CHARACTERIZATION OF THE RNA BINDING MODE OF A PLATINUM-ACRIDINE AGENT

By

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It is by the Love and Grace of God that this new path has been written in my life, and it is because of the people that have surrounded me by love that I have been able to recognize God’s signs.

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Pt-ACRAMTU (ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea) is a DNA-targeted anticancer agent that shows activity in a broad range of solid tumor cell lines. Unlike the classical platinum-based cross-linking drugs, this agent produces its cytotoxic effect by two synergistic DNA binding mechanisms: monofunctional platination and intercalation of the acridine chromophore.

The RNA binding of platinum drugs has been studied to a much lesser extent. Recently, the RNA interactions of platinum-containing drugs and drug conjugates have been reported. In the current study, an RNA hairpin has been generated through in vitro transcription and treated with Pt-ACRAMTU. Our goal was to determine if specific RNA structural motifs are susceptible to intercalator-driven platination. Acidic digestion assays in conjunction with liquid chromatography-electrospray mass spectrometry analysis (LC-ESMS) was used to determine the specific bases modified by platinum. In addition, the RNA conformational changes induced by ACRAMTU and PT-ACRAMTU were also analyzed by circular dichroism (CD) spectroscopy.
CHAPTER 1 - INTRODUCTION

1.1. Nucleic Acids Generalities

1.1.1. DNA characteristics

Described by Martin Kemp as the Mona Lisa of modern science, DNA includes numerous characteristics that make it both unique and mysterious. The discovery of its importance as the carrier of the genetic message by Oswald Avery at the beginning of the nineteenth century unleashed the curiosity of scientists such as Rosalind Franklin and Maurice Wilkins, who were able to study A-DNA fibers using X-rays for the first time. These studies led to the proposal of the DNA structure by James Watson and Francis Crick in 1953, and to the development of the “central dogma of life”, in which DNA works as the template to produce the messenger RNA (mRNA) in a process called transcription; this mRNA carries the genetic information for all the proteins in an organism, formed in a process called translation.

DNA structure is rather simple; it can be described as a double helical polymer with individual monomers called nucleotides. Each nucleotide is composed of a deoxyribose sugar; a nitrogenous base attached to the 1' carbon of the sugar, either a purine [adenine (A) or guanine (G)], or a pyrimidine [thymine (T) or cytosine (C)]; and a phosphate group attached to the 5’ carbon of the sugar. The phosphate group acts as a bridge between the first nucleotide and the 3' hydroxyl of the ribose sugar in the next nucleotide (Figure 1a).

Each of the bases in DNA is complementary to another, so A pairs to T, and G pairs to C. This results in one strand complementary to the other linked by Watson-Crick hydrogen bonds in an antiparallel fashion, with one strand going from 3’ to 5’ phosphate, and the other from 5’ to 3’
Figure 1a). Stacking interactions between the bases within one strand are a major contributor to DNA stability.

The attachment of the bases to the sugar through the glycosidic bond is asymmetrical, leading to the formation of a minor and a major groove. This asymmetry is mainly determined by the pucker of the sugar rings, B-DNA has a C2'endo sugar pucker, while A-DNA has a C3'endo sugar pucker (Figure 1b). In the B form of DNA, which is the hydrated form present more than 70% of the time, the major groove is wider than the minor groove. Conversely, in A-DNA, which is the dehydrated form, there is a wider minor groove (Figure 1c).

1.1.2. RNA characteristics

RNA has been known for almost 150 years; however, RNA research has not been nearly as widespread as DNA’s due mainly to the thought that RNA works only as a mediator of information or as an assistant for DNA. In addition, RNA’s single stranded primary structure has been considered less interesting than DNA’s; when in fact, it gives rise to a diverse array of secondary and tertiary structure when interacting with other molecules.

The presence of RNA in multiple fundamental biological processes, such as transcription and translation, led to the discovery of the catalytic properties of RNA by Thomas R. Cech and Sidney Altman, for which they received the Nobel Prize in 1989. This discovery supported the “RNA world” hypothesis, which introduced the idea of RNA as the primordial biomolecule and original carrier of information. In addition, interest in RNA has also increased due to the discovery of RNA interference (gene silencing by double-stranded RNA) by Andrew Z. Fire and Craig C. Mellow, who received the Nobel Prize in 2006.
In contrast to DNA, RNA is synthesized as a single strand with a ribose sugar instead of the deoxyribose where C2' has a hydroxyl group attached, and it uses a uracil base (U), instead of thymine, that lacks of the C5' methyl group. RNA often folds into complex secondary and tertiary structures; it is largely limited to the A-form mainly due to the 2'OH steric restriction which is
reflected on the presence of a C3′ sugar pucker. The A-form of RNA, just as A-DNA displays a wide minor groove and narrow major groove (Figure 1).

RNA is much more versatile in its functions than DNA; it has been categorized into four different groups based on biological activity. 1) RNAs involved in regulatory processes such as: mRNA, which is a copy of DNA carrying the genetic information out of the nucleus and is used as a codon (mRNA nucleotide triplets) in translation; tRNA (transfer RNA), which works as an adaptor that carries the anticodon for the recognition of the right amino acids in translation, and accounts for almost 15% of the RNA in the cell thus becoming the most common “soluble” RNA; and rRNA (ribosomal RNA), that forms part of the ribosome helping in translation, accounting for the remaining 80% of the total RNA in the cell; 2) small RNAs that help processing and modifying the three types of RNA mentioned before; 3) small RNAs that help in gene expression; and 4) the so-called “housekeeping” RNAs such as telomerase RNA. 4

Besides the mentioned RNA functions, there are additional RNAs involved in different cellular activities such as iron chelation and catalysis –like RNase P ribozyme–, and RNAs that have been recognized, but their functions are still unknown. RNA is also known to work as the primary genetic material for pathogenic viruses such as HIV-1. The versatility in both function and structure makes RNA a very important target for chemotherapy.

1.2. Cancer Chemotherapy

1.2.1. DNA in cancer chemotherapy

According to the American Cancer Society (ACS), cancer is the second most common cause of death in the US, just behind heart disease. Moreover, the rate of deaths due to cancer since
1950 to 2005 has decreased by only 5.2% compared to that of heart disease which has decreased by almost 65%.

These alarming statistics reflect the importance and necessity of new approaches to cancer chemotherapy research, which started in the 1940s, when nitrogen mustards were implemented as cancer treatment.

Different factors such as UV light, chemicals and viruses can damage DNA; if there is no repair or apoptosis (cell death) in the cells, then mutations lead to development of cancer cells (oncogenic transformation). For this reason, DNA is the most important target for cancer chemotherapy.

Two different levels of interaction have been described for drug-DNA binding. The first level relates to the interaction of a drug with the primary structure of DNA, and the second level is related to the change in DNA secondary structure due to drug binding. Three different types of compounds belong to the first category: alkylating agents, minor and major groove binders, and antibiotics.

Non-specific alkylation of DNA, meaning the substitution of one of the functional groups in a molecule for an alkyl group, was first achieved with nitrogen mustards. These agents form either genotoxic monoadducts when binding to the G-N7 which can end in carcinogenic products, or more potent and efficacious cytotoxic interstrand crosslinks between DNA chains (Figure 2).

Unfortunately, these compounds attack not only rapid proliferating tumors, but also healthy tissues.

![Figure 2. Mechanism of alkylation of nitrogen mustard.](image-url)
The sequencing of the human genome gave Dervan et al.\textsuperscript{7} the opportunity of synthesizing molecules that can read between 16-20 bases in a specific sequence to modulate mutant genes. These molecules are minor and major groove binders that, when combined with alkylating agents have shown inhibition of replication; there has also been description of competition of groove binders with transcription factors or covalent attachment to RNA polymerase in transcription.\textsuperscript{8}

Finally, antibiotics have been used to treat cancer as well; and although they have not achieved the same success as in bacterial infections, they are highly selective for cancer cells. Since cancer cells grow more rapidly than normal cells, it was discovered that this selectivity is due to protein-associated strand breaks that occur more frequently in cancer cells. This is the reason why transcription, replication and repair pathways are more susceptible to drug interaction in cancer cells than in normal cells.

Additionally, there are three different types of drugs that belong to the second level of interaction: platinum-based drugs such as cisplatin, drugs that interact with G-quadruplexes such as intercalators, and drugs that alter interaction with proteins involved in regulatory processes like transcription, translation or replication.

Cisplatin, an alkylating agent that forms predominantly intrastrand 5’-GG or 5’-AG crosslinks, induces a 32-34° bend in DNA (Figure 3).\textsuperscript{9} As a consequence, the cisplatin-DNA adduct stabilizes the binding of proteins such as the TATA binding protein (TBP), the human upstream binding factor, the high-mobility group (HMG) box protein, etc.\textsuperscript{10} Other repercussions of the DNA-cisplatin adduct are depletion of important proteins to start translation or interference with repair pathways. By having more than one mechanism of action, cisplatin has become a preferred drug in cancer chemotherapy.
Figure 3. a) Cisplatin structure. b) DNA-cisplatin crystal structure of the intrastrand adduct.

Another important feature of secondary structures of DNA in cancer chemotherapy is the G-quadruplex, a four guanine base stranded structure (Figure 4) found, for instance, in the telomeres. A telomere is a repetitive sequence (TTAGGG in humans) that protects the end of the chromosomes from eroding; yet, it is shortened after each replication cycle. Telomerase is the enzyme involved in regenerating the telomeres; in cancer cells, this enzyme is overexpressed, resulting in immortal cells. Several drugs have been synthesized to target telomere regeneration either by stabilizing the G-quadruplex conformation (alkylating such as porphyrines) or by inhibiting telomerase activity.

Figure 4. DNA quadruplex.
a) Representation of G-tetrad structure where G bases make Hoogsteen hydrogen bonds. b) Parallel G-quadruplex arrangement.

Besides DNA's importance as the carrier of genetic information, it is also the scaffold on which multiple proteins bind to start processes such as transcription, recombination, repair, etc. One important class of these proteins are topoisomerases; these enzymes are in charge of modulating the topological features of DNA, such as releasing the helical tension during nuclear activities, by inducing either single or double strand breaks. Drugs targeting topoisomerases inactivate these enzymes by binding, sometimes covalently, and changing the conformation of DNA to work as competitors or poisons for the enzyme.\textsuperscript{6,8}

Research in cancer chemotherapy targeting DNA has several drawbacks, with toxicity being one of the most important. Thus, targeting RNA seems as a good option to expand cancer-chemotherapy areas of research.

\subsection*{1.2.2. RNA in “cancer” chemotherapy}

For many years, researchers have preferentially targeted DNA over RNA based on the idea that targeting one gene can lead to the disability of all the RNAs related to it. However, as time goes by, obstacles in targeting DNA due to its limited function inside the nucleus, and the high ability of RNA to form tight complexes with other RNAs, proteins, peptides, antibiotics and small molecules has opened the way for RNA as a new and promising chemotherapeutical target.

More than 30 years ago, Paul Zamecnik and Mary Stephenson had the idea of attacking specific sequences of mRNA through the binding of small sequences of complementary DNA (antisense molecules), therefore inhibiting translation. This seemed like a good idea, since mRNA is less stable than DNA and, is therefore, more vulnerable to attack; however, it didn’t work in
clinical trials. Some time later, it was discovered that, indeed, RNA worked as an antisense molecule in nature. The discovery aroused hopes for a new technique; unfortunately it was not very successful due to the instability of transporting these molecules through the organism. Nevertheless, the impact on research concerning RNA was considerable.\textsuperscript{11}

1.2.2.1. RNA as a target in therapy

Sullenger and Gilboa\textsuperscript{12} have divided the targeting of RNA in two different categories: RNA-mediated inhibition of gene expression and RNA-mediated repair of genetic instructions; an additional category, targeting RNA with small-molecule drugs, has been included.

\textit{RNA-mediated inhibition of gene expression}

\textbf{Antisense molecules} are complementary RNAs that form Watson-Crick base-pairs with their target mRNA and decrease the expression of a particular gene product by the degradation of RNA or the inhibition of its translation. One disadvantage of this method is the need for an excess of antisense RNA to target the RNA of the cell. A new antisense-type molecule is catalytic RNA which has been applied \textit{in vitro} as a \textit{trans}-cleaving ribozyme; this binds to the target RNA, cleaves it, and is recycled for multiple uses. Clinical trials are being effected for the use of ribozymes as cancer and HIV therapy; the success of these therapies will be marked by the effective delivery and lifetime of the ribozyme to the cells \textit{in vivo}, and the correct targeting of specific mRNAs.

\textbf{RNA interference} involves small double-stranded RNA (siRNA for short interfering or silencing RNAs) and has been shown to specifically suppress gene expression. When siRNA enters the cell, it is recognized as an intruder by an enzyme complex called Dicer. Dicer cuts the siRNA into short pieces about 22 nucleotides long which are then recognized and unwound by the RNA-Induced
Silencing Complex (RISC). RISC carries the short antisense strands around the cell looking for matching sequences in mRNA; then the RISC/RNA complex binds to its complementary mRNA, targeting its cleavage and preventing its replication.\textsuperscript{13}

The main disadvantages of both antisense and interference strategies are the unspecific protein binding and inefficient metabolic stability and cellular uptake of the intruder RNAs. In addition, many mRNA sequences are not accessible to antisense agents because they are highly structured or bound by cellular proteins.\textsuperscript{14}

**RNA aptamers** are molecules obtained through SELEX (systematic evolution of ligands by exponential enrichment). In this *in vitro* engineering process, RNAs with the ability to bind specifically to an exclusive target molecule (protein, DNA or RNA) are isolated and amplified. The procedure is then repeated several times until only high-affinity RNAs are obtained. It has been demonstrated that when an RNA aptamer binds in the 5'-UTR region of mRNA, it disrupts translation by halting the mRNA in a rigid complex that cannot be read by the ribosome.\textsuperscript{15}

**RNA-mediated repair of genetic instructions**

Repair of RNA is achieved by a process known as trans-splicing that uses spliceosomes or ribozymes to replace the mutant part of an RNA strand with a healthy piece. The drawback of this method is its low specificity, which can have grave consequences.

**Targeting RNA with small-molecule drugs**

RNA's structural diversity and complexity have made it difficult to define the specific binding sites for RNA molecules, and thus mainly antibacterial drugs and their analogs have been widely studied as small molecules targeting RNA. Some of the best studied RNA targets are the Rev Response Element (RRE) and the Trans Activation Responsive Element (TAR) present in the HIV-
1 mRNA and the Internal Ribosome Entry Sites (IRES) present in the hepatitis virus C, polio and foot-and-mouth disease viruses (Figure 5).

Figure 5. Secondary structure representation of HIV-1 TAR, HIV-1 RRE and HCV IRES. Arrows show the sites of interaction with its respective protein.

RRE RNA binds to the arginine-rich binding domain of the Rev protein, which facilitates the export of HIV mRNA out of the host nucleus and protects it from splicing machinery. The HIV mRNA major coding regions **gag**, **pol** and **env**, can further be translated to proteins that are essential for the formation of new viruses; mRNA can also be packaged to work as the genome for new viral particles. The Rev protein is composed of 116 amino acids distributed in at least three functional domains; the arginine-rich motif composed of residues 34-50 is the region that binds to RRE with high affinity. When RRE is targeted by small molecules, Rev cannot bind with the same affinity and thus, viral proteins are not produced; this leads to highly spliced HIV mRNA unable to produce the viral genome. A low mutation rate is characteristic of RRE RNA due to its importance on binding both the Rev protein, and the gp41 Env protein which is essential for the viral infection of a host cell.¹⁵
TAR RNA is a 59-base stem-loop segment located downstream of the transcriptional start signal in the 5' region of HIV-1 mRNA. TAR forms a complex with a regulatory protein called Tat that activates transcription of all genes in the virus. When this complex is targeted with small molecules such as a neomycin-acridine conjugate, HIV-1 transcription is reduced. As a result, this 'receptor-ligand' interaction has become a very important target and an area of opportunity for the development of new drugs.

The IRES elements are 400 nucleotide structures located at the 5’ end of viral mRNA. Unlike eukaryotic translation, viral IRES elements allow translation to start without the need of a 5’-cap recognition site; additionally, their primary and secondary structure are highly conserved which makes them a good target for small molecules.

One important advantage of targeting RNA with anti-infective agents is that appearance of resistance is likely to be slow since the structure of the target sites is highly conserved. Four other targets for small molecules are human mRNAs, tRNA, rRNA and human telomerase RNA.

Finally, rRNA is the main component of the ribosome which is divided in two subunits, one large and one small. In eukaryotic cells, the large subunit 60S contains a 28S rRNA, a 5S rRNA and about 50 proteins, and the small subunit 40S is formed of a 18S rRNA and 33 proteins. In prokaryotic cells, the size of the subunits vary; the large subunit 50S contains a 23S rRNA, a 5S rRNA and 34 proteins; and the small subunit 30S is formed of a 16S rRNA and 21 proteins.

1.2.2.2. Mechanism of RNA recognition by small molecules

RNA has the capacity to form complex three-dimensional structures that correlate with its diverse biological functions. This structural and functional diversity makes RNA more similar to proteins than to DNA. Even though RNA’s four base primary structure is noticeably less diverse
than the 20 amino acid of proteins, small-molecule interactions with RNA can have a profound effect on cellular function by inhibiting or incrementing the production of proteins. One important difference with proteins is that RNA’s biologically relevant secondary structure and protein-binding properties prevail in subdomains, the smallest practical unit for drug binding; this may lead to broad diversity in drug targeting strategies.\textsuperscript{16}

Ribosomal RNA is a cluster of irregular double helical stems and loops organized in a complex tertiary structure that can bind drugs in diverse recognition mechanisms. These mechanisms can be interactions between polar groups of a drug, such as tetracycline, and the sugar-phosphate backbone of nearby RNA helices and loops. Magnesium ions may also be important for these interactions since they facilitate the folding and enzymatic activity of RNA. Drugs can also mimic an RNA dinucleotide and stack with nearby stem-loops like pactamycin. Others can interact only with the phosphate groups (streptomycin), or mainly with the bases (spectinomycin).\textsuperscript{17, 18} A specific example is the binding, mainly through electrostatic interactions and hydrogen bonding, of the negatively charged rRNA with the aminoglycoside paromomycin, a positively charged antibiotic with flexible chains of aminosugar rings. In this interaction, primary amines or hydroxyl groups of the aminoglycoside act as hydrogen bond donors to specific nucleotide acceptors on the wide major groove of the target (Figure 6).\textsuperscript{19} The sugar rings help orient the donor groups for correct recognition.
In less complex RNA structures, small-molecule binding sites are generally more flexible and exposed to solvent. This flexibility may be seen as a drawback for drug design, however the possibility of allosteric effects and direct competition make up for this disadvantage. Allosteric regulation may be achieved by forcing the RNA to a new conformation more favorable for the binding of the small molecule and unfavorable for RNA’s activity, as seen in the binding of neomycin B to HIV-1 TAR RNA.20

Base stacking interactions between small molecules and RNA were studied by Hamy et al.,21 who concluded that the potency of acridine derivatives to inhibit Tat-TAR binding was in all tested cases superior to that of other heterocycles. Tat binding to TAR produces an important conformational change in the major groove of the RNA, which allows accessible recognition of the
molecule. The interaction of an arginine residue with 3 bases of TAR (U23, A22 and G26) is important for the Tat-TAR complex formation. A similar structural change happens when an acridine compound binds to TAR (Figure 7); however, since this compound does not have an amino acid residue to interact with, it is the heterocyclic moiety that works as a stacker to stabilize TAR. Stabilization is possible due to the interaction of N7 of G26 and N4 of C39 with the N9 and oxygen of the ether in the acridine moiety, respectively.

![Figure 7. TAR-acridine interaction.](image)

Acridine stabilizes TAR through hydrogen bond interactions with N7 of G26 and N4 of C39 of the viral RNA.

In the end, electrostatic interactions are critical for small molecule binding to RNA; they may increase the affinity for certain desired drugs, but they also decrease the specificity of the binding and cellular uptake.
1.2.2.3. Chemistry of small molecules binding to RNA

According to Ecker and Griffey, small molecules targeting RNA should possess the following properties: sufficient affinity for RNA to produce biological consequences in the cell after binding to the target, high cell penetration, good pharmacokinetic properties and acceptable toxicological properties.

Among the small molecules targeting RNA that have shown at least the first characteristic are the aminoglycosides antibiotics, other antibacterial such as oxazolidinones, diphenylfuran aromatic ring molecules, and agents containing acridines and naphthalimides linked to a flexible cationic aminoalkyl moiety and other variations.

Aminoglycosides antibiotics

The aminoglycoside family is formed of natural products that interfere with prokaryotic protein biosynthesis, and includes neomycin, paromomycin, lividomycin, kanamycin and gentamicin. Its ability to form strong non-specifically electrostatic interactions with RNA was described almost 25 years ago. These interactions displace cations already present in RNA, such as magnesium ions. However, selectivity toward certain sequences in the ribosome has also been demonstrated; in experiments using ribosomal A-sites (binding location of an aminoacyl-tRNA) with the single mutation A1408G, cells acquired resistance to aminoglycosides. Aminoglycosides achieve their antibacterial function by decreasing the fidelity of prokaryotic translation; the A1408G nucleotide substitution results in drug resistance due to the disruption of invariant hydrogen bonds between drug and RNA.

As shown before, crystal structures have been useful to understand the mechanism of recognition of RNA towards aminoglycosides (Figure 6); these have revealed that a fully
dehydrated ammonium ion in aminoglycosides with a pKa of 5.7 in solution can form a hydrogen bond with RNA increasing this pKa value to 6.1-6.4 for the adduct.\textsuperscript{24} Wang and Tor\textsuperscript{25} suggested this variation in pKa as the most likely mechanism for modulating the RNA affinity; they also described the energetic contribution of the pendant hydroxyl groups of the aminoglycosides after binding RNA. The important role of electrostatic interactions can be seen when a reduction in the number of hydroxyl groups increases aminoglycoside binding affinity due to a decrease in the alkalinity of the neighboring amine groups. In addition, solvent molecules, such as water, play a key role in the correct orientation of the aminoglycosides bound through hydrogen bonds to RNA.

In an attempt to improve the efficiency of aminoglycosides by reducing their toxicity and increasing their anti-HIV activity, acridine conjugates and guanidinoglycosides were synthesized.

\textit{Acridine and naphthalimide containing molecules}

Both acridines and aminoalkyl-linked naphthalimides act as intercalators to the double-helical DNA; but in RNA, where many of the bases are unpaired, these chromophores show stacking interactions without classical intercalation. An example is the Tat-acridine already presented in Section 1.2.2.2. Furthermore, Kirk \textit{et al.}\textsuperscript{26} showed that the combination of strong ionic interactions
with intercalation ability leads to potent inhibitors of Rev-RRE, mainly because RRE RNA is purine rich; additionally, intercalating agents preferentially bind to duplex regions containing a bulged base. In the neo-acridine conjugate (Figure 8a), acridine adds a strong ionic interaction to the antibiotic neomycin B moiety, which by itself has low affinity to the RRE. The length of the linker in neo-acridine has been modified to produce Neo-N-acridine (Figure 8b); which due to its shorter linker has fewer degrees of freedom and higher specificity towards RRE RNA.

**Guanidinoglycosides**

Tan and Frankel\textsuperscript{27} demonstrated that guanidinium groups in the Rev protein’s arginine rich domain helped in the recognition of RRE; they substituted arginines 35, 38, 39 and 44 with lysines resulting in a 20-fold loss of \textit{in vivo} RRE binding. Therefore, Luedtke and Tor\textsuperscript{15} synthesized new aminoglycoside conjugates such as Guanidino-neomycin B (Figure 9) by changing the amino groups into guanidinium groups; guanidinylation led to an increase in affinity but a decrease in specificity towards RRE. Affinity (the fastness) and specificity (the correctness) are two major characteristics of any nucleic acid-based assay that determine its efficiency. From their experiments, Luedtke and Tor proposed a general rule relating the total charge of a ligand to its RNA affinity and specificity, where the total number of basic groups in a ligand is proportional to the affinity to RRE, and the higher the affinity the lower the specificity towards RRE.

Addition of guanidinium groups to the aminoglycoside molecule provides a stronger positive overall charge due to its high alkalinity which explains the higher affinity of guanidinylated compounds for RNA, but not the specificity. The specificity was discovered to come from the important electrostatic interactions formed with the RNA that are less dependent on total ionic strength and more dependent on stacking interactions; the guanidinium group is planar, thus, it can stack and make hydrophobic contacts.
Diphenylfuran aromatic molecules

With the intent to counteract the low cellular permeability and toxicity of neomycin B and tobramycin, two aminoglycosides that inhibit Rev in vitro and block production of HIV, Zapp et al.\textsuperscript{28} tested different aromatic heterocyclic compounds known to bind to RNA and identified specific diphenylfuran derivatives. These compounds exclusively inhibit Rev-RRE interaction by binding to RRE. In fact, they showed that tetracationic diphenylfuran (AK.A) inhibits Rev-RRE interaction at a 10-fold-lower concentration than neomycin B. In addition, to identify the chemical moieties important for such interaction, they studied in vitro Rev binding to structurally related heterocycles. The results showed that the alkylamine substituents are essential, but not sufficient, for inhibition; and that a central resonance-stabilized heterocycle considerably enhances inhibition. Interaction of AK.A with nucleotide C74 prevents the formation of the C46:G74 base pair in RRE, preventing the binding of Rev; this base pair has been shown to be essential for Rev function.

Figure 9. Structure of Guanidino-neomycin B.
**Oxazolidinones**

Oxazolidinones, such as linezolid, are the first new class of antibiotics to enter into the market in 30 years; this type of compound has been very effective against multidrug-resistant bacteria. Oxazolinezolid linezolid is known to interfere with translation by stabilizing U2585 in the A-site; this drug changes the base orientation to induce a nonproductive conformation of the peptidyl transferase center (PTC), where translation takes place. It also overlaps with the aminoacyl moiety of an A-site bound tRNA.

Linezolid contains 3 aromatic rings and an acetamidomethyl tail as shown in Figure 10. It is the oxazolidinone ring, Ring A, that forms the only hydrogen bond interaction with the N3 of U2585, relegating all the other contacts of the drug with the rRNA to less energy-rich Van der Waals and hydrophobic interactions.²⁹

![Figure 10. Interaction of Linezolid with U2585 in the A-site during translation.](image-url)
1.2.4. Screening methods

Compared to proteins, RNA has several advantages associated with its easy preparation through chemical and enzymatic synthesis. Furthermore, its (typically) smaller size allows mainly localized interactions with ligands, leading to straightforward investigation methods.

Fluorescence-based techniques are currently the most common techniques used to identify ligand binding to RNA. Also used for this purpose is mass spectrometry, which has the advantage of not requiring any modification of the ligand or RNA. Nuclear magnetic resonance (NMR) spectroscopy, can also be used to monitor the binding of the ligands, and to design new lead compounds. In addition, circular dichroism (CD) spectropolarimetry has been a powerful tool to study secondary structure changes.

1.3. Platinum Drugs

1.3.1. Cisplatin and Pt-ACRAMTU

Cisplatin was first synthesized and described by Michael Peyrone in 1845, who named the compound Peyrone’s chloride; yet, the structure was not worked out until 1893 by Alfred Werner. A long time passed without cisplatin being recognized; but in 1965 Barnett Rosenberg noticed that, while using platinum electrodes to investigate the effect of the electric and magnetic dipoles in cell division, two compounds prevented this process from happening in bacteria: Pt\(^{\text{III}}\) (NH\(_3\))\(_2\)Cl\(_2\) (cisplatin) and Pt\(^{\text{IV}}\) (NH\(_3\))\(_2\)Cl\(_4\). In 1968, Rosenberg showed that cisplatin caused tumor regression in mice; this led to further studies and to its use to treat cancer patients for the first time in 1971. The US Food and Drug Administration (FDA) approved cisplatin for clinical use in 1978.
Cisplatin is very effective for testicular and ovarian cancers; however, it presents two main drawbacks. It is highly toxic to the kidneys and the gastrointestinal tract; and cells become resistant after several cycles of treatment, or are intrinsically resistant to it. These disadvantages have led to the discovery of new analogues (Figure 11) such as oxaliplatin (less resistance), and carboplatin (less toxicity).

![Figure 11. Cisplatin analogs a) Oxaliplatin. b) Carboplatin.](image)

Overcoming the resistance to cisplatin has been a challenge due to the many variants of this process. Resistance has been described as mediated by two mechanisms: first, insufficient amount of cisplatin reaching DNA; and, second, failure to induce apoptosis after DNA-adduct formation (Figure 12). Before binding DNA, cisplatin has to make its way into the cell either through passive diffusion or through copper transporters called CTR1. Some cells develop a resistance mechanism where CTR1 transporter levels decrease, and as a consequence there is a decrease in the uptake of cisplatin. Once inside the cell, cisplatin has to be activated by aquation, where one molecule of water substitutes one of the chloride leaving groups at the low concentrations of chloride inside the cell. The two ammine ligands of cisplatin, also called carrier ligands or non leaving groups, remain intact. Another resistance pathway is through detoxification by the conjugation of cisplatin to sulfur-containing amino acids present in glutathione and metallothioneins. These complexes get excreted, preventing the anticancer activity of cisplatin.33
Figure 12. Life of cisplatin inside the cell.

Cisplatin can undergo resistance pathways by forming complexes with sulfur-containing biomolecules or by increased efflux; when it enters the nucleus it binds to DNA.

When the hydrolysis products formed by cisplatin enter the nucleus, they bind DNA at the N7 position either of guanine (G), or less frequently, of adenine (A), to form monofunctional or bifunctional adducts after loss of one or both aqua ligands, respectively. Cisplatin can form intrastrand adducts by cross-linking two G bases of the same strand of DNA around 60% of the time; or by cross linking an A and a G base around 20% of the time; cisplatin can also form interstrand adducts around 2% of the time, linking two G bases from different strands (Figure 12). Adducts formed by cisplatin on DNA affect its secondary structure; the formation of 1,2-GG or 1,2-AG intrastrand crosslinks of cisplatin leads to marked conformational alterations such as an
induced kink of 26-50° towards the major groove, and local duplex unwind of 9-11° (see Figure 3b).34

Exploring new structural classes of platinum antitumor drugs resulted in the synthesis of Pt-ACRAMTU (Figure 13a), which provides the possibility of a hybrid binding mode: monofunctional platination plus intercalation. In addition, it is able to attack mainly two sites through two different mechanisms. In the first mechanism, intercalation of the acridine group in the major groove leads to the attachment of platinum to the N7 of guanine. In the second mechanism, the acridine group intercalates in the minor groove (mainly between 5'-TA-3' and 5'-CG-3') prior to the covalent binding of platinum to the N3 of adenine. For this latter adduct, it is the intercalating moiety that drives Pt-ACRAMTU to the site of platination. Unlike cisplatin, Pt-ACRAMTU interaction with DNA generates untwisting of the right-handed DNA helix by 21° per adduct, which does not evolve into a major destabilization (Figure 13b).35, 36

The incorporation of the acridine pharmacophores to Pt-ACRAMTU increases the ability of the whole molecule to pass through the membranes, helping overcome the uptake problems.37 The length of the thiourea linker is important as well, since a rigid acridinylthiourea derivative has proven to be biologically inactive.38 In addition, it has been shown that Pt-ACRAMTU triggers cell death making it very effective against non-small-cell lung cancers; and may involve a unique mechanism independent of both tumor suppressor gene p53 and oncogene K-ras.39
1.3.2. RNA and platinum drugs

DNA was described by Jamieson and Lippard as the primary target of cisplatin in cells over both RNA and proteins. However, several research groups have invested their time on building platinum complexes with higher selectivity for RNA.

Just recently, Boer et al. decided to combine the high selectivity of the aminoglycoside and guanidinoglycoside antibiotics toward RNA with the covalent character of PtII complexes to create a derivative that permanently damages RNA (Figure 14).

Figure 13. a) Pt-ACRAMTU structure. b) Pt-ACRAMTU bound to B-DNA.

Figure 14. a) Neomycin-PtII conjugate. b) Guanidino-neomycin-PtII conjugate.
Both conjugates, neomycin-PtII and guanidino-neomycin-PtII, bind RRE; the reaction is rapid and adducts are stable under denaturing conditions. In addition, both compounds covalently modify RRE with comparable efficiencies, and there is a significant selectivity for RNA over DNA. Reaction is predominantly 1:1 drug:RNA yet there is also unmodified and doubly conjugated RNA. It was recognized through alkaline hydrolytic footprinting that the neomycin-PtII conjugate binds to A48, A63 and G67 of RRE, similarly to cisplatin; in contrast, guanidino-neomycin-PtII tends to form intramolecular crosslinks between G48 and G67 (See Figure 5), also consistent with the behavior of cisplatin.

Intercalative platinum compounds first studied on RNA by Barton and Lippard in 1978,42 were reconsidered by Cusumano et al43 who studied the interaction of a series of polypyridine PtII complexes (Figure 15) with a [poly(A)-poly(U)] duplex. The reduced size of the dipyridyl ligand makes it capable of easily slipping between adjacent base pairs, generating an intercalative interaction. However, it was noticeable that the binding constants were lower for RNA than for DNA, suggesting that the A-form of RNA makes the insertion of the complex inside adjacent pairs more difficult.

![Figure 15. Rotamer of [Pt(dipy)(2-Mepy)]2+](image-url)
Members of Berube’s group have studied a new type of highly biologically active Pt complexes that contain an estrogenic moiety (Figure 16) and their tRNA binding behavior. tRNA remains in its A-form, however reduction of the base-stacking interaction and tRNA aggregation are seen upon drug complexation. The binding constants (K) calculated for cisplatin-tRNA and Pt estradiol-tRNA were $1.72 \times 10^4$ M$^{-1}$ and $2.77 \times 10^4$ M$^{-1}$, respectively. This group concluded that H-bonding interactions of estradiol with RNA bases in addition to Pt-base (G and A) binding may be responsible for the larger K value observed for the estradiol complex.

In addition to the analysis of RNA as a target for platinum compounds; new methods utilizing RNA-platinum adducts have been developed. Hägerlöf et al. have made cisplatin- and oxaliplatin-siRNA complexes in order to modulate the melting properties of siRNAs. siRNAs are designed considering two factors: the accessibility towards the mRNA target and the use of siRNA duplexes with melting profiles favoring the recognition of the antisense strand by RISC. It has been demonstrated that melting of the 5'-end of the antisense strand is favored over the 3'-end. However, when there are highly symmetric siRNAs and the mRNA region of interest is sequence-limited it is sometimes possible to achieve either the sense- or antisense-mRNA interaction. To increase the efficacy of this process, pre-platination of the sense strand of siRNA followed by annealing with Wnt-5a mRNA, which is a potential binding site for HuR protein, was done. When comparing the melting temperatures of platinated and non-platinated siRNA, it was seen that the Tm decreased

![Chemical structure of estradiol-Pt hybrid molecule.](image-url)
by 3-10 °C for the platinated siRNA due to the increase in both ΔH and ΔS; this phenomenon decreased local duplex stability and increased the availability of antisense strand. In addition, platination improves the efficacy of the antisense selection over non-platinated siRNA by more that 100%; this effect happens because the presence of the platinated site on the sense strand of siRNA acts as a block for loading onto RISC.45,46

Rijal and Chow47 developed a method in which cisplatin is used to probe accessible purine residues in free 16S rRNA, or in the context of 30S subunits or 70S ribosomes both in vivo and in vitro; formation of RNA-cisplatin adducts were mapped by reverse transcription. This method could be used for in vivo probing of bacterial ribosomes, in order to find new target sites for novel compounds.

1.4. Biological processes as targets for nucleic acid-binding drugs

The purpose of forming platinum drug adducts with either DNA or RNA is to intervene and disrupt the critical biological processes in cancer cells such that apoptosis would occur.

1.4.1. DNA-platinum adducts in biological processes

DNA-drug adducts typically alter the DNA’s macromolecule structure, and this alteration interferes with DNA-processing enzymes and DNA binding proteins to induce apoptosis of the cell, through either a direct or an indirect mechanism. The direct mechanism includes interference with DNA replication and transcription, which are essential processes in rapidly proliferating cancer cells. The indirect mechanism involves the recognition of the DNA lesion by over 20 proteins including high mobility group (HMG) domain proteins and TATA-binding protein (TBP). HMG domain
proteins bind in the minor groove of the kinked DNA-cisplatin adduct providing a shield against recognition by repair proteins. Additionally, when cisplatin binds to the TATA box, it prevents the binding of TBP (critical for the initiation of transcription) on the minor groove of DNA, and thus the reduction of normal gene transcription.\textsuperscript{48}

Cisplatin inhibits DNA synthesis by prokaryotic and eukaryotic DNA polymerases both \textit{in vivo} and \textit{in vitro}; replication inhibition occurs mainly at sites where a bifunctional adduct has been formed. However, this inhibition is not always complete; there is a bypass repair mechanism called translesion synthesis that uses special polymerases such as Pol $\eta$ or Pol $\zeta$ to plow through a blocking lesion (Figure 17b). Classical DNA polymerases such as Pol $\delta$ and Pol $\epsilon$ stall in lesions due to their closed active site (Figure 17a); in contrast translesion polymerases have the unique ability to replicate through a variety of distorting lesions as a result of an expanded active site. Translesion synthesis may be accompanied by mutations due to the drug that stays bound to the DNA; this mutagenicity may lead to the development of secondary tumors.\textsuperscript{49}

\begin{figure}
\begin{center}
\includegraphics{DNA_polymerases.png}
\end{center}
\caption{DNA polymerases.}
\textit{a)} Normal DNA polymerase only replicates one nucleotide at a time. \textit{b)} Pol $\eta$ can replicate two.
\end{figure}

Although inhibiting DNA replication is an important part of the mechanism underlying the antitumor effects of cisplatin, it cannot fully explain the antitumor efficiency of this drug, suggesting
the presence of a more complex mechanism. Transcription is another essential function affected by cisplatin lesions; in contrast to DNA polymerases, RNA polymerases completely bypass monofunctional adducts, yet are strongly inhibited by bifunctional ones. From their experiments, Corda et al.\textsuperscript{50} concluded that platinum adducts may not only constitute a physical barrier to the progress of the enzymes on the template, but they alter the properties of transcription complexes due to the conformational change they induce in DNA.

Pt-ACRAMTU covalent adducts to DNA have also proven to stall T7 RNA polymerase; the importance of this compound against cisplatin is its noticeably different damage selectivity by binding 5′-TA, 5′-CG and 5′GA sites, instead of 5′-GG and 5′-AG by cisplatin.\textsuperscript{51} The ability of Pt-ACRAMTU to bind at the A-N3 in the major groove, as well as some bases in the major groove, has also proved useful by targeting the TATA box, and thus competing with TBP.\textsuperscript{48}

1.4.2. RNA adducts in biological processes

RNA participates in different biological processes than DNA, so when drugs target RNA they disrupt processes where RNA has a central function such as translation. Binding of aminoglycosides to RNA, especially rRNA, leads to a bactericidal action that occurs due to a misreading of the genetic code. This misreading culminates in abnormal production of membrane proteins which allow more drugs to enter the cell, leading to apoptosis.\textsuperscript{52}

During translation, tRNA and mRNA become the ligands of the ribosome. On the small subunit, tRNA anticodons form Watson-Crick hydrogen bonds to mRNA codons. On the large subunit, the 3′-ends of aminoacyl-tRNA are in close proximity for peptide bond formation at the peptidyl-transferase center (PTC).\textsuperscript{53} Both small subunit and large subunit rRNAs are critical for providing
tRNA binding sites and promoting accurate peptide bond formation. Mutations in rRNA alter translation fidelity; in particular, the PTC is entirely composed of rRNA.54

Figure 18. Peptidyltransefase activity site.
A-site tRNA (green) and P-site tRNA (red) docked in a ribosome subunit (adopted from ref. 53).

Multiple RNA-RNA interactions dictate the binding of the codon-anticodon pairing in the ribosome, and small changes in energy between the different cognate and non-cognate pairings and the rRNA result in highly accurate translation.

The PTC is one of the major targets within the cell for antimicrobials. A mechanism for the inhibitory action of oxazolidinone linezolid during translation was proposed by Wilson et al and is presented in Figure 19. Linezolid can bind to the free A-site in the PTC, where it stabilizes U2585 in a nonproductive conformation. This base is required for the correct positioning of the peptidyl-tRNA, so the presence of linezolid indirectly affects the binding or positioning of the initiator tRNA, but does not interfere with the binding of the tertiary complex including GTP and elongation factor Tu (EF-Tu). However, the entering tRNA cannot be accommodated efficiently into the A-site because it is blocked by the drug, and therefore the tRNA will dissociate from the ribosome.
Figure 19. Mechanism for the inhibitory action of linezolid during mRNA translation.

Aminoglycosides of the subclasses paromomycin, tobramycin and streptomycin interfere with translation by binding to the A-site on the 16S rRNA in the ribosome, which is in charge of decoding the aminoacyl-transfer RNA affecting the proofreading step. These aminoglycosides disturb the stability of the tRNA-mRNA interaction with the rRNA by mimicking the codon:anticodon conformation and stabilizing the rRNA site.55

Streptomycin is tightly bound to the phosphate backbone of 16S RNA from four different parts of the molecule through both salt bridges and hydrogen bonds. The interactions observed indicate that it preferentially stabilizes the ram (ribosomal ambiguity) or “error-prone” form during translation instead of the alternative hyperaccurate or “restrictive” form; this would lead to increase binding of non-cognate tRNAs. The preferential stabilization of the ram state would also make the transition to the restrictive state more difficult, thereby also affecting proof-reading (Figure 20). As shown in Figure 6, paramomycin interacts with the major groove of 16S RNA, increasing the error rate of the ribosome by reducing the dissociation rate of A-site tRNA from the ribosome.
Figure 20. Interaction of streptomycin with the 30S ribosomal subunit.

Translocation of tRNA and its associated mRNA to read a new codon can also be impaired by aminoglycosides such as sparsomycin which performs the activities usually carried out by elongation factor G; or hygromycin that blocks translocation without inducing misreading. Sparcomycin binds in the minor groove at the end of the H34 element of the 16S RNA, and makes a single contact with a 2′-OH, as well as hydrogen bonds to a number of bases. This drug sterically blocks movement of tRNA during translocation and prevents changes in the conformation of H34 (Figure 21). 18, 57
Figure 21. Interactions of spectinomycin with the 16S ribosomal subunit.

In analogy to the mentioned aminoglycoside cases, it is important to target other RNA regions besides the ribosome; tobramycin binds to tRNA altering its conformation and preventing the binding of the synthetase.\textsuperscript{58}

Cisplatin’s kinetic preference for the G-U wobble base pair in tRNA\textsuperscript{Met}, and preferred interaction with the stem over the anticodon sequence of tRNA\textsuperscript{Ala} provides an additional explanation of cisplatin’s mechanism of action; since this specific wobble pair and the stem element are important for specific aminoacylation of tRNA\textsuperscript{Ala} and can potentially inhibit protein synthesis.\textsuperscript{59,60}
1.5. Goals of Master Research

1. To synthesize nucleic acids, using different biochemical techniques, for their use in subsequent analyses.

2. To analyze the binding behavior of Pt-ACRAMTU to RNA using native polyacrylamide gel electrophoresis (PAGE).

3. To probe the binding behavior of Pt-ACRAMTU to RNA using circular dichroism.

4. To study the binding behavior of Pt-ACRAMTU to RNA using LC-MS of acidic digestion.

5. To compare the relative binding affinity of Pt-ACRAMTU to RNA and DNA.
2.1. Starting materials

Three different RNA hairpins and an RNA duplex were prepared for analysis (Figure 22). Two different methods were used: plasmid transformation for RNAH2 hairpin, and oligodeoxy-nucleotides transcription for GNRA and UUCG hairpins.

**Figure 22. RNA targets prepared for analysis.**

Hairpin RNAH2 contains 26 bases and a loop with 5 bases AGAGA, hairpin GNRA contains 24 bases and a loop with 4 bases GUGA, hairpin UUCG contains 24 bases and a loop with 4 bases UUCG, and the RNA duplex contains 20 bases in a palindromic duplex. Two DNA duplexes, DNA 5-26 with the same sequence as RNAH2 and DNAa with the same sequence of the duplex RNA, were also used for the analysis.
**RNAH2 hairpin synthesis**

A ligation reaction was done with 13.85 ng of insert 5′-P-**GAT** CC G AGC TCC TGG TAC AAG GTT CTC TAC CTT GTA CCT ATA GTG AGT GTT ATT A-3′ (where the bold type indicates the BamH1 recognition site), and 5′-P-**AAT** TCT AAT ACG ACT CAC TAT AGG TAC AAG GTA GAG AAC CTT GTA CCA GGA GCT CG-3′ (where the bold type indicates the EcoR1 recognition site and the underlined segment indicates the T7 RNA polymerase promoter), 400 ng of vector (pMet/BamH1/EcoR1), 1 μl T4 DNA ligase and 2 μl 10X T4 DNA Ligase Buffer (New England BioLabs) to a volume of 20 μl. Presence of the insert in the plasmid was determined by digestion with restriction enzymes BamH1 (Promega) and EcoR1 (Promega). Transformation of plasmid was carried out in Novo Blue competent bacteria which was grown on LB Agar plates with 50 μg/ml ampicillin and liquid LB-Amp cultures, followed by its purification using a QIAfilter Plasmid Purification Mega.

To obtain the double stranded DNA suitable for in vitro run off transcription (DNA5-26 with sequence 5′-GGTACAAGGTAGAGAACCTTGTACCA-3′ and 5′-TGGTACAAGGTCTCTACCTTG TACC-3′), the plasmid (545.4 μg of DNA) was digested with 1 μl BstN1(10000 U/ml) in 1x NE Buffer 2 and 10 μg/ml BSA to a volume of 100 μl at 60°C overnight; both enzymes and buffer were from New England BioLabs. dsDNA was ethanol precipitated and redissolved in H2O; its concentration was determined with an Eppendorf BioPhotometer.

Transcription was performed in a 10 ml reaction containing 500 μg dsDNA, 500 μl T7 RNA polymerase, 10X transcription buffer (250 mM MgCl2, 20 mM Spermidine, 0.1% TritonX-100, 300 mM tris pH 8.1), 200 mM DTT, 1 ml PEG-8000 50% W/V, 4 mM NTPs; the reaction mixture was incubated at 37°C for 8–10 h. The hairpin was purified by polyacrylamide gel electrophoresis (16%, 19:1 acrylamide:bisacrylamide/8 M urea) in 1% tris-borate-EDTA (TBE) buffer (90 mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Bands corresponding to full length deprotected
oligonucleotides were excised, homogenized and soaked in 0.5 M NH₄OAc, followed by ethanol precipitation.

**Plasmid transformation**

![Plasmid transformation diagram](image)

**Oligodeoxy-nucleotides transcription**

![Oligodeoxy-nucleotides transcription diagram](image)

Figure 23. Methodologies for RNA hairpins syntheses.

Two different methods were used for the synthesis of the oligonucleotides: plasmid transformation for RNAH2 hairpin, and oligodeoxy-nucleotides transcription for GNRA and UUCG hairpins.

**GNRA and UUCG hairpin syntheses**

Synthesis of both hairpins was done following Sherlin *et al.* methodology; oligodeoxy-nucleotides used as templates for enzymatic transcription were obtained from Integrated DNA
Technologies (Ceralville, IA) in PAGE-purified form; this methodology was more time and product efficient. The oligonucleotides used for construction of the UUCG hairpin were the complementary sequences: 5′-AAT TCC TGC AGT AAT ACG ACT CAC TAT AGG TAC AAG GTT TCG ACC TTG TAC C-3′, and 5′-mGmGT ACA AGG TCG AAA CCT TGT ACC TAT AGT GAG TCG TAT TAC TGC AGG AAT T-3′; and for the GNRA hairpin the complementary sequences were: 5′-AAT TCC TGC AGT AAT ACG ACT CAC TAT AGG TAC AAG GTG TGA ACC TTG TAC C-3′, and 5′-mGmGT ACA AGG TTC ACA CCT TGT ACC TAT AGT GAG TCG TAT TAC TGC AGG AAT T-3′, where mG represents an 2′-O-methyl nucleotide and the bold type indicates the T7 RNA polymerase promoter. Oligonucleotides were mixed to an equimolar concentration of 4 μM in a reaction solution containing 400 μM dNTPs, 10 mM Tris-HCl, pH 7.5, 10 mM MgSO4, 7.5 mM DTT, and 50 U/ml Klenow fragment polymerase (Promega). The mixture was cycled between 10°C and 37°C at 30s intervals for eight cycles, after which the DNA was ethanol precipitated, pelleted, and resuspended in H2O.

Transcription was performed in a 10 ml solution containing 500 μg dsDNA, 500 μl T7 RNA polymerase, 10X transcription buffer (25 mM MgCl2, 2 mM Spermidine, 0.01% TritonX-100, 30 mM tris pH 8.1), 200 mM DTT, 1 ml PEG-8000 50% W/V, 4 mM NTPs; the reaction mixture was incubated at 37°C for 8–10 h. The hairpin was purified by polyacrylamide gel electrophoresis (16%, 19:1 acrylamide:bisacrylamide/ 8 M urea) in 1% TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Bands corresponding to full length deprotected oligonucleotides were excised, homogenized and soaked in 0.5 M NH4OAc, followed by ethanol precipitation.

RNA duplex synthesis

The RNA duplex was synthesized and base deprotected by the WFUBMC DNA synthesis laboratory using Pac-protected phosphoramidites and was base deprotected with ammonia.
Further removal of the 2′-O-alkylsilyl protecting group was accomplished following Wincott et al. methodology; the base-deprotected oligoribonucleotide was resuspended in anhydrous TEA-HF/NMP solution (250 μl of a solution of 1.5 ml N-methylpyrrolidinone, 750 μl TEA and 1.0 ml TEA.3HF to provide a 1.4 M HF concentration) and heated to 65°C for 1.5 h. The resulting fully deprotected oligomer was precipitated directly from the desilylation reaction by addition of 3 M NaOAc (25 μl) followed by addition of n-BuOH (1 ml). The mixture was cooled to −20°C overnight and then centrifuged at 4°C, 13000 rpm for 30 min. The deprotected sample was estimated by analytical denaturing PAGE to be greater than 95% pure so no further purification was done. The solution was decanted, the pellet washed with 70% EtOH and then dried. Aqueous stock solutions of the oligonucleotides were stored at −20°C until used.

DNA duplex and platinum compounds

The DNA duplex was synthesized and desalted by IDT (Integrated DNA Technologies; Coralville, IA). The sequence is the same as the one for duplex RNA (Figure 22). They were dissolved in H₂O and stored at −20 °C. Synthesis and characterization of both ACRAMTU and Pt-ACRAMTU were published before. Stock solutions of both compounds (aqueous) were prepared at 25°C and stored in the dark at −4°C. Concentration of both compounds was calculated using ε = 9450 l/cm (mol).

2.2. PAGE Analysis

Platination of RNA and DNA.

Prior to the platination reactions, the oligonucleotides were annealed by heating to 90°C for 5 min followed by slow cooling to room temperature in a dry bath. Reactions were performed in
Buffer C (10 mM NaH$_2$PO$_4$, 0.1 mM EDTA, 2 mM MgCl$_2$) with RNA and DNA concentrations of 296 μM in 10 μl. Both RNA and DNA oligonucleotides were incubated with 0 – 10 molar equivalents (drug:duplex) of Pt-ACRAMTU, and the reactions were incubated at 25°C for 48 h in the dark.

**Polyacrylamide gel analysis**

Duplex-drug adducts were analyzed by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions (16% acrylamide, 19:1 acrylamide:bisacrylamide, 1xTBE) at 150 V for 1 h. The gel was then stained with ethidium bromide and dried for visualization.

### 2.3. Circular Dichroism Analysis

Reactions of Pt-ACRAMTU-oligonucleotide were prepared the same way as in PAGE experiments with hairpin and duplex concentrations of 296 μM and 740 μM in CD experiments.

CD spectra were recorded on an AVIV Model 215 spectrophotometer. The scanning wavelength ranged from 220 to 600 nm with scanning speed of 1 nm/s and response times of 2 to 5 s. CD profiles were base-line adjusted by subtracting buffer background.

### 2.4. Acidic Digestion

The DNA sequences [296 μM (nt)] were incubated with Pt-ACRAMTU at a platinum-to-nucleotide ratio (rb) of 0.4 and 0.6 at 37°C for 48 h in the dark. After incubation, the samples were dialyzed against Millipore water in a 28-well dialysis apparatus at 4°C for 24 h in the dark to remove unreacted drug. The molecular-weight cut-off (MWCO) of the dialysis membrane (regenerated cellulose, Spectrum Laboratories; Rancho Dominguez, CA) was 2000 Da. The pH of the samples was adjusted to 2.2–2.4 using formic acid. Acidified samples were digested at 55°C
for 16 h in the dark, centrifuged for 10 minutes at 13,400 rpm, and the supernatant collected and immediately injected into the HPLC system.

2.5. LC-MS analysis

*Chromatographic Separations*

The mixtures resulting from acidic digestion assays were directly injected into the HPLC using the LC module of an Agilent Technologies 1100 LC/MSD Trap system equipped with a multi-wavelength diode-array detector and autosampler. Two wavelengths, 254 nm and 413 nm, were used to monitor unmodified nucleic acid and ACRAMTU-containing fragments, respectively. The following eluents were used during the direct injection: solvent A, degassed water containing 1% formic acid, and solvent B, acetonitrile. The flow rate was 0.5 ml/min for both solvents at 50%. The integration of peaks was done using the LC/MSD Trap Control 4.0 data analysis software.

*Mass Spectrometry*

Mass spectra were recorded on an Agilent 1100 LC/MSD ion trap mass spectrometer. After separation by in-line HPLC, adducts were infused into the atmospheric-pressure electrospray source. Ion evaporation was assisted by a flow of N₂ drying gas (325°C) at a pressure of 55 psi and a flow rate of 10 l/min. For acidic digests, positive-mode mass spectra and tandem mass spectra (MS/MS) experiments were performed with a capillary voltage of 2800 V and a scan range of 100–800 m/z. Product-ion mass spectra in MS/MS experiments were generated by collision-induced dissociation (CID) with the assistance of helium as bath gas.
3.1. Polyacrylamide Gel Electrophoresis (PAGE)

3.1.1. Background

Electrophoresis separation techniques are widely used for biological and biochemical research because high separation efficiency can be achieved using relatively small amounts of inexpensive equipment. This method is based on the ability of charged molecules to migrate in the direction of an electrode bearing the opposite charge, under the influence of an electrical field. Electrophoretic separations are carried out in free solutions as in capillary and free flow systems, or in stabilizing media such as thin-layer plates, films or gels.

Acrylamide monomers are crosslinked in a co-polymerization reaction with TEMED (Tetramethylethylenediamine) and 10% APS (Ammonium persulfate) to produce a chemically inert and mechanically stable polyacrylamide gel; these types of gels were first used for electrophoresis by Raymond and Weintraub in 1959.

Polyacrylamide gels are placed in an apparatus in which the gel stands vertically supported in a sandwich between two glass plates; the top of the gel is connected by a salt solution to an electrode. A nucleic acid mixture is applied to the top of the gel slab, and the electrophoresis is started by applying a voltage (~150 V) across the gel. The rate of migration of a nucleic acid in the gel depends on two properties: the first is its net charge; molecules with the most charge (of a sign opposite to that of the far electrode) will migrate fastest to the far electrode. The second is the size, which is proportional to the molecular weight (MW) if spherical. The gel consists of a network of fibers; depending on the concentration of polyacrylamide, the network can be dense or tight, and
so nucleic acids have trouble migrating depending on how tangled the fibers are. The smallest and most highly charged nucleic acids will arrive to the bottom first. After the electrophoresis has been stopped, molecules will be distributed along the gel length according to these two characteristics (MW and net charge). For instance, a highly charged nucleic acid molecule, although pulled with a greater electromotive force, will go very far through the gel if it is relatively large (Figure 24).

Figure 24. Schematic of PAGE. Particles run at different speeds depending on size and charge.

Denaturing gels contain urea in order to disrupt the secondary structure of the nucleic acid that is being analyzed; in contrast, non-denaturing gels allow the nucleic acid to run without any change in its secondary structure. Using non-denaturing gels, change in the shape of RNA can be recognized in addition to a change in the charge and the molecular weight.

This method has been used before by Boer et al. to determine the binding behavior of neomycin-Pt\(^{II}\) and guanidino-neomycin-Pt\(^{II}\) to RRE. In this chapter, the binding behavior of Pt-
ACRAMTU to the shape and length diversified oligonucleotides is characterized using the PAGE technique.

### 3.1.2. Results

Lanes 1 and 2 of a non-denaturing polyacrylamide gel (Figure 25) show the oligonucleotide hairpin RNAH2 without the addition of Pt-ACRAMTU. RNA in lane 2 has been treated with urea and heat to denature it, yet no significant change in band migration is visible. Lanes 3 to 12 show hairpin RNAH2 treated with 1 equivalent up to 10 equivalents of Pt-ACRAMTU. Binding of one equivalent of Pt-ACRAMTU to the nucleic acid is indicated by the shift of the band in the gel, from the level of the free RNA to a higher level; this signifies a slower movement of RNA and indicates an increase in the molecular weight and/or a decrease in charge of the RNA hairpin. Addition of 2 equivalents of Pt-ACRAMTU to RNAH2 does not appear to shift the band, meaning no additional binding of Pt-ACRAMTU occurs. As the number of equivalents of Pt-ACRAMTU increases, so does the level of the nucleic acid in the gel which represents the binding of more than one molecule of Pt-ACRAMTU to the RNA hairpin. A decrease in band intensity starts after 3 equivalents of the drug have been added; this phenomenon suggests either that addition of more than 1 molecule of Pt-ACRAMTU to the hairpin leads to RNA degradation or may be due to decreased ethidium bromide binding because of the presence of acridine.

GNRA and UUCG present a similar pattern to that of RNAH2. Pt-ACRAMTU binds to GNRA after 1 equivalent has been added; however, the presence of two bands, one at the level of free RNA and 1 shifted to higher level suggests that GNRA hairpin does not bind completely to Pt-ACRAMTU after one equivalent of the drug has been added. This may be due to a slower reaction of GNRA with the drug. GRNA hairpin seems to be more stable to the binding of multiple molecules.
of drug than RNAH2. The same binding pattern is present for UUCG hairpin; however, it is important to notice that the bands still exhibit strong fluorescence despite the addition of 10 equivalents of the drug. This may indicate increased resistance to degradation. The presence of 4 different levels of bands in the gel for UUCG hairpin strongly suggests that this nucleic acid can bind to up to 4 molecules of Pt-ACRAMTU per hairpin.

RNA bands, in contrast to the RNA hairpins bands, are faint even after only 1 equivalent of Pt-ACRAMTU has been added, which suggests RNA degradation upon drug binding. However, binding of 1 equivalent of Pt-ACRAMTU does seem to be complete suggesting a fast reaction of the drug to the duplex, just as with RNAH2 hairpin. Further evidence of a faster binding of the drug to the oligonucleotide, compared to UUCG and GNRA hairpins, is the appearance of two bands after only 1 equivalent of Pt-ACRAMTU, which imply the formation of a 1:2 complex \[d(GCCAUAUGGC)_2(Pt-ACRAMTU)_2\].

DNA behaves in the same way as does RNA; degradation of the duplex or inhibition of ethidium bromide is noticed even after 1 equivalent of Pt-ACRAMTU has been added. One important difference compared to RNA is the significant formation of a multiple complex (more than one molecule of Pt-ACRAMTU per duplex) after 1 equivalent of Pt-ACRAMTU, which can be observed in the presence of 3 different bands in lane 3 of the DNA non-denaturing polyacrylamide gel. The presence of this multiple complex suggests a faster reaction of DNA with Pt-ACRAMTU.
Figure 25. PAGE data.

Non-denaturing polyacrylamide gels of hairpins RNH2, GNRA and UUCG, and duplexes RNA\textsubscript{d} and DNA\textsubscript{d}. Line 1 – control, 296 μM oligonucleotide in buffer C, line 2 – 296 μM denatured
oligonucleotide, line 3-12 – oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 -10 equivalents).

3.2. Circular Dichroism

3.2.1. Background

Circular Dichroism (CD) is a spectroscopic technique which measures differences in the absorption of left-handed circularly polarized light and right-handed circularly polarized light by a chiral molecule. The circular dichroism spectrophotometer is formed by a light source that emits an unpolarized light which is divided into its vertical and horizontal linearly polarized components of a single wavelength. This light is then passed through a silica photo-elastic modulator (PEM) which is made to oscillate typically at a frequency of 50 kHz to get birefringence. A birefringent material puts the two components of linearly polarized light out of phase producing circular polarized light (CPL); the components travel to different velocities inside the birefringent material due to the difference in indices of refraction. The CPL created has either a left-handed direction or a right-handed one. At specific wavelengths, chiral molecules can absorb the components of the CPL in different ratios due to the distinct extinction coefficients of the two polarized rays. If there is no circularly dichroic sample in the light path, the light hitting the detector is constant, but if there is a chiral sample in the light path, the recorded light intensity will be different for right- and left-CPL. Using a lock-in amplifier tuned to the frequency of the PEM, it is possible to measure the difference in intensity between the two circular polarizations. This signal difference is displayed in a spectrogram as molar extinction coefficient ($\Delta\varepsilon$) with units $\text{M}^{-1}\text{cm}^{-1}$ as a function of wavelength in nm (Figure 26).
The differential absorbance of left (ALCP) and right circularly polarized (ARCP) light can be expressed as: ΔA = ALCP – ARCP. However, taking into account the cell pathlength and compound concentration, molar circular dichroism (Δε) can be described as: Δε = εLCP - εRCP = ΔA/(C x l). Where εLCP and εRCP are the molar extinction coefficients for LCP and RCP light respectively, C = molar concentration, and l = pathlength in centimeters.

The chirality of a molecule produces electric and magnetic transition moments that lead to optical activity. Heterocyclic bases of RNA are not chiral; however, they become chiral when placed within the framework of the chiral sugar-phosphate backbone. Stereospecific coupling of degenerate or near-degenerate electronic transitions of neighboring base chromophores results in a CD signal. Many ligands are achiral, but they present an induced CD (ICD) signal when interacting with the chiral DNA. For a specific ligand, there are three possible binding modes to DNA: intercalation between base pairs, minor groove binding, and major groove binding. Intercalators generally exhibit small ICD signals (< 10 M⁻¹cm⁻¹); in contrast, large ICD signals are indicative of a groove-binding geometry. Calculations by Fornasiero et al. suggest that the ICD signal of a groove binder is 1-2 orders of magnitude greater than that of an intercalator. ICD signal
intensity and sign depend on the orientation of the ligand bound to RNA; they also depend on the ligand binding as a monomer or a dimer.68

Figure 27. Three modes of binding to DNA.
From left to right: intercalation between base pairs, minor groove binding, and major groove binding.

When there is an encounter between a chiral host and an achiral guest, two situations can occur: the chiral host transforms the guest into a chiral molecule by changing its structure; or the guest’s chiral transformation occurs without structural perturbation by an effective coupling between the electric transition moments of the two molecules.

Rotational strength (R) is used to define the electric (μ) and magnetic (m) transition moment vector contributions; R for an electronic transition from ground energy to a energy is called R_{0a}, and its definition is R_{0a} = μ • m = |μ| • |m| cosφ; where φ is the angle between the two vectors. It has been shown theoretically that R for the ICD bands is proportional to ΔΣ(μ^2GF/Δi) where GF_i equals the individual geometric arrangements between the transition moments of the host and the guest, and Δ_i is related to the absorption frequency differences between host and guest. The rapid decrease of R with increasing delta leads to a much smaller value of Δε in ICD as compared to
normal CD; usually, it is at least one order of magnitude smaller. Therefore, it is essential that the achiral component has a strong chromophore (large $\varepsilon$-value).

Platinum compounds’ binding behavior to DNA has been studied using Circular Dichroism. Spermidine platinum compounds such as the one depicted in Figure 28 have shown rapid binding to DNA; in addition, this type of compounds is able to induce both B $\rightarrow$ A and B $\rightarrow$ Z transition which can be verified using CD. B $\rightarrow$ A transitions can work as control mechanisms for transcription factors binding DNA because they recognize not only bases, but also the conformation of the helix among sequence. For the B $\rightarrow$ Z transition, there is an obvious shift from positive to negative CD due to the left handed nature of Z DNA.\textsuperscript{69}

\begin{center}
\includegraphics[width=0.5\textwidth]{figure28.png}
\end{center}

\textit{Figure 28. Spermidine platinum compound.}

Intercalation of acridine moiety of Pt-ACRAMTU to DNA has been characterized using CD. For the CD region (220 – 320 nm), the intensity of the spectrum changes when increasing the concentration of the drug, but its shape remains the same during the experiment. This suggests that the ligand binding mode is unchanged even when its amount is changing. However, when looking at the ICD region (320 – 480 nm), the appearance of a negative band represents a different binding mode for Pt-ACRAMTU which has been described as a $\pi - \pi^*$ transition for the acridine group intercalating between the bases.
3.2.1. Results

Circular dichroism spectra in Figure 29 show that upon titration of 1 equivalent of ACRAMTU into DNA 5-26, the adduct produces a CD spectrum typical of a B-form DNA with positive and negative Cotton effects at 268 nm and 249 nm, respectively, in accordance with previous biophysical studies of this compound. As shown by Baruah and Bierbach, addition of further equivalents of ACRAMTU to DNA 5-26 produces a pronounced decrease in the intensity, but no shifts. In contrast, CD spectra of DNA 5-26 titrated with 1 equivalent of Pt-ACRAMTU show the distinct hyperchromic effect and bathochromic shifts of the negative and positive bands to 279 and 252 nm (Figure 30). The red-shift is accompanied by an increase in intensity in both the negative and positive bands. These results agree with what has been published before by Baruah and Bierbach that the conformational changes in double-stranded DNA caused by ACRAMTU and Pt-ACRAMTU are distinctly different. For both the reversible (ACRAMTU) and irreversible (Pt-ACRAMTU) DNA binders, the duplexes appear to retain overall B-form parameters, and the observed spectral changes most likely reflect local perturbation of the helices at the adduct sites.

The induced circular dichroism (ICD) spectra in the 320–460 nm region is where the 9-aminoacridine chromophore absorbs (Figure 30b). While the intensity of the bands centered on 330 and 420 nm is characteristic of intercalators, the signs of the signal give important insight into the binding geometry of the planar chromophore with respect to the stacking base pairs. Previous studies have assigned the two $\pi - \pi^*$ transitions at 330 and 420 nm to electronic transitions polarized along the long and short axis of the 9-aminoacridine chromophore. The ICD band at 420 nm, which mimics the absorption spectrum of the chromophore by including its vibrational substructure, has a positive sign only for the duplex modified with PT-ACRAMTU. In ICD spectra of simple 9-aminoacridine, positive bands are observed for both transitions; this has
been suggested as evidence that the long axis of the chromophore is oriented parallel to the Watson-Crick hydrogen bonds.\textsuperscript{72} The negative sign for the long-axis transition observed for platinum-tethered ACRAMTU suggests a geometry in which the chromophore is twisted away from the parallel orientation, resulting in a more perpendicular penetration of the duplex with respect to one or both of the adjacent base pairs.\textsuperscript{73} An explanation of this phenomenon has been provided by Baruah and Bierbach who have demonstrated that intercalation of acridine in adducts of the platinum conjugate is restricted due to the nature of the covalent linkage. Molecular modeling data shows that even when the DNA retains its right-handed B-form with anti glycosidic torsion angles, the platinum adduct region presents a modification in the sugar pucker of some nucleotides where there has been a transition from the C2\textsuperscript{'-endo conformation to the C1\textsuperscript{'-exo or C3\textsuperscript{'-endo conformations. In addition, intercalation of the acridine moiety causes significant distortions in the base pair adjacent to the platinum binding site (G7-N7). A reduced twist angle of 18.9° is also present, which signifies that the duplex is noticeably unwound at the central base step; this effect extends into the neighboring base steps. In addition, the covalent G7-N7 linkage of the DNA-Pt-ACRAMTU adduct produces close contacts between the thiourea linker and the walls of the major groove, which prevents the planar chromophore from penetrating deeply into the base stack.

Unlike DNA, Pt-ACRAMTU turns the A-form RNA into an oligonucleotide with B-form characteristics. CD spectra for both hairpin and duplex RNA present the typical A-form RNA with positive and negative Cotton effects at 260 nm and 235 nm. Addition of Pt-ACRAMTU produces a pronounced bathochromic shift in the positive CD bands to 279 nm in all cases (Figure 31 - 33, 36 - 37). A stepwise transition from the A- to the B-form is more visible for the RNA duplex than for the RNA hairpins, this indicates a different binding mode for each type of nucleic acid. More information can be obtained with the ICD data that is only available for UUCG hairpin (Figure 33b) and RNAd (Figure 37b). The binding behavior of UUCG (Figure 33b) is similar to that of DNA 5-26:
signals at 330 and 420 nm correspond to electronic transitions polarized along the long and short axis of the 9-aminoacridine chromophore, and there appears to be a perpendicular interaction of the ligand with the bases represented by the negative signal at 330 showing there is restriction for the intercalation of the ligand. No ICD data was observed for RNAH2 and GNRA hairpins, strongly suggesting that the platinum moiety in Pt-ACRAMTU binds preferably to the A or G bases in the loop of these two hairpins. In order to understand the importance of the loop in the binding mode of Pt-ACRAMTU, the RNA duplex was designed. An ICD signal was present for RNAd as for UUCG. We conclude that the loop is the binding site for Pt-ACRAMTU in RNAH2 and GNRA hairpins, since there is evidence for binding but not intercalation. Duplex DNA shows the same Pt-ACRAMTU binding behavior as does DNA 5-26, which is expected since both oligonucleotide have the same secondary structure.

After addition of 5 equivalents of Pt-ACRAMTU, there seems to be a significant degradation of the hairpin, indicated by an almost quenched CD spectrum; this apparent degradation is faster for both RNAH2 (Figure 31a) and GNRA (Figure 32a) than for UUCG (Figure 33a). These data are consistent with the results seen for PAGE where more intense bands exist at 5 equivalents for UUCG, indicating the highest stability of this hairpin toward Pt-ACRAMTU.

Finally, Figure 38 shows that ACRAMTU does not intercalate in dRNA, this is also true for the 3 hairpins. This result is expected since A-RNA base pairs are inclined 15–20° to the overall helical axis, in contrast to B-RNA where base pairs are roughly perpendicular to the helical axis, and they impede the intercalation of acridine.
Figure 29. CD and ICD data for DNA5-26 treated with ACRAMTU.

a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified DNA5-26. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of ACRAMTU (1 – 5 equivalents).

b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified DNA5-26 duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of ACRAMTU (1 – 5 equivalents).
Figure 30. CD and ICD data for DNA5-26 treated with Pt-ACRAMTU.

a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified DNA5-26 duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 10 equivalents).

b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified DNA5-26 duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 10 equivalents).
Figure 31. CD data for RNAH2 hairpin treated with Pt-ACRAMTU.

CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified RNAH2 hairpin. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).
Figure 32. CD data for GNRA hairpin treated with Pt-ACRAMTU.

CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified GRNA hairpin. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).
Figure 33. CD and ICD data for UUCG hairpin treated with Pt-ACRAMTU.

a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified UUCG hairpin. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1–10 equivalents).  

b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified UUCG hairpin. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).
Figure 34. CD and ICD data for DNAd duplex (296 μM) treated with Pt-ACRAMTU.

a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified DNAd. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).

b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified DNA5-26 duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).
Figure 35. CD and ICD data for DNA duplex (740 μM) treated with Pt-ACRAMTU.
a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified DNA. Black line – 740 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 –5 equivalents). b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified DNA duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).
Figure 36. CD and ICD data for RNAd duplex (296 μM) treated with Pt-ACRAMTU.

a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified RNAd. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).

b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified RNAd duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).
Figure 37. CD and ICD data for RNAd duplex (740 μM) treated with Pt-ACRAMTU.

a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified RNAd. Black line – 740 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).

b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified RNAd duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of Pt-ACRAMTU (1 – 5 equivalents).
Figure 38. CD and ICD data for RNAd duplex (296 μM) treated with ACRAMTU.

a) CD spectra (220 – 320 nm) recorded at 25 °C of the unmodified and drug-modified RNAd. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of ACRAMTU (1 – 5 equivalents). b) ICD spectra (320 – 480 nm) recorded at 25 °C of the unmodified and drug-modified RNAd duplex. Black line – 296 μM oligonucleotide in buffer C, color coded lines show oligonucleotide treated with increasing concentration of ACRAMTU (1 – 5 equivalents).
3.3. Liquid Chromatography-Mass Spectrometry

3.3.1. Background

Mass spectrometry, in addition to being rapid, sensitive and useful for complex mixtures, may be relatively automated and is not affected by modified bases. It requires the formation of gas-phase ions to be separated by their mass-to-charge (m/z) ratio. The mass of the analyte can be calculated from the m/z ratio of the ion. Because of the multiple instruments used for the formation of ions and their separation, there are plenty of coupled devices on the market.

Both matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) mass spectrometry have been especially useful for the analysis of polar biomolecules such as proteins and peptides; as well as non-polar carbohydrates, lipids and metabolites.

Sample preparation is a key step in the mass spectrometry of RNA. The sensitivity of MALDI may be compromised if SDS, glycerol, EDTA and MgCl$_2$ among other common chemicals are used; also, the oligonucleotides must be desalted by utilizing ammonium-ion forms of cation-exchange beads, reverse-phase HPLC, or C$_{18}$ reverse-phase pipette tips (ZipTips), in order to avoid the formation of adducts between RNA and Na$^+$ or K$^+$ ions.

To carry out ESI mass spectrometry studies, the analyte is introduced into the ion source of the mass spectrometer embedded in a volatile binary aqueous-organic solvent phase. Ions are formed through high voltage generated in a metal-coated glass capillary in the injection chamber. While MALDI produces single charge ions, ESI generate ions with different charges of the form (M$+n$H)$^{n+}$ or (M$-n$H)$^{n-}$, consistently with the gain (positive ions) or loss (negative ions) of protons; M stands for the oligonucleotide analyte and n is the charge state according to the number of protons being attached or lost.
In order to reliably identify an RNA sequence it is necessary to use mass spectrometers with high mass accuracy and resolving power. It is known that the major limitation when sequencing oligonucleotides is that uridine and cytidine have a mass difference of only 1 Da which makes it very difficult to differentiate; in contrast, in DNA the smallest difference is of 9 Da between adenosine and thymine. This fact restricts the maximum length of an RNA chain correctly identified unambiguously. Among the additional weaknesses for this sequencing technique are the complicated procedures for making entire sequence ladders from digest, and the problems of ionizing and fragmenting large oligonucleotides during CID.74

In this section, acidic digestion as the treatment of the RNA samples has been used, and the detection of the Pt-ACRAMTU-purine adducts was achieved using direct injection in LC-MS.
3.3.2. Results

Electrospray ionization mass spectra of the dRNA complex [d(GCCAUAUGGC)₂(Plt-ACRAMTU)] (Figure 40) demonstrates that the main species released in this digestion assay is an isomer of the monofunctional platinum–adenine adduct, [Pt(ACRAMTU)(en)(adenine)-H]+ (M_r = 715.3). Mass spectra recorded in positive-ion mode show peaks characteristic of fragment ions produced by collisionally induced dissociation (CID). These include fragments resulting from loss of adenine base (m/z 579.3) and dissociation of the Pt–S bond (m/z 325.2). Based on the previously data published by Barry et al., it is difficult to determine where exactly Pt-ACRAMTU is binding in RNA. However, as RNA has a much wider, and thus more accessible minor groove than DNA, it is possible that Pt-ACRAMTU favors binding to N3 in the minor groove of dRNA.

Barry et al. described that, in DNA, protonation of guanine is prohibited due to the binding selectivity of the metal to the N7 position. In contrast, RNA binding of guanine seems possible as presented in Figure 41 where fragments resulting from loss of the guanine base (m/z 578.3 and m/z 153.1) are present. Figure 39 presents the acidic depurination reaction for the RNA duplex where platinated N3 of adenine is represented; in the same way, guanine can be platinated at the N7 position leading to depurination.

Mass spectra for adenine adduct formation with Pt-ACRAMTU in DNA (Figure 42) shows the lower abundance of this adduct in the sample compared to adenine adduct formation in RNA.

LC-MS data was not obtained for UUCG and GNRA hairpins since comparison with its DNA duplexes was not possible.
Figure 39. LC-MS of Acidic digested samples representation. Acridurination reaction where * represents the site of platination, and LC-MS apparatus diagram.
Figure 40. Positive-mode mass spectra and summary of fragments for adenine-Pt-ACRAMTU complex after RNA duplex acidic treatment.
Figure 41. Positive-mode mass spectra and summary of fragments for guanine-Pt-ACRAMTU complex after RNA duplex acidic treatment.
Figure 42. Positive-mode mass spectra and fragmentation pattern for adenine-Pt-ACRAMTU complex after DNA duplex acidic treatment.
CHAPTER 4 – DISCUSSION OF RESULTS

Binding of Pt-ACRAMTU to RNA is clearly different from its interaction with DNA as a result of RNA secondary structure. Even different RNA structures exhibit dissimilar modes of drug interaction. The lack of an ICD signal in RNAH2 and GNRA hairpins represents the absence of intercalation of Pt-ACRAMTU, meaning that the drug likely binds in the loop region as represented in Figure 45. This behavior can be explained taking into account the base conformations in the loop; a GNRA loop (5’-GUGA-3’ in this case) is highly stable due to the hydrogen bonding of 5’-G with A-3’, and the remaining uracil and guanine bases participate in stacking interactions. The solvent accessible representation of this loop (Figure 43) clearly shows the high accessibility of both G-N7 positions, this leads us to conclude that preference for loop binding may be driven by preference of platination at this position. In addition, the Pt-ACRAMTU molecule bound to guanine may be stabilized by electrostatic interactions as shown before in Figure 7 for the TAR-acridine interaction.

Figure 43. GNRA loop solvent accessible area representation (1R7W crystal structure). There is high accessibility of the for both G-N7 positions, the main site for platination.
The ICD signal for UUCG is consistent with the hypothesis of loop preference binding in RNH2 and GNRA loops; the low accessibility of the G in the UUCG loop (Figure 44), as well as absence of adenine bases for N3 adduct formation, highly diminishes the possibility of binding of Pt-ACRAMTU to this region and favors intercalation. Absence of a loop in the duplex also leads to intercalation which is corroborated with the ICD data. LC-MS data shows that there is a higher abundance of the adenine adduct for RNAd duplex, thus the representation of this adduct in Figure 45. In addition, A-N3 adduct formation may also influence the preference of loop binding in RNHA2 and GNRA since both hairpins have adenine bases in the loop.

Figure 44. UUCG loop solvent accessible area representation (1F7Y crystal structure).
There is low accessibility of the G-N7 position leading to no platination.
Figure 45. Representation of platinum modified RNA.

RNAH2 and GNRA seem to be modified in the loop while UUCG and the Duplex seem to be modified in the base paired region.

Despite the demonstrated high rigidity of A-RNA over B-DNA driven by interactions of the 2'OH with surrounding functional groups, ICD data provides strong evidence for a very important secondary structure change in RNA at the time of the binding of Pt-ACRAMTU. An A to B-form transition requires a dramatic change where the compact A-RNA structure, characterized by an 11-bp repeat, a 2.6-Å rise, and base pairs that are inclined up to 20° and displaced by ≈4 Å so they essentially wrap around the helix axis is converted into the long and narrow B-duplex—with its 10- to 10.5-bp repeat, 3.4-Å rise, and base pairs stacked at the center of the helix (Figure 46). The geometries of the ribose sugars are also converted from C3'-endo in A-RNA (with the C3'-carbon puckered above the furanose plane toward the nucleobase - Figure 1b) to C2'-endo in B-RNA.

The transition of the secondary structure in RNA may be driven, as described before by Baruah and Bierbach, by the unwinding of the duplex at the site of platination which may start at
the central base step and further extend to the neighboring base steps promoting a controlled transition.

Figure 46. A-RNA and B-RNA models.
Side view and projection along helical axis for A- and B-form RNA \( [d(GCCAUAUGGC)2] \); such transition is possible when binding of Pt-ACRAMTU.

The decreased ethidium bromide fluorescence of PAGE gel bands after several equivalents of Pt-ACRAMTU have been added was first attributed to degradation of RNA. Some bands faintness may also be due to the quenching of fluorescence of ethidium bromide by the intercalated acridine chromophore. CD data does provide further evidence for some degree of RNA degradation by
disappearance of all the CD spectra after the addition of several equivalents of Pt-ACRMTU. One possible explanation for RNA degradation could be the increase in B-helical character of RNA, which would result in exposure of reactive 2’-OH to solvent. Furthermore, the platinum lewis acid would promote ionization of water, leading to hydrolysis of the ribose-phosphate backbone.

The low presence of ICD when DNA5-26 was treated with ACRMTU (Figure 29) is due to the homopolymer character of the duplex, when it is known that acridine intercalates more efficiently between heteropolymers such as 5’-GA/TC.77
5.1. Conclusions

This study has been helpful to recognize the important role of RNA’s secondary structure when binding to Pt-ACRAMTU. We have established two main factors in the binding mode of this drug to RNA: the presence of a loop motif that promotes the binding to this region over intercalation, and the specific pattern of binding where the A-form of RNA contributes the probable formation of A-N3 adducts. We have also found evidence for Pt-ACRAMTU triggering an A to B-form transition in RNA; these results may have a significant application when targeting RNA in the different biological processes; one class of RNA target for small molecule drugs it the viral RNAs.

The bulge region of TAR HIV is an important site for the binding of the Tat protein (Figure 5); however, it is not the only site of interaction for this protein in TAR. Tat protein enhances the processivity of RNA polymerase II (pol II) elongation complexes that lead to virus transcription; the transactivation domain of Tat binds directly to the CycT1 subunit of the positive transcription elongation factor complex b P-TEFb and induces loop sequence-specific binding of P-TEFb onto nascent HIV-1 trans-activation responsive region (TAR) RNA. Neither CycT1 nor the P-TEFb complex binds TAR RNA in the absence of Tat. Richter et al.78 have found that residues 252–260 of CycT1 interact with one side of the TAR RNA loop and enhance interaction of Tat residue K50 to the other side of the loop. Secondary structure specificity of Pt-ACRAMTU over the loop region (Figure 47) may be an important asset to target TAR HIV; thus, preventing the binding of CytT1 transcription factor. Binding to adenine 35 seems more reasonable since the G-N7 of the three guanines may be difficult to access as shown in the solvent accessible area representation of the loop of TAR (Figure 48). In addition, the deformability of TAR due to A- to B-form transition may be
important determinant of its ability to interact with proteins, and can possibly contribute to transcription inhibition.

Figure 47. TAR-Pt-ACRAMTU adduct representation. Binding of K50 of Tat (PDB 1K5K) to TAR is inhibited due to the binding of Pt-ACRAMTU to the adenine 35 in the loop region.

Figure 48. TAR loop solvent accessible representation (1KIS crystal structure). Low accessibility towards G-N7.
As mentioned in section 1.3.2., Boer et al.\textsuperscript{41} have shown that neomycin-Pt\textsuperscript{II} and guanidino-neomycin-Pt\textsuperscript{II} bind to RRE at the bulge region; in a similar manner, it is feasible that Pt-ACRAMTU may bind into the same region disrupting the Rev-binding site and thus halting the active export of unspliced HIV genomic RNA from the nucleus. In addition, binding either to the bulge or the loop of RRE will lead to the A- to B-transition that may also prevent the binding of Rev (Figure 49).

![Figure 49. RRE-Pt-ACRAMTU adduct representation. Pt-ACRAMTU binding into the loop of RRE can prevent Rev binding. In this protein both A-N3 and G-N7 are accessible, so both adducts can form.]

5.2. Future work

Due to the potential importance of RNA as a drug target, it would be interesting to further investigate the interaction of intercalative platinum compounds through diverse experimental techniques such as fluorescence quenching or the selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE).

The former assay is used to study the binding affinity and global sequence selectivity of oligonucleotide binders. In this assay, a competing ligand (in our case acridine) directly or indirectly
binding to the oligonucleotide quenches the monitored fluorescence of DNA-bound ethidium. From this phenomenon $C_{50}$ values (concentration of acridine effecting 50% quenching of the ethidium fluorescence) can be obtained. Further information on the effect of secondary structure of RNA to the binding of Pt-ACRAMTU can be determine with fluorescence as well; for instance, if there is no quenching of the ethidium fluorescence then Pt-ACRAMTU is not intercalating, and so its binding into the loop.

The latter methodology, SHAPE, was first described by Weeks et al. as an alternative for the traditional sequencing methods which are unable to effectively discriminating between single-stranded and base-paired regions, and thus of providing information about RNA flexibility. Flexible nucleotides, due to the electronic influence of the adjacent 3'-phosphodiester group, present more conformations that enhance the nucleophilic reactivity of 2'-hydroxyl groups toward electrophiles such as N-methylisatoic anhydride (NMIA) than base-paired or constrained nucleotides. These modified nucleotides can work as stop sites during primer extension reaction, followed by electrophoretic fragment separation. The ability of this method to identify flexible sites yields very precise information regarding the pattern of base pairing and the formation of non-canonical tertiary interactions in RNA.

Molecular Modeling is also an interesting option to virtually characterize this ligand-RNA interaction since it can provide information about the energy contributions of these interactions and the potential binding mode. This can be done using the AMBER force field in the InsightII/Discover software. Kinetic assays to determine the rate constant for Pt-ACRAMTU to RNA with different sequences and secondary structure may also be helpful to expand our knowledge on this interaction.

Significance of Pt-ACRAMTU-TAR adduct when interacting to Tat can be analyzed using Western blot technique with antibodies to detect HA-Tat, CycT1(1–303), and CycT1(1–726). In
addition, electrophoretic mobility shift assay (EMSA) analyses can be performed to monitor the consequences of PT-ACRAMTU modification on Tat binding.\textsuperscript{48} The same methodology can be applied for Pt-ACRAMTU-RRE adducts with the Rev protein.
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