CYTOTOXIC PROPERTIES OF NOVEL PLATINUM COMPOUNDS, BBR3610-DACH AND TRANS-4-NBD IN TUMOR CELLS: CELLULAR EFFECTS OF 1, 2-DACH AND NBD LIGANDS

Vijay Menon
Virginia Commonwealth University

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CYTOTOXIC PROPERTIES OF NOVEL PLATINUM COMPOUNDS, BBR3610-DACH AND TRANS-4-NBD IN TUMOR CELLS:
CELLULAR EFFECTS OF 1, 2-DACH AND NBD LIGANDS

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

By

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Richmond, Virginia
April, 2013
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First, I would like to thank Dr. Nicholas Farrell for being an excellent advisor. I have no words to express my appreciation for his constant support and guidance during my graduate years at VCU. It has been a wonderful experience working in his laboratory where I was constantly encouraged to come up with my own ideas and had the complete freedom to design and carry out my own experiments. In a way, it was more like a post-doctoral training. All the time that I spent in the laboratory has been phenomenal and I could not wish for a much better place. Besides being a great mentor, Dr. Farrell has also been a good friend. I have always tried and succeeded in making him accustomed to the “Indian” ways, especially by recommending a couple of good Indian restaurants around.

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<tr>
<td>AG</td>
<td>adenine-guanine</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia Telangiectasia and Rad3 related</td>
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<tr>
<td>BER</td>
<td>base excision repair</td>
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<tr>
<td>BRCA</td>
<td>breast cancer type 1 susceptibility protein</td>
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<tr>
<td>°C</td>
<td>Celsius</td>
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<tr>
<td>Chk</td>
<td>checkpoint kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
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<tr>
<td>DAPI</td>
<td>4´, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
</tr>
<tr>
<td>DSBs</td>
<td>double strand breaks</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
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<tr>
<td>FOLFOX</td>
<td>Folinic acid, 5-fluorouracil, oxaliplatin</td>
</tr>
<tr>
<td>FAN1</td>
<td>Fanconi Anemia associated nuclease 1</td>
</tr>
<tr>
<td>Symbol</td>
<td>Term</td>
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<td>--------</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<tr>
<td>Hr</td>
<td>Hour</td>
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<tr>
<td>γ-H2AX</td>
<td>phosphorylated H2AX</td>
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<tr>
<td>GG</td>
<td>guanine-guanine</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HRR</td>
<td>homologous recombination repair</td>
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<tr>
<td>IR</td>
<td>ionizing radiation</td>
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<tr>
<td>ICL</td>
<td>interstrand crosslink</td>
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<tr>
<td>Kb</td>
<td>Kilobase</td>
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<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>KU-60019</td>
<td>ATM inhibitor</td>
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<tr>
<td>μL</td>
<td>Microliter</td>
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<tr>
<td>MDM2</td>
<td>mouse double minute 2 homolog</td>
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<td>min</td>
<td>Minutes</td>
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<td>MRN</td>
<td>Mre11/Rad50/Nbs1</td>
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<td>[3-(4,5-Dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide</td>
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<td>N7</td>
<td>Nitrogen 7</td>
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<tr>
<td>NBD</td>
<td>4-Chloro-7-nitrobenzofurazan</td>
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<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
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<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>OCT</td>
<td>organic cation transporter</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PARP 1</td>
<td>poly(ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RAD</td>
<td>family of RADiation sensitive genes</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RNase B</td>
<td>ribonuclease B</td>
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<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>TAT</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetramethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<td>Volts</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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<tr>
<td>XLF</td>
<td>XRCC4-like factor</td>
</tr>
<tr>
<td>XP</td>
<td>xeroderma pigmentosum</td>
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ABSTRACT

CYTOTOXIC PROPERTIES OF NOVEL PLATINUM COMPOUNDS, BBR3610-DACH AND TRANS-NBD IN TUMOR CELLS: CELLULAR EFFECTS OF THE 1, 2-DACH AND NBD LIGANDS

By Vijay Menon, M.Sc.

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

Virginia Commonwealth University, 2013

Advisor: Nicholas Farrell, Ph.D.
Professor, Department of Chemistry

Platinum-based chemotherapeutics are used for the treatment of a wide range of cancers and a number of attempts have been made toward developing compounds with better cellular stability and similar or enhanced cytotoxicity as compared to their predecessors.

The first part of the work reported here focuses on the cellular effects of the metabolically stable dinuclear platinum compound, BBR3610-DACH. Comet assay showed this compound to form interstrand crosslinks, a highly toxic DNA lesion in HCT116 cells, at equimolar concentrations to its parental compound, BBR3610. Cell cycle studies showed that BBR3610-DACH causes G1/S and G2/M cell cycle arrest with S phase depletion, which was p21 dependent and partially p53 dependent in contrast to BBR3610 which showed initial S phase accumulation followed by a classical G2/M arrest. BBR3610-DACH-induced G1/S and G2/M cell cycle arrest interestingly was found to be independent of the DNA damage response mediated via the activation of ATM.
and ATR kinases. Also, the cell cycle arrest culminated in apoptosis, although apparently through a non-canonical pathway.

The second project explores the cellular effects of trans-4-NBD which is a fluorescent derivative of transplatin. Like cisplatin, trans-4-NBD induced interstrand crosslinks in HCT116 cells as detected by the comet assay. Treatment with trans-4-NBD showed a G2/M arrest in HCT116 cells and a transient S phase accumulation in A2780 cells, with a marked increase in p53 and p21 protein levels. A robust apoptotic response is also seen via caspase activation and PARP cleavage in both the cell lines.

Finally, the focus is shifted toward the nucleolar targeting platinum complex, TriplatinNC. Confocal studies in TriplatinNC-treated HCT116 and A2780 cells showed disruption of rRNA transcription as an early event followed by a robust G1 cell cycle arrest. Apoptotic induction was observed with the onset of cellular morphological changes and apparent caspase activation which was independent of the p53 status of the cells.

Overall, these studies explore novel platinum based compounds that show promising anti-cancer activities by affecting various facets of cellular signaling.
CHAPTER 1

GENERAL INTRODUCTION

Cancer is one of the major contributors to world mortality. It is estimated that 1 in every 4 deaths in the United States is due to cancer. According to recent Cancer Statistics\textsuperscript{1} a total of 1,660,290 new cancer cases and 580,350 cancer deaths have been predicted to occur in 2013. However, cancer death rates have shown a 20% decline since 1991. This reduction could be attributed to different forms of treatments like surgery, radiotherapy, and chemotherapy.

Chemotherapeutic intervention has its roots as early as 1940 with the use of nitrogen mustards (alkylating agents) and antagonists against folic acid. Since then, the use of cancer drugs has constantly evolved starting with Zubrod’s initiatives that included the taxanes (anti-microtubule agents) and camptothecins and bleomycin (anti-tumor antibiotics) to the nitrosoureas (alkylating agents) and anthracyclines (anti-tumor antibiotics) to the more widely used transition metal complexes. These form some of the major classes of chemotherapeutics\textsuperscript{2} along with compounds that do not fall under these categories like monoclonal antibodies and signaling inhibitors. Although chemotherapy is a widely used treatment regimen along with radiation, most of the cancer drugs have been shown to be carcinogenic and induce toxic side effects.
Cisplatin and analogs

Platinum-based compounds are one of the major classes of cancer chemotherapeutics. Cisplatin (Fig 1-1), first discovered by Michael Peyrone in 1845, was later recognized serendipitously for its chemotherapeutic ability in 1964 by Barnett Rosenberg who observed an incredible elongation of *E.coli* cells when electricity was passed through platinum electrodes immersed in the bacterial solution. Eventually, cisplatin was found to be effective against tumors induced in mice and subsequently entered clinical trials in 1971. In spite of being an efficient anti-cancer agent, use of cisplatin was accompanied with serious side effects like nephrotoxicity and ototoxicity. Also, the development of intrinsic and acquired drug resistance hindered the efficacy of cisplatin in many forms of cancer. These mainly included decreased drug uptake, increased drug efflux, intracellular degradation by glutathione and metallothionein, increased DNA repair, and alterations in different signaling pathways. In order to circumvent these issues, carboplatin (Fig 1-1) was developed and introduced in the late 1980s. Although carboplatin decreased most of the toxic side effects of cisplatin, the major side effect associated with it was myelosuppression. Also, it was not very effective in cisplatin-resistant tumors and eventually its usage led to drug resistance as seen for cisplatin. In spite of these drawbacks, carboplatin is widely used for the treatment of lung cancer in combination with gemcitabine. The third generation drug, oxaliplatin (Fig 1-1), which was approved for clinical use (Europe, 1996 and United States, 2000) after cisplatin and carboplatin, was widely used for the treatment of colorectal cancers, owing to a different structure and mechanism of action compared to its predecessors. It was found to have an improved therapeutic index in colon cancers which could
be attributed to the rate of its uptake into cells. It was seen that the human organic cation transporters (OCT1 and OCT2) increased the uptake of oxaliplatin but not cisplatin or carboplatin. The anti-cancer activity of oxaliplatin was found to be markedly increased in combination with other compounds especially 5-fluorouracil and folinic acid (FOLFOX). However, as observed for cisplatin or carboplatin, the use of oxaliplatin led to side effects like hematological toxicity and GI tract toxicity. Also, drug resistance developed which was primarily due to increased DNA repair and increased drug efflux.
Figure 1-1. Structures of Cisplatin (Left), Carboplatin (Center), and Oxaliplatin (Right)
Types of DNA adducts

The main pharmacological target of platinum drugs is DNA. The cytotoxicity of cisplatin, carboplatin, and oxaliplatin is attributed to the formation of DNA adducts wherein the platinum moiety forms a bond with N7 of guanine or adenine. This leads to the formation of 1, 2-GG intrastrand crosslinks that form the primary adducts of cisplatin. The secondary adducts constitute 1, 2-AG intrastrand crosslinks, 1, 3-GXG intrastrand crosslinks, and 1, 2-GG interstrand crosslinks (Fig 1-2). While cisplatin and carboplatin form identical DNA adducts, oxaliplatin adducts are more bulky owing to the presence of the 1, 2-diaminocyclohexane moiety (Fig 1-1). These DNA adducts pose serious threats to different DNA transactions like replication, transcription, and recombination. As a result, one of the major resistance mechanisms elicited by cells in response to these drugs is increased DNA repair.
Figure 1-2. DNA adducts formed by cisplatin or carboplatin (Top) and oxaliplatin (Bottom)
Transplatin and Transplatinum Compounds

It is a well known fact that the \textit{trans} isomer of cisplatin, trans-[PtCl₂(NH₃)₂] or transplatin (Fig 1-3) is chemotherapeutically inactive\textsuperscript{29}. This inactivity is mainly attributed to kinetic instability leading to its degradation and the formation of DNA adducts that are stereochemically different from cisplatin induced adduct. In spite of this inactive nature of transplatin, a surge in novel platinum compounds with \textit{trans} geometry was seen when Farrell \textit{et al.} first reported a series of transplatinum compounds (Fig 1-3) with increased activity over transplatin and in most cases, equivalent to cisplatin. These were obtained by replacing the primary transplatin amines with N-donor heterocycles, aliphatic amines, heterocyclic aliphatic amines, and iminoethers\textsuperscript{30, 31}. It is believed that the substitution with bulky ligands retards the chloride ligand substitution, leading to increased kinetic stability. Also, the formation of DNA adducts, structurally different from cisplatin renders these compounds active in most of the cisplatin-resistant tumors.

One possible reason for the increased activity observed especially in transplatinum complexes containing heterocyclic derivatives is the intercalation or pi-stacking of these derivatives between DNA bases leading to the formation of distinct DNA adducts\textsuperscript{32}. Another modification that has been carried out is to conjugate platinum compounds with aromatic intercalators like anthraquinone and other cytotoxic moieties\textsuperscript{33}. One such modification discussed in Chapter 4, was the development of transplatinum complexes containing a fluorophore like NBD that also acts as a DNA intercalator. These compounds, designated as Trans-NBD (Fig 1-3), exhibit a dual mode of DNA interaction which is DNA crosslinking \textit{via} the transplatin moiety and DNA intercalation.
via the NBD fluorophore. Also, the presence of the fluorophore renders these compounds ideal for imaging studies in order to better understand their exact cellular distribution and subsequent cellular actions.
Figure 1-3. Structures of transplatin (Top), transplatinum compounds (Center), and trans-NBD (Bottom)
Polynuclear platinum compounds

Polynuclear platinum complexes constitute a distinct class of antitumor agents, different in structure and biological effects from their mononuclear counterparts represented by cisplatin and its congeners\(^3\). These compounds, containing two or more platinum coordination units were an important addition to the cancer armamentarium. \([\{\text{trans-PtCl(NH}_3\}_2\}_2\{l\text{-trans-Pt(NH}_3\}_2(H_2N(CH_2)_6NH_2)_2\}]^{4+}\) or BBR3464 (Fig 1-4), the first in this series of compounds, is a trinuclear, bifunctional DNA binding agent with a \(4^+\) charge and was the only non-cisplatin compound to enter phase II of clinical trials for the treatment of ovarian and lung cancers\(^3\).

The bifunctional DNA binding is characterized by the formation of long range intrastrand or interstrand crosslinks with more preference for the latter\(^3\). Since BBR3464 is structurally different than cisplatin or its congeners, the DNA adducts induced by BBR3464 are also structurally distinct. Initial studies with BBR3464 revealed a non-covalent preassociation of the central platinum with DNA. Some of the long range DNA adducts formed by BBR3464 are 1, 4 and 1, 6 interstrand crosslinks (Fig 1-5) that are not normally recognized by DNA repair proteins. As a result, these lesions prove to be major obstacles during DNA replication or transcription and at the same time escape DNA repair. Although BBR3464 exhibited enhanced antitumor effects against a wide range of cancer cell lines, it exhibited increased cellular toxicity which led to its failure in clinical trials. This was attributed to its increased biotransformation in the serum that contributed toward its instability\(^3\).
During the examination of the structure activity relationship of polynuclear platinum compounds, one of the main questions was whether the central platinum in BBR3464 could be replaced by other H-bonding groups and whether such a modification would retain its cytotoxicity. This led to the synthesis of linear polyamine-linked dinuclear compounds that displayed a similar mode of DNA binding and biological activity as BBR3464\(^40\). The inclusion of polyamine linkers within the alkanediamine chain of BBR3464 not only provided the required electrostatic interaction and H-bonding but also provided a means for increased uptake owing to the polycationic nature of the spermine or spermidine linkers. The first in this series of drugs, BBR3610 (Fig 1-4) containing a spermine linker, was found to be cytotoxic in the nanomolar range in gliomas\(^41\). However, BBR3610 was also found to be susceptible to serum breakdown which may have contributed to its narrow therapeutic index\(^42\). As a result, many modifications of BBR3610 were carried out to reduce its breakdown in serum and at the same time retain a considerable anti-tumor activity. Some of the modifications involved replacing the chloride ligands with carboxylate groups like butyrate and capronate\(^43\) (Fig 1-4). The most recent modification was BBR3610-DACH, discussed in detail in Chapter 2, wherein the primary amines at either ends of BBR3610 is replaced with the more stable 1, 2- diaminocyclohexane ligand which provided enhanced stability against glutathione degradation and showed remarkable cellular effects as compared to the parental BBR3610\(^44\).

The platinum drugs described so far bind to DNA covalently forming the specific adducts, although the polynuclear compounds had a non-covalent pre-association due to the presence of the central platinum or the polyamine linkers. An interesting modification carried out in
BBR3464 was the replacement of the chloride ligands by inert or dangling amines, \( \text{H}_2\text{N} (\text{CH}_2)_6\text{NH}_3 \). This resulted in a non-covalent trinuclear platinum compound, TriplatinNC\(^{45}\) (Fig 1-4). It showed a unique mode of DNA binding called the phosphate clamp which was characterized by two different DNA interactions namely “backbone tracking” which involved the association of the molecule with the phosphate backbone and “groove spanning” which involved interaction with both DNA strands by passing over the minor groove\(^{46}\). This structure is similar to the “arginine fork” seen in protein-DNA interactions. In addition to increased uptake and resistance to metabolic degradation\(^{47}\), this drug exhibited unique cellular effects, which is described in detail in Chapter 4.
Figure 1-4. Structures of polynuclear platinum compounds
Figure 1-5. DNA adducts formed by BBR3464.
Picture from *Polynuclear Platinum Drugs, Nicholas Farrell*
The formation of toxic DNA lesions by platinum drugs and other DNA targeting anti-cancer drugs is one of the key mechanisms responsible for their efficacy in cancer cells. Also, DNA is constantly under stress due to various endogenous processes like nucleotide misincorporation during replication, byproducts of various cellular processes, etc. In order to maintain the genome integrity and ensure normal development, organisms have evolved various DNA damage responses that mainly involve activation of cell cycle checkpoints to arrest cell cycle progression, various DNA repair pathways depending on the nature of DNA damage, and apoptotic pathways when the DNA damage is severe.
Cell Cycle Checkpoints and cell cycle arrest

Cell cycle arrest is primarily mediated via the activation of cell cycle checkpoints during various phases of the cell cycle (Fig 1.6). The key players involved in the activation of cell cycle checkpoints are the serine/threonine protein kinases, ATM (Ataxia Telangiectasia mutated) and ATR (Ataxia Telangiectasia and Rad3 related). Both these proteins have a catalytic C terminal motif containing a phosphatidylinositol 3-kinase domain. Although these proteins phosphorylate a myriad of cellular proteins, they function in response to specific DNA lesions or damage. ATM primarily recognizes DNA double strand breaks while ATR recognizes replication fork stalling due to UV and other agents that form bulky DNA adducts. During the G1 cell cycle checkpoint, one of the key steps is the stabilization and activation of the tumor suppressor protein, p53. Under normal circumstance, p53 levels are regulated by its interaction with MDM2 which targets it for nuclear export and subsequent degradation in the cytoplasm. Following DNA damage by UV or other agents, ATM activates Chk2 which in turn phosphorylates p53 at serine 20. Also, ATM can directly phosphorylate MDM2, still allowing its interaction with p53 but no nuclear export. ATR, on the other hand, activates Chk1 which can also phosphorylate p53 at serine 20. In addition to these events, p53 is also phosphorylated at serine 15 which is important for its transactivation activity. It then activates different downstream genes, one of them being p21 which is a cyclinE/Cdk2 inhibitor, eventually leading to G1 arrest. The S phase checkpoint is mainly activated in response to stalled replication forks due to obstructions in DNA replication. An important consequence is degradation of the Cdc25A phosphatase via phosphorylation by Chk2 which is activated by ATM at S123. The
absence of Cdc25A prevents the inhibitory phosphorylations from being removed. The Cdk2/cyclinE and Cdk2/cyclin A complexes become inactive and as a result DNA synthesis is stalled. Since most of the platinum drugs form DNA crosslinks, it is expected that DNA replication would stall leading to ATR activation and subsequent Chk1 activation. This concept is further explored in Chapter 2.

Finally, the G2 phase checkpoint is also activated during DNA damage in cells before S phase completion. ATR is eventually activated leading to Chk1 phosphorylation at S345. Chk1 then phosphorylates Cdc25C, creating a binding site for the 14-3-3 protein, either making Cdc25C catalytically inactive or sequestering it in the cytoplasm. This ultimately inhibits the mitotic cyclin B.Cdc2 kinase and prevents the cells from entering mitosis. In cells that have incurred DNA damage during the G2 phase, ATM is primarily activated leading to Chk2 phosphorylation at Thr68. Chk2 then phosphorylates Cdc25C at S216, ultimately bolstering the cyclinB.Cdc2 inhibition imposed by the ATR-Chk1 pathway. Most of the anti-cancer agents that cause DNA damage activate either of the above discussed checkpoints which are further explored in Chapter 2.
Figure 1-6. Cell Cycle Checkpoints
(Adapted from multiple sources)
DNA Repair Pathways

DNA repair pathways provide an ideal platform for tumor cells to survive DNA damage induced by chemotherapeutic agents. Depending upon the nature of the damage, organisms have evolved various mechanisms of DNA repair. As mentioned previously, one of the DNA damage responses is triggered following UV radiation that causes two types of lesions namely cyclobutane pyrimidine dimers and 6-4 photoproduc_3. These are potentially deleterious lesions that cause DNA bends and kinks, inhibiting DNA replication and transcription. These lesions are repaired by the nucleotide excision repair (NER) which involves over 30 proteins (XPC-hHR23B, TFIIH that includes XPD and XPB helicases, XPA, XPG, and XPF-ERCC1), defects in which are responsible for the human disorder, Xeroderma Pigmentosum_4. Bacteria and some other organisms repair UV induced damage by another mechanism called photoreactivation also called light repair_4 since it occurs only in the presence of light. It involves an enzyme, photolyase that binds pyrimidine dimers and a second molecule called chromophore that converts light energy into chemical energy, allowing the damaged DNA to revert to the normal form. In addition to NER, another predominant form of DNA repair, which also plays a pivotal role in the repair of mitochondrial DNA, is the base excision repair (BER) that repairs DNA damage induced by reactive oxygen species and free radicals_5. Mismatch repair is a post replication error correcting pathway that recognizes incorrectly incorporated nucleotides during replication. After the mismatch is recognized, the newly replicated strand including the mismatched base is excised and replaced with DNA repair synthesis and religation_6.
The most toxic DNA lesions encountered in the cells are the double strand break and the interstrand crosslink since they involve both strands of DNA. DNA double strand breaks (DSBs) are mainly resolved by two mechanisms, homologous recombination repair (HRR) and non-homologous end joining (NHEJ)\textsuperscript{70, 71}. The latter that occurs during all phases of the cell cycle is carried out principally by a small number of core proteins such as the Ku heterodimer, DNA-PKcs, Artemis, DNA Ligase IV, XLF, and XRCC4\textsuperscript{72}. In comparison, the HRR pathway requires a pair of homologous sequences like sister chromatids or homologous chromosomes to replace the nucleotides during repair. It involves a larger number of proteins including RAD51, RAD52, RAD54, RAD55, RAD57, BRCA1, BRCA2, XRCC2, XRCC3, and MRN complex. It is an error-free DNA repair mechanism and is most prevalent during the S/G\textsubscript{2} phases of the cell cycle\textsuperscript{73}.

Interstrand crosslink repair (Fig 1-7) is a highly complex DNA repair pathway responsible for resolving interstrand crosslinks (ICLs). As a result, it is one of the major factors responsible for platinum drug resistance. Early work in \textit{E.coli} has shown that interstrand crosslinks are repaired through combined action of the nucleotide excision repair pathway (NER) and the homologous recombination (HR) pathway\textsuperscript{74}. The mechanism of interstrand crosslink (ICL) repair in mammalian cells is much more complicated and most of the details are still unclear. Active mainly in replicative cells, it involves the sequential activation of an error-free homologous recombination process and an error-prone translesion bypass synthesis, along with a complex interplay of proteins involved in NER\textsuperscript{75, 76}. Briefly, when a replication fork collides with a crosslink, a double strand break is created via the activity of different endonucleases\textsuperscript{77}. The same
strand is then cleaved on the other side of the lesion\textsuperscript{78}, followed by unhooking of the crosslinking and finally culminating in translesion synthesis through the agency of specialized polymerases\textsuperscript{79}. The bypassed crosslink is later removed by the nucleotide excision repair machinery and the gap formed allows homologous recombination of the repaired chromatid with the sister\textsuperscript{80}.

The proteins associated with the pathophysiology of Fanconi Anemia have a crucial role in ICL repair. It is believed that these FA proteins assist in stabilizing replication forks and also assist the replication machinery in the removal of ICLs\textsuperscript{81-83}. The importance of the involvement of the FA proteins reached a new dimension with the identification and characterization of a nuclease, Fanconi Anemia associated nuclease 1 (FAN1), associated with platinum drug resistance\textsuperscript{84-87}. 
*Figure 1-7. Interstrand crosslink repair. (1) DNA with interstrand crosslink. (2) Stalling of replication fork 10-30 nucleotides away from the ICL. (3) A second replication fork stops near the ICL on the opposite side. (4) One of the replication forks continue forward and halt at the ICL. (5) Incision on one side of the ICL by endonuclease (Mus81-Eme1). (6) Second incision on the other side by second endonuclease (ERCC1-XPF). (7) Translesion bypass by polymerase (Rev1). (8) Nucleotide inserted is extended by DNA polymerase $\zeta$. (9) The flipped out crosslink is removed by the NER machinery. (10) End resection of parental strand forms a 3’ overhang. (11) Strand invasion by homologous recombination forming d-loop. (12) Strand switching. (13) The d-loop resolved via the FA pathway. (14) Repaired DNA

*Figure adapted and recreated from:
How the Fanconi Anemia Pathway Guards the Genome
Moldovan and D’Andrea
Annual Review of Genetics, Vol. 43: 223-249
**Apoptosis**

Cells that are arrested in different phases of the cell cycle eventually undergo programmed cell death or apoptosis. During an apoptotic response, a group of intracellular proteases called caspases get activated and eventually cause the disruption of cellular components into apoptotic bodies. These proteases are present in the cells as inactive zymogens that subsequently undergo proteolytic activation\(^{88,89}\). The two main pathways of apoptosis include: (1) the extrinsic pathway that occurs *via* the binding of death ligands to their respective death receptors\(^ {90}\), and (2) the intrinsic pathway activated following DNA damage. Death receptors activate caspase-8 which in turn either activates caspase-3 directly or cleaves Bid to give truncated Bid which then translocates to the mitochondria\(^ {91}\). Bid cleavage is also triggered *via* the intrinsic pathway which is primarily activated following DNA damage. During this phase, mitochondrial disruption occurs releasing cytochrome C into the cytoplasm which then complexes with APAF-1 forming the apoptosome\(^ {92}\). This complex then activates caspase-9 which then activates caspase-3. Both pathways converge on activated caspase-3 which then cleaves downstream cellular substrates like PARP which is also a DNA repair protein\(^ {93}\). The induction of apoptosis following treatment with platinum compounds will be discussed further in chapters 2, 3, and 4.
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CHAPTER 2

The dinuclear platinum agent, BBR3610-DACH, exhibits a significant anti-proliferative effect in human colorectal HCT116 cells via modulation of G1/S and G2/M cell cycle arrest and apoptosis induction: Cellular effects of the 1, 2-DACH carrier ligand

ABSTRACT:

BBR3610-DACH, a long-chain, bifunctional dinuclear Pt (II) complex with a similar DNA binding mode to the phase II clinical drug, BBR3464 and its potent analogue, BBR3610, was shown previously to be resistant to metabolic decomposition by sulfur nucleophiles. Comet analysis indicated that BBR3610-DACH formed interstrand crosslinks at equimolar concentrations as BBR3610. Interestingly, cytoflow analysis of HCT116 cells showed that BBR3610-DACH induced G1/S and G2/M cell cycle arrest with S phase depletion in contrast to BBR3610 which showed initial accumulation in S phase followed by a classical G2/M arrest. Immunoblotting revealed a stabilization of p53 and concomitant increased levels of p21 and p27 between 6-24 hr after treatment with BBR3610-DACH. Cell viability assays using MTT and subsequent cytoflow analysis with p53 and p21 null cells indicated that the cytotoxicity profile of BBR3610-DACH was p21 dependent and partially p53 dependent. However, increase in the levels of cyclin E was observed with steady state levels of CDK2 and Cdc25A which are G1 phase regulators. The G2/M block was corroborated with decreased levels of cyclin A and cyclin
B1. Surprisingly, BBR3610-DACH induced G₁ block appeared to be independent of the DNA damage response effectors, ATM and ATR. Finally, both compounds induced apoptosis with BBR3610-DACH showing a robust PARP-1 cleavage as early as 6 hr, independent of caspase-3/7 cleavage. In summary, BBR3610-DACH is a DNA binding platinum agent with unique inhibitory effects on cell cycle progression that could be further developed as a major chemotherapeutic.
INTRODUCTION:

Platinum drugs have been a mainstay of cancer therapeutics especially for the treatment of head and neck, testicular, ovarian, lung, and colorectal cancers. The design of platinum drugs stems from the basic structure of mononuclear platinum complexes, the efficacy of which is dictated by three main pharmacological factors; cellular uptake, metabolic deactivation, and the type of DNA adducts formed. Interaction of these drugs with cellular biomolecules like sulfur containing glutathione and metallothionein, render them inactive before reaching their pharmacological target, DNA. Subsequently, the multinuclear platinum compounds that are structurally different from cisplatin, and exhibit a different mode of DNA binding, were developed to circumvent the cellular resistance elicited toward the mononuclear compounds namely decreased uptake and increased efflux and increased DNA repair. Also, since most of the useful platinum compounds have the cis chemistry, the multi-nuclear platinum compounds showed a shift of paradigm in the overall structure-activity relationship. Dinuclear platinum complexes containing two reactive platinum centers were the first developed in this class of compounds to form long-range intrastrand and interstrand DNA crosslinks structurally different from adducts formed by cisplatin or its second-generation analogues. The DNA binding ability, cytotoxicity, and antitumor activity was further enhanced by introducing a third platinum within the alkanediamine framework of the dinuclear complexes. The first prototype of this class, BBR3464 [\{trans-PtCl(NH₃)₂\}_2µ-\{trans-Pt(NH₃)₂(H₂N(CH₂)_6NH₂)\}]^{4+} (Fig 2-1A), was the only non-cisplatin compound to have reached Phase II clinical trials. It was found to be cytotoxic in cisplatin resistant cell lines and showed high efficacy in p53 mutant tumor cells. However, a
low therapeutic index, possibly due to increased metabolism and biotransformation reactions in human plasma, rendered BBR3464 unsuitable for further evaluation\textsuperscript{6,7}. 

In investigating the structure-activity relationship of the multinuclear compounds, especially the trinuclear complexes, it was seen that the charged central platinum atom provided H-bonding and an electrostatic pre-association with duplex DNA in the minor groove. Replacement of this central platinum with linear polyamines resulted in second generation analogues of BBR3464 that retained its biological activity\textsuperscript{8}. BBR3610 (Fig 2-1B), which was developed by replacing the central platinum with a spermine linker, is one of the most cytotoxic platinum drugs with nanomolar toxicity in gliomas and colon cancer cells. However, no improvement was observed in the pharmacokinetic profile since like its predecessor, BBR3610 was also found to undergo serum biotransformation leading to its degradation\textsuperscript{9}. This was attributed to the substitution of the Pt-Cl bond by a trans-influencing S donor (from thiol containing molecules), resulting in bridge cleavage.

To improve the metabolic stability of the multinuclear compounds and also to circumvent the irreversible plasma protein binding, different analogues of BBR3610 were developed by modifying either the leaving groups or the carrier ligands. The first set of compounds were derived by the replacement of the chloride ligands in BBR3610 with alkylcarboxylates, resulting in a series of bis-platinum compounds, utilizing butyrate or capronate (CT-47518 and CT-47463 respectively, Fig 2-1C), with improved pharmacokinetic and pharmacodynamic profiles\textsuperscript{10}. These
compounds were found to overcome resistance due to defects in DNA mismatch repair and were also found to be highly effective in cisplatin and oxaliplatin resistant cell lines. This was subsequently followed by the development of another BBR3610 analogue containing the 1, 2-diaminocyclohexane (DACH) ligand (Fig 2-1D). The chelating effect of the DACH ring contributed to the increased metabolic stability of BBR3610-DACH in the presence of sulfur containing compounds at physiological pH. *In vitro* studies showed this compound formed DNA adducts that persisted longer, escaped DNA repair, and inhibited transcription\textsuperscript{11}.

Deregulation of cell cycle progression is one of the key contributors toward cancer development\textsuperscript{12}. Exposure to DNA damaging agents induces G\textsubscript{1}/S or G\textsubscript{2}/M cell cycle arrest by activating cell cycle checkpoint proteins that inhibit cyclin dependent kinases\textsuperscript{13}. The resulting cell cycle arrest can afford an opportunity for DNA repair and have negative effects on apoptosis\textsuperscript{14}. Thus, the pharmacologic disruption of the G\textsubscript{2}/M arrest induced by cytotoxic agents can increase drug sensitivity. Much of the earlier studies on the cell cycle effects of platinum drugs were carried out using cisplatin which showed a reduced DNA synthesis followed by S phase accumulation and finally culminating in G\textsubscript{2}/M arrest. With few exceptions, mononuclear and polynuclear platinum drugs, including BBR3464 and BBR3610 follow this trend of cell cycle arrest.

Cell cycle studies of BBR3610-DACH suggest a point of divergence from the conventional platinum drugs, and specifically its parental compound. BBR3610-DACH elicits a biphasic cell cycle arrest in G\textsubscript{1} and G\textsubscript{2}, with a complete depletion of cells in S phase. As our understanding of
cell cycle arrest increases, the ability to accurately sequence chemotherapy based on phase-specific sensitivity can have a great impact on therapeutic approach in the treatment of human cancers.
Figure 2-1. Structures of the polynuclear platinum compounds mentioned in the chapter.

(A) BBR3464; (B) BBR3610; (C) CT-47463 and CT-47518; (D) BBR3610-DACH
MATERIALS AND METHODS:

**Chemicals.** Cisplatin was obtained from Sigma, Cat# 479306. BBR3610 and BBR3610-DACH were synthesized as discussed earlier. The stock solutions of platinum compounds were prepared at the concentration of 1 mM in water and stored at -20°C. The ATM inhibitor, KU-60019, was obtained from Selleck Chemicals.

**Cell Culture and Drug treatments.** The human colorectal cancer cell line, HCT116, and its p53 and p21 knockout derivatives (denoted as p53-/- and p21-/-) were cultured in RPMI 1640 (Invitrogen, Cat# 11875-093) supplemented with 10% fetal bovine serum (Quality Biologicals, Cat# 110-001-101US) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Cat# 15140). The mismatch repair proficient HCT116+chr3 cells (a kind gift from Dr. Richard Boland) was cultured in IMDM (Invitrogen, Cat# 12440-053) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in logarithmic growth as a monolayer in T75 tissue culture flask at 37°C in a 5% CO₂ incubator. For the drug treatment, the molar drug:cell ratio was kept constant by maintaining a seeding density of 5X10⁵ cells/10 mL medium.

**Growth Inhibition Assay.** 5000 cells/well in 100 µL were seeded in a 96-well plate and allowed 24 hr to attach. Cells were then treated with the drugs for 72 hr. Following drug removal, the cells were treated with 0.5 mg/mL MTT reagent [(3, 4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Sigma, Cat# M2128] in RPMI medium for 3 hr at 37°C. The MTT reagent
was removed and 100 µL DMSO was added to each well. The plate was then incubated on a shaker at room temperature in the dark. The spectrophotometric reading was taken at 570 nm using a microplate reader.

**Analysis of cell cycle distribution.** HCT116, HCT116 p53-/-, HCT116 p21-/-, and HCT116 + chr3 cells were seeded in 100 mm tissue culture dishes at a density of 5X10^5 cells per dish. After 24 hr, cells were treated with an equimolar dose of the drugs (20 µM) for 6, 24, and 48 hr respectively. Both detached and floating cells were harvested at the different timepoints. One million cells were then suspended in 1 mL of propidium iodide solution (3.8 mM sodium citrate; 0.05 mg/mL propidium iodide; 0.1% Triton X-100) with added RNaseB at a final concentration of 7 Kunitz/mL and kept in the dark at 4°C. Cells were then analyzed using flow cytometry.

**Flow Cytometry.** A Becton Dickinson (San Jose, CA) FACSCanto II flow cytometer was used for these studies. The argon ion laser set at 488 nm was used as an excitation source. Cells having DNA content between 2N and 4N were designated as being in G₁, S, and G₂ phases respectively. Twenty thousand events were acquired for each sample and the data obtained were analyzed using the Modfit software.

**Cell Synchronization studies.** To obtain G₀ synchronization, HCT116 cells were first seeded at a density of 5X10^5 cells/10 cm dish and allowed 24 hr to attach. The cells were then serum-starved in medium containing 0.5% FBS for 96 hr. Flow cytometry analysis of synchronized cells showed approximately 80% cells arrested in G₀/G₁ phase.
**Antibodies.** The primary antibodies used are listed in Table 2-1 and were used at appropriate concentrations as recommended by the manufacturer.

**Immunoblotting.** Following drug treatments, both floating and adherent cells were harvested, washed once with ice-cold PBS and pelleted (10,000 rpm, 5 min, 4°C). The pellets were then lysed in 50-200 µL SDS lysis buffer [62.5 mM Tris-HCl, pH 7.5, 5% glycerol, 4% SDS, 4% complete protease inhibitor (Roche, Cat# 11873580001), 5% BME], passed through 21 gauge needle 10-15 times on ice, and centrifuged (12,000 rpm, 20 min, 4°C). Protein concentrations were determined by the Bradford Assay, resolved on 10%, 12%, or 4-20% gradient polyacrylamide gels, and transferred to PVDF membrane (90 V, 75 min, 4°C). The membrane was blocked in 5% non-fat dry milk in 1X Tris buffered saline containing 0.1% Tween-20 for 60-90 min. The membranes were then probed with the primary antibodies in blocking buffer overnight at 4°C, followed by secondary antibody (anti-rabbit or anti-mouse) conjugated to horseradish peroxidase (Cell Signaling, Thermo Scientific) for 1 hr at room temperature. Chemiluminescent protein bands were visualized on X-ray films.

**siRNA Transfection.** ATR expression was down-regulated using the ON-Target plus Smart Pool L-003202-00 and Non-targeting siGENOME control siRNA, D-001210-01-05 (Dharmacon, Thermo Scientific). 50 nM siRNAs were transfected 24 hr after seeding of HCT116 cells using Dharmafect 1 according to the manufacturer’s instructions.
**BrdU Incorporation Assay.** To determine the percentage of cells in the S phase, the cells were first treated with the drugs for the respective timepoints. They were then pulse-labeled with 10 µM BrdU for 3 hr prior to harvesting. The harvested cells were then fixed in 70% ethanol overnight. Following fixation, the cells were incubated in 2.5 M HCl for 30 min and then in 0.1 M sodium borate for 2 min at room temperature. These were then blocked in PBS, 2% BSA, 0.1% Tween-20, RT, 1 hr, incubated with mouse anti-BrdU antibody (BD Biosciences) for 1 hr and then probed with the secondary Alexa Fluor 488 rabbit anti-mouse antibody (Molecular Probes, Invitrogen) for 1 hr. Finally, the cells were stained with propidium iodide and analyzed by flow cytometry.

**Comet Assay.** Single cell gel electrophoresis or comet assay was carried out using a Trevigen kit to detect and evaluate the DNA damage. Briefly, HCT116 cells (1X10^5 cells/mL) were plated in 35 mm or 60 mm dishes and allowed to attach for 24 hrs. The cells were then treated with different concentrations of each drug for 1 hr. The medium was removed, the cells washed once, and further incubated in fresh medium for 6 hrs. Cells were then harvested by scraping and resuspended in 1 mL of ice-cold phosphate buffered saline (PBS, pH 7.4). Then, 50 µL of the cell suspension was mixed with 500 µL of low melting agarose at 37°C, and 50 µL of this mixture was spread on comet slides, and solidified in the dark for 30 mins at 4°C. The slides were then treated with ice-cold lysis buffer in the dark for 1 hr at 4°C and then incubated in an alkaline solution (pH>13) for 1 hr at room temperature to allow for alkaline unwinding. Electrophoresis was carried out under alkaline conditions at 21 V, 300 mA, and 30 min at 4°C.
Slides were washed twice with distilled water, once with 70% ethanol, and then allowed to dry for 10 min at around 37-45°C. The slides were then stained with SYBR green for 5 min at 4°C. Comet images were obtained using a fluorescence microscope (Olympus IX70). The comet analysis was performed using the Comet Score software from TriTek Corporation.

The extent of crosslinking was expressed as the percentage decrease in tail moment and was calculated using the formula:

\[
\% \text{ Decrease in tail moment} = \left[1 - \frac{(TM_{di} - TM_{cu})}{(TM_{ci} - TM_{cu})}\right] \times 100
\]

where, TM_{di} is the tail moment of the drug treated irradiated samples, TM_{ci} is the tail moment of the untreated irradiated samples, and TM_{cu} is the tail moment of the untreated, unirradiated samples. 40-50 comets were scored for each concentration and the reduction in comet tail length and intensity was apparent within each sample.
RESULTS:

**BBR3610-DACH induces interstrand crosslinks in HCT116 cells.** Polynuclear platinum compounds are known to cause long-range intrastrand and interstrand crosslinks on DNA. In this context, we investigated whether BBR3610-DACH induces interstrand crosslinks on the DNA by utilizing the alkaline comet assay. Typical comet images are shown in Fig 2-2. In the control, unirradiated, untreated HCT116 cells, no DNA damage was detected (Fig 2-2A). Following irradiation with 15 Gy to introduce a fixed level of DNA strand breaks, the shorter DNA fragments migrated from the bulk of DNA during alkaline electrophoresis to produce comet tails (Fig 2-2B). When the irradiated cells were treated with 50-250 µM BBR3610-DACH, a concentration-dependent decrease in the comet tail length and intensity was observed, in comparison to irradiated controls (Fig 2-2C, D, E). Also, the comet heads were larger and had higher intensity than the non-drug treated irradiated control possibly because of the retention of more DNA in the head due to the drug induced interstrand crosslinks.

By quantifying the decrease in the moment of the comet tails, BBR3610-DACH-induced interstrand crosslinks were compared to those formed by BBR3610 at similar concentrations and were found to be similar, although BBR3610 showed a slightly higher level of crosslinks (Fig 2-2F).
Figure 2-2. Comet images from HCT116 cells treated with BBR3610 or BBR3610-DACH. Following irradiation (15 Gy) of untreated cells, distinct comets were observed (B) in comparison to the untreated, unirradiated cells (A). Cells were then treated with 50, 100, and 250 μM BBR3610 or BBR3610-DACH for 1 hr followed by 6 hr drug free post-incubation for interstrand crosslink formation. After irradiation of drug treated samples, comet tails with decreased length and intensity could be observed due to the presence of the drug induced interstrand crosslinks (C-E). All samples stained with SYBR green and observed under a fluorescence microscope. Interstrand crosslink formation was represented as percentage decrease in the tail moment ± SE. *p-value > 0.05 (F).
**BBR3610-DACH exhibits a significant anti-proliferative effect.** BBR3610-DACH was evaluated in HCT116 and in isogenic cell lines deficient in p53 or p21. Cells were exposed to the platinum complex (0.78 – 50 µM) and the cytotoxicity was assessed using the MTT assay. In the case of BBR3610, the cells showed an approximately 50% reduction in cell viability at 1-10 nM, but were highly resistant to any further reduction (Fig 2-3A and Fig 2-4). The distinct biphasic response seen with BBR3610 was not seen in cells treated with BBR3610-DACH (Fig 2-3A) and as a result the IC₅₀ (5 µM) was lower than that for BBR3610. However, the IC₅₀ was higher in the p53-deficient cells than in parental cells (Fig 2-3B) indicating a pivotal role of p53 in the antiproliferative response. Surprisingly, in HCT116 p21-/- cells (Fig 2-3C), BBR3610-DACH showed similar cytostatic effect as in the wild type cells. This could indicate that p21-mediated cell cycle arrest in G₁/S led to enhanced DNA repair and a reduction in cell death that roughly balanced its direct anti-proliferative effect. The IC₅₀ concentration for the three cell lines is shown in Table 2-1.
Figure 2-3. Cytotoxicity curves in HCT116 and isogenic cell lines. Cytotoxicity curves showing the sensitivity of (A) HCT116; (B) HCT116 p53-/-; (C) HCT116 p21-/- to BBR3610 and BBR3610-DACH determined by MTT assay following exposure to micromolar concentrations of the drugs for 72 hr. Error bars indicate means ± SD from three independent experiments.
Figure 2-4. Percentage inhibition by BBR3610 plotted on a log scale. Error bars indicate means ± SD from independent experiments.
Table 2-1. Cytotoxicity of BBR3610-DACH in HCT116 and isogenic cell lines

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<tr>
<th>Cell Line</th>
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<tr>
<td>HCT116</td>
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<tr>
<td>HCT116 p53-/-</td>
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<tr>
<td>HCT116 p21-/-</td>
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</tr>
</tbody>
</table>

* IC50 values are means ± SD from 3 separate experiments, each conducted in triplicate
**BBR3610-DACH induces G₁ and G₂ arrest in HCT116 cells.** Following genotoxic stress induced by antitumor agents, the p53 levels are stabilized resulting in induction of p21 which mediates transient G₁/S arrest, or apoptosis, depending on the damage incurred. The platinum drugs such as cisplatin have been shown to activate the pathway leading to a G₂ arrest\textsuperscript{16, 17}. Accordingly, treatment of HCT116 cells with 20 µM cisplatin causes S phase accumulation of cells at 24 hr and finally a G₂ arrest at 48 hr (Data not shown). However, treatment with 20 µM BBR3610-DACH induced an early robust G₁ and G₂ phase arrest with a significant reduction in S phase as compared to BBR3610 which showed only G₂ arrest between 24-48 hr (**Fig 2-5A, B**). This was corroborated by the BrdU incorporation assay wherein a steady decrease in S phase population was seen following treatment with BBR3610-DACH (**Fig 2-5C**). Also, distinct cell cycle effects of the two analogues were observed over a range of concentrations, with the specific G₂ block detectable with as low as 0.5 µM BBR3610 and G₁ arrest with S phase depletion being apparent with 5 µM BBR3610-DACH treatment (**Fig 2-6**). Thus, there are qualitative differences between these compounds in cellular responses that cannot be ascribed to difference in uptake, overall potency, or stability.

Concurrently, a stabilization of p53 protein level was seen in BBR3610-DACH-treated cells, as early as 6 hr and continuing to 24 and 48 hr accompanied by an increase in p21 and p27 expression levels, which indicates the classical pathway being triggered for the G₁/S phase arrest and possibly the G₂/M arrest (**Fig 2-5D**). However, the induction of p21 was found to be independent of p53 (data not shown), indicating that in addition to p53 stabilization, other
mechanisms are operating that lead to an increased expression of p21. The essential role of p53 in the G₁/S phase arrest was confirmed by similar studies carried out in the HCT116 isogenic cell line lacking p53 or p21 (Fig 2-7). However, in these cases, the G₂/M arrest is independent of p53 and p21 indicating the role of other cell cycle proteins in triggering this pathway. Although BBR3610 also induced both p53 and p21, the response was slower and less robust.
Figure 2-5. BBR3610-DACH induces G1/S and G2/M cell cycle arrest in HCT116 cells with robust S phase depletion. (A) Cells were treated with 20 µM BBR3610 or 20 µM BBR3610-DACH and analyzed for cell cycle distribution using propidium iodide. (B) Modfit analysis of G1, S, and G2 populations. Asterisks indicate p-values obtained from t-test carried out between the treated and control populations. *p-value < 0.05. **p-value < 0.005.
Figure 2-5. BBR3610-DACH induces G₁/S and G₂/M cell cycle arrest in HCT116 cells with robust S phase depletion. (C) BrdU staining assay showing decrease in S phase population following treatment with 20 µM BBR3610-DACH as compared to BBR3610 or cisplatin.
Figure 2-5. BBR3610-DACH induces G₁/S and G₂/M cell cycle arrest in HCT116 cells with robust S phase depletion. (D) HCT116 cells were treated with 20 µM BBR3610 or 20 µM BBR3610-DACH for 6, 24, and 48 hr. Representative immunoblots are shown from two or three independent experiments.
Figure 2-6. BBR3610-DACH induces $G_1$ and $G_2$ cell cycle arrest in a dose dependent manner. HCT116 cells were treated with different concentrations of BBR3610 or BBR3610-DACH for 24 hr and analyzed for cell cycle distribution using propidium iodide.
Figure 2-7. The cell cycle effect of BBR3610-DACH is dependent on the p53 and p21 status in HCT116 cells. Following treatment with 20 µM BBR3610 or 20 µM BBR3610-DACH, cell cycle distribution was analyzed using propidium iodide.
**BBR3610-DACH specifically targets G₁ phase population of cells.** To define precisely the phase of the cell cycle where BBR3610-DACH exerts its anti-proliferative effect, we carried out a “cell mapping“ experiment. As shown in **Fig 2-9**, ~80% of the cell population was synchronized in G₁ phase after serum starvation (96 hr in 0.5% serum). The progression into S phase was seen at 12 hr after release from the block. Immediately following release of the block, treatment with 20 µM BBR3610 or BBR3610-DACH was carried out and cells were harvested at 8, 12, 16, and 24 hr respectively. The cells that were synchronized in G₀/G₁ continued to be arrested following treatment with 20 µM BBR3610-DACH (**Fig 2-8A**). However, with BBR3610 treatment, they progressed normally and halted in the S phase, possibly due to collision of replication forks with interstrand crosslinks. This result shows that the cells are specifically halted in the G₀/G₁ phase by BBR3610-DACH. To further confirm this finding, the cells were released from the block and allowed to progress through G₁ phase into S. A 4-hr treatment with the drug in the early S phase, ~8 hr after release of block, resulted in a significant blockade of cells at the G₂/M phases in the case of BBR3610-DACH and a significantly delayed accumulation in S phase in the case of BBR3610 (**Fig 2-8B**). These results corroborate the observation with the asynchronous population of cells wherein a prominent G₁/S and G₂/M arrest is seen following treatment with BBR3610-DACH, and further show that G₁ accumulation in BBR3610-DACH treated cells is due almost exclusively to a persistent and complete G₁/S arrest, and not to cells that successfully traversed mitosis to re-enter G₁.
Figure 2-8. BBR3610-DACH specifically inhibits the progression of G1 phase cells. (A) HCT116 cells were synchronized in G0/G1 by serum starvation using 0.5% serum for 96 hr. The cells were then released immediately into medium containing BBR3610 or BBR3610-DACH. Flow cytometric analysis was performed at different timepoints to determine the progression of cell cycle after drug treatment. (B) HCT116 cells were synchronized in G0/G1 by serum starvation, then released into medium and allowed to progress into S phase. They were then treated with BBR3610 or BBR3610-DACH and flow cytometric analysis was performed at different timepoints to determine the progression of cell cycle following the PPC treatment.
Figure 2-9. Cell Cycle Mapping. HCT116 cells were synchronized in G₀/G₁ phase by incubating in medium containing 0.5% serum for 96 hr. The cells were then released into complete medium and harvested at specific timepoints to check for entry into S phase.
BBR3610-DACH induced S phase depletion is independent of ATM and ATR activation. ATM and ATR are DNA damage sensor protein kinases that activate a signal transduction cascade involving Chk1 and Chk2 and culminating in cell cycle checkpoints\textsuperscript{18}. To check whether the cell cycle perturbations elicited by BBR3610-DACH were ATM/ATR dependent, we inhibited ATM/ATR activation by, (i) 2 mM caffeine treatment (inhibits both ATM and ATR), (ii) KU-60019, a small molecule inhibitor of ATM (3 µM), and (iii) knockdown of ATR by 50 nM ATR-targeting siRNA.

Treatment with 2 mM caffeine, a pharmacological inhibitor of ATM/ATR, did not abrogate the G\textsubscript{2} arrest induced by BBR3610 treatment. However, following treatment with BBR3610-DACH, the G\textsubscript{1} phase arrest was maintained, but a reduction in the G\textsubscript{2} population was observed (Fig 2-10A). In order to decipher this further, we individually inhibited ATM and ATR activation using an ATM inhibitor and ATR-targeting siRNA respectively. Inhibition of ATM using 3 µM KU-60019 did not abrogate the cell cycle effect elicited either by BBR3610 or BBR3610-DACH (Fig 2-10B), indicating that ATM activation was not a pre-requisite for cell cycle arrest by these agents. Next, we investigated the involvement of ATR in the observed cell cycle effects induced by the drug. ATR knockdown by siRNA (Fig 2-10D) allowed faster progression of BBR3610 treated cells through S phase to G\textsubscript{2}/M arrest, but did not relieve the G\textsubscript{1} block in BBR3610-DACH treated cells at all (Fig 2-10C). Instead a reduction in G\textsubscript{2} phase cells was seen, as observed with the caffeine treatment, suggesting partial abrogation of the G\textsubscript{2}/M arrest. The absence of ATR activation by BBR3610-DACH was further confirmed by examining P-Chk1 levels\textsuperscript{19}, which were increased dramatically after 24 hr in the cells treated with BBR3610 (Fig 2-10E) or
cisplatin (Fig 2-11A). This shows a significant S phase arrest following treatment with these drugs which is then resolved, allowing cells to progress into the G2 phase. However, BBR3610-DACH treatment did not induce any P-Chk1, indicating the absence of ATR activation, which is consistent with the lack of delay in progression through S phase. In summary, ATM/ATR activation is not a pre-requisite for the observed BBR3610-DACH induced S phase depletion in comparison to BBR3610 wherein the S phase accumulation is abrogated following knockdown of ATR (Fig 2-10C)
Figure 2-10. BBR3610-DACH induced cell cycle arrest is independent of ATM or ATR activity. (A) Effect of caffeine on BBR3610 or BBR3610-DACH induced cell cycle arrest. Cells were pretreated with 2 mM caffeine for 1 hr and then treated with 20 µM BBR3610 or BBR3610-DACH for 10 hr or 24 hr as indicated. They were then analyzed for cell cycle distribution using propidium iodide.
Figure 2-10. BBR3610-DACH induced cell cycle arrest is independent of ATM or ATR activity. (B) Effect of ATM inhibition on BBR3610 or BBR3610-DACH induced cell cycle arrest. Cells were pretreated with 3 µM KU-60019, an ATM inhibitor, for 1hr and then treated with 20 µM BBR3610 or BBR3610-DACH for 10 hr and 24 hr respectively. As a control, cells were also treated with 0.05% DMSO (ATMi solvent).
Figure 2-10. BBR3610-DACH induced cell cycle arrest is independent of ATM or ATR activity. (C) Effect of ATR knockdown on BBR3610 or BBR3610-DACH induced cell cycle arrest. Cells were transfected with 50nM siRNA against ATR for 3 days. Drug was then added and the cells incubated for 24 hr.
Figure 2-10. BBR3610-DACH induced cell cycle arrest is independent of ATM or ATR activity. (D) Immunoblot showing ATR knockdown in HCT116 cells using 50 nM siRNA in comparison to non-targeting siRNA control and Dharmafect Reagent Control. DNA-PK was used as a loading control. Blots are representative of three independent experiments.
Figure 2-10. BBR3610-DACH induced cell cycle arrest is independent of ATM or ATR activity. (E) Immunoblot analysis of P-Chk1 levels in HCT116 cells following treatment with 20 µM BBR3610 or BBR3610-DACH. Blots are representative of three independent experiments.

Figure 2-11. (A) Time course analysis of P-Chk1 levels in 20 µM cisplatin treated HCT116 cells.
**Effect of BBR3610-DACH on G1/S and G2/M phase cell cycle regulators.** The interaction of cyclin-dependent kinases (CDKs) with their corresponding cyclins mediates the normal cell cycle progression from G1 to S, S to G2, and G2 to M phases. To assess their role in cell cycle perturbations, we investigated the effect of BBR3610-DACH treatment on the protein levels of G1 CDKs and cyclins. Immunoblot analysis revealed that 6, 24, and 48 hr treatment with BBR3610-DACH which elicits a G1 arrest was accompanied by an increase in cyclin E which normally accumulates in G1 and is degraded in S. CDK2 and Cdc25A were maintained at steady state levels, while phospho-Rb decreased markedly after 24 and 48 hr of drug treatment. Thus, in BBR3610-DACH treated cells CDK2/cyclin E complexes presumably were formed, but kinase activity was apparently suppressed by p21, resulting in Rb dephosphorylation and G1 arrest. An increase in cyclin D1 and cyclin D3 was observed, which could be due to cells exiting the G0 phase and getting arrested in G1. Also, the levels of cyclin A and cyclin B drastically decreased after 24 and 48 hr of BBR3610-DACH treatment, which may result from p53 mediated suppression of their synthesis and may contribute to G2 arrest.
Figure 2-12. Effect of BBR3610-DACH on $G_1/S$ and $G_2/M$ regulators. Cells were treated with 20 µM BBR3610 or BBR3610-DACH for 6, 24, or 48 hr. Protein lysates were prepared and 30 µg protein was resolved on SDS-PAGE and detected by probing the immunoblot with antibodies against (A) pRb, CDK2, Cdc25A, cyclin E, cyclin D1 and cyclin D. β-actin was used as a loading control. Blots are representative of three independent experiments.
Figure 2-12. Effect of BBR3610-DACH on $G_1/S$ and $G_2/M$ regulators. Cells were treated with 20 μM BBR3610 or BBR3610-DACH for 6, 24, or 48 hr. Protein lysates were prepared and 30 μg protein was resolved on SDS-PAGE and detected by probing the immunoblot with antibodies against (B) cyclin A and cyclin B1. β-actin was used as a loading control. Blots are representative of three independent experiments.
BBR3610-DACH induces an early PARP cleavage independent of caspase-3 and caspase-7 activation. Following prolonged arrest, damaged cells either recover or eventually undergo programmed cell death or apoptosis. During this latter event, activated cysteine aspartate proteases (caspases) primarily caspase-3 and caspase-7 cleave downstream substrates such as the DNA repair protein, PARP, while activated caspase-6 mediates cleavage of nuclear lamins. Treatment with BBR3610-DACH elicited a significant increase in PARP cleavage (~89 kDa) as early as 6 hr (Fig 2-13). This was accompanied by a concomitant cleavage of both caspase-8 (45 kDa) which is a marker for the extrinsic pathway of apoptosis and caspase-9 (37 kDa and 35 kDa). However, no caspase-3 or caspase-7 cleavage was observed, suggesting a caspase-independent PARP cleavage. For comparison and to rule out any technical errors, cells were treated with 20 μM cisplatin as a positive control. A robust caspase-3/7 cleavage can be seen between 24-48 hr of cDDP treatment (Fig 2-11B). There was no internucleosomal DNA fragmentation in BBR3610-DACH treated cells, as analyzed by agarose gel electrophoresis (Data not shown). Together, our results suggest that BBR3610-DACH induces a non-classical apoptotic pathway.
### Figure 2-13. BBR3610-DACH induces an early apoptotic response.

Time course analysis of caspase-8, caspase-9, full length and cleaved caspase-3, full length and cleaved caspase-7, and cleaved PARP in HCT116 cells treated with 20 µM BBR3610 or 20 µM BBR3610-DACH for 6, 24, or 48 hr. β-actin was used as a loading control. Arrow indicates the 37 kDa fragment of cleaved caspase-9. Blots are representative of three independent experiments.
Figure 2-11. (B) Time course analysis of cleaved caspase-3 and cleaved caspase-7 in HCT116 cells treated with 20 µM cisplatin for 6, 24, and 48 hr. β-actin was used as a loading control. Representative immunoblots are shown from three independent experiments. Asterisks indicate cleaved fragments of caspase-3 (17 and 19 kDa) and caspase-7 (20 kDa) respectively. Blots are representative of three independent experiments.
BBR3610-DACH-induced $G_1$/S and $G_2$/M arrest is independent of the DNA mismatch repair system. DNA mismatch repair is a post-replication DNA repair mechanism which removes the mismatch from newly synthesized daughter strands$^{25,26}$. Defects in mismatch repair are one of the hallmarks of resistance to platinum drugs especially cisplatin. These defects allow resistant tumor cells to tolerate DNA damage and complete DNA replication instead of undergoing cell cycle arrest and apoptosis. Earlier reports have shown cells defective in the mismatch repair system to be 1.5-2 fold resistant to cisplatin$^{27}$. However, they do not show any resistance to oxaliplatin, another mononuclear platinum compounds containing the DACH ligand. This is attributed to the fact that the mismatch repair system recognizes cisplatin diadducts more efficiently than the Pt-DACH diadducts$^{28}$. In this context, we investigated whether the disparate cell cycle effects elicited by BBR3610 and BBR3610-DACH were dependent on the mismatch repair status of the cells. We used HCT116+chr3 cells wherein the absence of the mismatch repair $hMLH1$ gene is complemented by the transfer of an additional copy of chromosome 3, which carries the MLH1 gene$^{29}$. Treatment of HCT116+chr3 cells with BBR3610 or BBR3610-DACH resulted in cell cycle profiles similar to those of parental HCT116 cells (Fig 2-14). Treatment with cisplatin elicited a classical $G_2$/M arrest 24-48 hr after drug treatment (Data not shown) that was similar for the two cell lines. This shows that similar to cisplatin induced adducts, the DACH adducts are not recognized by the mismatch repair machinery. Further experiments would be aimed toward deciphering whether another mismatch repair gene, $hMSH2$ can recognize these adducts and elicit a repair response.
Figure 2-14. The cell cycle response elicited by BBR3610 or BBR3610-DACH is independent of the DNA mismatch repair. Wild-type HCT116 and hMLH1 complemented cells, HCT116+chr3, were treated with 20 µM BBR3610 or BBR3610-DACH for 6, 24, and 48 hr. They were then analyzed for cell cycle distribution using propidium iodide.
Table 2-2. Antibodies Used in this study

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<th>Antibody</th>
<th>Description</th>
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DISCUSSION:

Platinum-based drugs constitute one of the major classes in the armamentarium of cancer therapies. However, their use is often compromised by inherited or acquired drug resistance in cells. Presence of thiol-containing compounds such as glutathione and metallothionein is one of the major factors contributing toward platinum drug inactivation\textsuperscript{3, 4}. The dinuclear platinum compound, BBR3610-DACH, used in this study, was previously shown to be resistant to metabolic decomposition by glutathione. Subsequent \textit{in vitro} studies showed the DNA adducts formed by BBR3610-DACH persisted longer, escaped DNA repair, and inhibited RNA pol II transcription much more efficiently than cisplatin adducts\textsuperscript{11}. Taking these aspects into consideration, the primary aim of this paper was to decipher the cellular effects elicited by BBR3610-DACH in colorectal cancer cells.

Most of the polynuclear platinum compounds exhibit their anti-proliferative effect \textit{via} a classical G\textsubscript{2}/M cell cycle arrest mechanism. In the present study, BBR3610 induced a G\textsubscript{2}/M arrest between 24-48 hr of treatment in HCT116 cells (\textbf{Figure 2-5A}). An identical profile was also seen in glioma cells wherein BBR3610 induces an early G\textsubscript{2}/M arrest and autophagy subsequently culminating in apoptosis\textsuperscript{30}. However, BBR3610-DACH showed an interesting deviation from the expected results wherein a biphasic G\textsubscript{1}/S and G\textsubscript{2}/M cell cycle arrest was seen with a robust decrease in S phase (\textbf{Figure 2-5A-C}) after 24 hr drug treatment.
DNA damage activates the tumor suppressor protein p53 which plays a crucial role in eliciting a potent cell cycle arrest in both G\textsubscript{1} and G\textsubscript{2} phases of the cell cycle\textsuperscript{31}. Platinum compounds target and damage DNA which leads to activation and stabilization of p53. As expected, treatment with both BBR3610 and BBR3610-DACH showed an increase in p53 levels, with BBR3610-DACH showing more robust p53 stabilization after 24 hr, accompanied by increased levels of its transcriptional target, p21. The role of p21 in conferring the aforementioned cell cycle effect was confirmed in experiments with isogenic HCT116 p21\textsuperscript{-/-} cells wherein the drug treatment failed to arrest the normal G\textsubscript{1} to S phase transition. However, the cells arrested in G\textsubscript{2} phase, indicating that p21 is not required for the drug mediated G\textsubscript{2} arrest. Interestingly, similar set of experiments with HCT116 p53\textsuperscript{-/-} cells indicated a delayed S phase progression culminating in a late G\textsubscript{2} arrest. Binding of p21 to the proliferating cell nuclear antigen (PCNA) in p53\textsuperscript{-/-} cells causes both G\textsubscript{1} and G\textsubscript{2} arrest\textsuperscript{32}. Interestingly, in HCT116 p53\textsuperscript{-/-} cells, we see increased expression of p21 following treatment with both BBR3610 and BBR3610-DACH (data not shown), possibly pointing to a p53-independent mechanism for induction of p21 in eliciting bimodal cell cycle arrest after treatment with platinum drugs.

One of the key features that distinguish polynuclear platinum complexes (PPC) from its mononuclear counterparts is the formation of long range DNA intrastrand and interstrand crosslinks. It is known that although some of the PPC-induced crosslinks cause conformational changes in DNA, they are not recognized by many of the DNA binding proteins and repair proteins that bind to adducts formed by cisplatin or its congeners. Earlier work using plasmid DNA has shown that both BBR3610 and BBR3610-DACH form a high proportion of interstrand
crosslinks (23% and 26% of total adducts respectively). Interstrand crosslinks are considered to be highly toxic, since like DNA double strand breaks, they affect both strands of DNA, and can irreversibly block major DNA transactions like replication, transcription, and recombination. Similar to the in vitro studies, a significant amount of interstrand crosslinks were induced in cells, as detected and analyzed by the comet assay (Figure 2-2). Thus, while it is likely that the intracellular effects observed after treatment with these compounds are a direct consequence of the crosslinks formed, there are no obvious qualitative or quantitative differences in adducts formed that would explain the dramatic differences in cellular effects of BBR3610 and BBR3610-DACH.

Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3-related (ATR) are members of the PI3K-like family of serine/threonine protein kinases playing critical roles as DNA damage sensors and contributing toward cell cycle arrest, DNA repair, and apoptosis induction. The role of ATR in sensing stalled replication forks owing to the presence of DNA interstrand crosslinks caused by platinum drugs has been demonstrated earlier. Initial experiments with caffeine (pharmacological inhibitor of both ATM/ATR), the ATM inhibitor KU-60019, and ATR knockdown using siRNA showed that BBR3610-induced cell cycle arrest was ATM-independent but ATR-dependent (Figure 2-10A-E). ATM activation occurs in cells exposed to ionizing radiation like X-rays leading to the formation of DNA double strand breaks, activating ATM which then phosphorylates serine 15 on p53 and stabilizes p53 levels. In the case of BBR3610, the crosslinks formed by the compound could be leading to DNA double strand breaks due to a potential transcriptional arrest, which is then detected by ATM. Also,
following BBR3610 treatment, the observed S phase accumulation at 24 hr could be possibly due to replication fork arrest which is subsequently recognized by ATR which then activates P-Chk1 (Figure 2-10E). However, after 24 hr treatment, BBR3610-DACH induced a p53-dependent but ATM- or ATR-independent G₁ and G₂ arrest, but the exact mechanism behind the dual phase arrest is not known. Although increase in P-Chk2 levels (data not shown) is seen after BBR3610-DACH treatment, the absence of S phase accumulation (Figure 2-5C) and ATR activation (Figure 2-10E) excludes the possibility of known mechanisms of adduct recognition and suggests a novel mechanism which detects DNA adducts directly and causes the recruitment of DNA damage checkpoints and cell cycle arrest proteins. In this context, it has been reported earlier that adducts formed by the mononuclear platinum compound, oxaliplatin containing a DACH ligand, is not recognized by ATR which points out to the fact that probably DACH adducts are not recognized by the conventional DNA damage checkpoints.

The normal progression through the different phases of the cell cycle depends on the pivotal interaction of cyclin dependent kinases (CDKs) with the cyclins. In our study, the steady state levels of CDK2 and Cdc25A remain unchanged while the level of cyclin D1 increases at 48 hr of BBR3610-DACH treatment (Figure 2-12A), in comparison to BBR3610. A possible explanation for the increased cyclin D1 levels could be due to the cells exiting the G₀ phase and getting arrested in G₁. Interestingly, an increase in cyclin E levels is observed which could be a result of a small subset of cells escaping the G₁ arrest and entering into S phase. Earlier studies with fibroblasts have shown an increase in the levels of cyclin D1 in cells undergoing replicative
senescence\textsuperscript{38}. It remains to be determined whether treatment with BBR3610-DACH could be triggering the cancer cells to undergo a state of drug induced cellular senescence.

The G\textsubscript{2}-M transition is primarily governed by the activities of cyclin A and cyclin B. Following treatment with BBR3610-DACH, a robust decrease in levels of both cyclins (Figure 2-12B) is observed, indicative of a possible mitotic catastrophe. Treatment of HepG2 cells with 5-fluorouracil or methotrexate reduced cyclin B2 mRNA levels accompanied with increased p53 levels\textsuperscript{22}. Likewise, DLD-1 colorectal cancer cells harboring a p53 mutation showed decreased cyclin B1 and B2 mRNA levels after expression of a wild-type p53\textsuperscript{22}. On a similar basis, stabilization of p53 following BBR3610-DACH treatment could be interfering with the transcription of cyclin B or cyclin A\textsuperscript{21} since the latter consists of a p53 binding site in the 5’ untranslated region.

Earlier reports have shown that p53 could be activated independent of DNA damage especially during metabolic stress. A p53-dependent G\textsubscript{1} arrest is observed following depletion of nucleotide pools caused by antimetabolites\textsuperscript{39}. Experiments with synchronized cells show the G\textsubscript{1} population to be more sensitive toward BBR3610-DACH treatment (Figure 2-8). In this context, it would be interesting to determine whether BBR3610-DACH could be acting as nucleotide biosynthesis inhibitor, wherein p53 function helps maintain genomic stability by preventing the G\textsubscript{1} to S phase transition, in response to metabolic stress. Also, a cell cycle profile similar to that of BBR3610-DACH has been reported in breast cancer cells treated with microtubule depolymerizing agents.
or in replicatively senescent cells, emphasizing the fact that BBR3610-DACH might be targeting cellular components other than DNA\textsuperscript{40}.

Cells arrested in different phases of the cell cycle for prolonged periods of time eventually undergo cell death. In our study, BBR3610-DACH causes both caspase-8 and caspase-9 activation, similar to the parental compound BBR3610 or cisplatin (data not shown). Also, a caspase independent PARP-1 cleavage (\textbf{Figure 2-13}) is observed which indicates the possible involvement of cathepsins or TGF-\(\beta\) activation\textsuperscript{41}. Since this effect is mechanistically different from other platinum based compounds, it would be interesting to decipher whether this activity is specific to compounds harboring a polyamine linker like spermine (BBR3610, BBR3610-DACH) or spermidine (BBR3571). In this paper, we have not looked at the mitochondrial disruption (loss of \(\Delta\Psi_m\), permeability transition, cytochrome C release) following drug treatment and which would be likely aspects to be considered for future studies.

In summary, BBR3610-DACH induces DNA interstrand crosslinks, eventually leading to a dual mode of cell cycle arrest, and apoptotic induction. Since this compound exhibits significant anti-proliferative activity and also has been shown previously to be metabolically stable to nucleophilic attack, it would be an ideal candidate to be developed further and tested for clinical trials. Also, it shows a promising paradigm shift for the structure-activity relationship of novel platinum based compounds, wherein a ligand modification in the original compound, BBR3610 yields a structurally similar compound with a different pharmacokinetic and pharmacological profile.
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37. Lewis KA, Lilly KK, Reynolds EA, Sullivan WP, Kaufmann SH, Cliby WA. Ataxia telangiectasia and rad3-related kinase contributes to cell cycle arrest and survival after cisplatin but not oxaliplatin. Mol Cancer Ther 2009; 8:855-63.


CHAPTER 3

The platinum-intercalator conjugate, Trans-4-NBD, exhibits a mechanistic behavior in cells similar to cisplatin: Cytotoxicity, cell cycle arrest, DNA damage, and apoptosis

ABSTRACT:

Cisplatin has been one of the primary drugs utilized in the treatment of a variety of malignancies like ovarian, colorectal, testicular, head, neck, and bladder cancers etc. However, recently, a number of transplatinum compounds have been shown to be clinically active. The current work explores the activity of a novel trans-NBD compound of structure trans-[PtCl₂ (N-NBD) X (NH₃)] where X is butane-1,4-diamine, on the growth and viability of HCT116 human colorectal carcinoma and A2780 human ovarian carcinoma cells, in addition to its possible mechanisms of cytotoxicity. Comet analysis indicated that trans-4-NBD formed interstrand crosslinks at equimolar concentrations as cisplatin in HCT116 cells. Cell cycle analysis in HCT116 shows a significant G₂ arrest at 24 and 48 hr of drug treatment whereas in A2780 cells, following 9 μM (IC₉₀) drug treatment after 24 hr, there is a small elevation in the number of cells in S-phase which eventually recedes after 48 hr. We observed a marked increase in p53 protein levels in both HCT116 and A2780 cells followed by an increase in p21 protein level. Both intrinsic as well as extrinsic pathways of apoptosis are induced via activation of caspase-8, caspase-9, and
caspase-3 and concomitant PARP cleavage. Also, a time-dependent activation of \( \gamma \)-H2AX is seen which might be indicative of DNA damage response triggered following the formation of double strand DNA breaks. In summary, trans-4-NBD is an active derivative of the therapeutically inactive transplatin, showing cellular mechanisms similar to cisplatin.
INTRODUCTION:

Platinum-based anti-cancer agents like cisplatin, carboplatin, and oxaliplatin have been highly effective chemotherapeutics against a wide range of malignancies like testicular, ovarian, breast, colorectal, head and neck, bladder cancers etc\textsuperscript{1-4}. The cytotoxic activity of these platinum compounds is attributed to their ability to form toxic bifunctional 1,2-intrastrand crosslinks between the N7 atoms of guanine bases on DNA, inhibiting important DNA transactions like replication, transcription, and recombination\textsuperscript{5-8}. However, transplatin, the trans isomer of cisplatin, is therapeutically inactive, since the trans geometry prevents the formation of 1, 2-intrastrand crosslinks\textsuperscript{9-11}. Eventually, the focus was shifted toward activating the trans geometry\textsuperscript{12}, \textsuperscript{13} to develop compounds with cytotoxicity greater than transplatin and similar to or greater than cisplatin. These compounds were synthesized by replacing the NH\textsubscript{3} ligand of transplatin with planar heterocyclic ligands like pyridine, thiazole, quinoline, etc\textsuperscript{14, 15}. Additionally, these complexes were found to be effective in cisplatin and oxaliplatin-resistant cell lines \textsuperscript{16}, suggesting a mechanistic difference in activity as compared to the clinically approved platinum compounds.

Platinum compounds exhibiting a dual mode of DNA binding through the use of DNA intercalators have been explored earlier\textsuperscript{17}. These compounds interact with DNA through conventional crosslinking and intercalation. Some of these compounds have been designed earlier that utilized aromatic intercalators like anthraquinone\textsuperscript{18} and other cytotoxic moieties.
Also, some of the intercalators employed are fluorophores that allow the visualization of intracellular distribution of platinum drugs and their subsequent trafficking. One such fluorophore, NBD (4-Chloro-7-nitrobenzofurazan), was recently used in the synthesis of a fluorescent derivative of the polynuclear platinum compound, TriplatinNC.\textsuperscript{19} Previously, cisplatin derivatives containing the FITC or Alexa tags have been developed which unfortunately exhibited biological properties of the tags utilized rather than the platinum compound itself\textsuperscript{20, 21}. The advantage of NBD is that it avoids difficult chemical modifications and involves direct addition to the primary amine on the platinum compounds, giving analogs mimicking the parental compound. Also, NBD by itself is inactive and exhibits fluorescent properties only when it comes in contact with aliphatic amines or thiol compounds.

In this paper, we have reported the cytotoxic activity and cellular mechanism of a novel transplatinum compound, trans-4-NBD (\textbf{Fig 3-1}). Interestingly, treatment of HCT116 and A2780 cells with trans-4-NBD showed similar cytotoxicity and biological properties to that of cisplatin, suggesting a paradigm shift in the structure-activity relationship of platinum compounds.
Figure 3-1. Structures of: (A) Cisplatin; (B) Transplatin; (C) Trans-NBD
MATERIALS AND METHODS:

Chemicals:

Synthesis of the N-(7-nitro-2,1,3-benzoxadiazol-4-yl) butane-1,4-diamine 4-NBD Ligand. BOC–diaminoethane/BOC–diaminobutane/BOC–diaminohexane was dissolved in ethanol containing a 10% w/w of Na₂CO₃, and NBD-Cl was added. The mixture was stirred at room temperature overnight, then water was added and the product was extracted with diethyl ether. The organic phase was washed with brine, dried over Na₂SO₄ and purified by flash chromatography (CHCl₃: EtOAc, 75:25). The BOC protecting group of the purified intermediate was removed by using trifluoroacetic acid (2 ml) at room temperature. After the mixture had been stirred overnight, excess TFA was evaporated, and the oily product was treated with diethyl ether to yield the trifluoroacetate salt as orange crystals. The compound was neutralized by dissolving it in a mixture H₂O/Ethanol 1:1 and addition of 1 equivalent of NaOH. The neutral ligand was then extracted with CH₂Cl₂. The organic fractions were dried over K₂CO₃ and the solvent removed under vacuum. The product was dried under vacuum overnight. The absence of TFAcetate was confirmed by ¹⁹F NMR.

Synthesis of Trans-[PtCl₂ (N-NBD) butane-1,4-diamine(NH₃)] (Trans4-NBD). Cisplatin (26 mg, 0.087 mmol) was suspended in 5 ml of deionized water and one equivalent of AgNO₃ was added. The mixture was stirred at 45°C for 4.5 hr, and then cooled to 5°C for 2 hr to allow full precipitation of AgCl. The silver white salt was double filtered through a nylon membrane.
with a porous size of 0.45 µm. To that pale yellow filtrate, the amino ligand was added (19.34 mg, 0.087 mmol). That solution was stirred at room temperature overnight, and then filtered. 1.5 mL of HCl 37% was added and the resulting solution was stirred at 75°C for 5 hr. The orange clear solution was concentrated under vacuum until a precipitate appeared (~ 3 ml) and then cooled to 5°C to complete the precipitation of the final product. The orange powder was filtered, washed with ice-cold water, ice-cold ethanol and ether, and then dried under vacuum for 24 hours. $^1$H NMR D$_2$O δ (ppm): 8.36(d, 1H); 6.23 (d, 1H); 3.49 (m, 2H); 2.91 (m, 2H); 1.69 (m, 4H). $^{195}$Pt NMR D$_2$O δ (ppm): 2158(s). EA calculated for C$_{10}$ H$_{16}$ N$_6$ O$_3$ Pt Cl$_2$: C, 22.53; H, 3.03; N, 15.75. Found: C, 22.16; H, 3.10; N, 15.65.

Cisplatin was obtained from Sigma, Cat# 479306. BBR3610 and BBR3610-DACH were synthesized as discussed earlier. The stock solutions of platinum compounds were prepared at the concentration of 1 mM in water and stored at -20°C.

**Cell Culture and Drug treatments.** The human colorectal cancer cell line, HCT116 and isogenic HCT116 p53-/-, and the ovarian carcinoma cell line, A2780 were cultured in RPMI 1640 (Invitrogen, Cat# 11875-093) supplemented with 10% fetal bovine serum (Quality Biologicals, Cat# 110-001-101US) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Cat# 15140). Cells were maintained in logarithmic growth as a monolayer in T75 tissue culture flask at 37°C in a 5% CO$_2$ incubator. For the drug treatment, the molar drug:cell ratio was kept constant by maintaining a seeding density of 5X10$^5$ cells/10 mL medium.
**Growth Inhibition Assay.** 5000 cells/well in 100 µL were seeded in a 96-well plate and allowed 24 hr to attach. Cells were then treated with the drugs for 72 hr. Following drug removal, the cells were treated with 0.5 mg/mL MTT reagent [(3, 4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Sigma, Cat# M2128] in RPMI medium for 3 hrs at 37°C. The MTT reagent was removed and 100 µL DMSO was added to each well. The plate was then incubated on a shaker at room temperature in the dark. The spectrophotometric reading was taken at 570 nm using a microplate reader.

**Analysis of cell cycle distribution.** HCT116 and A2780 cells were seeded in 100 mm tissue culture dishes at a density of 5X10^5 cells per dish. After 24 hr, cells were treated for 6, 24, and 48 hr with an equimolar dose of the drugs (20 µM) for HCT116 cells or (9 µM) for A2780 respectively. Both attached and floating cells were harvested at the different timepoints. One million cells were then suspended in 1 mL of propidium iodide solution (3.8 mM sodium citrate; 0.05 mg/mL propidium iodide; 0.1% Triton X-100) with added RNaseB at a final concentration of 7 Kunitz/mL and kept in the dark at 4°C. Cells were then analyzed using flow cytometry.

**Flow Cytometry.** A Becton Dickinson (San Jose, CA) FACSCanto II flow cytometer was used for these studies. The argon ion laser set at 488 nm was used as an excitation source. Cells having DNA content between 2N and 4N were designated as being in G1, S, and G2 phases respectively. Twenty thousand events were acquired for each sample and the data obtained were analyzed using the Modfit software.
**Immunoblotting.** Primary antibodies used were p53 (Cell Signaling, Cat # 9282), p21 (Cell Signaling, Clone 12D1, Cat # 2947), caspase-8 (Cell Signaling, Clone 1C12, Cat # 9746), caspase-9 (Cell Signaling, Cat # 9502), Caspase-3 (Cell Signaling, Cat # 9662), Cleaved PARP (Cell Signaling, Cat # 9541), and β-actin (Cell Signaling, Clone 13E5, Cat # 4970). Following drug treatments, both floating and adherent cells were harvested, washed once with ice-cold PBS and pelleted (10,000 rpm, 5 min, 4°C). The pellets were then lysed in 50-200 μL SDS lysis buffer [62.5 mM Tris-HCl, pH 7.5, 5% glycerol, 4% SDS, 4% complete protease inhibitor (Roche, Cat# 11873580001), 5% BME], passed through 21 gauge needle 10-15 times on ice, and centrifuged (12,000 rpm, 20 min, 4°C). Protein concentrations were determined by the Bradford Assay and proteins were resolved on 10%, 12%, or 4-20% gradient polyacrylamide gels, and transferred to PVDF membrane (90 V, 75 min, 4°C). The membrane was blocked in 5% non-fat dry milk in 1X Tris buffered saline containing 0.1% Tween-20 for 60-90 min. The membranes were then probed with the primary antibodies in blocking buffer overnight at 4°C, followed by secondary antibody (anti-rabbit or anti-mouse) conjugated to horseradish peroxidase (Cell Signaling, Thermo Scientific) for 1 hr at room temperature. Chemiluminescent protein bands were visualized on X-ray films.

**Comet Assay.** Single-cell gel electrophoresis or comet assay was carried out using a Trevigen kit to detect and evaluate the DNA damage. Briefly, HCT116 cells (1X10^5 cells/mL) were plated in 35 mm or 60 mm dishes and allowed to attach for 24 hr. The cells were then treated with different concentrations of each drug for 1 hr. The medium was removed, the cells washed once, and further incubated in fresh medium for 9 hr. Cells were then harvested by scraping and
resuspended in 1 mL of ice-cold phosphate buffered saline (PBS, pH 7.4). Then, the cell suspension was mixed with low melting agarose at 37°C in 1:5 ratio (e.g. 50 µL of cell suspension with 500 µL agarose), and 50 µL of this mixture was spread on comet slides, and solidified in the dark for 30 min at 4°C. The slides were then treated with ice-cold lysis buffer in the dark for 1 hr at 4°C and then incubated in an alkaline solution (0.6 g NaOH, 250 µL 2 mM EDTA, pH>13) for 1 hr at room temperature to allow for alkaline unwinding. Electrophoresis was carried out under alkaline conditions (12 g NaOH, 2 mL 500 mM EDTA, pH 8.0) at 21 V, 300 mA, and 30 min at 4°C. Slides were washed twice with distilled water, once with 70% ethanol, and then allowed to dry for 10 min at around 37-45°C. The slides were then stained with SYBR green for 5 min at 4°C. Comet images were obtained using a fluorescence microscope (Olympus IX70).

Immunofluorescence. 2.5X10^4 HCT116 or A2780 cells were seeded on tissue culture treated 8-well chamber slides (Lab Tek II) in 0.5 mL medium. Cells were then treated with 20 µM cisplatin or trans-4-NBD for the indicated times. After removing the medium, cells were washed with PBS and then fixed by incubating with 0.5 mL 3 % paraformaldehyde for 15 min at RT. The cells were then washed with PBS and permeabilized with 0.5 % Triton-X/PBS for 10 min at RT. The slides were then washed two times with 0.5 mL PBS/well followed by blocking in 1X PBS/Casein buffer (Bio-Rad, Cat# 161-0783) for 2.5-3 hr. The primary antibody was diluted appropriately in the blocking buffer, 0.5 mL was added to each well and allowed to incubate overnight at 4°C on a rocker. Primary antibody used for immunostaining was anti-γ-H2AX (Millipore) at 1:500 dilution. The primary antibody was then removed and the slides washed
three times with PBS for 15 min each. The secondary antibody was diluted appropriately in the blocking buffer, 0.5 mL added to each well and allowed to incubate for 3 hr at RT. The secondary antibody used was Alexa Fluor 488 goat anti-mouse. The secondary antibody was removed and the slides washed three times with PBS for 15 min each. The antibodies were then fixed by treating each well with 0.5 mL 3 % paraformaldehyde for 15 min at RT, which was then removed by 2 X 0.5 mL PBS washes. The chambers were removed with the tool provided with the kit and the slides were then allowed to air dry (using the blower inside the tissue culture hood). The slides were then mounted with Vectashield mounting medium containing DAPI (4’, 6-diamidino-2-phenylindole). A coverslip was placed over all the wells and sealed with clear nail polish. Images were captured using Olympus fluoview 500 confocal microscope, using a 430 nm diode laser with a 605 nm band pass filter (DAPI) and a 510 nm laser with a 530 nm band pass filter (Alexa fluor 488).
RESULTS:

**Trans-4-NBD induces interstrand crosslinks.** Mononuclear platinum compounds like cisplatin form DNA interstrand crosslinks that constitutes 1% of the total DNA adducts formed. Cellular uptake and DNA binding studies showed that trans-4-NBD is taken up by the cells and binds to DNA to the same extent as cisplatin in HCT116 cells (Martinez et al., unpublished data). In this context, we investigated whether trans-4-NBD induces interstrand crosslinks on the DNA by utilizing the alkaline comet assay. Three concentrations of trans-4-NBD were chosen (20, 50, and 100 µM). Typical comet images are shown in **Fig 3-2.** In the control, unirradiated, untreated HCT116 cells, no DNA damage was detected (**Fig 3-2A**). Following irradiation with 15 Gy to introduce a fixed level of DNA strand breaks, the shorter DNA fragments migrated from the bulk of DNA during alkaline electrophoresis to produce comet tails (**Fig 3-2B**). When the irradiated cells were treated with trans-4-NBD, a concentration dependent decrease in the comet tail length and intensity was observed, in comparison to irradiated controls (**Fig 3-2C,D,E**). Also, the comet heads were larger and had higher intensity than the non-drug treated irradiated control possibly because of the retention of more DNA in the head due to the drug induced interstrand crosslinks.
Figure 3-2. Comet images from HCT116 cells treated with cisplatin or trans-4-NBD. Following irradiation (15 Gy) of untreated cells, distinct comets were observed in comparison to the untreated, unirradiated cells. Cells were then treated with 20, 50, or 100 μM cisplatin or trans-4-NBD for 1 hr followed by a 9 hr drug free post-incubation for interstrand crosslink formation. After irradiation of treated samples, comet tails with decreased length and intensity could be observed due to the presence of the compound induced interstrand crosslinks. All samples were stained with SYBR green and observed under a fluorescence microscope.
Trans-4-NBD exhibits a significant anti-proliferative effect. Trans-4-NBD was evaluated in HCT116 and A2780 cells. These cells were exposed to the platinum complex (0.78 – 50 µM) and the cytotoxicity was assessed using the MTT assay. Remarkably, both cell lines showed sensitivity to trans-4-NBD in the same range as cisplatin with IC$_{50}$ values of 7.6 µM and 2.3 µM in HCT116 and A2780 cells respectively (Fig 3-3, Table 3-1).
Figure 3-3. Cytotoxicity curves in (A) HCT116; (B) A2780 cells treated with 0.78 – 50 µM cisplatin or trans-4-NBD for 72 hr, determined by MTT assay. Error bars indicate means ± SD from three independent experiments carried out in triplicates.
Table 3-1. Cytotoxicity of cisplatin and trans-4-NBD in HCT116 colorectal and A2780 ovarian cancer cell lines

<table>
<thead>
<tr>
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<th>IC$_{50}$ (μM)</th>
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<tr>
<td></td>
<td>HCT116</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>7.6 ± 1.7</td>
</tr>
<tr>
<td>Trans-4-NBD</td>
<td>7.6 ± 0.6</td>
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*IC50 values are means ± SD from 3 separate experiments, each conducted in triplicate*
Trans-4-NBD induces G₂ arrest in HCT116 cells and a transient S phase accumulation in A2780 cells. Many DNA damaging drugs lead to stabilization of p53 protein levels which in turn induces the transcription of p21, leading to G₁/S arrest or apoptosis, depending on whether the damage is repaired. The platinum drugs like cisplatin have been shown to activate the pathway leading to a G₂ arrest. Accordingly, treatment of HCT116 cells with 20 µM cisplatin induced a transient S phase accumulation at 24 hr and finally a G₂ arrest at 48 hr (Fig 3-4A). Treatment of A2780 ovarian carcinoma cells with cisplatin has been shown earlier to cause S phase accumulation following 24 hr drug treatment. In our studies too, we see a similar pattern of cell cycle arrest following treatment with cisplatin. HCT116 or A2780 cells treated with trans-4-NBD showed an identical cell cycle profile as cisplatin (Fig 3-4A, B). Concurrently, a time dependent stabilization of p53 protein level is seen accompanied by an increase in p21 expression levels (Figure 3-5A, B), which indicates the classical pathway being triggered for the G₂/M arrest. This emphasizes the fact that both drugs might be activating the same set of cell cycle checkpoints in different tumors.
Figure 3-4. Trans-4-NBD induces $G_2$ arrest in HCT116 cells and a transient S phase accumulation in A2780 cells. (A) HCT116 cells treated with 20 µM cDDP or 20 µM trans-4-NBD and analyzed for cell cycle distribution using propidium iodide. (B) A2780 cells treated with 20 µM cDDP or 20 µM trans-4-NBD and analyzed for cell cycle distribution using propidium iodide.
Figure 3-5. Trans-4-NBD induces stabilization of p53 with concomitant upregulation of p21 in HCT116 and A2780 cells. (A) HCT116 cells were treated with 20 µM trans-4-NBD for 18, 30, and 50 hr. Representative immunoblots are shown from three independent experiments. (B) A2780 cells were treated with 20 µM trans-4-NBD for 18, 30, and 50 hr. Representative immunoblots are shown from three independent experiments.
Trans-4-NBD induces both the extrinsic and intrinsic apoptotic pathways. Cytotoxic transplatinum compounds have been shown earlier to induce apoptosis in different cell types. One of the hallmarks of apoptosis is the activation of the cysteine serine proteases called caspases. The extrinsic pathway triggers the activation of caspase-8 and the intrinsic or mitochondrial dependent pathway activates caspase-9. Both pathways converge on the activation of caspase-3 which then cleaves cellular substrates downstream. Treatment of HCT116 with 20 µM trans-4-NBD promoted cleavage of caspase-9 (Fig 3-6A) that probably indicates extensive DNA damage triggering the intrinsic pathway. This was also accompanied by caspase-8 cleavage indicating the activation of the death receptor-dependent apoptotic pathway. However, apoptotic induction was found to be slightly lower compared to cells treated with 20 µM cisplatin for 55 hr (Fig 3-6A). The induction of apoptosis was further proved by the activation of caspase-3 (Fig 3-6A) and the subsequent cleavage of its downstream substrate, PARP (Fig 3-6C) which is a DNA repair protein. Treatment of A2780 cells with trans-4-NBD showed a similar pattern of apoptotic induction (data not shown). Also, similar experiments were carried out in the isogenic p53-null HCT116 cells to check whether the apoptotic induction was dependent on the p53 status of the cell. Interestingly, treatment of HCT116 p53-/- cells with 20 µM trans-4-NBD (Fig 3-6B) showed identical apoptotic induction as the wild-type cells. This shows that trans-4-NBD functions in a p53-independent manner and could prove to be a useful drug candidate against cancer cells harboring mutant p53.
Figure 3-6. Trans-4-NBD induces both the extrinsic and intrinsic apoptotic pathways. (A) Time course analysis of caspase-8, caspase-9, and caspase-3 activation in HCT116 p53+/+ cells treated with 20 µM trans-4-NBD for indicated timepoints. Treatment with 20 µM cisplatin for 50 hr is used for comparison. β-actin was used as a loading control.
Figure 3-6. Trans-4-NBD induces both the extrinsic and intrinsic apoptotic pathways. (B) Time course analysis of caspase-8, caspase-9, and caspase-3 activation in HCT116 p53-/cells treated with 20 µM trans-4-NBD for indicated timepoints. Treatment with 20 µM cisplatin for 50 hr is used for comparison. β-actin was used as a loading control.
Figure 3-6. **Trans-4-NBD induces both the extrinsic and intrinsic apoptotic pathways.**
(C) Time course analysis of PARP cleavage in HCT116 p53+/+ and HCT116 p53-/- cells treated with 20 µM trans-4-NBD for indicated timepoints. Representative immunoblots are shown from two or three independent experiments.
Trans-4-NBD triggers the formation of DNA double strand breaks. As seen earlier, trans-4-NBD treatment caused the formation of interstrand crosslinks followed by a robust stabilization of p53. To check whether there are other forms of DNA damage, we checked for the formation of γ-H2AX foci, which is DNA damage repair protein recruited to sites harboring DNA double strand breaks. HCT116 (Fig 3-7A) or A2780 (Fig 3-7B) cells treated with trans-4-NBD showed a time-dependent induction of γ-H2AX foci, reaching maximum levels at 16-24 hr after drug treatment. This shows that trans-4-NBD induces a variety of DNA damage responses mainly due to the formation of interstrand crosslinks and DNA double strand breaks.
Figure 3-7. Trans-4-NBD triggers the formation of DNA double strand breaks. (A) Fluorescence microscopy of HCT116 cells treated with 20 µM cDDP or 20 µM trans-4-NBD, showing an increase in γ-H2AX foci. (B) Fluorescence microscopy of A2780 cells treated with 20 µM cDDP or 20 µM trans-4-NBD, showing an increase in γ-H2AX foci.
DISCUSSION:

The replacement of the NH$_3$ ligand in transplatin with planar amines and intercalating fluorophores has led to the development of transplatinum compounds with enhanced biological activity. Earlier studies have shown that the transplanaramine complexes have similar cytotoxicity as cisplatin and also retain their activity in cisplatin-resistant tumors$^{22}$. Although trans-4-NBD shows similar cytotoxicity as cisplatin in both HCT116 and A2780 cells, it remains to be seen whether the trans-4-NBD discussed in this paper is effective in cisplatin or oxaliplatin-resistant tumors. Also, one of the factors contributing toward the cytotoxicity of platinum compounds is cellular accumulation. Cellular uptake studies (Martinez et al., unpublished data) showed that trans-4-NBD accumulated in HCT116 or A2780 cells in a similar fashion as compared to cisplatin. This was confirmed from the identical IC$_{50}$ values of both drugs in HCT116 (5 µM) and A2780 (0.5 µM). Interestingly, in earlier studies it has been reported that cellular accumulation does not necessarily correlate to cytotoxicity of transplatinum complexes$^{23}$.

DNA damage induced by cisplatin and the transplanaramine complexes cause a significant stabilization of p53 in both HCT116$^{24}$ and A2780 cells$^{25}$. Likewise, treatment of HCT116 or A2780 cells with trans-4-NBD showed an increase in p53 protein levels accompanied with increased p21 levels. Like cisplatin, it showed a S phase accumulation in HCT116 after 24 hr and a subsequent G$_2$/M arrest at 48 hr whereas in A2780 cells, it showed an initial S phase accumulation after 24 hr. Transplatinum complexes containing either a cyclohexylamine or a propylamine ligand were shown earlier to cause a complete S phase blockade$^{26}$. Therefore, it
appears that most of the transplatinum complexes have a common mechanism of disrupting cellular progression. The differential activation of various cell cycle checkpoints remains to be elucidated following treatment with trans-4-NBD, although it might be mimicking cisplatin-induced signal transduction.

The stabilization of p53 following trans-4-NBD treatment could be attributed to the DNA interstrand crosslinking ability of the compound in cells. Since the cytotoxicity and cell cycle effects elicited by trans-4-NBD are identical to cisplatin, it would be interesting to determine the nature of DNA bending and unwinding since trans-4-NBD is structurally different from cisplatin. Also, interstrand crosslinks formed by transplanaramine complexes have been shown to be poor substrates for nucleotide excision repair\textsuperscript{27}. On a similar basis, it would be exciting to see whether the trans-4-NBD-induced interstrand crosslinks are recognized by the conventional repair pathways and whether they are repaired to the same extent as cisplatin adducts.

The induction of apoptosis by transplatinum complexes has been verified in the past using propidium iodide, annexin V binding, or caspase activation assays\textsuperscript{28}. Treating HCT116 or A2780 cells with trans-4-NBD-induced both the extrinsic as well as intrinsic apoptotic pathways. This could be due to the DNA damage caused by the compound \textit{via} the formation of interstrand crosslinks and DNA double strand breaks as seen from the increase in $\gamma$-H2AX foci. A similar accumulation of $\gamma$-H2AX was seen in the transplanaramine complexes containing thiazole\textsuperscript{29}. 
Trans-4-NBD provides a distinct advantage over cisplatin and other transplatinum complexes wherein it is an excellent tool to visualize the distribution of the compound within the cells. Experiments with a NBD-tagged polynuclear platinum compound, TriplatinNC, have shown a distinct nucleolar localization of the compound, which ultimately is responsible for disruption of rRNA transcription, leading to cell cycle arrest and apoptosis. Also, mononuclear platinum complexes containing the NBD fluorophore\textsuperscript{30} were shown to trigger a non-apoptotic cell death mechanism called paraptosis which occurred through extensive cytoplasmic vacuolization. Although trans-4-NBD has been shown to induce apoptosis \textit{via} activation of caspases and PARP cleavage, cellular imaging studies will reveal distinct morphological changes induced after drug treatment and whether it resembles classical apoptotic features or whether other mechanisms of cell death are additionally involved.
REFERENCES:


CHAPTER 4

TriplatinNC selectively targets the nucleolus and disrupts rRNA transcription, which leads to G₁ phase cell cycle arrest and p53-independent apoptosis

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(Paper in submission to Molecular Pharmaceutics)

ABSTRACT:

Cationic peptides, such as polyarginine, penetrate cellular membranes and localize specifically to the nucleolar region of cells. TriplatinNC is a highly charged (+8), noncovalent derivative of the phase II clinical platinum drug, BBR3464. TriplatinNC exhibits a distinct mode of high affinity DNA interaction, increased cellular accumulation, and a significant cytotoxic profile in mastocytoma, ovarian carcinoma, and cisplatin-resistant ovarian carcinoma cell lines. Here, it is shown, through use of fluorescent-tagging and confocal colocalization experiments that TriplatinNC rapidly localizes to the nucleolar compartment in HCT116 and A2780 cells. Due to the cationic nature of TriplatinNC, it was considered likely to interact with negatively charged nucleic acid components of the nucleolus, that is, ribosomal DNA/RNA. ³²P-metabolic labeling of HCT116 cells determined that the production rate of 47S rRNA precursor transcripts is drastically reduced as an early event after drug treatment, followed by a robust G₁ cell cycle arrest. Morphological characteristics of the apoptotic process are apparent within 24 hr,
including: reduction in cell size, membrane blebbing, and cytosolic vacuolization. TriplatinNC induces cleavage of mitochondrial-dependent and -independent initiators of apoptosis, procaspase-9 and -8, followed by activation of the effector procaspase-3, and downstream target, PARP-1. Furthermore, utilizing the isogenic HCT116 p53+/+ and p53-/- cell lines, it was determined that these processes, are not dependent upon the presence of p53 protein. The results presented here support a basis for selective targeting of rRNA processes as a strategy for p53-independent induction of apoptosis in cancer cells and pave the way for an entirely new class of platinum-based anticancer therapeutics.

AUTHOR CONTRIBUTIONS:

Designed Research – Erica Peterson, Nicholas Farrell

Experiments performed – Erica Peterson, Vijay Menon, Peyman Kabolizadeh, Brad Benedetti

Analyzed Data – Erica Peterson, Vijay Menon, Peyman Kabolizadeh, Ralph Kipping, John Ryan, Lawrence Povirk, Nicholas Farrell

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Wrote the paper – Erica Peterson, Nicholas Farrell, Vijay Menon
INTRODUCTION:

The increased proliferative rate of cancer cells relies on a concomitant upregulation of ribosomal biogenesis in order to meet the cellular demands for protein synthesis (1-4). The biogenesis of ribosomes is a coordinated process that largely takes place in the nucleolar compartment of the cell. The nucleolus is formed around tandem arrays of more than 400 copies of ribosomal RNA (rRNA) genes as they are transcribed, processed, and assembled into ribosomal subunits (5). Recently, rRNA synthesis has emerged as a shared target of many anticancer agents (6). The platinum-based drugs, oxaliplatin, and to a lesser extent, cisplatin, inhibit the transcriptional rate of the 47S rRNA precursor transcript, while the antimetabolite, 5-fluorouracil (5-FU), disrupts processing of the precursor into mature 28S, 18S, and 5.8S rRNA transcripts. In each case, the effects are surprisingly early events; observed within hours after cell treatment. This implies that inhibition of rRNA processes may be an early determinant of the antiproliferative activity of these drugs. Of course, the effects of oxaliplatin, cisplatin, and 5-FU are not limited to rRNA processes. They are nonselective, genotoxic drugs that modify, or incorporate into the total pool of nucleic acid (7-9). Limiting genotoxic events by drug-targeting only nucleic acid within the nucleolus and specifically, rRNA synthesis, is an interesting challenge for small molecule therapeutics.

Unlike the nucleus and other membrane-bound organelles, there is no evidence of a membrane or membrane-like structure separating the nucleolus from the surrounding nucleoplasm. In theory, any soluble molecule could diffuse in and out of the nucleolar compartment. Because of
sequence similarities in nuclear and nucleolar localization signals, it is currently accepted that targeting of a specific molecule to the nucleolus results from its direct or indirect interaction with components of the nucleolus, that is, ribosomal DNA and its transcripts (10). Positive charge is a major factor in localization and retention of proteins to the nucleolus. Mutagenesis studies of nucleolar proteins, such as nucleolin, fibrillarin, and the viral HIV TAT, determined that clusters of the positively charged amino acids arginine, and lysine serve as nucleolar localization signals (11, 12). Furthermore, poly-arginines and -lysines, containing more than six amino acids, rapidly penetrate cellular membranes and localize specifically to the nucleolar region of cells (13-15).

TriplatinNC is a highly positively charged, (+8) non-covalent derivative of the phase II clinical platinum drug, BBR3464 (16) (Fig. 4-1A, B). The crystal and molecular structure of TriplatinNC associated with a double-stranded B-DNA dodecamer 5′-d(CGCGAATTCGCG)2 at 1.2 Å resolution (PDB:2DYW), shows formation of phosphate clamps, a discrete third mode of DNA binding, distinct from intercalation or minor groove binding. The phosphate clamp involves two modes of DNA binding: “backbone tracking” by following the phosphate backbone of one strand, and "groove-spanning" across the minor groove to interact with both strands (17). These interactions are uniquely mediated through hydrogen bonding, and are analogous to the “arginine fork”, an important motif for protein-DNA interactions, where positively charged guanidino groups of arginine, interact with negatively charged oxygens of DNA phosphate (18-20). The polyarginine analogy has also allowed identification of heparan sulfate proteoglycans as mediators of TriplatinNC cellular accumulation by pursuing
the isostructural analogy of sulfate and phosphate and the accepted molecular mechanism of polyarginine uptake (21). We have now extended the conceptual analogy of TriplatinNC as a small molecule polyarginine mimic by examining its localization to the nucleolar compartment within cells. This report examines the biology of the phosphate clamp and reveals remarkable differences in the cellular localization pattern of TriplatinNC compared to cisplatin by comparing the fluorophore-drug conjugates, TriplatinNC-NBD and its cisplatin-NBD analog (22). TriplatinNC rapidly accumulates within the nucleolar region of the cell, and as an early event upon cellular treatment there is a resulting decrease in the production rate of 47S rRNA precursor transcripts. Further studies detailed in this report set a timeline of the subsequent morphological, antiproliferative, and apoptotic events.
Figure 4-1. Structures of (A) BBR3464 and (B) TriplatinNC. The noncovalent polynuclear compound, TriplatinNC, is a direct structural analog of the Phase II drug BBR3464, where the labile chloride ion is replaced by the amine group, NH$_2$(CH$_2$)$_6$NH$_2$. 
MATERIALS AND METHODS:

Drug Synthesis. Cisplatin was synthesized as previously described; [Wong, E.S.Y.; Giandomenico, C.M., Patent No. 09678595]. TriplatinNC was synthesized as described previously (16). The synthesis of TriplatinNC-NBD and Cisplatin-NBD are described later in this chapter.

Cell Culture and Drug Treatments. HCT116p53+/+, HCT116 p53-/−, and A2780 cell lines were cultured in RPMI 1640 (Invitrogen), supplemented with 10% calf serum (Atlanta Biologicals) and 1% penicillin/streptomycin (Invitrogen). Cells were maintained in logarithmic growth as a monolayer in T75 culture flasks at 37°C in a humidified atmosphere containing 5% CO2. For drug treatment studies, unless otherwise noted, the molar drug: cell ratio was kept constant by seeding 5x10⁴ cells/mL media throughout.

Confocal Fluorescence Microscopy. 5x10⁴ cells were seeded on chamber slides (Lab-Tek II) in media. 24 hr after seeding, cells were treated with TriplatinNC-NBD or Cisplatin-NBD at the indicated times and concentrations. After removing the media, cells are washed with PBS and mounted as described below. For Immunofluorescence of fibrillarin, cells were permeabilized with 0.5% Triton-X in PBS for 10 min. and blocked in PBS/Casein for 1 hr at room temperature. 1:200 dilution of primary antibody, anti-fibrillarin (cell signaling, clone C13C3) was added overnight at 4°C. The primary antibody was then removed and wells were washed 4 times with 0.5 mL PBS for 15 min each followed by incubation with a 1:500 dilution of secondary antibody.
anti-rabbit IgG Alexa 647 conjugate (cell signaling) for 3 hr at room temperature. After removing the secondary antibody, the wells are washed again 4 times with 0.5 mL PBS and antibodies are then fixed with 3% paraformaldehyde for 15 min. at room temperature. Paraformaldehyde is then removed by 2 X 0.5 mL washes. All slides were mounted in VectaShield mounting medium containing DAPI (4', 6-diamidino-2-phenylindole, Vector Labs) and viewed using a Zeiss LSM 510 confocal microscope.

**Flow Cytometry.** HCT116 cells were drug treated for 6, 24, and 48 hr. 1x10^6 cells were suspended in 1 ml of propidium iodide solution (3.8 mM sodium citrate; 0.05 mg/ml propidium iodide; 0.1% Triton X-100) with added RNase B (7000 units/ml) and kept in the dark at 4°C. Cells were analyzed by flow cytometry on a CoulterElite XL-MCL (Beckman Coulter). Twenty thousand events were acquired and analyzed using Modfit software.

**Metabolic Labeling and rRNA analysis.** 2x10^5 HCT116 cells were grown in 6-well plates in 4 ml RPMI/10% FBS media for 24 hr. Cells were drug treated for 5 hr. For phosphate depletion, complete media was replaced with phosphate-free DMEM/10% dialyzed FBS containing drug and incubated for 30 min. before the addition of 15 μCi/ml 32P-orthophosphate (Perkin Elmer) and further 30 min. incubation. Medium was again changed to RPMI/10% FBS drug-containing medium for 3 hr and total RNA was isolated using RNeasy (Qiagen). RNA concentration was determined using a Nanodrop. 1.5 μg of total RNA was separated on a 1% agarose-formaldehyde gel. After electrophoresis, 28S rRNA quantities were visualized with Ethidium Bromide as a
loading control. Gels were placed on Whatman paper and dried for 2 hr at 80°C under vacuum suction. Dried agarose gels were exposed to X-ray film.

**Electromobility Shift Assay.** 2 nM end-labeled dsDNA (24mer 5’-GAAGGGGGGCTCTAAAAGGGGGTG-3’ containing the AdML TATA box.) was incubated for 15 min. at 30°C with or without drug in a final volume of 10 µl reaction buffer (20 mM Hepes-KOH, pH 7.9, 25 mM KCl, 10% glycerol, 0.025% NP-40, 100 µg/mL BSA, 0.5 mM DTT, 0.1 mM EDTA, and 2 mM MgCl₂). Where indicated, 100 ng rTBP (Santa Cruz) was added to the reactions and was further incubated for 30 min. The reactions were separated by electrophoresis in a 6% native polyacrylamide gel in 0.5X TBE buffer (45 mM Tris-HCl, 44 mM boric acid, and 2 mM MgCl₂). The gel was dried and exposed to X-ray film.

**Growth Inhibition Assay.** Cells were seeded in a 96-well plate at 5x10³ cells/well in 100 µl media and allowed 24 hr to attach. Cells were then drug treated for a period of 72 hr. After removal of drug, the cells were treated with 0.5 mg/mL MTT reagent [(3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] in media for 3 hr at 37°C. The MTT reagent was removed and 100 µl of DMSO was added to each well. The plate was then incubated on a shaker at room temperature in the dark. The spectrophotometric reading was taken at 570 nm using a microplate reader.
Clonogenic Survival Assay. 2x10^4 HCT116+/+ and HCT116-/- cells were seeded in 3 ml of medium in a 6-well plate. After 24 hr of incubation, cells were then treated with 20 µM drug for a 24 hr period. 250 or 2500 cells were seeded into 10 cm dishes and allowed to grow for 10-14 days to form colonies. These were then fixed by methanol, stained by 0.1% crystal violet, rinsed with water, and air dried. Colonies consisting of more than 50 cells were counted. Plating efficiency and surviving fraction were determined for each drug.

Immunoblot Analysis. Primary antibodies used were p53 (Cell signaling, # 9282), p21 (Santa Cruz, clone F-5), p27 (Cell signaling, clone SX53G8.5), total caspase-3 (Cell-signaling, 9662), total caspase-8 (Cell signaling, clone IC12), cleaved caspase-9 (Cell signaling, #9501), cleaved PARP (Cell signaling, # 9541), and β-Actin (Abcam, ab8226). After drug treatments, both floating and adherent cells were harvested. Cells were washed with ice-cold PBS, repelleted, and, resuspended in SDS lysis buffer (62.5 mM Tris-HCl, pH 7.5, 5% glycerol, 4% SDS, 4% complete protease inhibitor (Roche), 5% BME). After homogenization, proteins were resolved on 7.5-15% polyacrylamide gels, transferred to PVDF membrane, and blocked in 5% non-fat milk at room temperature for 1 hr. The membranes were probed with primary antibodies overnight, followed by secondary anti-rabbit, or anti-mouse antibodies conjugated to horseradish peroxidase (Cell Signaling, Thermo Scientific). Chemiluminescent protein bands were visualized on X-ray film.
RESULTS AND DISCUSSION:

TriplatinNC rapidly accumulates within the nucleolus. To determine differences in the cellular localization pattern of TriplatinNC compared to Cisplatin, each compound was labeled with the small fluorophore, 7-nitrobenzoxadiazole (NBD) (22). The resulting drug-NBD conjugates, Cisplatin-NBD and TriplatinNC-NBD (Fig. 4-2A), retain charge properties and cell growth inhibition values (IC) comparable to that of the parent compounds (Fig. 4-3). Confocal microscopy showed that TriplatinNC-NBD, but not Cisplatin-NBD, rapidly accumulates to punctate regions of the nucleus in HCT116 colon carcinoma and A2780 ovarian carcinoma cell lines (Fig. 4-2B, C). In co-localization studies, the distinct nuclear localization patterns of TriplatinNC overlaps the signal of the commonly used nucleolar marker, Fibrillarin, a ribonucleoprotein methyltransferase involved in the first steps of ribosomal RNA (rRNA) processing (Fig. 4-2D). Due to this unique pattern of localization, and the charged nature of the drug, it was considered that TriplatinNC was likely to interact with ribosomal DNA/RNA within the nucleolus.
Figure 4-2. Fluorescently-tagged TriplatinNC localizes to the nucleolus.
(A) Structures; Fluorophore conjugates Cisplatin-NBD and TriplatinNC-NBD. 
(B) Confocal Microscopy; The cellular distribution of NBD-Cisplatin (green) in A2780 cells after 24 hr incubation in 10 µM drug. 
(C) NBD-TriplatinNC (green) in A2780 and HCT116 cells after 2 hr incubation with 20 µM drug, respectively. The DNA is subsequently stained with DAPI (blue) as reference. 
(D) Colocalization (orange) of NBD-TriplatinNC (green) with the nucleolar marker, fibrillarin (red), in HCT116 cells after 4 hr incubation in 20 µM drug. Anti-fibrillarin signal was detected using a secondary antibody conjugated to Alexa-fluor 647.
Figure 4-3. MTT Assay; A2780 or HCT116 cells were treated with the indicated concentration of drug for 72 hr. In each graph the drug-NBD derivative is denoted in dark gray and the parent compound in light gray. (A) A2780 cells treated with TriplatinNC-NBD and TriplatinNC. (B) HCT116 cells treated with TriplatinNC-NBD and TriplatinNC-NBD. (C) A2780 cells treated with Cisplatin-NBD and Cisplatin. (D) HCT116 cells treated with Cisplatin-NBD and Cisplatin. Percent Inhibition is calculated as: 1-(N/N₀), where N=treated samples and N₀= untreated control samples. Data are shown as mean ± S.D. representative of at least 2 independent experiments.
As an early event, TriplatinNC disrupts the rate of rRNA transcription. Initiation of rDNA transcription requires assembly of a specific multiprotein complex that binds to the rDNA promoter containing RNA polymerase I (Pol I), and two Pol I specific transcription factors, upstream binding protein (UBF), and the promoter selectivity factor complex, SL1 (23). The binding specificity of Pol I at rDNA promoters is conferred by SL1 (24), comprised of the transcription factor TBP (TATA-Box Binding Protein) and several auxiliary TBP-associated factors (25, 26). The ability of TriplatinNC to inhibit TBP-DNA interactions was examined in vitro using the Electromobility Shift Assay (EMSA). TriplatinNC reduced the TBP-DNA complex by 50% using between 0.63 and 1.25 µM drug concentrations (Fig. 4-4A). These results suggest that TriplatinNC has the ability to inhibit RNA transcription by physical inhibition of DNA-binding proteins required for this process. This process is distinct from the effects of covalently-binding platinum drugs (27-30).

To determine whether TriplatinNC affects the rate of rRNA transcription in vivo, HCT116 cells were treated with varying drug concentrations for 5.5 hr, and then metabolically labeled according to the scheme depicted in Fig. 4-4B. The 47S rRNA precursor transcript is sequentially cleaved to yield the mature 28S, 18S, and 5.8S rRNAs. As cells are pulsed with 32P-radiolabeled orthophosphate for 30 min. followed by a 3 hr chase, the abundance of newly formed 47S precursors, 32S intermediate cleavage products, and mature 28S and 18S rRNAs are sufficiently labeled for visualization by autoradiography (6). It was evident that treatment of cells with TriplatinNC inhibits the production rate of 47S rRNA precursor transcripts in a dose dependent manner (Fig. 4-4B). It does not appear that TriplatinNC affects the rate of 47S rRNA
processing, as the abundance of 32S, 28S, and 18S rRNAs decreases proportionally to that of the precursor.

The transcriptional activity of rRNA genes has been shown to change in accordance with the cell cycle (25). rRNA transcription levels are highest in S and G2 phases, nonexistent in mitosis, and rebounding in G1 (26, 27). Therefore, it was important to consider whether the inhibitory effect of TriplatinNC on the rate of rRNA transcription was direct, or if rRNA transcription levels were merely downregulated as an indirect effect of changes within the cell cycle. The latter would be likely if there were an increase in the population of cells in G1 (when rRNA levels are lower). For this purpose, HCT116 cells treated with TriplatinNC were subjected to cell cycle analysis by flow cytometry. In cells treated with 20 µM TriplatinNC [(IC90) (Fig. 4-3B)] for 6 hr, there were modest changes that occurred within the cell cycle. The population of cells in G1 decreases slightly from 37% to 30% compared to untreated control cells, while the population of cells within S+G2 increases slightly from 63% to 70%. These results imply the disruption of rRNA transcription is an early event of cellular treatment with TriplatinNC, and does not result from changes in the cell cycle.
Figure 4-4. TriplatinNC competitively inhibits TBP-DNA interaction and interferes with rRNA transcription.

(A) EMSA; Lanes 3-9; 2 nM $^{32}$P-labeled DNA incubated with 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, and 5 µM drug, respectively, followed by 100 ng rTBP protein. Lane 2 is the ‘TBP/DNA complex’ positive control. Lane 1 is the ‘free DNA’ control containing DNA only.
Figure 4-4. TriplatinNC competitively inhibits TBP-DNA interaction and interferes with rRNA transcription.

(B) $^{32}$P-Pulse/Chase Metabolic Labeling; Samples were treated with or without drug according to the experimental outline. Lanes 1-8 were treated with 0.78, 1.6, 3.1, 6.3, 12.5, 25, 50, 100 µM drug, or without drug in lanes 1 and 2. 1.5 µg total RNA was analyzed for each sample. Ethidium Bromide (EtBr) staining of 28S rRNA was used as a loading control.
At 24hr, TriplatinNC induces a G1 cell cycle arrest. Anticancer therapeutics cause proliferative arrest generally through induction of a classical signaling pathway (28). Central to this pathway is the stabilization of p53 protein by serine/threonine kinases, followed by transactivation of the cyclin-dependent kinase (CDK) inhibitor, p21. Increased protein levels of p21 inhibit CDK activities resulting in cell cycle arrest. This pathway is induced by cisplatin, which has been shown to arrest cells at the G2 checkpoint as an attempt to repair DNA damage before cells enter mitosis (29, 30). In agreement with these studies, HCT116 cells treated with 20 µM cisplatin [(IC90), (Fig. 4-3D)] were shown to induce S-phase accumulation at 24 hr, and finally G2 arrest at 48 hr (Fig. 4-6). Treatment of HCT116 cells with 20 µM TriplatinNC, on the other hand, induced an earlier arrest in G1 at 24 hr continuing to 48 hr. The increase in the number of cells in G1 was mostly at the expense of the proportion of cells undergoing DNA replication in S-phase, which decreased 52% at 24 hr and 77% at 48 hr compared to the control (Fig. 4-5A, B). As expected, there is a substantial stabilization of p53 protein levels leading into the G1 arrest at 12 and 24 hr after treatment with TriplatinNC (Fig. 4-5C). However, p21 expression levels are not concomitantly upregulated in the classical manner. Surprisingly, p21 protein expression levels decreased at 6 and 12 hr, and increase only to basal level at 24 hr. Furthermore, the protein expression levels of p27, a similar CDK inhibitor with the potential to cause cell cycle arrest (31), also decreased. These data suggest that the G1-arrest induced by TriplatinNC may not depend on classical signaling events. The fact that TriplatinNC induces G1-arrest in the HCT116 isogenic cell lines lacking either p53 or p21 (Fig. 4-7), furthers this point. It is tempting to speculate that TriplatinNC may disrupt the cell cycle at G1, because S-phase has the highest requirements for rRNA production (32). The consideration that G1-cyclin proteins
and others may be synthesized in insufficient amounts to allow for the transition into S-phase is a subject of continuing research.
Figure 4-5. TriplatinNC induces G₁ cell cycle arrest. (A) HCT116 cells were treated with 20 µM TriplatinNC (IC₉₀) for 6, 24, and 48 hr and analyzed for cell cycle distribution using propidium iodide. (B) Modfit software analysis of G₁, S, and G₂ populations. Data points are shown as mean ± S.D. of 2 independent experiments with 2 replicates per sample.
Figure 4-5. TriplatinNC induces $G_1$ cell cycle arrest. (C) HCT116 cells were treated with 20 μM TriplatinNC for 3, 6, 12, and 24 hr. The levels of p53, p21, and p27 were detected using western blotting. $\beta$-Actin is used as a loading control. Representative immunoblots are shown from three independent experiments.
Figure 4-6. HCT116 cells treated with 20 µM cisplatin for 0, 24, and 48 hr. They were then RNase B treated and analyzed for cell cycle distribution using propidium iodide.
Figure 4-7. HCT116, HCT116 p53-/-, and HCT116 p21-/- cells were treated with 20 μM TriplatinNC for 0, 24, and 48 hr. They were then RNase B treated and analyzed for cell cycle distribution using propidium iodide.
TriplatinNC causes rapid proliferative arrest and cell death independently of p53 status. In cells undergoing apoptosis, activated cysteine proteases, caspases-3, -6, -7, result in cleavage of cytoskeletal proteins and fragmentation of nuclear DNA. These events result in visible changes to the morphology of the cell including cell shrinkage, chromatin condensation, cytosolic vacuolization, and membrane blebbing (33). Changes in physiology of HCT116 cells treated with 20 µM TriplatinNC-NBD were visualized using confocal microscopy after 2, 10, and 30 hr of treatment (Fig. 4-8A). After 10 hr of treatment, cells are much smaller in size and exhibit prolific cytosolic vacuolization. After 30 hr of treatment, blebbing and disintegration of the outer membrane is apparent.

Nearly all cancers harbor genetic defects that directly, or indirectly, inhibit normal proapoptotic, or tumor suppressor functions of p53 (28). For this reason, we asked whether the absence of p53 affects the ability of TriplatinNC to effectively induce proliferative arrest and cell death. HCT116 p53+/+ and p53-/- cells were treated with 20 µM cisplatin or TriplatinNC for 12, 24, 48, or 72 hr. Using the MTT assay, it was determined that the kinetics and extent of cisplatin inhibition on cell growth was significantly limited in the absence of p53 (Fig. 4-8B). However, the inhibitory effects of TriplatinNC on cell growth was unaffected by the absence of p53 protein (Fig. 4-8B). In support of these results, we asked further whether the absence of p53 affects the ability of TriplatinNC to inhibit colony-formation, or reproductive viability, using the clonogenic survival assay. HCT116 p53+/+ and HCT116 p53-/- cells were treated with 20 µM TriplatinNC for 24, 48, and 72 hr (Fig. 4-8C, Fig. 4-9). The percentage of cells that failed to replicate was
determined as 69±6%, 88±2%, 92±7% after 24, 48, and 72 hr, respectively. There was no significant difference in cells with or without p53.
Figure 4-8. TriplatinNC causes rapid proliferative arrest and cell death. (A) HCT116 cells were treated with 20 µM TriplatinNC for 2, 10, and 30 hr. Confocal microscopy was carried out to observe morphological effects of apoptosis like reduction in cell size, membrane blebbing, and cytosolic vacuolization (white arrows).
Figure 4-8. TriplatinNC causes rapid proliferative arrest and cell death. 
(B) Comparison of growth inhibition in HCT116 p53+/+ (light gray) and p53-/- (dark gray) cells treated with 20 µM cisplatin or TriplatinNC was carried out using the MTT assay. Percent inhibition is calculated as: \[1 - (N/N_0)\], where \(N\)=treated samples and \(N_0\)=untreated control samples. Data points are shown as mean ± S.D. of 2 independent experiments with 3 replicates per sample. **\(p<0.05\), ***\(p<0.005\). (C) Clonogenic survival assay was carried out to compare the reproductive viability in HCT116 p53+/+ cells and p53-/- cells treated with 20 µM TriplatinNC for 24 and 48 hr. Plates are representative of 4 independent experiments with 2 replicates per sample; mean ± S.D. of the combined data is shown in Fig. 4-9.
Figure 4-9. Clonogenic Survival Assay. Comparison of colonies formed in HCT116 p53+/+ cells (black) and HCT116 p53-/- cells (gray) treated with 20 μM TriplatinNC. Percent Inhibition is calculated as: 1-(N/N₀), where N=treated samples and N₀= untreated control samples. Data are shown as mean ± S.D. representative of 4 independent experiments.
After 48 hr, TriplatinNC induces a robust apoptotic signaling cascade. TriplatinNC induces apoptosis in mast cells through activation of the mitochondrial-dependent pathway initiator, procaspase-9, and the downstream effector, procaspase-3 (34). In extension of these studies, it was determined whether TriplatinNC induced accumulation of the activated forms of procaspase-9 and procaspase-3 in p53+/+ as compared to p53/- HCT116 isogenic colon carcinoma cell lines. Both cells lines show a time-dependent increase in active caspase-9 and caspase-3 after treatment with 20 µM TriplatinNC (Fig. 4-10A). At 30 or 55 hr after treatment in p53/- cells, there is less accumulation of active caspase-9 compared to the untreated control than in p53+/+ cells. However, the abundance of active caspase-3 appears to be unaffected by the absence of p53. Therefore, it was asked whether caspase-3 may be activated also by the initiator of the mitochondrial-independent pathway of apoptosis, caspase-8.

Caspase-8, has the ability to activate procaspase-3 through both mitochondrial-dependent and -independent apoptotic pathways. In the mitochondria-dependent pathway, caspase-8 cleaves BID to tBID, which translocates to the mitochondria and causes damage by culminating an efflux of death promoting proteins such as cytochrome-C. These events, in turn, lead to activation of procaspase-9, followed by procaspase-3. In the mitochondrial-independent pathway, caspase-8 instead directly activates procaspase-3, which cleaves downstream substrates such as PARP-1, eventually leading to cell death (35). In HCT116 cells treated with 20 µM cisplatin for 48 hr, it was determined that the abundance of active caspase-8 (p18) and downstream target, Parp-1, is reduced in cells lacking p53 as compared to the wild-type control (Fig. 4-10B). However, treatment with 20 µM TriplatinNC for 48 hr induced similar levels of active caspase-8 and
PARP-1 in p53+/+ and p53-/- cells (Fig. 4-10C). Furthermore, TriplatinNC does not induce the cleavage of BID into tBID (Fig 4-11), therefore, the mitochondrial-dependent and independent pathways are likely activated independent of each other (36).
Figure 4-10. TriplatinNC induces activation of caspases independent of p53. (A) Western Blot Analysis; Time course analysis of cleaved caspase-9 and -3 protein in HCT116 p53+/+ and p53-/- cells treated with 20 µM TriplatinNC for 5, 15, 30, and 55 hr. (B, C) Comparison of cleaved caspase-8 and PARP-1 protein in HCT116 p53+/+ and p53-/- cells treated with 20 µM Cisplatin or TriplatinNC at 48 hr. β-Actin was used as a loading control.
Figure 4-11. HCT116 p53+/+ cells were treated with 20 μM Cisplatin or TriplatinNC for 48 hr and comparison of BID (p22) and the active form tBID (p15) proteins were carried out using immunoblotting. β-Actin was used as a loading control.
Appendix:

*Synthesis of Cisplatin-2-NBD and TriplatinNC-NBD

N-(7-nitro-2, 1, 3-benzoxadiazol-4-yl)hexane-1,6-diamine

Ligand was synthesized by a modification of a described procedure (A). BOC–diaminohexane hydrochloride was dissolved in ddH₂O and NBD-Cl (5eq) and DIPEA (2eq) was added. After the mixture had been stirred at room temperature overnight the product was extracted with diethyl ether. The organic phase was washed with brine (4x10 mL), dried over Na₂SO₄ and purified by flash chromatography (CHCl₃/EtOAc, 90:10). The BOC protecting group of the purified intermediate was removed by using trifluoroacetic acid (1 mL) at room temperature. After the mixture had been stirred overnight, excess TFA was evaporated, and the oily product was treated with diethyl ether to yield the product as orange crystals.

N-(7-nitro-2,1,3-benzoxadiazol-4-yl)ethane-1,2-diamine(L)

BOC–diaminoethane (0.79 ml, 4.99 mmol) was dissolved in 20 ml ethanol containing a 10% w/w of Na₂CO₃, and NBD-Cl (0.50 g, 2.51 mmol) was added. The mixture was stirred at room temperature overnight, then 50 ml of water were added and the product was extracted with diethyl ether (3 x 30 ml). The organic phase was washed with brine (3 x 30 ml), dried over Na₂SO₄ and purified by flash chromatography (CHCl₃/EtOAc, 70:30). The BOC protecting group of the purified intermediate was removed by using 2 ml of trifluoroacetic acid at room
temperature. After the mixture had been stirred overnight, excess TFA was evaporated, and the oily product was treated with diethyl ether to yield the trifluoroacetate salt as orange crystals.

Both ligands were neutralized by dissolving in a mixture of H2O/Ethanol 1:1 and addition of 1 equivalent of NaOH. The neutral ligand was then extracted with CH2Cl2. The organic fractions were dried over K2CO3 and the solvent removed under vacuum. The product was dried under vacuum overnight. TriplatinNC-NBD2: BBR3464 Cl- salt was treated with 5.97eq of AgNO3 in ddH2O and allowed to stir at room temperature for 4 hr. AgCl was filtered off and to the clear solution was added NBD-diaminohexane (5eq) and DIPEA (5eq). The solution was allowed to stir at 50°C overnight. The solution was then evaporated to dryness and the resulting orange oil was re-dissolved in ddH2O and excess NBD-diaminohexane was removed by extraction with EtOAc (5x50 mL). The aqueous layer was removed and the product was redissolved in ddH2O and lyophilized. The resulting orange powder was washed with EtOAc, CHCl3 and diethyl ether to yield TriplatinNC-NBD2 (1.7mg, 30%). 1H NMR D2O δ (ppm): 8.54 (d, 2H); 6.23 (d, 2H); 3.00 (t, 12H); 2.72 (m, 4H); 1.67 (m, 16H); 1.42 (m, 16H).

Cis-[PtCl2((N-NBD)ethane-1,2-diamine)(NH3)]

The synthesis of this compound was achieved by means of a modification of a patented procedure (B). K[PtCl3(NH3)] (30.0 mg, 0.084 mmol) was dissolved in 5 ml of a 15 mM aqueous solution of KCl. A solution of KI (41 mg, 0.248 mmol) in 1 ml of water was prepared. The KI solution was added to the K[PtCl3(NH3)] solution, followed by a slight excess of the NBD amino ligand L (20.5 mg, 0.091 mmol). The combined solution was stirred in the dark for 5 hr at room
temperature. A reddish precipitate formed which was collected, washed with cold water, cold methanol, and dried under vacuum at room temperature overnight. The product was Cis-[PtI₂((N-NBD)ethane-1,2-diamine)(NH₃)], confirmed by NMR. The Cis-[PtI₂((N-NBD)ethane-1,2-diamine)(NH₃)] (40 mg, 0.067 mmol) was suspended in 2 ml of a mixture H₂O/Acetone 85:15 and 1 eq of silver nitrate was added to the suspension. It was then stirred for 24 hr in the dark at room temperature. The suspension was filtered and 200 µL of HCl (~12 M) were added to the filtrate. The solution was stirred at room temperature for 2 hr, during which time a black precipitate formed. The precipitate was filtered and the solution concentrated to obtain the product as a brownish powder, which was washed with ice cold methanol, ether and dried under vacuum. ¹H NMR D₂O δ (ppm): 8.45 (d, 1H); 6.33 (d, 1H); 3.81 (t, 2H); 3.27 (t, 2H). ESI-MS: m/z: (M+H)+507.22; (M-Cl)+471.23


(B) Wong, E.S.Y., Giandomenico, C.M., Patent No.09678595

*Compounds were synthesized by Brad Benedetti*
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CHAPTER 5

FUTURE PERSPECTIVES

Platinum-based compounds that are clinically active and those currently undergoing clinical trials exhibit conventional mechanisms of inducing cytotoxicity and subsequent downstream processes. These include activation of multiple cell cycle checkpoints leading to growth arrest and further activation of cell death mechanisms. The primary mode of cell death exhibited by these compounds is through the extrinsic or intrinsic pathways of apoptosis which is mediated by the activation of cysteine aspartate proteases called caspases. However, published results indicate the role of a second form of cell death or autophagy following treatment with these platinum compounds. The role of autophagy in cancer progression has been controversial since depending upon the cellular conditions it either promotes carcinogenesis or prevents cell proliferation. For instance, earlier reports have shown that inhibition of autophagy prevents senescence which is believed to be an anti-tumorigenic mechanism\(^1\). Another example is seen in the case of metformin treatment that prevents tumorigenesis through AMPK activation and mTOR signaling\(^2\). In contrast, it has been shown that cancer cells expressing an oncogenic Ras gene can upregulate autophagy in response to stress and starvation, providing a pro-survival advantage\(^3\). It is postulated that tumor cells depend on autophagic induction to meet the metabolic demands arising through cellular proliferation and different forms of environmental and therapeutic stress\(^4\). From the perspective of platinum based compounds, it has been shown recently that the
clinically active oxaliplatin induces a cytoprotective autophagic response in colorectal cancer cells through the production of reactive oxygen species and induction of endoplasmic reticulum stress. Inhibiting autophagy by pharmacological intervention using 3-methyladenine or bafilomycin increased the cytotoxic properties of oxaliplatin in these cells. A similar activity was seen in hepatocellular carcinoma cells and xenografts treated with oxaliplatin wherein cytotoxicity was potentiated using pharmacological inhibition of autophagy. However, a recent modification of oxaliplatin called E Platinum has been shown to exhibit anti-tumor properties in gastric carcinoma BGC-823 cells through the activation of autophagy, suppressing mTOR activation and other pro-survival signaling pathways. It is quite interesting to see that structural modifications in a compound yield a derivative that behaves differently from its parental compound. Although there are no significant studies done to see whether polynuclear platinum complexes induce autophagy, it has been shown that the dinuclear platinum compound, BBR3610 activates an early autophagic and a late apoptotic response in glioma cells, although the autophagy induced was found to be neither cytotoxic or cytoprotective. BBR3610-DACH is a derivative of BBR3610 and both these compounds seem to be activating a non-classical mechanism of apoptosis in colorectal carcinoma cells as seen from our data wherein PARP cleavage is observed (more robust after BBR3610-DACH treatment) in the absence of caspase-3 or caspase-7 activation. In this context, some of the experiments that can be carried out in colorectal carcinoma as well as glioma cells would be to check whether BBR3610-DACH mimics its parental compound in activating an autophagic response. Also, it would be interesting to see whether autophagy induction has any role to play in PARP cleavage and whether pharmacological inhibition of autophagy or genetic knockdown of autophagy related genes
prevents PARP cleavage or could possibly lead to the induction of a more robust apoptotic response accompanied with concomitant caspase activation.

As mentioned earlier, the activity of platinum drugs is attributed to the formation of different kinds of DNA adducts that interfere with normal DNA processes. One of the major hurdles encountered by DNA binding agents is the chromatin into which the DNA is packaged. The basic building block on chromatin is the nucleosome which comprises two each of the histone proteins H2A, H2B, H3, and H4 wrapped around ~146 bp of DNA. Adjacent nucleosomes on the chromosome are joined by ~20-50 bp of linker DNA. As such, a major proportion of the genomic sequence is comprised of histone octamers. However, not much is known about the role of DNA binding agents in modulating the nucleosome dynamics. An earlier in vitro study showed selective disruption of nucleosomes by minor groove binding agents like DAPI which bind to the AT rich region in the nucleosomes and disrupt the nucleosome by preventing histone binding or by increasing DNA stiffness. One of the key factors governing nucleosome dynamics is the extent of nucleosome mobility. Higher nucleosome mobility destabilizes the chromatin arrangement whereas lesser nucleosome mobility has been found to stabilize the same, thus decreasing the accessibility of DNA to non-histone components and DNA binding agents. The formation of cisplatin or oxaliplatin adducts reduced the mobility of the nucleosomes but at the same time, allowed to maintain the nucleosome positioning. Although adducts form randomly across the genome, the effects of these drugs on nucleosome positioning allow these adducts to be protected from DNA repair. The apparent role of the polynuclear platinum
compounds in regulating the nucleosome dynamics has not been studied in detail and it would be interesting to see the impact of some of these complexes like BBR3464, BBR3610-DACH, or TriplatinNC on the nucleosome positioning or mobility. Also, earlier studies using atomic force microscopy have elucidated the extent of DNA condensation by drugs like cisplatin, oxaliplatin\textsuperscript{14}, or BBR3464\textsuperscript{15} which accounts for much of the DNA modification by these drugs. Using a similar approach, it would be exciting to study similar effects on DNA by the dinuclear platinum complexes, BBR3610 and BBR3610-DACH.

The design and development of novel anti-cancer drugs is an ongoing process and it would be essential to understand the detailed mechanism of action of these agents for further therapeutic intervention. In addition to the studies outlined above, an important avenue to shift focus would be to see the effect of these platinum compounds on the mitochondrial DNA. The surprising find that colorectal cancers have decreased mitochondrial DNA mutagenesis compared to the normal cells\textsuperscript{36}, begs the question as to whether some of these platinum compounds could cause mitochondrial DNA damage \textit{via} the formation of adducts leading to increased mitochondrial mutagenesis. Overall, there are different cellular aspects that remain to be explored toward the development of more potent and efficacious cancer therapeutics.
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Vita

Vijay Menon was born on November 15, 1980 in Mumbai, India and is an Indian citizen. He received his Bachelor of Science degree in Life Sciences from Ramnarain Ruia College, University of Mumbai, India, in May 2001 and further continued at the same place to earn his Master of Science degree in Life Sciences with Biotechnology as the field of specialization in April 2003. He worked as a lecturer at Ramnarain Ruia College from 2005-2007 and later came to the United States of America in August 2008 to pursue his doctorate in Molecular Biology and Genetics at Virginia Commonwealth University. He joined the laboratory of Dr. Nicholas Farrell in August 2009 and went on to become a Ph.D. candidate under the combined mentorship of both Dr. Nicholas Farrell and Dr. Lawrence Povirk who is a professor in the Department of Pharmacology and Toxicology. Vijay currently resides in Richmond, Virginia.