CELLULAR EFFECTS OF PLATINUM CHEMOTHERAPEUTICS: ALTERATIONS BY ANTIDEPRESSANTS AND HEPARAN SULFATE PROTEOGLYCANS

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CELLULAR EFFECTS OF PLATINUM CHEMOTHERAPEUTICS: ALTERATIONS BY ANTIDEPRESSANTS AND HEPARAN SULFATE PROTEOGLYCANS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Biology at Virginia Commonwealth University.

By

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I would like to thank Dr. Farrell for all the guidance and training he has given me over the past three years. I would also like to thank Dr. Ryan for allowing me to work in his lab and for training me as one of his own.
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List of Abbreviations

ERK extracellular signal-regulated kinase
JNK c-Jun NH2-terminal kinase
WT wild type
P53/- p53 knockout
HCT116 wt a human colon carcinoma cell line, wild type p53 status
HCT116 p53/- a human colon carcinoma cell line, p53 knockout
HT-29 a human colon carcinoma cell line
A2780 a human ovarian carcinoma cell line
A2780/CP a platinum resistant ovarian carcinoma cell line
SKOV-3 a human ovarian carcinoma cell line, platinum resistant
CHO Chinese Hamster ovary cells
SAOS-2 a human osteosarcoma cell line
2008/C13*5.25 a platinum resistant ovarian carcinoma cell line
IGROV-1/CP a platinum resistant ovarian carcinoma cell line
BBR3464, BBR $\left\{\text{trans-PtCl(NH}_3\text{)}_2\right\}_2\mu-\left\{\text{trans-Pt(NH}_3\text{)}_2(H_2N(CH_2)_6-NH_2)_2\right\}^{4+}$
Carboplatin, Carbo cis-diammine-1,1'-cyclobutane dicarboxylate platinum(II)
Cisplatin, c-DDP cis-diamminedichloro platinum(II)
FBS Fetal Bovine Serum
Fondaparinux, Fonda Decasodium methyl 2-deoxy-6-O-sulfonato-2-(sulfonatoamino)-α-D-glucopyranosyl-(1->4)-β-D-glucopyranuronosyl-(1->4)-2-deoxy-3,6-di-O-sulfonato-2-(sulfonatoamino)-α-D-glucopyranosyl-(1->4)-
2- O-sulfonato-α-L-idopyranuronosyl-(1->4)-2-deoxy-6-O-sulfonato-2-(sulfonatoamino)-α-D-glucopyranoside

<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
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<tr>
<td>HMG</td>
<td>High mobility group</td>
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<tr>
<td>Ox</td>
<td>Oxaliplatin, Ox, 1,2-diaminocyclohexanecyclohexaneoxalatoplatinum(II), [Pt(ox)dach])</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>Pt</td>
<td>Platinum</td>
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<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
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<tr>
<td>OCT</td>
<td>Organic Cation Transporter</td>
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<tr>
<td>[Di(OC₆)₃]</td>
<td>3,3’–dihexyloxacarbocyanine iodide</td>
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<tr>
<td>ICP-OES</td>
<td>Inductively Coupled Plasma Optical Emission Spectroscopy</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-ADP ribose polymerase</td>
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<tr>
<td>C.I.</td>
<td>Combination Index</td>
</tr>
<tr>
<td>RPMI medium</td>
<td>Roswell Park Memorial Institute medium</td>
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<tr>
<td>cRPMI medium</td>
<td>completed RPMI</td>
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<td>W7</td>
<td>N-(6-Aminoheptyl)-5-chloro-1-naphthalenesulfonamide hydrochloride</td>
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<tr>
<td>WST-1</td>
<td>2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt</td>
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<tr>
<td>Δψₘ</td>
<td>mitochondrial membrane potential</td>
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<tr>
<td>GnTV</td>
<td>N-acetylglucosaminyltransferase V</td>
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Abstract

CELLULAR EFFECTS OF PLATINUM CHEMOTHERAPEUTICS: ALTERATIONS BY ANTIDEPRESSANTS AND HEPARAN SULFATE PROTEOGLYCANS

By Brigitte J Engelmann, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Biology at Virginia Commonwealth University.

Virginia Commonwealth University 2012

Major Director: Nicholas P. Farrell/Professor of Chemistry and Director of the Chemical Biology Ph.D. Program

Co-Director: John J. Ryan/Professor of Biology

The work discussed here is divided into two projects. The first project involves the interactions between antidepressants and the platinum based chemotherapeutics while
the second project begins to investigate possible implications of a recently discovered uptake mechanism for positively charged platinum drugs.

Gaining understanding of the interactions between antidepressants and platinum-based chemotherapeutics is important due to the frequency with which they are prescribed together. Although using a combination regimen of antineoplastics is beneficial to the patient, not all drug interactions are. For instance, many of the serotonin reuptake inhibitors have been shown to decrease the efficacy of tamoxifen.

Desipramine, a tricyclic antidepressant used to treat neuropathic pain, has been shown to increase the cytotoxicity of cisplatin, oxaliplatin and carboplatin in the human colon carcinoma cell line, HCT116 wt. To study this interaction, the cell line specificity as well as the drug specificity with regard to both the platinum-based chemotherapeutic and the antidepressant were investigated. The data show that the effect is both cell line specific as well as drug specific with respect to both types of drugs.

To elucidate the mechanism behind the alteration in cytotoxicity of the platinum drugs, the effect of p53 status was investigated. A reduction of the effect is observed in the absence of p53, suggesting that there is a p53 dependent mechanism as well as a p53 independent mechanism. The tricyclic antidepressants and fluoxetine are known to be calmodulin inhibitors. Calmodulin inhibition mirrored some of the effects seen with the antidepressants suggesting that calmodulin inhibition might also play a role in the mechanism.
The second project is based on the discovery that heparan sulfate proteoglycans mediate the uptake of positively charged platinum complexes. Heparan sulfate proteoglycans are important in cell-cell as well as cell-extracellular matrix adhesion. In cancer, heparanase, the enzyme that cleaves heparan sulfate, is over expressed creating a pro-angiogenic and pro-metastatic state. This work demonstrates that the positively charged platinum complexes can inhibit heparanase activity by binding to the substrate (heparan sulfate proteoglycans). This suggests that this class of drugs may have the capacity to be anti-angiogenic and anti-metastatic as well as cytotoxic.
Chapter 1: Introduction

1.1 Cancer

Cancer is not a single disease. Instead, the word cancer refers to a group of diseases that are characterized by uncontrolled growth of abnormal cells. The abnormal cells spread throughout the body and this can result in death. In fact, in the United States, cancer is the cause of almost 25% of all deaths. This makes cancer the second most common cause of death in the United States. It is second only to heart disease.\(^1\) Although cancer is second to heart disease in the annual number of deaths, heart disease is second to cancer in years of potential life lost.\(^2\)

The American Cancer Society estimates that this year in the United States, 1,638,910 new cancer cases will be diagnosed and 577,190 people will die from cancer.\(^1\) The individual and societal financial burdens due to cancer are enormous. According to the NIH Fact Book Fiscal Year 2011, in 2008, the direct cost in medical care for cancer patients was 77.4 billion dollars. This includes costs for doctors’ visits and hospital stays, prescribed medications and home care but excludes costs associated with nursing home care. The NIH further estimates the indirect cost of mortality to be 124 billion dollars. This is an estimate of the cost of loss of productivity due to premature
death. In total, cancer cost the United States 201.4 billion dollars in 2008. This is second only to the costs associated with heart disease.\textsuperscript{2}

Although cancer refers to many diseases, there are certain traits that are consistent across them all. Malignant tumors are poorly differentiated (the tissue no longer resembles the parent tissue), monoclonal (derive from a single cell), the growth rate is often increased and the nuclear:cytoplasmic ratio is increased compared to benign tumors or normal tissue. Malignant tumors are also able to locally invade tissue as well as metastasize to distant tissues.\textsuperscript{3}

It is thought that tumorigenesis is a multi-step process that reflects genetic alterations. Hanahan and Weinberg suggest that there are six alterations in cell physiology that are necessary for malignant tumorigenesis. These alterations are 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) the ability to evade apoptosis, 4) the ability to replicate and divide forever, 5) sustained angiogenesis, and 6) the ability to invade tissues and metastasize.\textsuperscript{4}

Cancer is usually treated with a combination of surgery, radiation, immunotherapy and chemotherapy. An example of immunotherapy is the use of Rituximab in the treatment of non-Hodgkin’s lymphoma. Rituximab is a monoclonal antibody that binds to a surface protein on the lymphoma cells and leads to cell death.\textsuperscript{5} Chemotherapy is the treatment of cancer with cellular toxins. Examples of chemotherapeutic agents are the platinum-based chemotherapeutics: cisplatin, oxaliplatin and carboplatin (Figure 1.1).
1.2 Platinum Drugs

1.2.1 Cisplatin, Oxaliplatin and Carboplatin

Cisplatin was discovered in 1845 by Michel Peyrone. Despite its early discovery, its anticancer properties were not discovered until the 1960s. It was then that Barnett Rosenberg discovered that cisplatin caused the elongation of *Escherichia coli* (*E. coli*) cells by inhibiting cellular division. It was due to this observation that cisplatin was tested for its anticancer properties. In 1978, it was approved by the FDA. Today, cisplatin is used to treat ovarian, bladder, cervical and head and neck cancers as well as melanoma and lymphomas. In fact, cisplatin in combination with other chemotherapeutic agents cures over 90% of testicular cancer cases.

It is generally believed that the cytotoxic activity of cisplatin and the other platinum-based chemotherapeutics is due to their interactions with DNA leading to apoptosis. Cisplatin is usually administered to the patient intravenously. The chloride concentration in the plasma is quite high (approximately 100 mM). This is a high enough concentration that it will limit the replacement of the chloride ligands by water.
molecules. Although hydrolysis of the chloride ligand is inhibited, cisplatin is still reactive and binds to albumin in the plasma.\textsuperscript{9} This binding is quite extensive and is thought to deactivate the cisplatin.\textsuperscript{6} In fact, it has been reported that one day after cisplatin intravenous infusion, 65-98\% of the platinum in the plasma is bound to albumin. This binding is irreversible.\textsuperscript{9} Any remaining intact cisplatin is able to enter the cells where there is a much lower chloride concentration (approximately 4 mM). Hydrolysis of the chloride ligand then occurs and the cisplatin is able to bind to the DNA at adjacent guanines on the same strand of DNA (intrastrand adduct) or at two guanines one on each strand of the DNA double helix (interstrand adduct). The cell will then either proceed to apoptosis or the platinum-DNA lesion will be repaired and the cell will continue living.\textsuperscript{6} As shown in Figure 1.2. The limiting toxicity of cisplatin is neurotoxicity resulting in peripheral neuritis, tinnitus and hearing loss. Cisplatin is also quite nephrotoxic.\textsuperscript{10}

Since the discovery of cisplatin, two other platinum-based chemotherapeutics have been added to clinical use: carboplatin and oxaliplatin (Figure 1.1). Carboplatin is administered intravenously.\textsuperscript{11} It is less toxic than cisplatin and causes much less neuro- and nephro-toxicity.\textsuperscript{10, 11, 12} The lower toxicity allows it to be given in a higher dose, but unfortunately, it is active in the same range of tumors as cisplatin.\textsuperscript{11} The limiting toxicity of carboplatin is myelosupression.\textsuperscript{10, 12} Oxaliplatin is used in advanced colon cancer.\textsuperscript{10, 13} It has less nephrotoxicity and myelosuppression than cisplatin and carboplatin, but its dose limiting toxicity is peripheral sensory neuropathy.\textsuperscript{10, 12} Patients reported dysaesthesias of the arms, legs, mouth and throat. In one patient the
dysaesthesia of the throat was accompanied by laryngospasm (a spasmodic closing of the larynx which results in the inability to get air into the lungs). Other patients reported ataxia (loss of coordination) and gait disturbances. Although these side effects are disturbing, patients often experience complete recovery after stopping oxaliplatin therapy. This is a distinct advantage over cisplatin where the neuropathy may be permanent.¹²

Figure 1.2 The cytotoxic pathway for cisplatin. Once cisplatin enters the cell, the chloride ligands are replaced with aqua groups. Cisplatin is then able to bind to the DNA. If the cell cannot repair the DNA damage, the cell will proceed to apoptosis down path a). If the DNA can be repaired, the cell will proceed down path b). This figure was taken from Alderden RA, Hall MD, and Hambley TW, (2006) J Chem Ed 83:728-734.
1.2.2 Polynuclear Platinum Compounds

In an effort to circumvent resistance against cisplatin and its analogues, researchers developed platinum-based compounds that form very different platinum-DNA adducts. To this end, researchers have developed multinuclear platinum complexes that are connected with linkers.\textsuperscript{11} An example of this type of drug is BBR3464 (Figure 1.3), a trinuclear platinum complex where the platinum coordination units are linked by alkanediamine linkers.\textsuperscript{14} BBR3464 has been shown to have increased cellular accumulation compared to cisplatin in the osteosarcoma cell lines U2-OS and U2-OS/Pt.\textsuperscript{14} It has also been shown to be more cytotoxic than cisplatin and to be effective in the treatment of cisplatin-resistant cell lines and tumors \textit{in vitro} as well as \textit{in vivo}.\textsuperscript{14, 15}

While BBR3464 covalently binds to DNA, the trinuclear platinum chemotherapeutics AH44 and AH78 (Figure 1.3) non-covalently bind to DNA. In the case of AH44 and AH78, the reactive chloride ligands of BBR3464 are replaced with inert ammonia or amine groups.\textsuperscript{16, 17}
Factors Affecting Cytotoxicity

There are three major factors that affect the cytotoxicity of the platinum drugs. The first factor is the nature of the platinum-DNA adducts formed and the frequency with which these adducts are formed. The second factor is deactivation of the platinum drugs by reactions with sulfur-containing nucleophiles such as glutathione. The third factor is alteration of the cellular uptake and efflux of the platinum drugs.

1.3.1 Platinum-DNA Adducts

Cisplatin can form monofunctional and bifunctional adducts to DNA (Figure 1.4). The majority of adducts formed are 1,2 intrastrand adducts at adjacent guanines. Cisplatin also forms 1,2-intrastrand adducts between an adenine and a guanine as well as 1,3-intrastrand adducts between two guanines with another base in between the two

\[
\text{BBR3464 } X=\text{Cl}; \quad n=4
\]
\[
\text{AH44 } X=\text{NH}_3; \quad n=6
\]
\[
\text{AH78 } X=\text{NH}_2(\text{CH}_2)_6\text{NH}_2; \quad n=8
\]

Figure 1.3 Structures of the polynuclear platinum complexes BBR3464, AH44 and AH78.
guanines. Cisplatin is also able to form 1,2-interstrands adducts between two guanines.\(^\text{18, 19}\)

BBR3464 forms long range 1,4 and 1,6-interstrand cross-links.\(^\text{20, 21, 22}\) The non-covalently binding platinum drugs, AH44 and AH78, have two unique DNA binding features, backbone tracking and minor groove spanning.\(^\text{17}\) Backbone tracking describes the electrostatic interactions between the non-covalently binding platinum drugs and the phosphate oxygen atoms of the DNA backbone. Minor groove spanning describes how the non-covalently binding platinum drugs can interact with two phosphate oxygens on different DNA strands via two phosphate clamps thereby spanning the minor groove of the DNA duplex.\(^\text{17}\)

The different types of platinum-DNA adducts formed by these drugs are not treated the same way by the cells. For instance, the high mobility group (HMG) proteins recognize
cisplatin-DNA adducts. It is thought that this may modulate the cytotoxicity of cisplatin in two ways. First, many HMG proteins are transcription factors and if they are binding to cisplatin-DNA adducts, they are not binding to their normal binding sites. This would disrupt cellular function. Second, the HMG proteins may actually shield the cisplatin-DNA adducts from damage recognition resulting in a reduction of cisplatin-DNA adduct repair. The BBR3464-DNA adducts are not recognized by the HMG proteins.

These platinum-DNA adducts cause DNA damage which initiates biochemical signaling pathways. The platinum-DNA adducts can activate p53 which can lead to cell cycle arrest. Once the cell is arrested, the cell can either repair the DNA damage or proceed to apoptosis.

1.3.2 Deactivation of the Platinum Drugs

Although DNA is thought to be the cytotoxic target of the platinum based chemotherapeutics, they are also able to bind to sulfur-containing molecules. Two examples of sulfur-containing molecules that bind to the platinum based chemotherapeutics are human serum albumin and glutathione. When the platinum drugs bind to these sulfur-containing molecules, the platinum drugs are deactivated and are no longer able to bind to their DNA target. An increased cellular level of glutathione is an important resistance mechanism that has been observed in many platinum-resistant cell lines.
1.3.3 Cellular Uptake and Efflux of Platinum-Based Chemotherapeutics

Some resistant cell lines have been shown to have decreased accumulation of the platinum drugs when compared to their platinum-sensitive counterparts. Resistance to cisplatin and the other platinum-based chemotherapeutics is a frequent reason for treatment failure. Platinum-resistance may be intrinsic to the cell or tissue type or it may be acquired through exposure to the drug over time, during treatment for instance.\(^7\) Differences in accumulation are either caused by changes in platinum uptake or by changes in platinum efflux.

1.3.3a Role of CTR1 in Platinum Drug Uptake

While early experiments suggested that cisplatin and its analogues entered cells by passive diffusion, later studies suggested that these platinum based chemotherapeutics likely use transporters as well as passive diffusion to enter the cell.\(^25\) CTR1 (copper transporter 1) is a copper influx transporter that also transports platinum based chemotherapeutics.\(^25\) Holzer \textit{et al.} investigated the role of human CTR1 on cisplatin accumulation in human ovarian carcinoma cells. A2780 cells were either transfected with an empty vector or with a vector containing the human CTR1 cDNA. Interestingly, although the human CTR1 increased cisplatin accumulation there was only a marginal increase in cytotoxicity.\(^26\)

Kabolizadeh \textit{et al.} also investigated the role of human CTR1 in cisplatin and BBR3464 mediated apoptosis in A2780 cells and A2780 cells that were transfected with a vector containing the human CTR1 cDNA. In this study, the expression of human CTR1 gave
increased accumulation of cisplatin and BBR3464 as well as increased cytotoxicity for both of the platinum drugs.\textsuperscript{27}

Beretta \textit{et al.} studied the role of human CTR1 in the cisplatin-resistant cervix squamous cell carcinoma A431/Pt. In contrast to the studies discussed above, A431/Pt cells that were transfected with human CTR1 did not show changes in platinum accumulation when compared to A431/Pt cells that were not overexpressing human CTR1. Furthermore, no alteration in cisplatin sensitivity was observed in the A431/Pt cells that overexpressed human CTR1 compared to the A431/Pt cells that were not overexpressing human CTR1.\textsuperscript{28}

\subsection*{1.3.3b Organic Cation Transporters}

The organic cation transporters are transmembrane transporters that are members of the SLC22A family. There are three organic cation transporters known as OCT1, 2 and 3.\textsuperscript{25} OCT1 is primarily expressed on the basolateral membrane of hepatocytes.\textsuperscript{29} OCT2 is primarily expressed in the kidney\textsuperscript{25} while OCT3 is expressed in many tissues including the kidney, liver, placenta, skeletal muscle and heart.\textsuperscript{25, 29} These transporters transport drugs, endogenous metabolites and toxins and have been shown to be important in cellular accumulation of platinum based chemotherapeutics.\textsuperscript{30} Cisplatin has been shown to be transported by OCT2 while oxaliplatin has been shown to be transported by OCT1, 2 and 3.\textsuperscript{31} Carboplatin has not been shown to be a substrate for the organic cation transporters. In fact, it has been shown that the transport of cisplatin by OCT2 is responsible for the nephrotoxicity observed during cisplatin therapy.\textsuperscript{30} It is
interesting to note however, that although oxaliplatin is also transported by OCT2, nephrotoxicity was not observed with oxaliplatin.\(^{30}\) The fact that OCT1 and OCT2 are expressed in a variety of colon cancer cell lines may explain why oxaliplatin is more effective in treating colon cancer than cisplatin.\(^{23}\)

### 1.3.3c Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans have been shown to mediate cell entry for positively charged tri-platinum compounds.\(^ {32}\) Due to the fact that nonaarginine is the most efficiently transported ‘protein transduction domain’\(^ {33}\), Silva et al. used a TAMRA (5- (and 6-) carboxytetramethylrhodamine) tagged nonaarginine to investigate how different platinum drugs competed with the nonaarginine for cellular internalization in wild type CHO cells. The neutral platinum compounds cisplatin and oxaliplatin did not inhibit the internalization of the nonaarginine. However, the positively charged tri-platinum compounds BBR3464, AH44 and Triplatin NC (aka AH78) prevented internalization of the nonaarginine in a charge-dependent manner. BBR3464 with a charge of +4 and AH44 with a charge of +6 decreased the nonaarginine internalization while Triplatin NC with a charge of +8 completely inhibited internalization of the nonaarginine. This was also examined in two cancer cell lines: the human colon carcinoma HCT116 and the osteosarcoma SAOS-2. Interestingly, the positively charged tri-platinum drugs showed increased inhibition of nonaarginine internalization in the tumor cell lines compared to the normal CHO cells. This suggests that these drugs may have some tumor selectivity. To confirm that heparan sulfate proteoglycans were responsible for these observations, Silva et al. investigated the cellular accumulation of the platinum drugs in
CHO cells, heparan-sulfate deficient CHO cells and heparan- and chondroitin-sulfate deficient CHO cells. The cellular accumulation of BBR3464, AH44 and Triplatin NC was reduced in the CHO mutants confirming the role of proteoglycans in the uptake of positively charged platinum drugs. Furthermore, endocytosis is known to be a mechanism of cell entry for heparan sulfate proteoglycans. Silva et al. demonstrated that macropinocytosis, a type of endocytosis, is responsible for the uptake of Triplatin NC and to a lesser extent BBR3464.

1.3.3d ATP7A and ATP7B Copper Efflux Transporters

ATP7A and ATP7B are copper efflux transporters that have been shown to have a role in platinum drug resistance. Although ATP7A and ATP7B are very similar proteins, they are expressed in different tissues. ATP7A is expressed in the intestines while ATP7B is expressed in the liver and kidney. ATP7B expression is a poor prognostic factor for chemotherapy success in patients with solid carcinomas.

Samimi et al. transfected Me32a fibroblast cells to express either ATP7A or ATP7B. The Me32a cell line does not normally express ATP7A or ATP7B. The expression of ATP7A or ATP7B resulted in resistance to cisplatin and carboplatin. Surprisingly, the resistance was also accompanied by an increase in platinum cellular accumulation but not an increase in platinum-DNA binding. Increased levels of platinum were measured in vesicles suggesting that ATP7A and ATP7B could sequester the cisplatin and carboplatin into vesicles but that they could not re-localize to the cell membrane and efflux the platinum drugs. Interestingly, although ATP7A and ATP7B increased the
cellular accumulation of oxaliplatin, the cells became more sensitive to oxaliplatin as opposed to more resistant as was observed with cisplatin and carboplatin. The cells expressing ATP7A did not have increased oxaliplatin in vesicles when compared to the parental cell line, but they did show increased oxaliplatin-DNA binding. The cells expressing ATP7B showed increased levels of oxaliplatin in the vesicles as well as increased oxaliplatin-DNA binding when compared to the parental cell line. This demonstrates that oxaliplatin is not as efficiently sequestered by ATP7A as it is by ATP7B. Samimi et al. suggest that although ATP7B can sequester oxaliplatin, the sequestration does not inactivate the oxaliplatin. This was supported by the observed increase in oxaliplatin-DNA binding in cells expressing ATP7B.\(^{34}\)

In contrast, Katano et al. observed that in cisplatin-resistant cells that were cross resistant to copper, platinum accumulation was reduced compared to the platinum sensitive parental lines. The resistant ovarian carcinoma cell lines used, A2780/CP, 2008/C13*5.25 and IGROV-1/CP, all exhibited increased levels of the copper efflux transporters when compared to their parental cell lines. A2780/CP and 2008/C13*5.25 overexpressed ATP7A. IGROV-1/CP overexpressed ATP7B. The 2008/C13*5.25 cell line also showed increased copper and platinum efflux compared to the 2008 parental cell line.\(^{35}\)

Taken together, these data show that the copper efflux transporters, ATP7A and ATP7B are important in platinum resistance. They have been shown to decrease the cytotoxicity of platinum drugs by either sequestering the drugs into vesicles so that they
are unable to reach their DNA target or by decreasing the cellular accumulation of the platinum drugs.

1.4 Doctoral Projects

This thesis describes two projects that are the result of different platinum cellular accumulation pathways and the implications of these pathways. The first project, addresses the interactions between antidepressants and platinum-based chemotherapeutics. This project began with the hypothesis that desipramine, a tricyclic antidepressant and known organic cation transporter inhibitor, might inhibit the effects of cisplatin, oxaliplatin and the experimental positively charged platinum drug BBR3464. Not only did desipramine fail to block cellular accumulation of all three platinum drugs, but it also increased the cytotoxicity of cisplatin, oxaliplatin and BBR3464. Chapters 2 and 3 examine potential mechanisms to explain the observed augmentation in cytotoxicity of the platinum drugs. They also address how cell specific and drug specific the observed interactions are. Chapter 2 has been published in the Journal of Biological Inorganic Chemistry.

The second project began with the hypothesis that positively charged platinum-based chemotherapeutics bind to heparan sulfate proteoglycans on the cell surface and use this interaction as a mechanism for cellular uptake. This new uptake mechanism was demonstrated by Silva et al. Chapter 4 addresses the role of glycosaminoglycans and proteoglycans in both normal and pathological states. It also investigates the potential implications of platinum drug binding to heparan sulfate in cancer treatment.
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2.1 Abstract

A unique potentiation of platinum drug cytotoxicity is noted in the presence of the tricyclic antidepressant desipramine. Desipramine is used for treating neuropathic pain, particularly in prostate cancer patients. The clinically used drugs cisplatin (cis-[PtCl₂(NH₃)₂], c-DDP), oxaliplatin (1,2-diaminocyclohexaneoxalatoplatinum(II),
[Pt(ox)dach]) and the cationic trinuclear agent BBR3464 \[{\text{\{trans-PtCl(NH}_3\}_2\mu-\{trans-Pt(NH}_3\}_2(H}_2N(CH}_2)\_6NH}_2\}_2\}]^{4+}\), which has undergone evaluation in Phase II for activity in lung and ovarian cancers, were evaluated. Surprisingly, desipramine greatly augments the cytotoxicity of all the platinum-based chemotherapeutics in the HCT116 colorectal carcinoma cell line. Desipramine enhanced cellular accumulation of cisplatin, but had no effect on the accumulation of oxaliplatin or BBR3464, suggesting that enhanced accumulation could not be a consistent means by which desipramine altered the platinum drug-mediated cytotoxicity. The enhancement of cytotoxicity due to desipramine resulted in increased expression of p53, mitochondrial damage, caspase activation and PARP cleavage. The study shows that desipramine may be a means of enhancing chemo-responsiveness of platinum drugs and the results warrant further investigation. The results emphasize the importance of understanding the differential pharmacology of adjuvants employed in combinations with cancer chemotherapeutics.

2.2 Introduction

Cancer chemotherapy usually involves treatment with drug combination regimens. Platinum-based drugs play an important part as components of these regimens. Cisplatin is curative in testicular cancer while oxaliplatin (Eloxatin) is recommended with 5-fluorouracil (FOLFOX) for treatment of metastatic colon cancer. The pharmacological action of any drug must be monitored in light of proposed combinations. The pharmacological parameters affecting platinum drug cytotoxicity and antitumor activity are generally accepted to be (i) the nature and extent of target (DNA) binding; (ii) the extent of platinum drug cellular accumulation and (iii) metabolizing (destabilizing)
interactions with sulfur-containing biomolecules such as Human Serum Albumin (HSA) and glutathione (GSH). Reduced cellular accumulation is consistently being seen as a major cause of development of clinical resistance.\textsuperscript{1, 2} Cellular accumulation can be affected by drug combinations and this point may be an important feature in designing potential combination regimens for new, emerging agents. In pre-clinical studies, the Raf kinase inhibitor BAY43-9006 reduces cellular accumulation of cisplatin and oxaliplatin\textsuperscript{3} whereas, in contrast, the 20S proteasome inhibitor Bortezomib enhances cisplatin accumulation by blocking the cisplatin-induced down-regulation of the hCTR1 transporter in a concentration-dependent manner.\textsuperscript{4} Interestingly, the concomitant administration of imatinib with cisplatin prevents cisplatin-induced nephrotoxicity by inhibiting cisplatin renal accumulation.\textsuperscript{5}

Adjuvant therapy with antidepressants is also common standard of care for cancer patients.\textsuperscript{6} People with cancer are three times more likely than the general population and almost two times more likely than other hospitalized medical patients to develop major depression.\textsuperscript{7} Untreated, this can lead to decreased compliance with medical care as well as increasing the psychological toll on patient and family. Patients will also most likely be treated with combination chemotherapy with a variety of anticancer drugs. There is therefore an important need to examine how the combination of an adjuvant drug interacts with the chemotherapeutic agents - the combination may be additive or antagonistic, where the drug interference can result in decreased efficacy of the treatment. Recent discussion on the negative effect of antidepressants on tamoxifen therapy in breast cancer patients highlights the necessity to understand the
pharmacology of drug combinations. The differential effects noted above on cellular platinum accumulation, and hence efficacy, could also extend to adjuvant therapies.

The choice of a specific antidepressant depends on a number of factors including the nature of the depressive symptoms, medical problems present, and side effects of the specific drug. Low doses of tricyclic antidepressants can be especially useful as adjuvant pain medications in patients with neuropathic pain syndromes. Desipramine (Figure 2.1), is one such tricyclic and is important for treating neuropathic pain from cancer chemotherapy. It is also relevant as an inhibitor of organic cation transport accumulation pathways, a possible mechanism for cellular accumulation of cisplatin. As such, it was anticipated that desipramine might antagonize the effects of cisplatin and oxaliplatin, Figure 2.1, and their side effects of nephrotoxicity and neuropathy respectively. The tumor cell line selected for study was human colon HCT116. We also examined the pre-clinical drug BBR3464 because a survey of cytotoxicity across the NCI tumor panel showed enhanced sensitivity of colon cancers to this drug. This work demonstrates the role of desipramine in modulating the biological effects of platinum drugs and the unexpected potentiation of cytotoxicity in the presence of the antidepressant.
2.3 Materials and Methods

2.3.1 Compound Synthesis

BBR3464 and cisplatin were synthesized as previously described.\textsuperscript{15} Oxaliplatin and desipramine were obtained from Sigma-Aldrich (St. Louis, MO).

2.3.2 Cell Culture Conditions

The colorectal carcinoma cell lines HCT116 were the kind gift of Professor Bert Vogelstein (Johns-Hopkins University, Baltimore, MD). HCT116 cells were cultured with...
RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mmol/L sodium pyruvate (cRPMI, all from Biofluids, Rockville, MD) in humidified air with 5% CO₂. For the assays, cells were cultured in 6-well plates at an initial density of 7.0 × 10⁴ cells/mL. Different concentrations of drugs were added to each well as indicated. Total cells (adherent and non-adherent cells) were collected. BBR3464, c-DDP, and oxaliplatin concentrations were adjusted to achieve approximately 30%-40% apoptosis after 72 hours of treatment, allowing measurement of enhancement or inhibition in presence of the antidepressant.

2.3.3 Propidium Iodide DNA Staining and Analysis of Apoptosis

Samples were fixed in a solution containing 35% 1x PBS (vol/vol), 12.48% fetal bovine serum (vol/vol), and 52.52% 70% ethanol (vol/vol). The samples were then washed with phosphate buffered saline (PBS), and stained with a solution containing 94% 1x PBS (vol/vol), 0.1 mg/mL RNase A, 0.0001 M EDTA, and 0.04 mg/mL propidium iodide (PI). The samples were stained for two hours. Samples were then analyzed for subdiploid DNA content using a FACScan flow cytometer (BD Biosciences, San Jose, CA). It is noteworthy that this protocol differs significantly from the more common PI-based exclusion, which only differentiates live versus dead cells. Through fixation and RNase A treatment, we were able to detect intact versus fragmented DNA, revealing discrete stages of the cell cycle and the percentage of the population undergoing apoptosis.
2.3.4 Assessment of Mitochondrial Membrane Potential

Alteration in mitochondrial membrane potential was assessed by staining with 3,3’–dihexyloxacarbocyanine iodide [Di(OC₆)₃]; Molecular Probes, Eugene, OR. Di(OC₆)₃ was added to 200 µL of cells at 40 nmol/L final concentration. Cultures were incubated for 30 minutes at 37°C in a CO₂ incubator. The cells were then washed twice with PBS and resuspended in 200 µL PBS for flow cytometric analysis using a forward and side scatter gate sufficiently open to include apoptotic/dying cells.

2.3.5 Assessment of Caspase Activation

Staining for active caspases was performed with caspase kits (Immunochemistry Technologies, LLC, Bloomington, MN), as specified by the manufacturer. Cells were incubated with a cleavable substrate that binds to the active caspases-3 and -7. Subsequent substrate cleavage results in increased fluorescence intensity, interpreted as caspase-positive cells. The percentage of caspase-positive cells was measured by flow cytometry.

2.3.6 Platinum Accumulation Assays

Cells were plated at 2.0×10⁶ cells/mL. Platinum drug was added in different concentrations alone or 60 minutes after the addition of desipramine. After 8 or 16 hours, cells were harvested and washed twice with PBS. The cell pellets were then dissolved in hot nitric acid followed by the addition of hydrogen peroxide and hydrochloric acid, according to the United States Environmental Protection Agency procedure 3050b (all volumes reduced by 1/10) and diluted with Milli-Q water (Millipore...
Corporation, Billerica, MA). Platinum analysis was performed on a Vista-MPX simultaneous inductively coupled plasma optical emission spectroscopy (ICP-OES) at 265 nm (Varian Inc., Palo Alto, CA). Standards and blank were prepared the same as the samples.

2.3.7 Platinum Drug Binding to Human Serum Albumin

A stock solution containing 0.33 mM human serum albumin (HSA) was prepared in phosphate buffered saline (PBS). Individual 1 mM stock solutions of cisplatin, oxaliplatin, BBR3464, and desipramine were also prepared in phosphate buffered saline (PBS). Final sample solutions contained the platinum drugs, desipramine and HSA in a stoichiometric 3:3:1 platinum drug:desipramine:HSA ratio. Final control solutions contained the platinum drugs and HSA in a stoichiometric 3:1 platinum drug:HSA ratio. To keep the volume of the controls the same as the samples, the volume of desipramine was replaced with an equal volume of PBS. These samples were prepared by adding desipramine or PBS to the HSA solution and allowing the resulting solution to incubate for 1 hour at 37°C. The platinum drugs were then added and 150 μL aliquots were taken at 0 minutes, 30 minutes, 2 hours, 6 hours, 24 hours and 48 hours. The samples were pipetted into filter tubes and centrifuged for 10 minutes at 14000 rpm. The liquid that came through the filter was the free drug portion. The filter was then flipped over; placed in another tube and centrifuged at 1000 rpm for 5 minutes to give the portion with drug-bound protein. After the samples were centrifuged, all portions were then digested with acid according to the United States Environmental
Protection Agency procedure 3050b (all volumes reduced by 1/10) as described previously\(^\text{15}\) and analyzed using ICP-OES.

**2.3.8 Measurement of Platinum Accumulation in DNA**

Cells were plated at 2.0×10\(^6\) cells/mL. Platinum drug was added in 160 µM concentrations alone or 60 minutes after the addition of desipramine. After 36 hours cells were harvested and washed twice with PBS. The DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. The concentration and the purity of the extracted DNA were calculated using a Nanodrop spectrophotometer and the 260/280nm absorbance ratio. The samples were then digested with acid according to the United States Environmental Protection Agency procedure 3050b (all volumes reduced by 1/10) and analyzed using a Varian 820-MS Axial Simultaneous inductively coupled mass spectrometer (Varian, Palo Alto, CA).

**2.3.9 Western Blotting**

Whole-cell lysates were blotted with a mouse monoclonal antibody against human p53 (BD Bioscience), monoclonal antibody against poly-ADP ribose polymerase (PARP) (Trevigen, Inc.), mouse monoclonal antibody against ERK (Cell Signaling Technology), and, subsequently resolved with secondary antibody conjugated with horseradish peroxidase. Blots were then treated with a chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to film. Band intensity was measured by densitometry with an Eagle Eye II system (Stratagene, La Jolla, CA).
2.3.10 Colony Formation Assay

Cells were cultured (250-1500 cells per well in a 6 well plate) and 12 hours after plating, cells were treated with drugs for 48 hours. Afterward, the drug containing media was carefully removed, the cells were washed once, and fresh media lacking drugs was added. Colony formation assays were cultured for an additional 10-14 days after which the media was removed, and the cells were fixed with methanol, stained with crystal violet and counted manually.

2.3.11 Statistical Analysis

Statistical analysis was performed using t-test for two data points using SysStat9 software (SPSS, Chicago, IL, USA). \( p < 0.05 \) was considered to be significant.

2.4 Results

2.4.1 Effect of Desipramine on Platinum Drug-Induced Cytotoxicity. Potentiating Effects of Desipramine.

Desipramine surprisingly augmented cytotoxicity of all three platinum drugs studied (Figure 2.2). The enhancement of apoptosis in the presence of desipramine is time and concentration-dependent. The highest percent apoptosis was observed at a desipramine concentration of 20 \( \mu \text{mol/L} \) in combination with BBR3464 or c-DDP but for oxaliplatin, the percent apoptosis peaked with 40 \( \mu \text{mol/L} \) desipramine. At this latter concentration, desipramine alone has very little effect on HCT116 survival and this concentration was therefore used for time-dependence studies. To determine concentration-dependence, cells were treated with platinum drugs and different
concentrations of desipramine for 72 hours, and percent apoptosis was measured by PI-DNA staining. Concentrations of the platinum drugs were chosen to give approximately 30-40% apoptosis as single agents after 72 hours. In the case of cisplatin, the enhancement reached a maximum at 48 hours after treatment, where desipramine increased the c-DDP-induced apoptosis from 27% to approx. 80% (Figure 2.2A). For oxaliplatin, a quantitatively smaller but significant increase in apoptosis from 49% to 76% was observed after 72 hours of treatment in the presence of the antidepressant (Figure 2.2B). The BBR3464-desipramine combination at 72 hours also showed a significant increase to approximately 80% in comparison to that induced by either desipramine or BBR3464 alone.

2.4.2 Mechanistic Studies on the Effect of Desipramine on Platinum Drug Cellular Accumulation and DNA Binding.

To examine the possible mechanism(s) of the desipramine effect, several assays were employed including measurement of HSA interactions, total cellular platinum accumulation and platinum-DNA binding, as well as the effects of the platinum drug/antidepressant combination on downstream signaling pathways.
Figure 2.2 Time course dependence of the effect of desipramine on platinum-drug-induced apoptosis in HCT116 colorectal carcinoma cells. Sub-diploid cell content was detected by PI-DNA staining. In A, B, C, HCT116 cells were cultured with 10 µmol/L c-DDP, 30 µmol/L oxaliplatin or 50 µmol/L BBR3464, respectively for the indicated time points in the absence and presence of 40 µmol/L desipramine. Platinum-drug concentrations were adjusted to achieve approximately 20-30% apoptosis after 48h, allowing measurement of enhancement or inhibition. Platinum drugs were added to the media after 1h of treatment with desipramine. Each point represents the average (+/- SEM) of three independent experiments. *, p<0.05 when comparing cells treated with and without desipramine, by Student’s t-test. All points after 48h have p <0.05 for Pt drug with desipramine vs platinum drug alone
2.4.2a Interactions with Human Serum Albumin

Serotonin-specific reuptake inhibitors (SSRIs), which have become first-line therapy for treatment of depression in cancer patients, are strongly protein-bound, and therefore consideration must be given to their interaction with anticancer agents.\textsuperscript{16} Binding to human serum albumin (HSA) may also affect the cytotoxicity and cellular accumulation of platinum drugs as well as their structural integrity.\textsuperscript{17, 18, 19} In a cell-free assay, the presence of equimolar concentrations of desipramine did not affect the binding of any of the platinum drugs to human serum albumin (Figure 2.3). There are slight differences between cisplatin and oxaliplatin at early time points but overall the antidepressant does not affect binding of platinum drugs. Simultaneous binding of both types of drug to albumin may well still occur – the binding sites may simply be different.

2.4.2b Cellular Platinum Accumulation

Platinum drug cellular accumulation in HCT-116 colon cancer cells was measured in cells treated with platinum drug +/- desipramine (Figure 2.4 A,B,C). Desipramine surprisingly enhanced the cellular accumulation of cisplatin more than 2 fold but in contrast, neither oxaliplatin nor BBR3464 showed any difference in cellular accumulation in the presence of the tricyclic (Figure 2.4). The somewhat higher accumulation of oxaliplatin relative to cisplatin in human colon cancer cells at equimolar concentrations was confirmed.\textsuperscript{20} Because of the more rapid accumulation of BBR3464 at early time points, accumulation for this agent was measured after 8 hours whereas a 16-hour time-point was used for the mononuclear drugs. The overall results did confirm
the high cellular accumulation of the 4+ positively charged compound relative to the neutral cisplatin and oxaliplatin at early time points in the absence of desipramine.\textsuperscript{15, 21}

2.4.2c Cellular Platinum-DNA Binding

Another important parameter for platinum cytotoxicity is the extent of DNA binding. No enhancement of platinum-DNA binding was found for either cisplatin or oxaliplatin in the presence of desipramine (Figure 2.5). Some increase was found for BBR34364 but, by itself, is unlikely to explain the enhancement of cytotoxicity by the anti-depressant. Since cellular accumulation in the presence of desipramine is only enhanced in the case of c-DDP (Fig. 2.4A), there appears to be no direct correlation between enhanced accumulation and the levels of platinum-DNA adducts.
Figure 2.3 Cisplatin, oxaliplatin and BBR3464 binding (with standard deviations) to Human Serum Albumin (HSA) in the presence and absence of desipramine. The platinum drugs and desipramine are in a 1:1 molar ratio. The drugs are in a 1:3 molar ratio to HSA. BBR3464 concentration is divided by 3 to reflect the trinuclear nature of the drug.
Figure 2.4 The effect of desipramine on platinum-compound cellular accumulation. In A, B, C, HCT116 cells were treated with equimolar concentrations (20µmol/L) c-DDP and Oxaliplatin for 16h and BBR3464 for 8h, with the indicated concentrations of desipramine. Cellular platinum content was measured by ICP-OES as described in method and materials. Each point represents the average (+/-SEM) of three independent experiments. *, p<0.05 as compared to drug alone as determined by t test
Figure 2.5 Nanograms platinum per µg DNA (with standard deviations) after a 36 hour exposure time to the platinum drugs and 40 µM desipramine. The concentration of the platinum drugs was chosen so that the moles platinum drug: cell ratio was $4 \times 10^{-12}$ moles/cell
2.4.3 Cellular and Signalling Effects of Desipramine on Platinum Drug Combination.

2.4.3a Platinum Drug-Mediated p53 Expression

Pharmacological effects such as plasma protein binding, cellular accumulation and/or the extent of cellular platinum-DNA binding cannot explain the desipramine-mediated effects on platinum drug cytotoxicity. We have therefore investigated potential global biological responses that could be augmented by desipramine. Many apoptotic signaling pathways converge at the transcription factor p53. p53 causes cell death in part by inducing mitochondrial damage that activates the death effector caspase enzymes.\textsuperscript{22} Since all platinum drugs have been argued to elicit apoptosis in certain cell lines \textit{via} a p53-dependent pathway\textsuperscript{23, 24}, the level of p53 was measured by Western blot analysis and quantified by densitometry (Figure 2.6). Interestingly, p53 levels in the presence of desipramine alone were low – in agreement with the observation that, while desipramine can induce apoptosis in a mitochondrial-dependent manner, HCT116 cells are not especially sensitive to this agent.\textsuperscript{25} However, the combination of the platinum drugs with desipramine showed an increase in p53 level. This could be due to increased p53 production or p53 stabilization. The increase in p53 in the presence of desipramine resembled its augmentation of apoptosis with a measured increase for all three agents.
Figure 2.6 Effect of desipramine on p53 stabilization by cisplatin, and oxaliplatin and BBR3464. HCT116 cells were cultured with 10µmol/L cisplatin, 30µmol/L oxaliplatin or 50µmol/L BBR3464, in the absence and presence 40µmol/L desipramine. The expression of p53 was detected by Western blotting. The same membrane was stripped and re-probed for ERK to show protein loading. The assay was consistently repeated three times. The fold change (by densitometry) was 1.2 in all cases for desipramine control and 6.1, 1.0 and 4.1 for cisplatin, oxaliplatin and BBR3464 respectively.
2.4.3b Importance of the p53 Dependent Pathway in Induced Apoptosis

If the effects of desipramine on p53 expression are functionally significant, they should be consistent with increased mitochondrial damage, activation of the downstream death-inducing caspase enzymes and increased cleavage of the caspase substrate PARP, all of which can be triggered by p53. The same drug concentrations employed in the apoptosis assays of Figure 2.2 were used in these experiments. The effects of desipramine on mitochondrial damage, caspase activation and total PARP levels mirrored the effects on apoptosis and p53 expression (Figure 2.7). The integrity of the mitochondrion was examined by measuring desipramine/platinum drug-mediated loss of mitochondrial membrane potential (Δψ_m), which leads to apoptosis. Desipramine/platinum drug treatment significantly decreased Δψ_m, increasing the percentage of cells showing reduced Δψ_m by approximately two orders of magnitude compared to those treated with cisplatin, oxaliplatin and BBR3464 alone (Figure 2.7A). In a similar manner, caspase 3 activation was also increased in HCT116 carcinoma cells treated with desipramine prior to cisplatin, oxaliplatin or BBR3464 addition (Figure 2.7B). Finally, the expression of uncleaved PARP was decreased in HCT116 carcinoma cells treated under the same experimental conditions (Figure 2.7C). These latter effects mimic the effect of desipramine on p53 stabilization, mitochondrial damage and caspase activation (Fig. 2.6C).
Figure 2.7 Effect of desipramine on downstream signaling pathways of p53 activated by c-DDP and oxaliplatin and BBR3464. A. Effect on mitochondrial damage. HCT116 cells were cultured with 10µmol/L c-DDP, or 30µmol/L oxaliplatin or 50µmol/L BBR3464 in the absence or presence of desipramine for 72h. Reduction in mitochondrial membrane potential ($\Delta \Psi_m$) was assessed by staining with [Di(OC6)3] as described in Materials and Methods. B. Effect of desipramine on Platinum-drug-induced caspase activation. HCT116 cells were cultured as in (A). Cells were stained for active caspase-3/7 as described in Materials and Methods. Data shown are the percent of the population displaying active caspase-3/7. Each point represents the average (+/-SEM) of three independent experiments. C. The effect of desipramine on uncleaved PARP expression. HCT116 were cultured as in (A). The expression of uncleaved PARP was detected by western blotting. The same membrane was stripped and re-probed for ERK to show protein loading. Each experiment was consistently repeated three times. *, p<0.05 when comparing cells treated with and without desipramine, by Student's t-test. The fold change (by densitometry) in Panel C was 0.92 for desipramine control and 0.13, 0.56 and 0.81 for cisplatin, oxaliplatin and BBR3464 respectively.
2.5 Discussion

These results complement the published reports of anti-depressant mediated interference of tamoxifen efficacy in breast cancer treatment. Tamoxifen is standard adjuvant treatment for women with estrogen receptor-positive breast cancer and is a preventative agent for women at high risk of developing breast cancer. Studies have shown that three antidepressants—paroxetine, fluoxetine and bupropion—may interfere with tamoxifen treatment by inhibiting the action of CYP2D6, the principal metabolic enzyme that converts tamoxifen into the active form endoxifen. Given the seriousness of these findings it is imperative to examine the generality of these findings across the anti-cancer armamentarium. The results presented here show that the tricyclic antidepressant desipramine actually augments the cytotoxicity of platinum anti-cancer agents. To understand this interesting result, with potential clinical importance, we have explored the comparative pharmacology and cellular biology of the platinum drugs in the presence and absence of the antidepressant. Desipramine does not interact with either cisplatin or [PtCl(dien)]Cl as monitored by $^1$HNMR experiments.

It is noteworthy that desipramine has differential effects on cellular accumulation of the platinum agents yet enhances cytotoxicity of all platinum agents studied. Only the cellular accumulation of c-DDP is affected by the presence of desipramine—accumulation was increased 2.7 fold by desipramine, which may explain the more rapid kinetics of apoptosis seen (48 hours for c-DDP vs. 72 hours for BBR3464 and oxaliplatin) (Figure 2.4). It is plausible that increased accumulation of c-DDP is due to a reduced recycling of a potential c-DDP importer or perhaps inhibition of an efflux transporter. Desipramine inhibits the serotonin transporter and affects trafficking of
transporters such as β-adrenoreceptors. Desipramine had no consistent effect upon platinum-DNA binding– only BBR3464 showed measurable increases in the number of DNA adducts in the presence of the anti-depressant. This selective effect on BBR3464 further demonstrates the distinct cellular pharmacology of this trinuclear platinum compound but more importantly, emphasizes the broad effects of desipramine on platinum chemotherapeutics. The kinetics of DNA binding and indeed cellular uptake of all three platinum agents are different and it is difficult therefore to examine identical time points for both accumulation and DNA platination assays, nevertheless the results are consistent in that, unlike the tamoxifen case, there is no direct correlation of cellular pharmacology (plasma protein binding, cellular accumulation and/or cellular platinum-DNA binding) with the enhancement of cytotoxicity by desipramine.

Within the realm of cell cycle arrest and apoptosis, perhaps no cell signaling pathway is more relevant than p53 activation. All three platinum compounds stabilized p53 expression in HCT116 cells and desipramine further enhanced this effect. Desipramine itself induces apoptotic cell death through both mitochondrial and non-mitochondrial pathways in different colon carcinoma cells. Notably, p53 levels due to desipramine in HCT116 cells is also not as significant as that caused by platinum agents and is considered to cause apoptotic cell death primarily through disturbance of mitochondrial function, although HCT116 sensitivity in general is less than that of HT29 cells. The molecular mechanism of desipramine apoptosis however remains to be elucidated but is unlikely to be a consequence of direct DNA modification. Thus, complementary signaling pathways may be activated in the presence of both drugs. In this scenario, the
role of p53 may lie in enhancement of two pathways – one through direct DNA modification and the second a multiple effect on signaling pathways through the presence of p53, adding to the cellular stress.

The viability of HCT116 cells is not significantly altered in the presence of up to 50μM desipramine\(^{25}\), suggesting that the results obtained here at the concentrations used may have genuine pharmacological significance. The optimal concentration of desipramine was well within the range of clinically relevant doses achieved in patients, although there is some discrepancy in literature values - free desipramine concentration in the serum has been reported at approximately 9.5 μM\(^{33}\), but toxicity has been noted at 3.8 μM\(^{34}\).

In conclusion, an unexpected result is the potentiation of cytotoxicity shown by desipramine. The tricyclic organic compound, a safe and effective antidepressant already in use for cancer treatment, greatly augments the cytotoxicity of platinum-based chemotherapeutics. While the molecular mechanism of enhancement is clearly complicated, and may indeed not be strictly the same for all platinum agents, the results to date suggest that enhancement of weakly induced signaling pathways in the case of desipramine may be exerted with greater efficiency, leading to the observation of drug enhancement. These effects correlated to some extent with enhanced activation of the p53-mitochondrial death pathway. The study suggests that desipramine may be a means of enhancing chemo-responsiveness to platinum-based anticancer agents, and warrants further investigation for its clinical utility. The results also stress the desirability
of surveying similar cellular effects for tamoxifen, beyond the effect of antidepressants on the drugs metabolic activation.
List of References
List of References


3.1 Abstract

This work examines the interactions of cisplatin, carboplatin and oxaliplatin with desipramine in two human colon carcinoma cell lines (HCT116 wt and HT-29) as well as two ovarian carcinoma cell lines (SKOV-3 and A2780). The effect of desipramine on the cytotoxicity of the platinum drugs was both cell line specific and platinum drug specific. Specificity with regard to the antidepressant was also investigated. The tricyclic antidepressant imipramine and the selective serotonin re-uptake inhibitors citalopram and fluoxetine were investigated with cisplatin, carboplatin and oxaliplatin in HCT116 wt cells. The two tricyclic antidepressants and fluoxetine had the largest effects on the cytotoxicity of cisplatin, carboplatin and oxaliplatin. The role of p53 was investigated by using HCT116 p53-/- cells. The data show that the observed effects on platinum drug cytotoxicity by desipramine have both a p53-dependent and a p53-independent portion. Interestingly, the tricyclic antidepressants and fluoxetine are known calmodulin inhibitors. The role of calmodulin inhibition was investigated using W7 (a known calmodulin inhibitor, N-(6-Aminohexyl)-5-chloro-1-
naphthalenesulfonamide hydrochloride) along with the platinum drugs in HCT116 wt and HCT116 p53/- cells. Calmodulin inhibition effected the cytotoxicity of the platinum chemotherapeutic agents in a similar manner to the antidepressants.

3.2 Introduction

Many cancer chemotherapeutic regimens include a combination of drugs. The platinum-based chemotherapeutic agents are frequently included in these combinations. For instance, cisplatin in combination with other chemotherapeutic agents cures over 90% of testicular cancer cases\(^1\) while oxaliplatin in combination with 5-fluorouracil is used to treat metastatic colon cancer.\(^2\)

Many cancer patients receive adjuvant antidepressant therapy for depression as well as to treat side effects of the cancer therapy, such as neuropathic pain. It is estimated that 25% of cancer patients will experience major depression at some point during their illness.\(^3\) Given the frequency with which these drugs are concurrently prescribed, it is very important to understand how antidepressants and antineoplastics interact – the combination may be additive or it may be antagonistic. The importance of this issue has been highlighted by the recent discussion on the effects of selective serotonin reuptake inhibitors on the efficacy of tamoxifen in breast cancer therapy.\(^4,5\)

We have previously shown that the tricyclic antidepressant desipramine (Figure 3.1) augments the cytotoxicity of cisplatin, oxaliplatin and the pre-clinical platinum drug BBR3464 in HCT116 wt cells. The data also demonstrated that only cisplatin had an
increase in cellular accumulation while only BBR3464 had an increase in platinum-DNA binding in the presence of desipramine. Furthermore, desipramine did not affect platinum drug binding to human serum albumin.  

This study builds on the work of Kabolizadeh et al. focusing on the clinically used platinum drugs cisplatin, oxaliplatin and carboplatin. The cell specificity, drug specificity and potential mechanisms of the previously observed augmentation of cytotoxicity were examined. The structures of all the compounds investigated in this study are shown in Figure 3.1.

![Figure 3.1](image-url)  

**Figure 3.1** Structures of the clinically used platinum drugs, the antidepressants and the calmodulin inhibitor, W7. A. Structures of the three clinically used platinum anti-cancer drugs. B. Structures of the tricyclic antidepressants. C. Structures of the selective serotonin reuptake inhibitor antidepressants. D. Structure of W7, a known calmodulin antagonist.
3.3 Materials and Methods

3.3.1 Materials
HCT116 human colon carcinoma cells were a gift from Bert Vogelstein (Johns Hopkins University, Baltimore MD). The HT-29, A2780 and SKOV3 cells were purchased from ATCC (Manassas, Virginia, USA). McCoy’s 5a Medium Modified, RPMI, Fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, HEPES buffer and sodium pyruvate were all purchased from Biofluids (Rockville, MD). N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) was purchased from Axxora (Farmingdale, New York, USA).

3.3.2 Cell Systems and Culture Conditions
HCT116 wt, HCT116 p53-/-, HT-29, A2780 and SKOV-3 cells were used. The medium used for the HT-29 cells was McCoy’s 5a Medium Modified with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES buffer and 1 mmol/L sodium pyruvate (McCoy’s, all from Biofluids, Rockville, MD). The medium used for all other cell types was RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES buffer and 1 mmol/L sodium pyruvate (cRPMI, all from Biofluids, Rockville, MD). The cells were cultured in 175 cm² Cell Star tissue culture flasks from Greiner Bio-One (Frickenhausen, Germany) in an incubator with 5% CO₂ and humidified air.
3.3.3 Apoptosis Studies

Cells were cultured in 6 well plates with $7.0 \times 10^4$ cells per well. 3 mL medium was put in each well. Cells were treated for 24 hours, 48 hours or 72 hours with these conditions: untreated, platinum drug by itself, antidepressant by itself, and platinum drug plus antidepressant. The platinum drugs were added to the medium after a one-hour treatment period with the antidepressant. At the time points, all cells (adherent and non-adherent) were collected. Samples were fixed in a solution containing 35% 1x PBS (vol/vol), 12.48% fetal bovine serum (vol/vol), and 52.52% 70% ethanol (vol/vol). The samples were then washed with phosphate buffered saline (PBS), and stained with a solution containing 94% 1x PBS (vol/vol), 0.1 mg/mL RNase A, 0.0001 M EDTA, and 0.04 mg/mL propidium iodide (PI). The samples were stained for two hours. Samples were then analyzed for subdiploid DNA content using a FACScan flow cytometer (BD Biosciences, San Jose, CA). It is noteworthy that this protocol differs significantly from the more common PI-based exclusion, which only differentiates live versus dead cells. Through fixation and RNase A treatment, we were able to detect intact versus fragmented DNA, revealing discrete stages of the cell cycle and the percentage of the population undergoing apoptosis.

3.4 Results

3.4.1 Desipramine Enhances Carboplatin Cytotoxicity

The interaction of desipramine with carboplatin was investigated in HCT116 wt cells in order to expand on the previous work that demonstrated that desipramine enhances the cytotoxicity of cisplatin and oxaliplatin. Although carboplatin alone had low cytotoxicity
in this cell line, the combination of carboplatin and desipramine was highly cytotoxic. At 72 hours, carboplatin alone caused 12% apoptosis while the combination of carboplatin and desipramine caused 53% apoptosis (Figure 3.2). The combination was significantly different from the percent of apoptosis caused by carboplatin alone at both the 48 and 72 hour time points. The p-values were $4.7 \times 10^{-9}$ and $1.8 \times 10^{-12}$ respectively.

**Figure 3.2** Desipramine enhances the cytotoxicity of carboplatin in HCT116 wt cells. Carboplatin was 40 µM and desipramine was 40 µM. The error bars are ± the SEM. * denotes a p-value < 0.05.
3.4.2 Survey of Cell Lines

3.4.2a HT-29 Cell Line

The interaction of desipramine with cisplatin, oxaliplatin and carboplatin was investigated in HT-29 cells. The HT-29 cell line is another human colon carcinoma that in the literature has been shown to respond differently to desipramine than HCT116 wt cells.\(^8\) The results of the combination of the platinum based chemotherapeutics and desipramine in the HT-29 cells were quite different from those seen in the HCT116 wt cells. Desipramine decreased the cytotoxicity of cisplatin and increased the cytotoxicity of oxaliplatin. At 72 hours, cisplatin alone caused 25% apoptosis while cisplatin plus desipramine only caused 18% apoptosis. The difference between the percent apoptosis due to cisplatin and cisplatin plus desipramine has a p-value of \(8.2 \times 10^{-5}\) (Figure 3.3 A). Oxaliplatin alone was significantly different from oxaliplatin plus desipramine at both 24 and 72 hours but not at 48 hours. The combination of oxaliplatin plus desipramine initially causes more apoptosis than oxaliplatin alone. By 48 hours, the percent apoptosis caused by oxaliplatin alone has caught up to the percent apoptosis caused by oxaliplatin plus desipramine. By 72 hours the percent apoptosis caused by oxaliplatin alone has decreased slightly from what was observed at 48 hours (from 10% to 9%) but this difference is not significant. The percent apoptosis from oxaliplatin plus desipramine on the other hand has increased to 23% (Figure 3.3 B). In the case of carboplatin, the addition of desipramine has little effect (Figure 3.3 C). Although it is statistically different from carboplatin alone at 72 hours (p-value=0.01), killing approximately 5% of a tumor is unlikely to have clinical significance.
Figure 3.3 Effect of desipramine on the cytotoxicity of cisplatin, oxaliplatin and carboplatin in HT-29 cells. Cisplatin plus desipramine. B. Oxaliplatin plus desipramine. C. Carboplatin plus desipramine. D. Concentrations of drugs used in this cell line. The error bars are ± the SEM. * denotes a p-value < 0.05.

3.4.2b A2780 Cell Line

The interaction of desipramine with cisplatin, oxaliplatin and carboplatin was investigated in A2780 cells. The A2780 cell line is a human ovarian carcinoma cell line. In this cell line desipramine augmented the cytotoxicity of cisplatin and carboplatin at 24, 48 and 72 hours. In the case of cisplatin, cisplatin alone caused 5% apoptosis at 24 hours while cisplatin plus desipramine caused 9% apoptosis. This has a p-value of 0.01. At 48 hours the difference was even more significant. Cisplatin alone caused 17% apoptosis while the combination with desipramine caused 50% apoptosis with a p-
value of $2.4 \times 10^{-8}$. At 72 hours, cisplatin alone caused 42% apoptosis while the combination of cisplatin plus desipramine caused 66% apoptosis with a p-value of $3.0 \times 10^{-4}$ (Figure 3.4 A).

In the case of oxaliplatin, the combination of oxaliplatin plus desipramine is only significantly different from oxaliplatin alone at 48 hours. At 48 hours, oxaliplatin alone caused 49% apoptosis while the combination of oxaliplatin plus desipramine caused 67% apoptosis with a p-value of $8.0 \times 10^{-4}$. At 72 hours, there is no statistical difference between oxaliplatin and oxaliplatin plus desipramine. The percent apoptosis due to the combination of oxaliplatin plus desipramine appears to decrease below that of oxaliplatin alone. This is due to two samples which did not have as much apoptosis as the rest of the samples. Although the samples were not statistical outliers by the Q test and therefore could not be removed, if the data was analyzed without them the end result was the same: the two conditions are not significantly different at 72 hours (Figure 3.4 B).

In the case of carboplatin, the combination of carboplatin plus desipramine is significantly different from carboplatin alone at all three time points investigated. At 24 hours, carboplatin alone caused 4% apoptosis while carboplatin plus desipramine caused 10% apoptosis with a p-value of $3.0 \times 10^{-4}$. At 48 hours, carboplatin alone caused 8% apoptosis while the combination with desipramine caused 16% apoptosis with a p-value of $2.4 \times 10^{-6}$. At 72 hours, carboplatin alone caused 25% apoptosis while
carboplatin plus desipramine caused 40% apoptosis with a p-value of 1.9 x 10^-5 (Figure 3.4 C).

**Figure 3.4** Effect of desipramine on the cytotoxicity of cisplatin, oxaliplatin and carboplatin in A2780 cells. A. Cisplatin plus desipramine. B. Oxaliplatin plus desipramine. C. Carboplatin plus desipramine. D. Concentrations of drugs used in this cell line. The error bars are ± the SEM. * denotes a p-value < 0.05.

### 3.4.2c SKOV-3 Cell Line

The interaction of desipramine with cisplatin, oxaliplatin and carboplatin was investigated in SKOV-3 cells. The SKOV-3 cell line is a platinum-resistant human ovarian carcinoma cell line. Desipramine was unable to reverse resistance in this cell line. Cisplatin plus desipramine, oxaliplatin plus desipramine and carboplatin plus
Desipramine were not significantly different from the platinum drugs by themselves at any of the three time points investigated.

Figure 3.5 Desipramine has no effect on the cytotoxicity of cisplatin, oxaliplatin and carboplatin in SKOV-3 cells. A. Cisplatin plus desipramine. B. Oxaliplatin plus desipramine. C. Carboplatin plus desipramine. D. Concentrations of drugs used in this cell line. The error bars are ± the SEM. * denotes a p-value <0.05.

In summary, the effect of the individual drugs as well as the combination of drugs is cell-line specific. Table 3.1 shows a summary of these data.
Table 3.1 Summary of Cell Line Survey

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>cDDP</th>
<th>Ox</th>
<th>Carbo</th>
<th>Des</th>
<th>cDDP + Des</th>
<th>Ox + Des</th>
<th>Carbo + Des</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116 wt</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>HT-29</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>A2780</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>SKOV3</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Note:** + Indicates that the percent apoptosis is greater than in the untreated control. ++ Indicates that the combination is better than either drug alone. – Indicates that the percent apoptosis is not significantly different from that observed in the untreated control. - - Indicates that the combination causes less cytotoxicity than the platinum drug alone.

### 3.4.3 Platinum Drugs with Other Antidepressants

#### 3.4.3a Imipramine in HCT116 wt Cells

In order to determine if the observed effect is specific to desipramine the interaction of the platinum drugs with imipramine (Figure 3.1) was investigated. Imipramine is another member of the tricyclic antidepressant family. It differs from desipramine by the addition of one methyl group. In combination with cisplatin, imipramine behaves very much like desipramine. Imipramine enhances the cytotoxicity of cisplatin and the combination is significantly different from cisplatin alone at 24, 48 and 72 hours. At 24 hours, cisplatin alone caused 22% apoptosis while the combination of cisplatin and
imipramine caused 34% apoptosis. This has a p-value of 0.009. At 48 hours, cisplatin alone caused 53.8% apoptosis while the addition of imipramine caused 84% apoptosis with a p-value of $4.6 \times 10^{-8}$. At 72 hours, cisplatin alone caused 65% apoptosis but the combination of cisplatin plus imipramine caused 90% apoptosis with a p-value of $8.1 \times 10^{-9}$ (Figure 3.6 A).

Interestingly, imipramine inhibits the cytotoxicity of oxaliplatin and this is significant at all three time points investigated. At 24 hours, oxaliplatin alone caused 7% apoptosis while the combination of oxaliplatin plus imipramine caused only 3.5% apoptosis. This has a p-value of 0.001. At 48 hours, oxaliplatin alone caused 35% apoptosis while the combination with imipramine only caused 14% apoptosis. At 72 hours the difference is even larger. Oxaliplatin alone caused 60% apoptosis whereas oxaliplatin plus imipramine only caused 34% apoptosis with a p-value of 0.0005 (Figure 3.6 B).

The combination of carboplatin and imipramine is not impressive (Figure 3.6 C). Carboplatin plus imipramine is only significantly different from carboplatin alone at 48 hours. At 48 hours, carboplatin alone caused 6% apoptosis while the combination of carboplatin plus imipramine caused 11% apoptosis (p=0.005). This is unlikely to be clinically significant.
3.4.3b Citalopram in HCT116 wt Cells

Citalopram is a member of the selective serotonin reuptake inhibitors (Figure 3.1). The selective serotonin reuptake inhibitors are the most commonly prescribed antidepressants. Citalopram augments the cytotoxicity of cisplatin at 24 and 48 hours but not at 72 hours. At 24 hours, cisplatin alone caused 17% apoptosis while the combination of cisplatin plus citalopram caused 24% apoptosis with a p-value of 0.01. At 48 hours, cisplatin alone caused 44% apoptosis while the combination with
Citalopram caused 62% apoptosis with a p-value of 0.002. By 72 hours however, the percent apoptosis caused by cisplatin alone was no longer significantly less than that caused by the combination of cisplatin plus citalopram (Figure 3.7 A).

Oxaliplatin plus citalopram was not significantly different from oxaliplatin at 24 or 48 hours. At 72 hours however, oxaliplatin plus citalopram caused significantly less apoptosis than oxaliplatin alone. Oxaliplatin alone caused 55% apoptosis whereas the combination of oxaliplatin plus citalopram caused only 40% apoptosis (Figure 3.7 B). This has p-value of 0.02.

The combination of carboplatin with citalopram is unimpressive. Like oxaliplatin plus citalopram, the combination of carboplatin plus citalopram is not significantly different from carboplatin alone at 24 or 48 hours but at 72 hours, the combination causes less apoptosis than carboplatin alone. At 72 hours, carboplatin alone caused 12% apoptosis while carboplatin plus citalopram caused 7% apoptosis with a p-value of $2 \times 10^{-4}$ (Figure 3.7 C). While this is a significant difference, killing 12% or 7% of a tumor is not likely to be clinically useful.
Citalopram has little effect on the cytotoxicity of cisplatin, oxaliplatin and carboplatin in HCT116 wt cells. A. Cisplatin plus citalopram. B. Oxaliplatin plus citalopram. C. Carboplatin plus citalopram. D. Concentration of drugs used in this cell line.

3.4.3c Fluoxetine in HCT116 wt Cells

Another selective serotonin reuptake inhibitor is fluoxetine, which is better known as Prozac (Figure 3.1). Fluoxetine augments the cytotoxicity of cisplatin in HCT116 wt cells at all three time points investigated. At 24 hours, cisplatin alone caused 22% apoptosis while the combination of cisplatin plus fluoxetine caused 46% apoptosis with a p-value of 1.5 x 10^{-7}. At 48 hours, cisplatin alone caused 54% apoptosis while the combination with fluoxetine caused 85% apoptosis with a p-value of 5.3 x 10^{-8}. At 72
hours, cisplatin alone caused 65% apoptosis whereas cisplatin plus fluoxetine caused 87% apoptosis with a p-value of $2.2 \times 10^{-6}$ (Figure 3.8 A). Fluoxetine has no effect on the cytotoxicity of oxaliplatin in HCT116 wt cells (Figure 3.8 B).

Fluoxetine augments the cytotoxicity of carboplatin in HCT116 wt cells. At 24 hours, carboplatin alone causes 6% apoptosis while the combination of carboplatin plus fluoxetine causes 11% apoptosis with a p-value of $9.8 \times 10^{-7}$. At 48 hours, carboplatin alone caused 9% apoptosis whereas carboplatin plus fluoxetine caused 24% apoptosis with a p-value of $2.3 \times 10^{-6}$. At 72 hours, carboplatin alone caused 19% apoptosis while the combination of carboplatin plus fluoxetine caused 60% apoptosis with a p-value of $5.1 \times 10^{-10}$ (Figure 3.8 C).

3.4.4 Mechanistic Studies

3.4.4a The Role of p53

3.4.4a1 Platinum Drugs and Desipramine in HCT116 p53-/- Cells

The tumor suppressor gene p53 is often mutated in cancer cell lines, so it is important to examine whether or not wild type p53 is required for the observed effect of antidepressants on the cytotoxicity of platinum drugs. To investigate the role of p53, the interaction of tricyclic antidepressants on the cytotoxicity of platinum drugs was studied in the HCT116 p53-/- cell line. In the case of desipramine, augmentation of cytotoxicity was observed with cisplatin, oxaliplatin and carboplatin (Figure 3.9). However, the augmentation observed in the p53 knockout cell line was less than was observed in the p53 wild type cell line. In the case of cisplatin, the combination of cisplatin plus
**Figure 3.8** Fluoxetine in HCT116 wt cells. A. Fluoxetine augments the cytotoxicity of cisplatin. B. Fluoxetine has no effect on the cytotoxicity of oxaliplatin. C. Fluoxetine augments the cytotoxicity of carboplatin. D. Concentrations of drugs used in these experiments. Error bars are ± the SEM. * denotes a p-value < 0.05.
desipramine augmented the cytotoxicity of cisplatin at 24, 48 and 72 hours but was only significantly different at 48 and 72 hours. At 48 hours, cisplatin alone caused 35% apoptosis whereas cisplatin plus desipramine caused 58% apoptosis with a p-value of $5.1 \times 10^{-5}$. At 72 hours, cisplatin alone caused 34% apoptosis while the combination of cisplatin plus desipramine caused 70% apoptosis with a p-value of $9.8 \times 10^{-8}$ (Figure 3.9 A).

Desipramine also augments the cytotoxicity of oxaliplatin in the HCT116 p53-/− cell line. At 24 hours, oxaliplatin alone caused 9% apoptosis while oxaliplatin plus desipramine caused 14% apoptosis. This difference has a p-value of 0.02. At 48 hours, oxaliplatin alone caused 26% apoptosis whereas oxaliplatin plus desipramine caused 39% apoptosis with a p-value of 0.006. At 72 hours, oxaliplatin alone only caused 21% apoptosis while the combination with desipramine caused 50% apoptosis (Figure 3.9 B). This difference has a p-value of $7.2 \times 10^{-5}$.

Desipramine also enhances the cytotoxicity of carboplatin in the HCT116 p53-/− cell line. At 24 hours, carboplatin alone caused 6% apoptosis while carboplatin plus desipramine caused 14% apoptosis with a p-value of 0.0002. Although the enhancement over carboplatin alone is significant at 24 hours, carboplatin plus desipramine is not significantly different from desipramine alone at 24 hours. At 48 hours, carboplatin alone caused 10% apoptosis while the combination of carboplatin plus desipramine caused 30% apoptosis with a p-value of $6.1 \times 10^{-6}$. At 72 hours, carboplatin alone caused 13% apoptosis whereas carboplatin plus desipramine caused 52% apoptosis with a p-value of $1.4 \times 10^{-11}$ (Figure 3.9 C).
In the HCT116 p53-/— cells, less of an increase in cytotoxicity was observed due to the desipramine plus platinum drug combination than was observed in the HCT116 wt cells. For cisplatin plus desipramine, the difference between the percent apoptosis observed in the HCT116 wt cells and the HCT116 p53-/— cells was significantly different at both 48 and 72 hours with p-values of $1.9 \times 10^{-4}$ and $1.5 \times 10^{-4}$ respectively. For oxaliplatin plus desipramine, the difference between the percent apoptosis observed in the HCT116 wt cells and the HCT116 p53-/— cells was also significantly different at both 48 hours and 72 hours with p-values of $4.2 \times 10^{-3}$ and $1.2 \times 10^{-3}$ respectively. In the case of carboplatin plus desipramine, the difference between the percent apoptosis observed in the HCT116 wt cells and the HCT116 p53-/— cells is only significantly different at 48 hours with a p-value of $1.1 \times 10^{-4}$.

### 3.4.4a2 Platinum Drugs and Imipramine in HCT116 p53-/— Cells

Given that in HCT116 wt cells, the platinum drugs with imipramine did not behave exactly the same way as the platinum drugs with desipramine, it was important to investigate whether or not the same effects were observed in the HCT116 p53-/— cells. Imipramine augments the cytotoxicity of cisplatin. At 24 hours, cisplatin alone caused 6% apoptosis while the combination with imipramine caused 12% apoptosis. This difference had a p-value of 0.005. At 48 hours, cisplatin alone caused 15% apoptosis while cisplatin plus imipramine caused 42% apoptosis with a p-value of $2.7 \times 10^{-7}$. At 72 hours, cisplatin alone caused 29% apoptosis while the combination of cisplatin plus imipramine caused 69% apoptosis. This difference had a p-value of $4.1 \times 10^{-7}$ (Figure 3.10 A).
Figure 3.9 Desipramine increases the cytotoxicity of cisplatin, oxaliplatin and carboplatin in HCT116 p53-/ cells. A. Desipramine augments the cytotoxicity of cisplatin. B. Desipramine augments the cytotoxicity of oxaliplatin. C. Desipramine augments the cytotoxicity of carboplatin. D. Concentrations of drugs used in these experiments. Error bars are ± the SEM. * denotes a p-value < 0.05.
Imipramine had very little effect on the cytotoxicity of oxaliplatin in HCT116 p53 -/- cells. Although the combination of oxaliplatin plus imipramine did appear to trend towards increased apoptosis, it was not significantly different from the percent apoptosis caused by oxaliplatin alone at any of the three time points investigated (Figure 3.10 B).

Imipramine enhanced the cytotoxicity of carboplatin in HCT116 p53 -/- cells at 48 hours and 72 hours. At 48 hours, carboplatin caused 4 % apoptosis while carboplatin plus imipramine caused 9% apoptosis. This difference had a p-value of $2.1 \times 10^{-4}$. At 72 hours, carboplatin alone caused 12% apoptosis whereas the combination of carboplatin plus desipramine caused 18% apoptosis. This difference had a p-value of $1.3 \times 10^{-4}$ (Figure 3.10 C).

The comparison of the percent apoptosis due to the imipramine plus platinum drug combination in the HCT116 wt cells and the HCT116 p53 -/- cells is interesting. In the case of cisplatin plus imipramine, the difference between the percent apoptosis observed in the HCT116 wt cells and the HCT116 p53 -/- cells was significantly different at both 48 and 72 hours with p-values of $2.4 \times 10^{-7}$ and $9.1 \times 10^{-6}$ respectively. For oxaliplatin plus imipramine, the difference between the percent apoptosis observed in the HCT116 wt cells and the HCT116 p53 -/- cells was not significantly different. In the case of carboplatin plus imipramine, the percent apoptosis observed in the HCT116 p53 -/- cells was significantly decreased from what was observed in the HCT116 wt cells at 48 hours (p=0.01). However, there was no significant difference at 72 hours.
3.4.4b Role of Calmodulin Inhibition

The tricyclic antidepressants\(^9\) and fluoxetine\(^10\) are known to be calmodulin inhibitors. Given this, it was important to investigate the role of calmodulin inhibition in the observed effects on cytotoxicity. To do this, the percent apoptosis due to the platinum drugs was measured in the presence and absence of \(N\)-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), a known calmodulin inhibitor.\(^11\) This was done using both HCT116 wt cells and HCT116 p53/- cells.
3.4.4b1 Role of Calmodulin Inhibition in HCT116 wt Cells

In the HCT116 wt cells, the combination of the platinum drugs with W7 mirrors the results observed with imipramine. W7 augments the cytotoxicity of cisplatin at 24, 48 and 72 hours. At 24 hours, cisplatin alone caused 14% apoptosis while cisplatin plus W7 caused 25% apoptosis with a p-value of 0.01. At 48 hours, cisplatin caused 36% apoptosis while the combination with W7 caused 70% apoptosis. This difference had a p-value of $4.67 \times 10^{-6}$. At 72 hours, the percent apoptosis caused by cisplatin remained at 36% while the percent apoptosis due to cisplatin plus W7 increased to 77% (Figure 3.11 A). This difference had a p-value of $1.8 \times 10^{-6}$.

W7 inhibited the cytotoxicity of oxaliplatin at both 48 and 72 hours. At 48 hours, oxaliplatin alone caused 51% apoptosis while oxaliplatin plus W7 caused 34% apoptosis. This difference had a p-value of 0.001. At 72 hours, oxaliplatin caused 65% apoptosis whereas the combination of oxaliplatin plus W7 only caused 47% apoptosis with a p-value of 0.01 (Figure 3.11 B). W7 has very little effect on the cytotoxicity of carboplatin in HCT116wt cells. The percent apoptosis due to carboplatin plus W7 was not significantly different from the percent apoptosis due to carboplatin alone at any of the three time points investigated (3.11 C).
3.4.4b2 Role of Calmodulin Inhibition in HCT116 p53/- Cells

In the HCT116 p53/- cells, the results are a little different than they are in the HCT116 wt cells. W7 still augments the cytotoxicity of cisplatin, but not as much as it does in the HCT116 wt cells. At 24 hours, cisplatin alone caused 6% apoptosis while cisplatin plus W7 caused 9% apoptosis with a p-value of 0.002. At 48 hours, cisplatin caused 15% apoptosis while the combination of cisplatin plus W7 caused 36% apoptosis. This difference had a p-value of $9.3 \times 10^{-4}$. At 72 hours, cisplatin caused 29% apoptosis.
whereas cisplatin plus W7 caused 60% apoptosis with a p-value of $3.1 \times 10^{-9}$ (Figure 3.12 A).

The percent apoptosis due to the combination of oxaliplatin plus W7 was not significantly different from the percent apoptosis due to oxaliplatin alone at any of the three time points investigated (3.12 B). It is interesting to note that W7 inhibits the cytotoxicity of oxaliplatin in HCT116 wt cells but not in HCT116 p53-/cells. The reason for this is unclear.

W7 enhanced the cytotoxicity of carboplatin at 48 hours. At 48 hours, carboplatin alone caused 4% apoptosis while the combination of carboplatin plus W7 caused 7% apoptosis (3.12 C, p=3.1 x 10^{-5}). Although statistically significant, this is unlikely to be clinically significant.
Figure 3.12 Effect of calmodulin inhibition on the cytotoxicity of cisplatin, oxaliplatin and carboplatin in HCT116 p53-/- cells. A. W7 augments the cytotoxicity of cisplatin. B. W7 has no effect on the cytotoxicity of oxaliplatin. C. W7 slightly augments the cytotoxicity of carboplatin at 48 hours. D. Concentrations of drugs used in these experiments. Error bars are ± the SEM. * denotes a p-value < 0.05.

3.5 Discussion

Previous work has shown that desipramine augments the cytotoxicity of cisplatin and oxaliplatin in HCT116 wt cells. This work has built upon that by showing that desipramine also augments the cytotoxicity of carboplatin in HCT116 wt cells.

It has also been shown that the observed effects are cell line specific as well as drug specific with regard to the platinum drugs. Two colon carcinoma cell lines, HCT116 wt
and HT-29, as well as two ovarian carcinoma cell lines, SKOV-3 and A2780, were investigated. In the HCT116 wt cells, desipramine augmented the cytotoxicity of all three platinum drugs investigated. In the HT-29 cells however, desipramine only augmented cisplatin at 72 hours and oxaliplatin at 24 and 48 hours. The SKOV-3 cell line is a platinum resistant cell line. The addition of desipramine was unable to overcome this resistance. In the A2780 cell line however, desipramine augmented both cisplatin and carboplatin. It also augmented oxaliplatin cytotoxicity at 48 hours.

This work also demonstrated that the observed effects are also drug specific with regard to the antidepressants. Imipramine, another tricyclic antidepressant, differs from desipramine by one methyl group but behaves quite differently with the three platinum drugs investigated. In HCT116 wt cells, imipramine enhances the cytotoxicity of cisplatin but inhibits the cytotoxicity of oxaliplatin. Imipramine has little effect on the cytotoxicity of carboplatin. It is important to keep in mind however that desipramine is an active metabolite of imipramine. Therefore, in a clinical setting this data may not replicate exactly or may appear to be more of a mix between the results observed with desipramine and the results observed with imipramine.

The selective serotonin reuptake inhibitors, citalopram and fluoxetine were also investigated due to the frequency with which they are prescribed. Citalopram only enhances the cytotoxicity of cisplatin at the early time points. Although there is enhancement of cytotoxicity, it is less than was observed with desipramine and imipramine. By 72 hours, cisplatin and cisplatin plus citalopram cause the same
amount of apoptosis. Although citalopram has no effect on the cytotoxicity of oxaliplatin at the early time points, it does inhibit the cytotoxicity of oxaliplatin at 72 hours. Citalopram has little effect on the cytotoxicity of carboplatin. Fluoxetine on the other hand, strongly enhances the cytotoxicity of cisplatin. It also augments the cytotoxicity of carboplatin in HCT116 wt cells but it does not augment the cytotoxicity to the same extent that desipramine does. Fluoxetine has no effect on the cytotoxicity of oxaliplatin.

To investigate the mechanisms behind these observations, the role of p53 was examined. In the HCT116 p53-/- cells, desipramine augmented the cytotoxicity of cisplatin, oxaliplatin and carboplatin. Although enhancement of cytotoxicity was observed in both the p53 wild type cells and the p53 knock out cells, the enhancement observed in the p53 knock out cells was less than what was seen in the wild type cells. This suggests that the observed effect has two mechanisms at work: one involving p53 and one that is p53 independent. Similar results are seen with imipramine when combined with cisplatin in the HCT116 p53-/- cells. In the case of oxaliplatin plus imipramine in the HCT116 p53-/- cells, the percent of apoptosis observed was not significantly different from the percent apoptosis due to oxaliplatin alone. In the case of carboplatin plus imipramine, the percent apoptosis observed in the HCT116 p53-/- cells was significantly decreased from what was observed in the HCT116 wt cells at 48 hours. By 72 hours, this difference is no longer observed. Taken together, these data suggest that there are at least two mechanisms at work. One of these mechanisms appears to p53 independent while the other appears to be p53 dependent. The
platinum drugs themselves may use these mechanisms to different extents which could explain the oxaliplatin data.

Since the tricyclic antidepressants\textsuperscript{9} and fluoxetine\textsuperscript{10} are known to be calmodulin inhibitors, it was important to investigate the role of calmodulin inhibition. In both the HCT116 wt and HCT116 p53\textsuperscript{-/-} cells, the results of calmodulin inhibition were similar to the results due to imipramine. Calmodulin inhibition augmented the cytotoxicity of cisplatin in both cell lines, however the enhancement was less in the HCT116 p53\textsuperscript{-/-} cells. Calmodulin inhibition by W7 also inhibited oxaliplatin cytotoxicity in HCT116 wt cells while having little effect in HCT116 p53\textsuperscript{-/-} cells. W7 had no effect on carboplatin cytotoxicity in HCT116 wt cells but did enhance carboplatin cytotoxicity at 48 hours in the HCT116 p53\textsuperscript{-/-} cells.

In conclusion, it has been shown that these drug effects on cytotoxicity of platinum drugs are both drug and cell line specific. The data suggest that there are multiple mechanisms involved in the observed effects. It is clear that there are both p53 dependent and independent components to this mechanism. The platinum drugs themselves may utilize these different mechanisms to different extents. This work has also shown that calmodulin inhibition plays a role in the mechanism.

To further determine the mechanism(s), Western blots for pro-apoptotic proteins such as p21 and Bax could be performed. This would allow comparison of protein levels in cells that have been treated with the platinum drugs alone, the antidepressants alone,
or the combination of platinum drug plus antidepressant. These experiments may show differences in protein activation/induction between the different combinations. This would help elucidate whether one pathway or set of pathways is involved in the effects observed with all platinum drug/antidepressant combinations or whether different drug combinations utilize different pathways that happen to result in similar effects on cytotoxicity. Since the data suggests that there is a p53 independent mechanism as well, the role of the death receptors should be investigated. Once the mechanism is fully elucidated, members of the responsible pathway(s) could be used as new drug targets.

It is also possible that if more cell lines were investigated, patterns with regard to cytogenetic abnormalities or over-expression of a particular protein may be observed. Observations of this type would suggest which cell features are necessary to observe the augmentation of platinum drug cytotoxicity due to the presence of an antidepressant. This would be particularly useful in moving cancer chemotherapy towards personalized medicine.
List of References
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CHAPTER 4: Heparanase Inhibition by Positively Charged Platinum-Based Chemotherapeutics

4.1 Abstract
This work examines the interaction between the positively charged platinum-based chemotherapeutics (BBR3464, AH44 and AH78) and the model substrate fondaparinux. The positively charged platinum based chemotherapeutics blocked fondaparinux cleavage by both heparinase and heparanase. The enzyme inhibition with respect to AH44 and AH78 was charge dependent. Surprisingly, BBR3464 inhibited enzyme activity more than AH44 despite being less positively charged. Time course studies showed that the interaction between the positively charged platinum-based chemotherapeutics and the fondaparinux is instantaneous. It further suggested that BBR3464 may have an initial electrostatic binding followed by covalent binding whereas AH44 and AH78 can only bind electrostatically. The results suggest that the positively charged platinum-based chemotherapeutics may be able to reduce the increased angiogenesis and metastasis observed with heparanase over-expression in cancer.

4.2 Introduction
Platinum-based chemotherapeutics are often used in cancer therapy but acquired resistance is a frequent problem. This acquired resistance can lead to patient relapse.
Decreased drug accumulation is a common cause of clinical chemoresistance.\textsuperscript{1} For this reason, we have focused on the uptake mechanisms of positively charged platinum-based chemotherapeutics and the possible implications of these uptake mechanisms.

Poly(arginine) sequences are commonly used as a recognition motif in protein-nucleic acid recognition. This interaction is mediated by hydrogen bonds between the charged guanidine groups of the arginine and the phosphate backbone oxygens of the oligonucleotide to form what is known as an arginine fork.\textsuperscript{2} A similar binding interaction has been characterized in between duplex DNA and the non-covalent polynuclear platinum complexes (AH78 and AH44). In this case, there is hydrogen bonding to the phosphate oxygen from two cis-oriented amine (RNH\textsubscript{2}) and ammine (NH\textsubscript{3}) groups to form what is known as a phosphate clamp.\textsuperscript{3, 4} The arginine fork and phosphate clamp are shown in Figure 4.1.

It is also known that proteins containing poly(arginine) sequences are efficiently taken up by cells due to interactions with proteoglycans on the cell surface.\textsuperscript{5} The similarity between the arginine fork and the phosphate clamp raises the question: Do the positively charged platinum-based chemotherapeutics interact with proteoglycans in a similar way to the arginine? Silva \textit{et al.} have discovered that the answer to this question is: Yes.\textsuperscript{6} The positively charged platinum-based chemotherapeutics were shown to bind to heparan sulfate proteoglycans on the cell surface. Furthermore, the heparan sulfate proteoglycans were necessary for uptake of the positively charged platinum-based chemotherapeutics. This is a newly discovered mode of uptake for platinum drugs.\textsuperscript{6}
4.2.1 Glycosaminoglycans

Glycosaminoglycans are complex carbohydrate molecules that are present on the cell surface as well as in the extracellular matrix and the basement membrane. They are linear polysaccharides made up of repeating disaccharide units, which are composed of uronic acid (D-glucoronic acid or L-iduronic acid) and a hexosamine (D-galactosamine or D-glucosamine). Glycosaminoglycans are often highly sulfated which makes them negatively charged. Hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratin sulfate and heparan sulfate are examples of glycosaminoglycans.

Glycosaminoglycans may be in solution or may be bound to a protein core yielding a proteoglycan.

Proteoglycans have enormous heterogeneity. This is due to different protein cores, variations in the length of the glycosaminoglycan chains as well as in the number of chains attached, and the different types of glycosaminoglycan chains. Further variation is inherent due to different possible sulfation patterns on the repeating disaccharide units of the glycosaminoglycan chains.

Glycosaminoglycans are able to interact with many proteins and are involved in both normal physiological processes as well as pathological processes. Glycosaminoglycans and their proteoglycan counterparts have been shown to have a role in cell signaling and development, angiogenesis, anti-coagulation, anti-coagulation, axonal pathfinding, neurotransmission, tumor progression and metastasis. Heparan sulfate proteoglycans play a role in the pathology of amyloid diseases such as
They also facilitate host cell invasion by pathogens including meningococcus, *Bordetella pertussis*, *Mycobacterium tuberculosis*, herpes simplex virus, *Plasmodium*, Dengue fever and HIV.

### 4.2.2 Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans are present on both the cell surface as well as in the extracellular matrix and the basement membrane. Soluble heparan sulfate proteoglycans are bound in the extracellular matrix and the serum. Heparan sulfate proteoglycans are very important for many normal physiological functions. They are involved in cell-cell adhesion as well as cell-extracellular matrix adhesion. They can also act as co-receptors for growth factors and they can facilitate the storage of heparin-binding proteins for future mobilization.

Heparan sulfate proteoglycans are found in very high concentrations (micromolar range) and can have half-lives of hours to days. For this reason, the signaling events mediated by heparan sulfate proteoglycans are often long lasting. These events may be paracrine or autocrine in nature.

Heparan sulfate proteoglycans can act as receptors and modulate protein signaling in several ways. One mechanism involves the heparan sulfate proteoglycan binding several ligand molecules resulting in their oligomerization. The ligands on the heparan sulfate proteoglycan-ligand complex can then monovalently bind to several receptors. The receptors are able to dimerize and signaling results. An important point of this
mechanism is that the heparan sulfate proteoglycans also bind to the receptors as well as the ligands. This interaction stabilizes the ternary complex.\textsuperscript{19} This mechanism is used by fibroblast growth factors.\textsuperscript{18} A second very similar mechanism involves the heparan sulfate proteoglycans acting as a template for ligand dimerization. The dimerized ligand is then able to bind to two receptors leading to receptor dimerization and signaling. This is thought to be the mechanism used by many growth factors.\textsuperscript{9} A third mechanism involves the heparan sulfate proteoglycans inducing a conformational change in the ligand upon binding which causes the ligand to go from an inactive form to an active form. An example of a ligand that utilizes this mechanism is antithrombin. When antithrombin binds to heparan sulfate glycosaminoglycans, the antithrombin becomes an active inhibitor of thrombin and factor Xa. Because thrombin and factor Xa are involved in the coagulation cascade, this example also demonstrates how heparan sulfate glycosaminoglycans are involved in hemostasis.\textsuperscript{9} As mentioned earlier, heparan sulfate proteoglycans are involved in both cell-cell adhesion and cell-extracellular matrix adhesion. In cell-extracellular matrix adhesion, heparan sulfate proteoglycans bind to fibronectin in the extracellular matrix. This along with integrin binding to fibronectin causes the formation of focal adhesions.\textsuperscript{20, 21}

Heparan sulfate proteoglycans are also important in cellular uptake of polycationic macromolecules. HIV-TAT is an example of a protein that uses this entry mechanism. HIV-TAT has six arginine residues that are positively charged under physiological conditions.\textsuperscript{5} The arginines bind to heparan sulfate proteoglycans on the cell surface resulting in internalization of the HIV-TAT peptide.\textsuperscript{22}
Heparan sulfate proteoglycans have also been shown to mediate cell entry for positively charged tri-platinum compounds.\(^6\) Due to the fact that nonaarginine is the most efficiently transported 'protein transduction domain'\(^5\), Silva et al. used a TAMRA (5-(and 6-) carboxytetramethylrhodamine) tagged nonaarginine to investigate how different platinum drugs competed with the nonaarginine for cellular internalization in wild type CHO cells. The neutral platinum compounds cisplatin and oxaliplatin did not inhibit the internalization of the nonaarginine. However, the positively charged tri-platinum compounds BBR3464, AH44 and Triplatin NC (aka AH78) prevented internalization of the nonaarginine in a charge-dependent manner. BBR3464 with a charge of +4 and AH44 with a charge of +6 decreased the nonaarginine internalization while Triplatin NC with a charge of +8 completely inhibited internalization of the nonaarginine. This was also examined in two cancer cell lines: the human colon carcinoma HCT116 and the osteosarcoma SAOS-2. Interestingly, the positively charged tri-platinum drugs showed increased inhibition of nonaarginine internalization in the tumor cell lines compared to the normal CHO cells. This suggests that these drugs may have some tumor selectivity. To confirm that heparan sulfate proteoglycans were responsible for these observations, Silva et al. investigated the cellular accumulation of the platinum drugs in CHO cells, heparan-sulfate deficient CHO cells and heparan- and chondroitin-sulfate deficient CHO cells. The cellular accumulation of BBR3464, AH44 and Triplatin NC was reduced in the CHO mutants confirming the role of proteoglycans.\(^6\)
4.2.3 Heparanase and Heparinase

Heparanase is a mammalian endoglycosidase, which cleaves heparan sulfate by a hydrolysis mechanism. It is important in the degradation and remodeling of the extracellular matrix. Heparanase is also secreted by normal invasive cells such as cytotrophoblasts in the placenta as well as by malignant cells. Heparinase, the bacterial form of the mammalian enzyme heparanase, cleaves heparan sulfate by an elimination mechanism. There are three heparinases. Heparinase I cleaves the glycosidic linkage to the non-reducing end of iduronic acid. Heparinase II can cleave at the same point as heparinase I as well as cleaving the heparan sulfate next to the glucuronic acid, the site of cleavage by heparinase III. Heparinase I is frequently used in the literature as an in vitro model for heparanase. The cleavage of fondaparinux (a pentasaccharide used as a model for heparan sulfate), by the mammalian heparanase and the bacterial heparinase I is shown in Figure 4.2.

4.2.4 Glycosaminoglycans, Proteoglycans and Cancer

Cancer, the uncontrolled division of abnormal cells in the body, arises when cells incur mutations in proto-oncogenes and tumor suppressors that would normally regulate the cell cycle and control DNA repair. Many tumors have alterations in their expression of glycosaminoglycans. They may express epitopes that are typically expressed in embryonic tissues and only a few adult cell types. These are known as oncofetal antigens. In other cases, tumors may under express certain glycosaminoglycans or they may have truncated or altered versions of glycosaminoglycans when compared to normal tissues.
Figure 4.2 Comparison of fondaparinux cleavage by heparanase and heparinase I. Cleavage of fondaparinux by heparanase (A). Cleavage of fondaparinux by heparinase I (B).
Heparan sulfate proteoglycans are quite important in cancer. For example, pancreatic carcinoma cells over express glypican-1, a glycosylphosphatidylinositol anchored heparan sulfate proteoglycan. It is also over expressed by the fibroblasts surrounding the tumor. Glypican-1 binds fibroblast growth factor 2 and heparin-binding EGF-like growth factor, two growth factors that are also commonly over expressed by pancreatic carcinomas. Glypican-1 is necessary for cell proliferation to result from these two growth factors.\textsuperscript{25}

Heparan sulfate proteoglycans may also have a role in epithelial differentiation and in some cases tumor suppression.\textsuperscript{7} For example, if there are mutations or deletions in the gene for glypican-3, a glycosylphosphatidylinositol anchored heparan sulfate proteoglycan, a congenital overgrowth syndrome called Simpson-Golabi-Behmel syndrome results.\textsuperscript{7} Among many other symptoms, these patients have an increased risk of developing embryonal cancers. They also have an increased risk of developing neuroblastoma and hepatocellular carcinoma.\textsuperscript{26}

An important glycoprotein in cell-cell adhesion is E-cadherin. E-cadherin mediates cell-cell adhesion through homotypic interactions.\textsuperscript{7} The cytoplasmic domain of E-cadherin is attached to actin filaments of the cytoskeleton by catenins.\textsuperscript{27} In cancer, hypermethylation of the promoter region of the E-cadherin gene is frequently observed. This leads to silencing of the E-cadherin gene and reduced cell-cell adhesion. The reduction in cell-cell adhesion makes it possible for cancer cells to escape the primary cancer site. Loss of function mutations in the genes for E-cadherin and the catenins
have also been reported in cancer. Furthermore, a glycoprotein known as dysadherin is frequently expressed in colorectal carcinomas. Dysadherin down regulates E-cadherin. All of these abnormalities decrease cell-cell adhesion allowing cancer cells to become more invasive.27

Integrins are very important proteoglycans involved in cell-extracellular matrix adhesion. They mediate cellular attachment to fibronectin and laminin in the extracellular matrix.7 The integrin α5β1 is a primary fibronectin receptor.28 In many types of malignant cells, the enzyme N-acetylglucosaminyltransferase V (GnTV) is over expressed.7 N-acetylglucosaminyltransferase V is an enzyme that transfers N-acetylglucosamine to glycans and increases β1,6-branches on the growing glycans. It has been shown that GnTV increases the β1,6-branches on the β1 subunit of the α5β1 integrin. The increase in β1,6-branching on the β1 subunit reduces the ability of α1β5 integrins to cluster in the cell membrane. This results in the inhibition of attachment and spreading of cells on fibronectin as well as the stimulation of cell migration and invasion.28

Glycosaminoglycans and their proteoglycan counterparts also play an important role in tumor metastasis.7 Metastasis is tumor cell dissemination to areas that are distant from the primary tumor. This dissemination can occur by two mechanisms: 1) Cancer cells can invade the blood or the lymph vessels of the tumor. 2) Cancer cells can also directly invade into body cavities or spaces around the organs.29 When cancer cells invade the blood stream, selectins (intercellular adhesion molecules that bind to glycosaminoglycans) on platelets and leukocytes form complexes with the tumor cells.
This complex formation helps the tumor cells lodge in blood vessels and also helps the tumor cells avoid immune effector cells in the circulation.\textsuperscript{30, 31} Furthermore, tumors can shed individual cells or clumps of cells containing cancer cells as well as stromal cells such as fibroblasts. Interestingly, it has been found that the heterotypic cell clumps contained a higher percentage of viable cancer cells than the individual or doublet cancer cells in the circulation. The clumps of cells are more efficient at forming metastatic sites and the stromal cells survive the circulation and are involved in metastatic growth at the secondary site.\textsuperscript{32} Circulating cancer cells have also been shown to self-seed established tumor sites. These seeder cells are able to accelerate tumor growth. One of the chemoattractants responsible for the self-seeding was tumor derived IL-8.\textsuperscript{33} IL-8 binds to heparan sulfate\textsuperscript{7, 34} stabilizing it in an active conformation\textsuperscript{34} so the observed self-seeding is also mediated by glycosaminoglycans.

**4.2.5 Heparanase and Cancer**

Many tumors overexpress heparanase.\textsuperscript{35} As mentioned earlier, heparan sulfate proteoglycans in the extracellular matrix can bind large quantities of heparin binding growth factors such as fibroblast growth factor 2 and vascular endothelial growth factor. This protects the growth factors from proteolysis or denaturation. When tumor cells secrete heparanase, the heparanase cleaves the heparan sulfate proteoglycans and releases the bound growth factors. The released growth factors can then be utilized by the invading tumor cells.\textsuperscript{7} Heparanase also opens up the extracellular matrix and the basement membrane making it possible for cancer cells to migrate.\textsuperscript{29} In patients, this overexpression of heparanase has been correlated with increased tumor vasculature,
increased metastasis to lymph nodes as well as to more distant sites, and decreased post-operative survival.\textsuperscript{24}

Given that the over-expression of heparanase leads to a pro-angiogenic and pro-metastatic state\textsuperscript{24}, the inhibition of heparanase activity is a potential target for anti-cancer therapies. The work of Silva \textit{et al.} raises the question: Since the positively charged platinum based chemotherapeutics bind to heparan sulfate, can they also inhibit heparanase activity by inhibiting the enzyme-substrate interaction?

The work presented in this chapter examines the relationship between heparanse and the positively charged platinum-based chemotherapeutics. The data show that the positively charged platinum-based chemotherapeutics bind to the model substrate fondaparinux (Figure 4.3) and inhibit the enzymatic activity of both heparinase (the bacterial form of the enzyme) and heparanse (the human form of the enzyme). Inhibition of heparanase activity represents a new mechanism of action for platinum antitumor compounds. We hypothesize that the positively charged platinum-based chemotherapeutics may offer a way to treat resistant cancer cells as well as reducing angiogenesis and metastasis.
Figure 4.3 Structures of fondaparinux, cisplatin and the positively charged platinum complexes BBR3464, AH44 and AH78.
4.3 Materials and Methods

4.3.1 Materials

Fondaparinux was manufactured by Gland Pharma Limited (India) for Dr. Reddy's Laboratories Limited (Bachepalli, India). The heparinase I, cytodex 3 microcarriers, cold collagen type I, fibrinogen and aprotinin were purchased from Sigma Aldrich (St. Louis, Mo, USA). Thrombin was purchased from ZymoGenetics (Seattle, Washington, USA). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kamimashiki-gun, Japan). All platinum drugs were synthesized in the lab according to published methods. The R9-Tat protein was purchased from AnaSpec (Fremont, California, USA).

4.3.2 Heparinase Dose Response and Inhibition in Relation to Charge

In order to determine what concentration of the platinum drugs are necessary to inhibit heparinase activity and how the charge on the platinum drugs effects heparinase activity, an in vitro colorometric assay was used. To do this, a standard curve was constructed using fondaparinux in a 40 mM sodium acetate buffer (pH 5.0) in each 96-well plate. The standard curve was over the range 0 uM – 200 uM fondaparinux. For the samples, 40 µM fondaparinux was placed in the wells of a 96-well plate. The appropriate concentration of each platinum drug or R9-Tat protein was then added to the wells. The plate was then incubated at 37 °C for 23 hours. After the incubation period, heparinase I (Sigma Aldrich, from Flavibacterium heparinum) was added to each well so that the final concentration was 0.28 µM. The plate was then incubated at 37 °C for 3 hours. A 1.69 mM solution of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was then produced in a 0.1 M
NaOH solution. A 100 µL aliquot of this WST-1 solution was added to each well to stop the assay. The plates were covered with Parafilm and developed at 60 °C for 60 minutes. The absorbance was measured at 584 nm using a µQuant plate reader (Bio-Tek Instruments, Inc).

4.3.3 Determination of the Length of Fondaparinux-Platinum Drug Pre-Incubation Necessary for Heparinase Inhibition

In order to determine how long it takes for the platinum drugs to inhibit heparinase activity, the *in vitro* colorimetric assay was once again used. In each 96-well plate, a standard curve was constructed using fondaparinux in a 40 mM sodium acetate buffer (pH 5.0). The standard curve was over the range 0 µM – 200 µM fondaparinux. For the samples, 40 µM fondaparinux was placed in the wells of a 96-well plate. The appropriate concentration of each platinum drug was then added to the wells. At 0, 0.17, 3 and 6 hours heparinase I (Sigma Aldrich, from *Flavibacterium heparinum*) was added to each well so that the final concentration was 0.28 µM. The plate was then incubated at 37 °C for 3 hours. A 1.69 mM solution of WST-1 was then made in a 0.1 M NaOH solution. A 100 µL aliquot of this WST-1 solution was added to each well to stop the assay. The plates were covered with Parafilm and developed at 60 °C for 60 minutes. The absorbance was measured at 584 nm using a µQuant plate reader (Bio-Tek Instruments, Inc).
4.3.4 HCT116 wt and p53-/- Growth Conditions

HCT116 wt and HCT116 p53-/- cells were grown in RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 mmol/L sodium pyruvate (cRPMI, all from Biofluids, Rockville, MD) in humidified air with 5% CO₂.

4.3.5 Heparanase with Cells

15 x 10³ HCT116 wt or HCT116 p53-/- cells/well were plated in a 96-well plate. Cells were allowed to grow in cRPMI for 72 hours until nearly confluent before treating. After cells had been allowed to adhere, the medium was removed and the cells were washed with PBS. The cells were then treated. Conditions were either Untreated (PBS), 100 µM fondaparinux, 120 µM AH78 or 100 µM fondaparinux plus 120 µM AH78. All samples were in PBS and the fondaparinux and AH78 were pre-incubated before being placed on the cells. After treatment, the plates were incubated at 37°C for 5 hours. The supernatants were then transferred to another 96-well plate that had a series of known fondaparinux standards in it to create a standard curve. A 1.69 mM solution of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was then produced in a 0.1 M NaOH solution. A 100 µL aliquot of this WST-1 solution was added to each well to stop the assay. The plates were covered with Parafilm and developed at 60 °C for 60 minutes. The absorbance was measured at 584 nm using a µQuant plate reader (Bio-Tek Instruments, Inc).
4.4 Results

4.4.1 Inhibition of Bacterial Heparinase by Platinum Drugs

4.4.1a Inhibition of Heparinase Activity

Four platinum drugs, cisplatin, BBR3464, AH44 and AH78 were examined (Figure 4.3). Of these four drugs, only the positively charged drugs (AH44, BBR3464 and AH78) inhibit heparinase activity. BBR3464 has a charge of +4, AH44 has a charge of +6 and AH78 has a charge of +8. As expected, AH78 inhibited heparinase activity more than AH44. Surprisingly, BBR3464 also inhibited heparinase activity more than AH44 (Figure 4.4).

Figure 4.4 Inhibition of heparinase activity. Pt drugs and fondaparinux were co-incubated at 37 °C for 23 hours. The heparinase was then added and incubated for 3 hours. Error bars are ± the SEM. * denotes a p-value <0.0001.
4.4.1b Determination of the Length of Fondaparinux-Platinum Drug Pre-Incubation Necessary for Heparinase Inhibition

A time course dependence study of heparinase inhibition by platinum drugs with the substrate fondaparinux was done to investigate how long it takes for the platinum drugs to interact with the fondaparinux. The AH44 and AH78 instantly inhibited the heparinase as would be expected with electrostatic binding to fondaparinux. BBR3464 instantly inhibited heparinase activity to approximately 20% of the control but the amount of inhibition increased with time. In fact, at 3 hours of co-incubation of fondaparinux with BBR3464, heparinase activity was inhibited to approximately 2% of the control (Figure 4.5). The values for all three positively charged platinum complexes are significantly different from the untreated control at all time points investigated ($p<0.0001$).

4.4.1c Heparinase Inhibition with Increasing Platinum Drug Concentration

To see how much drug was required to inhibit heparinase activity, the dose response curves for BBR3464, AH44 and AH78 were determined (Figure 4.6). It is interesting to note that more AH44 is required to inhibit heparinase than BBR3464 or AH78. Due to the fact that proteins with arginine rich sequences are known to bind to heparan sulfate, an R9-Tat protein was used as a control. At high concentrations, the R9-protein also inhibits heparinase activity but not to the same extent as BBR3464 or AH78.
Figure 4.5 Determination of the length of fondaparinux-platinum drug pre-incubation necessary for heparinase inhibition. The positively charged platinum complexes were pre-incubated for the indicated times before the addition of heparinase. The inhibition of heparinase activity due to BBR3464 increased with time much more than was observed with AH44 and AH78. This supports the idea that BBR3464 may be able to initially bind electrostatically to fondaparinux and then covalently bind with time, whereas, AH44 and AH78 can only bind electrostatically. Error bars are ± the SEM.
Figure 4.6 Inhibition of heparinase due to increasing concentrations of R9-Tat protein, BBR3464, AH44 and AH78. Fondaparinux was 40 µM. The R9-Tat protein initially increases heparan sulfate cleavage slightly but at higher concentrations, it inhibits enzyme activity. BBR3464 begins to inhibit enzyme activity between 10 and 30 µM. AH44 begins to inhibit heparinase activity between 60 and 120 µM. AH78 begins to inhibit heparinase activity between 10 and 30 µM. Error bars are ± the SEM.
4.4.2 Heparanase Inhibition

The previous experiments have shown that the positively charged platinum compounds can inhibit heparinase I activity, but can they also inhibit heparanase activity? Heparanase is secreted from human cancer cells so a cell line could be used as a source of heparanase. Although it would be ideal to investigate this using the colorimetric assay that was used for the *in vitro* studies with heparinase I\(^{38}\), this assay method was only used in a completely *in vitro* system in the literature. In an effort to modify the assay to work with cells, several conditions were investigated. It was determined that the medium used to grow the cells contained too many sugars that could react with WST-1. Therefore, the incubation of the fondaparinux and AH78 had to be done in PBS. To alter the amount of enzyme secreted, the initial number of cells plated was examined. The concentrations of fondaparinux and AH78 were also varied to determine the ideal concentrations. The length of time that the fondaparinux and AH78 were exposed to the cells was also investigated. Two cell lines were investigated: HCT116 wt and HCT116 p53\(^{-/-}\). These two cell lines differ only in their p53 status. The HCT116 p53\(^{-/-}\) cells were chosen due to the fact that they should over express heparanase because p53 is a negative regulator of heparanase expression.\(^{24}\)

The finalized method involved growing the cells in a 96-well plate until they were nearly confluent. The medium was then removed and the cells were washed with PBS. The cells were then treated with PBS (untreated), 100 µM fondaparinux, 120 µM AH78 or the combination of 100 µM fondaparinux and 120 µM AH78 which were mixed together before putting it on the cells (Figure 4.7). The drug conditions were all in PBS. In the
HCT116 wt cells, the addition of fondaparinux increases the amount of color formation over the baseline from the cells alone. This indicates that the fondaparinux is indeed being cleaved by heparanase secreted from the cells. Although it is possible that there may be other endoglycosidases that can cleave fondaparinux, the literature does not report such endoglycosidases. AH78 alone does not change the amount of color formation compared to the cells alone. However, when AH78 was added to the fondaparinux, the amount of color formation was almost decreased to the level of the untreated cells. This suggests that AH78 inhibited fondaparinux cleavage. The data were normalized to the control. This gives the fold change relative to the control, which is shown as fold control in the figure. The difference in fold control between fondaparinux alone and fondaparinux plus AH78 has a p-value of 5.5 \times 10^{-7}. In the HCT116 p53-/- cells, there was a higher background from the untreated cells than was observed with the HCT116 wt cells. Even so, with the addition of fondaparinux, more color formation was observed. Once again, AH78 alone did not alter color formation when compared to the untreated cells. When AH78 was added to the fondaparinux, the amount of color produced was even closer to baseline levels than what was observed with the HCT116 wt cells. The difference in fold control between fondaparinux alone and fondaparinux plus AH78 has a p-value of 3.3 \times 10^{-9}. Taken together, the data show that AH78 inhibits fondaparinux cleavage by heparanase in both the HCT116 wt and HCT116 p53-/- cell lines.
Figure 4.7 Heparanse assay with cells. A) HCT116 wt. The combination of fondaparinux and AH78 reduces fondaparinux cleavage almost down to the untreated baseline. B) HCT116 p53-/- The combination of fondaparinux and AH78 reduces fondaparinux cleavage down to the untreated baseline. The error bars are ± the SEM. * Denotes p-value < 0.0001
4.5 Discussion

This research has shown that only the positively charged platinum drugs, BBR3464, AH44 and AH78 are able to inhibit heparinase I activity. Interestingly, BBR3464 inhibited heparinase I activity more than AH44. This is somewhat surprising because AH44 has a charge of +6 while BBR3464 has a charge of +4. The increased inhibition due to BBR3464 is most likely due to the chloride leaving groups, which could allow covalent binding to the fondaparinux whereas the AH44 and AH78 can only bind electrostatically. The results of the time course study further support this hypothesis. The AH44 and AH78 instantly inhibited the heparinase, as would be expected with electrostatic binding to fondaparinux. The fact that BBR3464 had an initial inhibition that increased over the course of 3-hours, supports the hypothesis that BBR3464 has an initial electrostatic interaction with fondaparinux followed by covalent binding over time.

The in vitro colorimetric assay used to measure heparinase I inhibition was successfully modified to investigate heparanase inhibition in cell culture. These studies showed that although the cells secreted components that would react with WST-1, the addition of fondaparinux would increase the amount of color formation, presumably due to fondaparinux cleavage. Furthermore, the addition of AH78 with the fondaparinux, decreased the color formation almost to cellular baseline. This shows that AH78 can inhibit heparanase activity in a cellular environment.
In conclusion, the positively charged platinum-based chemotherapeutics can inhibit fondaparinux cleavage by both heparinase I and heparanase. The enzyme inhibition with respect to AH44 and AH78 was charge dependent. BBR3464 inhibited enzyme activity more than AH44 despite being less positively charged. Time course studies showed that the interaction between the positively charged platinum-based chemotherapeutics and the fondaparinux is instantaneous. It further suggested that BBR3464 may have an initial electrostatic binding followed by covalent binding whereas AH44 and AH78 can only bind electrostatically. In total, the results prove the concept that positively charged platinum-based chemotherapeutics can inhibit heparanase activity and may be able to reduce tumor angiogenesis and metastasis.

To confirm that BBR3464 covalently binds to fondaparinux, mass spectrometry studies are currently underway. To gain insight into the reaction, isothermal titration calorimetry experiments could also be performed. As understanding of the interaction between the positively charged platinum complexes and heparan sulfate proteoglycans is gained, compounds with a higher affinity for heparan sulfate could be designed. These compounds would be able to elicit the same heparanase inhibition at lower doses.

In the future, the functional relevance of the findings discussed in this chapter need to be investigated using angiogenesis and metastasis assays. Angiogenesis and metastasis could be investigated using angiogenesis bead assays or by doing a flank tumor model in mice. Although functional relevance remains to be proven, this work does suggest a new way to target heparanase activity in cancer patients.
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Appendix 1

Effect of Imipramine on BBR3464 Cytotoxicity and the Effect of Clomipramine on the Cytotoxicity of the Platinum Drugs

A.1.1 Abstract

In continuation of the work discussed in chapters two and three, the percent apoptosis due to the cationic trinuclear agent BBR3464 \([\{\text{trans-PtCl(NH}_3\text{)}\}_2\mu\-(\text{trans-Pt(NH}_3\text{)}_2(\text{H}_2\text{N(CH}_2\text{)}_6\text{NH}_2\text{)}}\}_2\text{]}^{4+}\) was investigated in the presence and absence of imipramine in HCT116 wt cells. It was found that imipramine inhibits the cytotoxicity of BBR3464. Another tricylic antidepressant, clomipramine, was also investigated with cisplatin, oxaliplatin, carboplatin and BBR3464. Clomipramine augmented the cytotoxicity of cisplatin and oxaliplatin. Although clomipramine appears to augment the cytotoxicity of carboplatin and BBR3464, clomipramine is far more toxic than the other antidepressants used and the observed augmentation was not significantly different from the cytotoxicity due to clomipramine alone.

A.1.2 Introduction

The work discussed in this appendix is a continuation of the work discussed in chapters two and three. Given how much desipramine enhanced the cytotoxicity of the cationic
tri-nuclear agent BBR3464 \([\{\text{trans-PtCl(NH}_3\}_2\mu-(\text{trans-Pt(NH}_3)_2(\text{H}_2\text{N(CH}_2\text{CH}_2\text{NH}_2)_2}\}]^{4+}\), it was important to investigate how specific the effect was to desipramine. To this end, the percent apoptosis due to BBR3464 was investigated in the presence of two other tricyclic antidepressants, imipramine and clomipramine (Figure A.1.1). As shown in Figure 3.1, imipramine and desipramine differ by one methyl group. As can be seen in Figure A.1.1 clomipramine differs from imipramine by the addition of one chloride group. Although these structural differences are minor, it is important to determine how they effect the observed augmentation of cytotoxicity due to platinum anti-cancer drugs. To further investigate this, the percent apoptosis due to cisplatin, oxaliplatin and carboplatin were also investigated in the presence and absence of clomipramine.

![Structures of imipramine and clomipramine.](image)

**Figure A.1.1** Structures of imipramine and clomipramine.
A.1.3 Materials and Methods

A.1.3.1 Materials

HCT 116 human colon carcinoma cells were a gift from Bert Vogelstein (Johns Hopkins University, Baltimore MD). McCoy’s 5a Medium Modified, RPMI, Fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, HEPES buffer and sodium pyruvate were all purchased from Biofluids (Rockville, MD).

A.1.3.2 Cell Systems and Culture Conditions

HCT116 wt cells were used. The medium used types was RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES buffer and 1 mmol/L sodium pyruvate. (cRPMI, all from Biofluids, Rockville, MD). The cells were cultured in 175 cm$^2$ Cell Star tissue culture flasks from Greiner Bio-One (Frickenhausen, Germany) in an incubator with 5% CO$_2$ and humidified air.

A.1.3.3 Apoptosis Studies

Cells were cultured in 6-well plates with 7.0 x 10$^4$ cells per well. A 3 mL aliquot of medium was put in each well. Cells were treated for 24 hours, 48 hours or 72 hours with these conditions: untreated, platinum drug by itself, antidepressant by itself, and platinum drug plus antidepressant. The platinum drugs were added to the medium after a one-hour treatment period with the antidepressant. At the time points, all cells (adherent and non-adherent) were collected. As described previously, the cells were fixed with an ethanol/fetal bovine serum solution and stained with a propidium
The samples were analyzed for subdiploid DNA content using a Becton Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA). This protocol allows the detection of intact versus fragmented DNA, which allows cell cycle analysis.

**A.1.4 Results**

Imipramine significantly inhibited the cytotoxicity of BBR3464 at 24, 48 and 72 hours. At 24 hours, BBR3464 caused 13% apoptosis while the combination of BBR3464 plus imipramine only caused 7% apoptosis with a p-value of 0.05. At 48 hours, BBR3464 alone caused 19% apoptosis while BBR3464 plus imipramine only caused 9% apoptosis. This difference has a p-value of 0.005. At 72 hours, BBR3464 alone caused 24% apoptosis while the combination of BBR3464 and imipramine only caused 12% apoptosis with a p-value of 0.02 (Figure A.1.2).

Another tricyclic antidepressant, clomipramine was also investigated. Clomipramine augmented the cytotoxicity of both cisplatin and oxaliplatin. At 24 hours, cisplatin alone caused 8% apoptosis while the combination of cisplatin plus clomipramine caused 17% apoptosis with a p-value of 0.05. At 48 hours, cisplatin caused 42% apoptosis while cisplatin plus clomipramine caused 44% apoptosis. Although there is only a 2% difference between these two values, it is a statistically significant difference and has a p-value of $1.4 \times 10^{-8}$. At 72 hours, the percent apoptosis caused by cisplatin increased to 48% while the percent apoptosis due to cisplatin plus clomipramine increased to 95%. This difference has a p-value of $1.1 \times 10^{-9}$ (Figure A.1.3 A).
Figure A.1.2 Imipramine inhibits the cytotoxicity of BBR3464 in HCT116 wt cells. Error bars are ± the SEM. BBR3464 was 10 µM and imipramine was 40 µM.
Clomipramine also enhances the cytotoxicity of oxaliplatin. At 24 hours, oxaliplatin alone caused 4% apoptosis while the combination of oxaliplatin plus clomipramine caused 17% apoptosis. This difference has a p-value of 0.0005. At 48 hours, oxaliplatin alone caused 22% apoptosis whereas oxaliplatin plus clomipramine caused 44% apoptosis with a p-value of $3.1 \times 10^{-8}$. At 72 hours, oxaliplatin caused 28% apoptosis while the combination with clomipramine caused 67% apoptosis with a p-value of $5.4 \times 10^{-6}$ (Figure A.1.3 B).

The results are different with carboplatin and BBR3464. In both cases, the combination of the platinum drug with clomipramine is in fact significantly different from the percent apoptosis due to the platinum drug alone at all three time points investigated. Unfortunately, clomipramine by itself is quite a bit more cytotoxic than the other antidepressants that were investigated in Chapter 3. So although the combination of platinum drugs with clomipramine is significantly different from the percent apoptosis due to the drug alone, it is not significantly different from the percent apoptosis due to clomipramine alone (Figure A.1.3 C and D).
**Figure A.1.3** Effect of clomipramine on the cytotoxicity of cisplatin, oxaliplatin, carboplatin and BBR3464 in HCT116 wt cells. Error bars are ± the SEM. Cisplatin was 10 µM, oxaliplatin was 10 µM, Carboplatin was 10 µM, BBR3464 was 10 µM and clomipramine was 40 µM.

**A.1.5 Discussion**

This work has demonstrated that imipramine augments the cytotoxicity of BBR3464 although the increase is not as much as what was observed with desipramine (Figure 2.2). This is further evidence that desipramine and imipramine do something different in the cells.
Although clomipramine only differs from imipramine by one chloride group the results were quite different. Clomipramine augmented the cytotoxicity of both cisplatin and oxaliplatin whereas imipramine augmented cisplatin and inhibited oxaliplatin. Clomipramine did not enhance the cytotoxicity of BBR3464 or carboplatin. Due to the cytotoxicity of clomipramine, even at lower doses, further work with this antidepressant was abandoned.
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Vita

Brigitte Jacqueline Engelmann was born on December 16, 1983 in Elkhart, Indiana and is an American citizen. She graduated from Portage Central High School in Portage, Michigan in 2002. She attended Western Michigan University in Kalamazoo, Michigan and graduated with a B.S. in Biochemistry in 2007. In 2007, she entered the MD/PhD program at Virginia Commonwealth University in Richmond, Virginia. She has published an article in the Journal of Biological Inorganic Chemistry. The reference is shown below.