A.6). However, neither was pursued as both of these compounds were found to inhibit PARP-1 to a significant degree.
Figure A.6  ChemBridge VPARP hits from high throughput screen. These are the twenty compounds that were ordered from ChemBridge. Compounds 5759604 and 5758375 were positive in assays for VPARP inhibition, but also inhibited PARP-1.
A.3 Conclusions

Despite utilizing three different strategies, a specific VPARP inhibitor remains elusive. High-throughput screening and synthesis of focused libraries have not produced an isozyme-specific VPARP inhibitor. In conclusion, this project is currently not active.

A.4 Acknowledgements

I would like to thank Dr. Valerie Kickhoefer for her kind gift of pET28b-p193cat plasmid (catalytic fragment of VPARP). Also thank you to Dr. Jerome Baudry for his assistance with \textit{in silico} screening.
A.5 References


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