The Design and Evaluation of Catalytic MetalloDrugs Targeting HCV IRES RNA:
Demonstration of a New Therapeutic Approach

DISSERTATION

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Abstract

Traditional drug design has been very effective in the development of therapies for a wide variety of disease states but there is a need for new approaches to drug design that will not only be able to tackle new challenges but also complement current approaches. The use of metals in medicine has had some success and allows for the introduction of new properties that are unachievable using only organic compounds but also introduces new challenges that can be addressed by careful design and an understanding of inorganic chemistry. Toward this end, catalytic metallodrugs are being developed for the irreversible inactivation of a therapeutically relevant target. A catalytic metallodrug consists of a metal-binding domain that mediates chemistry and a target recognition domain that provides specificity for the therapeutic target of interest. This approach has a number of advantages including a potential for higher specificity leading to lower doses as well as a unique mechanism of action that will complement current therapies and help combat resistance. Previous work has shown the inactivation of enzymes by irreversible modification of key residues. This approach was then extended to RNA where the backbone is more likely to be susceptible to hydrolytic and oxidative cleavage. Phenylalanine transfer RNA was used as a starting point to look at reactions with RNA and moderate chemistry was observed. This was then further extended to the study of
TAR, a therapeutically important RNA found in HIV. Activity was not observed for the degradation of TAR despite the presence of the RNA binding domain, further supporting a double filter mechanism where binding alone is not sufficient for chemistry to occur. A metallopeptide, Cu-GGHYrFK-amide (1-Cu), targeting stem loop IIb of the internal ribosomal entry site (IRES) of the hepatitis C virus (HCV), was tested and found to exhibit good activity both in vitro and in a cellular replicon assay. In the absence of an accepted animal model for hepatitis C infection, this replicon assay is accepted by the FDA as a measure of drug efficacy. A reduction of HCV RNA levels in this replicon assay was confirmed by real-time polymerase chain reaction (RT-PCR). This compound also showed additive to slightly synergic activity when given in combination with the current therapy, recombinant interferon α-2b. Derivatives of this peptide were studied to provide insights into the mechanisms of binding and reactivity as well as to enhance properties in vivo. Another set of peptides based on the human La protein was also studied in terms of their interaction with both stem loop IIb and stem loop IV and were shown to have good activity both in vitro and in the same cellular replicon assay. These metallopeptides represent a new class of compounds for the treatment of hepatitis C infection and demonstrate the potential of catalytic metallodrugs as a novel approach to drug design.
This dissertation is dedicated to my father, Craig Bradford, for raising me and being the best role model I could hope for, to my mother, Patricia Lanzilotta, for always being there for me and looking out for my best interests, and to my girlfriend Isabel Averill for being so understanding and good to me.
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Chapter 1- Introduction

1.1 Drug Discovery and Design

History is filled with descriptions of diseases and conditions as well as attempts to combat them. The use of drugs in the past probably started out as observations of cause and effect, such as the observation that a plant or plant extract had certain healing properties or that it relieved pain. For example, the use of salicylic acid-rich plants for pain and inflammation dates back to before the Common Era but it wasn’t until the mid to late 1800’s that the active component was identified as acetylsalicylic acid and marketed as aspirin. More recent advances in the physical and biological sciences contributed to the determination of the chemical composition of distinct drug molecules as well as an appreciation of the importance of understanding the drug target and the development of techniques to study the effects of a given drug both in the test tube and in vivo. This required many advancements including the development of the microscope and discovery of a variety of microorganisms including bacteria by Antony Leeuwenhoek in the 1600’s, the discovery of cells in 1665 by Robert Hooke, the development of staining procedures, such as the Gram stain, for cells and tissues developed in the 1700’s and 1800’s, and the identification of viruses by Dmitri Ivanovsky and Martinus Beijerinck in the late 1800’s.
Treatment also required an understanding of the cause of a disease. Malaria has been known for over 4000 years but the malaria parasite wasn’t identified until 1880 by Charles Louis Alphonse Laveran. As the molecular understanding of drugs and drug targets has improved, so have the methodologies for optimizing and testing them. Current approaches to the discovery of natural products rely on an understanding of the biological and physical sciences to affect the screening, separation, characterization, and synthesis of the specific molecules involved in achieving the desired response. The use of drugs derived from nature is still important, especially since nature can still design drugs that humans cannot, but advances in the sciences have allowed for the better understanding of how drugs work as well as how best to use and design them.

The rational design of drugs derives from a basic understanding of the importance of specific biological targets to the cause and treatment of a condition as well as the ability to alter the processes that ultimately lead to that disease state. As advances in chemistry and the biological sciences continued and a deeper understanding of drug interactions with a target was developed, scientists began to design and modify drugs rationally. A compound with a specific type of activity could be modified to alter its properties to generate higher activity, lower toxicity, improved specificity, better stability, or an improved half life in vivo. Modifications could be made in a systematic way to generate structure activity relationships (SARs) that help to guide further optimization. One of the earliest examples of a medicinal chemistry approach to drug design was the work of Paul Ehrlich which was studies of arsenic compounds for the treatment of syphilis. Ehrlich’s concept of a “magic bullet” that selectively targets a bacterium over other
organisms is an important concept even today and achieving this selectivity is still one of the most promising, yet challenging, aspects of drug design.

Currently, the development of drugs often consists of a combination of screening compounds to understand mechanism and identify lead compounds as well as rational design to further optimize these compounds. Rational design acts as a guiding hand to focus research but the complexities ultimately make prediction an uncertain process and there is still a great deal of trial and error involved. A pharmaceutical company will often have access to a large number of compounds that then will be tested for activity. The number of possible compounds to test can be very large and often will be narrowed down according to other parameters such as computational results or physical properties. Lipinski’s rule of five is a commonly used guideline to determine the “druglikeness” of molecules. For example, it doesn’t matter how active a compound is in an assay if it is insoluble or has other properties which would preclude it from being used in vivo. It was found that most of the medications in use consisted of compounds with no more than five hydrogen bond donors and 10 hydrogen bond acceptors, a molecular weight less than 500 grams per mole, and an octanol-water partition coefficient (log P) of less than five. These rules are used as a guideline to focus the screening process. New drugs tend to be small organic molecules with high affinity for their target as well as good stability and solubility in vivo. It is important to keep in mind, however, that these are only guidelines. The recently approved protease inhibitor for the treatment of hepatitis C, telaprevir, is a notable example of a compound that breaks all of Lipinski’s rules.
The most common examples of targets for therapeutic intervention are enzymes. For example, a common approach to blood pressure regulation is inhibition of angiotensin converting enzyme (ACE). ACE is an enzyme that increases blood pressure by converting angiotensin I to angiotensin II, a vasoconstrictor, and also by degrading bradykinin which is a vasodilator.\textsuperscript{10} ACE and other enzymes that affect blood pressure need to be properly regulated or else the blood pressure can get too high or too low, both of which cause health problems. In situations where blood pressure is high, inhibition of ACE by small molecules is a common treatment. This is how some commonly used blood pressure medications such as captopril (Capoten)\textsuperscript{11} and lisinopril (Zestril, Tensopril),\textsuperscript{12} shown in Figure 1.1, work. The general scheme for this type of inhibition is shown in Figure 1.2 and is the most common approach to drug design. The small molecule inhibitor (I) binds to the active site of the enzyme (E) and blocks it so that the substrate (S) cannot bind.

Figure 1.1 Known small molecule inhibitors of angiotensin converting enzyme.
There are many advantages to traditional drug design and the vast majority of the drugs currently on the market were discovered and developed according to these same basic principles. The process shown in Figure 1.2 is a reversible process dictated by equilibrium and therefore requires enough of the inhibitor (I) to shift the equilibrium and achieve the desired effect. This can lead to higher amounts of drug to be administered, potentially more frequently, leading to toxicity and therefore issues with patient compliance. Also, because it is reversible the inhibitor will come off and be cleared by the body, leaving the enzyme to start converting substrate to product all over again.

The limited use of elements, primarily C, H, N, O, and S, leads to a limited range of properties that can be achieved using these small organic molecules. The shapes that can be achieved, for example for fitting into an enzyme active site, will also be limited to geometries available to those atoms where higher coordination numbers, and hence more diverse shapes, cannot be achieved. The use of metals in medicine is a less traditional approach which overcomes some of these limitations while introducing disadvantages of its own, such as considerations of the stability and toxicity of the complex. Proper ligand

![Figure 1.2 Reversible inhibition of an enzyme.](image-url)
design and application of basic inorganic principles allows for the design of stable compounds with a wide array of properties available to them.

1.2 Metals in Medicine

The importance of metals in biology has become apparent over time as more enzymes and other biological molecules have been identified that contain metals that are important for structure or function in some way. Metals can perform functions that cannot otherwise be achieved, such as electrolyte balance important for a variety of processes including the firing of neurons, as well as oxygen transport, electron transfer, and catalysis. The importance of metals to health is made clear by the abundance of diseases caused by an excess or deficiency of a metal. For example, iron is essential for life but in humans too much iron can lead to haemochromatosis and too little iron can lead to iron deficiency. Therefore, iron regulation is very important. Other examples of diseases related to metal ion regulation include Wilson’s disease,\textsuperscript{13} Friedrich’s ataxia,\textsuperscript{14} and hypozincemia.\textsuperscript{15} Diseases relating to metal overload can often be treated by use of metal chelating compounds, and British anti-Lewisite (dimercaprol, Figure 1.3) was the first chelating compound used medicinally.\textsuperscript{16} Metal overload can also be caused by intake of metals either involuntarily or voluntarily, such as was often the case in historical uses of metals to treat diseases. Silver has found a variety of uses in medicine most notably as an antibiotic although its mechanism of action is still unclear. Prior to the development of antibiotics around the time of World War I, silver was used as a disinfectant and it still
finds use today in the form of silver sulfadiazine (Figure 1.3) for the treatment of burn wounds.\textsuperscript{17} The use of mercury is another common example of a compound that was used in the past without a full understanding of how it works.\textsuperscript{18} Probably the most commonly known use of mercury was in the processing of felt hats which caused toxicity and led to the term “mad as a hatter.”\textsuperscript{19} Medicinally it was used as a disinfectant or to treat syphilis without fully understanding the toxicity of the treatment itself. These examples illustrate the potential for activity from inorganic compounds but also the possible pitfalls when used without taking proper precautions and without understanding their mechanism of action.

\begin{center}
\textbf{Figure 1.3} Metal related therapies used throughout history.
\end{center}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1_3.png}
\end{figure}

While there are some metal based therapeutics currently used, they often tend to be the exception rather than the rule and tend to be discovered rather than rationally designed. The potential for toxicity from the metal center is a very important concern but one that can be addressed by proper ligand design and knowledge of coordination chemistry. Nevertheless, it is important that the use of a metal center is necessary for the chosen application, such as by the introduction of properties that cannot be achieved by a traditional organic compound. Examples include the use of metals for imaging, such as
by the use of a positron emitting metal for positron emission tomography (PET) or a gadolinium complex for MRI contrast, or for the introduction of new reactivity such as in bleomycin. Another potential use of metals is to generate new shapes that can more effectively explore structure activity space, such as the use of an inert metal center to introduce an octahedral geometry that cannot be otherwise achieved in simple organic molecules. In the current market, past issues with metal toxicity are less likely, due to an increased emphasis on proper testing and understanding of the mechanism of potential therapeutics. There are several examples of metal based drugs that are still currently in use and these compounds illustrate both the potential of this class of compounds and the need for further work to optimize their uses in a clinical setting.

![Chemical structures](image)

Gadoteridol (Prohance)  
Auranofin  
Ibritumomab  
Zevalin  
Bismuth Subsalicylate

**Figure 1.4** Clinically used inorganic based drugs.
1.2.1 Overview of Currently Used Metal Based Therapeutics

The number of metal-based therapeutics that have received FDA-approval is limited and this is a reflection both of the challenges involved and the lack of interest of pharmaceutical companies in inorganic compounds. There is precedent, however, for a willingness to approve a compound quickly especially if it fits an identified need that is not being met. A good example of this is the large number of imaging agents on the market. One example of an imaging agent is Prohance (Figure 1.4), which was approved in 1992 and is an MRI contrast agent used for imaging of the central nervous system. Gadolinium contains seven unpaired electrons and interacts with water to enhance its relaxivity. This enhancement occurs only where the gadolinium is present and can be used to image where the complex localizes in the body. The history of FDA approved metal compounds used for therapy is more limited. The use of lithium carbonate for the treatment of depression, primarily bipolar disorder, was approved in 1970. Its mechanism of action is still not well understood. In 1985, the gold compound auranofin (Ridaura, Figure 1.4) was approved for the treatment of rheumatoid arthritis. Its mechanism of action is thought to be due primarily to inhibition of an esterase that breaks down cartilage tissue but this mechanism of action is still debated. Bismuth salicylate (Figure 1.4), the active ingredient in Pepto-Bismol, was approved in 1996 for use as an antacid and antidiarrheal. In 1997, Sclerosol Intrapleural Aerosol, Mg$_3$Si$_4$O$_{10}$(OH)$_2$, was approved for malignant pleural effusion in lung cancers. Malignant pleural effusion is when an abnormal amount of fluid collects in the lungs, and this compound is
thought to induce an inflammation response and act as a sealant to prevent the further accumulation of liquids. Trisenox is a formulation of As$_4$O$_6$ and was approved in 2000 for administration in combination with all-trans retinoic acid (ATRA) for the treatment of some forms of leukemia.$^{29}$ The metal-based radiopharmaceutical Zevalin (Figure 1.4) was approved in 2002 for the treatment of low-grade, B-cell, Non-Hodgkin’s Lymphoma.$^{30}$ This compound consists of the monoclonal mouse IgG1 antibody ibritumomab linked to the metal binding domain tiuxetan, and either indium-111 or yttrium-90 is used. Zevalin binds to the CD20 antigen found on the surface of B cells and selectively kills them. This range of drugs shows the potential for generating compounds that have unique activity but also shows the limitations and potential challenges in terms of toxicity and achieving a detailed understanding of mechanism of action.

1.2.2 Cisplatin

Probably the most successful metal based drugs are represented by cisplatin and its analogs. Like many of the drugs mentioned, and many drugs in general, cisplatin (structure shown in Figure 1.5) was not rationally designed but serendipitously discovered by Barnett Rosenberg in 1965.$^{31}$ Cisplatin was approved by the FDA in 1978 for the treatment of testicular and ovarian cancers. Cisplatin when given is a neutral compound but is susceptible to hydrolysis as shown in Figure 1.5. In the blood, the chloride concentration is sufficiently high that the equilibrium lies to the neutral
compound on the left. Upon entering the cell, the [Cl\textsuperscript{-}] drops and the cisplatin hydrolyzes to form the charged complex on the right which then cannot cross the cell membrane and therefore is trapped inside the cell. This is the active form of cisplatin. The platinum compound then reacts with DNA to generate inter- and intrastrand crosslinks that then lead to apoptosis.\textsuperscript{32} The selectivity of cisplatin derives from an enhanced uptake in cancer cells relative to healthy cells as well as the higher accessibility of the DNA in cells that are undergoing division. This is why other cells that are continuously dividing, such as hair cells or cells in the gastrointestinal tract, are also damaged by cisplatin and this explains some of the side effects such as hair loss and nausea. Therefore, while cisplatin does have a preference for killing cancer cells, it does not have a high level of specificity.

\[
\begin{align*}
\begin{array}{c}
\begin{array}{c}
\text{H}_3\text{N} \\
\text{Cl}
\end{array} \\
\text{H}_3\text{N} \\
\text{Cl}
\end{array} 
\end{align*}^0 + 2 \text{H}_2\text{O} & \rightarrow 
\begin{align*}
\begin{array}{c}
\begin{array}{c}
\text{H}_3\text{N} \\
\text{OH}
\end{array} \\
\text{H}_3\text{N} \\
\text{OH}_2
\end{array}^\oplus + 2 \text{Cl}^- + \text{H}^+
\end{align*}
\]

Cisplatin

**Figure 1.5** Hydrolysis of cisplatin to generate the active form.

Many derivatives of cisplatin have been studied and two have received approval by the FDA. Carboplatin\textsuperscript{33} (Figure 1.6) was approved in 1989 for the treatment of ovarian cancer and oxaliplatin\textsuperscript{34} was approved in 2002 for the treatment of advanced colorectal cancer. Both compounds replace the chloride leaving group of cisplatin with a bidentate dicarboxylate ligand that is less reactive. This leads to less side reactions, and therefore lower toxicity, particularly with proteins, and also better retention in the body relative to cisplatin.
Cisplatin and its derivatives are a good example of the promise of inorganic pharmaceuticals and also demonstrate the concerns that need to be addressed by future metal based drugs. Their effectiveness and contributions to human health are undeniable but there is much room for improvement in specificity and therefore a reduction in toxicity. Side reactions and the fact that the drug is consumed in the mechanism lead to a requirement for high doses. Achieving selective targeting to the cells of interest is still one of the biggest challenges in the use of metals in medicine as well as drug design in general.

**Figure 1.6** Clinically approved derivatives of cisplatin.

![Carboplatin and Oxaliplatin](image)
1.2.3 Bleomycin

Bleomycin (Figure 1.7) was isolated from *Streptomyces verticillus* in 1966 and is an example of a drug designed by nature that takes advantage of the reactivity of metals. It received approval in the United States in 1973 and is used for the treatment of squamous cell carcinomas, testicular cancer, and Hodgkin’s lymphoma. Bleomycin is not actually administered as a metal complex but instead consists of several domains including one that is important for binding to DNA and another that is for metal binding. The mechanism of action of bleomycin is the induction of DNA strand breaks due to recruitment of a metal, probably iron, and subsequent generation of reactive oxygen species which react with the DNA. Much like cisplatin, bleomycin shows a preference for cancer cells but its mechanism of action is not specific to them. It is a good example,
though, of a drug incorporating a metal ion to do chemistry on a target. It is also another example of a metal based drug that would benefit greatly from enhanced selectivity for cancer cells.

1.3 Catalytic Metallo drugs

Cisplatin and bleomycin represent two examples of toxic compounds that show some level of selectivity for a disease state but would benefit greatly from more specific targeting to the site of interest. Catalytic metallo drugs are a class of metal based drugs developed in the Cowan lab that help to address some of these concerns. The basic design strategy is shown in Figure 1.8 and consists of a metal binding domain linked to a target recognition domain. The target recognition domain binds to the therapeutic target of interest and provides selectivity while the metal binding domain performs chemistry on that target. This simple modular design allows for the variation of the different domains to modify the properties of the metallee drug and can be quickly adapted to a variety of different therapeutic targets. As shown in the reaction scheme at the bottom of Figure 1.8, the metal-ligand complex (ML) binds to its target (T) in a process that is under equilibrium and analogous to the enzyme inhibition strategy described above. The next step, however, involves irreversible reaction with the target to generate the product (P). In this way it acts like an enzyme. This irreversible step will help to pull the equilibrium to the right according to Le’chatelier’s principle and aid the progress of the reaction even under situations where binding might not be as tight as would be required
for a traditional drug. The final step involves release of the metal-ligand complex from the target to regenerate the metallodrug. Hence, another important design feature is the ability to act catalytically.

\[
T + ML \leftrightarrow T-ML \rightarrow P-ML \leftrightarrow P + ML
\]

**Figure 1.8** (Top) Basic catalytic metallodrug design. (Bottom) Reaction scheme for catalytic inactivation of target T by the catalytic metallodrug consisting of a metal binding domain (M) and a target recognition domain (L).

One of the major advantages of this approach is its ability to address some of the issues previously mentioned in terms of specificity and toxicity. One feature that emerges from the design as described is a double filter mechanism for reactivity, as shown in Figure 1.9. Drugs will often have side effects and toxicity due to nonspecific interactions with macromolecules other than their therapeutic target. For catalytic metallodrugs, however, there is a requirement for both binding and proper positioning of the metal binding site for reactivity. Even if nonspecific binding occurs, as shown on the right, if the metal binding domain isn’t properly oriented to react with the target then it will not be active.
This extra level of selectivity helps to address one of the biggest issues with the currently used metal based drugs described above, which is their inherent toxicity. This feature combined with the fact that these metallodrugs are designed to act catalytically allows for higher selectivity at a lower dose. Lower dosages should also contribute to lower costs and, combined with the reduced toxicity, better patient compliance. The unique mechanism of action also makes catalytic metallodrugs a perfect complement to current therapies both in terms of combination therapy and to combat resistance. The novel mechanism of action makes it very difficult for resistance to develop against both a catalytic metallodrug and another drug at the same time. Often resistance can occur by mutation of the therapeutic target to prevent or reduce binding. In the case of catalytic metallodrugs, however, this reduction in binding could potentially not affect, or even
enhance, catalyst activity depending on what the rate limiting step is for a particular system.

1.3.1 The Metal Binding Domain

As mentioned, the role of the metal binding motif is to perform chemistry on the therapeutic target. In order to be useful as a metallodrug, it must also bind to the metal very tightly and be stable both kinetically and thermodynamically. The primary metal binding domain to be used will be the amino terminal copper and nickel binding (ATCUN) motif derived from the N-terminus of serum albumins.\textsuperscript{38} It has been shown to bind to copper and nickel very tightly (\(K_d\) for copper of 1.18 \(\times\) 10\(^{-16}\) M).\textsuperscript{39} There is also precedent for this complex reacting with nucleic acids.\textsuperscript{40, 41} This metal binding domain is shown in Figure 1.10. Metal coordination occurs by binding of the N-terminal amine, the two backbone amides, and the histidine. Therefore, any peptide containing a histidine in the third position is capable of binding to copper or nickel. NMR, EPR, visible spectroscopy and X-ray crystallography are consistent with a divalent metal ion coordinated in a square planar configuration,\textsuperscript{39, 42-44} leaving two faces accessible for binding to a target molecule. This design allows for variation of the properties of this domain by changing the R groups coming off the first two residues. It also is convenient for incorporation of a target recognition domain that is a peptide because the sequence of that peptide can simply be added to the C-terminus without interfering with metal binding. Another important feature of this metal binding domain is that it is well
characterized as undergoing redox chemistry through a Cu\(^{3+/2+}\) couple rather than the more common Cu\(^{2+/+}\) couple.\(^{45}\) The hard deprotonated backbone amide nitrogens help to stabilize the Cu\(^{3+}\) which is a d\(^8\) metal ion and also prefers a square planar geometry which this ligand can accommodate. A Cu\(^+\) has a d\(^{10}\) configuration and prefers a tetrahedral geometry which is not as compatible with the ligand environment. This feature is important not only due to the higher oxidizing power of the Cu\(^{3+}\) but also in terms of the in vivo stability. Standard copper binding ligands such as the polyamine cyclen can be reduced to Cu\(^+\) in vivo and, since the ligand set isn’t set up properly to bind in a tetrahedral geometry, the copper ion can fall out of the ligand. For Cu-GGH, however, the metal complex is stable in both the Cu\(^{2+}\) and Cu\(^{3+}\) forms.

![Figure 1.10 The amino terminal copper and nickel binding (ATCUN) motif.](image)

The reactions involving the metal complex are generally assayed in the presence of both ascorbic acid and hydrogen peroxide under aerobic conditions. The exact pathway involved is not yet fully elucidated but is expected to involve the generation of reactive oxygen species such as through the reduction pathways shown below and could involve superoxide, peroxide, hydroxyl radicals, or a metal bound oxygen species. These
coreagents can also be important for cycling and regenerating the metal complex in the starting state, such as by reduction of Cu$^{3+}$ back to Cu$^{2+}$ by ascorbic acid. These conditions are expected to be present in some form under physiological conditions since ascorbic acid is vitamin C and both it and hydrogen peroxide are available *in vivo*. Even if these compounds are not readily accessible or are not available at high enough concentrations *in vivo*, there are expected to be a variety of other reducing or oxidizing agents present that could perform the same functions.

![Reduction potentials diagram](image)

**Figure 1.11** Relevant reduction potentials (relative to NHE) for oxidative chemistry of catalytic metallo-drugs.

1.3.2 The Target Recognition Domain and Linker

The target recognition domain is anything that can bind to and recognize a specific
target of interest. If it is a peptide, it is easily incorporated into the ATCUN motif as described above, but any compound can be used as long as the chemistry for conjugation to the metal binding domain can be worked out. Also, depending on how it is designed, there will be a linker group between them which also can be varied to tailor the chemistry. For example, the introduction of flexibility could either enhance or reduce chemistry at the target by affecting its placement and orientation relative to potential scissile bonds. This linker can also be varied to tailor other properties, such as water solubility, partition coefficient, or half-life in vivo. Depending on the mode of binding, other functional groups can be incorporated to further tailor the properties of the catalytic metallodrug, such as for the optimization of in vivo properties.

1.4 Conclusions

Catalytic metallodrugs fill a current need in drug design by introducing new properties and providing a new class of drugs that act by a unique mechanism of action. They also address some of the current drawbacks with metal based drugs, such as a requirement for high doses and poor selectivity leading to high toxicity. The design described above utilizes an ATCUN motif to introduce reactivity while also showing good stability. The modular design also allows for the variation of different components to optimize the properties of the drug. The targeting domain represents a way to achieve enhanced selectivity and also makes it generally applicable to any systems of interest. A new approach like this also requires a development of the methodology to characterize it and
this information can be used to further understand mechanism of action and how best to optimize activity. The mechanism could occur by a variety of different pathways and is likely to vary depending on the system being studied. In order to understand this process, the approach was applied to several different biological systems to show proof of principle and to obtain a basic understanding of how reactivity typically occurs in these types of systems, as well as to develop the methodology required to study these systems.
Chapter 2- Preliminary Work

2.1 Introduction

It is important not only to show that the catalytic metallodrug approach to drug design can work but also to understand how it works. Preliminary work has looked at showing that irreversible chemistry can be performed on a biological target, and understanding how this works will also aid in the design of future catalytic metallodrugs. Previous efforts to study the reactivity of these metallopeptides with enzymes will be described and then initial studies to apply this to tRNA\textsuperscript{Phe} as a model system, as well as TAR, a therapeutically important structured RNA in HIV, will be described.

2.1.1 Catalytic Inactivation of Enzymes

The catalytic metallodrug approach has previously been applied to enzymes to show the irreversible inactivation of angiotensin-converting enzyme (ACE).\textsuperscript{46} ACE is a zinc metallopeptidase that degrades bradykinin, a vasodilator, and also catalyzes the conversion of angiotensin I to angiotensin II, a known vasoconstrictor. It is a common target for the design of inhibitors to treat high blood pressure and there are a large number of ACE inhibitors on the market, including lisinopril (shown in Figure 2.1 bound
to a model of the active site) and captopril (Figure 2.1). Cu-KGHK-COOH, shown in Figure 2.1, is an ATCUN motif previously observed to oxidatively inactivate human ACE with a \( k_{\text{obs}} = 2.9 \times 10^{-2} \text{ min}^{-1} \). Activity was also demonstrated for the cobalt complex. It was also found that Cu-KGHK-COOH showed similar activity towards human endothelin-converting enzyme (hECE), another zinc containing enzyme implicated in blood pressure regulation by catalyzing the formation of a vasoconstrictor, in this case endothelin. The mechanism of inactivation for this system, however, is unclear. Activity was also observed for the peptide Cu-CGHK-COOH against thermolysin (TLN), another zinc metalloenzyme in the same family as ACE and ECE.\(^4\) No activity against TLN was observed for Cu-KGHK-COOH and it is expected that cysteine coordination to the active site zinc is important, consistent with other known inhibitors such as captopril.

Figure 2.1 Schematic of lisinopril bound in the active site of angiotensin converting enzyme (top left), captopril (top right), and KGHK-COOH (bottom).
In order to further probe the mechanism of enzyme inactivation by this class of compounds, human carbonic anhydrase I (CA-I), another zinc metalloenzyme, was used as a model system.\(^{48}\) Carbonic anhydrase is readily obtainable and can be used to carry out experiments that require large quantities of material, such as mass spectrometry. It is a very important enzyme physiologically and catalyzes the conversion of CO\(_2\) and water to bicarbonate and protons. A variety of sulfonamides have been characterized as binding to carbonic anhydrase and one of the simplest, sulfanilamide (SLN, Figure 2.2), was coupled to GGH to generate the catalytic metallodrug Cu-GGHSLN (Figure 2.2). Time dependent inactivation was observed much like for ACE, ECE, and TLN (Figure 2.3, left). Enzyme cleavage was not observed in SDS-PAGE studies. Capillary-liquid chromatography-nanospray tandem mass spectrometry was performed to look at the enzyme before and after inactivation by Cu-GGHSLN. It was found that the enzyme was modified at specific residues as opposed to cleavage of the peptide backbone (Figure 2.3, right). These modifications were mostly centered around the zinc active site (distances listed for residues are the distance from the catalytic zinc) and occurred primarily at histidine residues but modification of tryptophan was also observed. Prior reports indicate that histidine residues are effectively converted to 2-oxo-histidine upon exposure to Cu-ATCUN derived oxygen based radicals.\(^{49-51}\) Three of the modified residues (His64, His67, and His200) have been implicated in proton transfer within the active site and are important for catalysis by the enzyme.\(^{52,53}\) The data, therefore, are consistent with the mechanism of inactivation being via irreversible modification of residues that are important for the mechanism of the enzyme.
2.1.2 Enzyme Inactivation Summary

Overall, the potential for catalytic metallodrugs to react with, and inactivate, enzymes has previously been demonstrated. The reaction shows time dependence and is...
irreversible. The mechanism of action seems to be due to modification of key residues in the enzyme rather than cleavage of the peptide backbone. These compounds show potential for use as catalytic metallodrugs and should have the advantages described in chapter 1, including lower dosages and higher selectivity relative to traditional drugs. One potential concern, especially for the examples described, is that the enzymes described are essential to human health and their activity needs to be reduced in disease states but not completely abolished. It is expected that the reactivity of the metallodrug as well as the dosage can be tuned to achieve a proper balance to maintain a therapeutic effect while minimizing toxicity. Future compounds can also be designed to inactivate proteins that are unique to an invading organism such as a virus or a bacterium.

2.1.3 RNA as a Drug Target

Another important target molecule for the design of catalytic metallodrugs is RNA. The increasing awareness of the functional importance and structural complexity of RNA makes it an attractive target for therapy. Including the discovery of ribozymes in the late 1980’s there have been many examples of functional RNA molecules. Many viruses, bacteria, and other pathogens contain RNA that is unique to that organism and functionally important to its life cycle, and most aminoglycosides target ribosomal RNA. Especially with the completion of the Human Genome Project, there was excitement about potential therapies targeting nucleic acids. As an example, antisense therapy has great promise but so far has had limited success. Similarly, RNA
interference has found great application in the lab but its uses in therapy so far have not lived up to initial expectations. The three dimensional complexity of RNA, much like an enzyme, makes it ideal for targeting based on shape rather than sequence. DNA, on the other hand, has a more regular structure that is difficult to target with a high level of selectivity. Also, there are no known repair mechanisms for RNA and there is an extensive literature detailing the hydrolysis of RNA as well as the oxidation of nucleic acids in general. Therefore it is expected that the targeted recognition and cleavage of a therapeutically relevant RNA could be an effective application of catalytic metallodrugs.

2.1.3.1 Nucleic Acid Cleavage

There is great interest in the design of small molecules that can efficiently cleave nucleic acids for applications in genetics as well as for the design of therapeutics. RNA is notoriously difficult to work with in the lab due its susceptibility to hydrolysis. DNA, on the other hand, is much more stable to hydrolysis. The presence of the 2’-OH in RNA provides a pathway for attack of the nearby phosphate leading to strand cleavage as shown in Figure 2.4. This reaction can be catalyzed by a positively charged metal ion by stabilizing the negative charge on the deprotonated 2’-OH to facilitate attack of the phosphate or by stabilizing the negative charges being formed in the transition state. A common approach is to use multiple metal centers, such as in the dieuropium compounds developed in the lab of Janet Morrow, and in this way both mechanisms can be exploited to catalyze RNA hydrolysis. These studies commonly use activated substrates and there
is a severe lack of studies describing detailed kinetic analysis of systems that degrade natural structured RNA sequences.

Aside from hydrolysis, the other possible pathway for nucleic acid cleavage is oxidation. For RNA, oxidation pathways are not well characterized and it is expected that RNA is more difficult to oxidize than DNA. For DNA there are a variety of known mechanisms for DNA oxidative strand scission. These pathways generally involve hydrogen abstraction on the ribose ring and result in distinct products depending on where this hydrogen abstraction occurs. These distinct products can be detected and used to determine the pathway, such as C-2’ or C-4’ hydrogen abstraction. For example, DNA cleavage by Fe-bleomycin under aerobic conditions occurs by H-4’ abstraction as shown in Figure 2.5. The phosphoglycolate product can be determined by polyacrylamide gel electrophoresis (PAGE) and the base propenal reacts with 2-thiobarbituric acid to yield a product that can be assayed colorimetrically. Other distinct products, such as free bases,
can be observed as well depending on the site of hydrogen abstraction. Pathways for oxidative degradation of RNA are less well characterized but may be expected to follow pathways similar to that for DNA but with lower reactivity. It is also possible that the presence of the 2’-OH could cause new pathways to occur or prevent others.

![Figure 2.5 Mechanism for oxidative degradation of DNA by bleomycin under aerobic conditions.](image)

2.1.3.2 Previous Use of Catalytic Metallodrugs Targeting RNA

Previous work in the Cowan lab has explored the use of metal based compounds for reaction with RNA. The first studies involved the coordination of a metal ion to aminoglycosides, such as in Cu$^{2+}$-kanamycin A as shown in Figure 2.6.$^{60, 61}$ The site shown for copper coordination is known to be tolerant of modification while still maintaining binding. It was demonstrated using agarose gel electrophoresis that this
compound could cleave DNA under physiological conditions. Aminoglycosides such as kanamycin and neomycin act as antibiotics and are known to interact with a variety of RNA structures including the HIV Rev response element (RRE). Site specific cleavage of RRE by copper aminoglycosides was also demonstrated and showed specificity for RRE over tRNA\(^{\text{Phe}}\). For this system, the mechanism of action was determined to be hydrolysis. The activity of copper kanamycin A was also demonstrated in a cell based fluorescence assay.\(^{60}\)

![Cu\(^{2+}\)-kanamycin A](image_url)

**Figure 2.6** Copper kanamycin A as an example of an early catalytic metallodrug targeting RNA.

The targeting of RNA was further applied to demonstrate the more specific cleavage of HIV RRE RNA by a metallopeptide based on the combination of the GGH ATCUN motif with the Rev peptide, which is known to bind to bind to the RRE with high affinity (K\(_d\) = 1.6 nM).\(^{62}\) Incorporation of a metal-binding ATCUN motif into the RNA binding Rev peptide did not significantly affect RRE binding. Variation of the metal binding domain was also explored and this system is one of the first examples of a structure activity relationship study with catalytic metallodrugs. The most active metal binding domain used was found to be one where the reduction potential (E\(^{\circ}\)) was between that of
the two coreagents used, ascorbate \((E^\circ = -66 \text{ mV vs. NHE})\) and \(\text{H}_2\text{O}_2\) \((E^\circ = +380 \text{ mV vs. NHE})\), as shown in Figure 2.7. This may be important for the cycling of the oxidation state of the metal, where it is poised to be both reduced by the ascorbic acid and oxidized by the hydrogen peroxide. The highest activities were observed for the Rev copper complexes of nitrilotriacetic acid (NTA, Figure 2.7) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), with rate constants of 2.1 nM RNA/min and 0.8 nM RNA/min, respectively.

**Figure 2.7** An example of a metallodrug based on the Rev protein for catalytic degradation of HIV RRE RNA (left). The most reactive derivatives showed a reduction potential between that of ascorbic acid and peroxide (right).

2.1.3.3 Phenylalanine Transfer RNA

Important for the development of catalytic metallodrugs is a more detailed understanding of the mechanism for metal-promoted cleavage of RNA, particularly when mediated by oxidative damage. While the pathways for oxidative degradation of DNA are well established, the influence of RNA structure and the presence of 2’-OH
functionality on reaction mechanism is not clearly defined. Eric Long and coworkers have examined the interaction of Ni-ATCUN complexes with RNA and observed cleavage only under conditions that included the use of oxone and treatment with aniline acetate. The cleavage of phenylalanine transfer RNA (tRNA\textsuperscript{Phe}, Figure 2.8) by Cu-ATCUN complexes in the presence of ascorbate and dioxygen is studied and compared with findings for related nickel ATCUN complexes, and the influence of amidation of the C-terminus of the ATCUN motif on both binding and chemistry is reported.

Figure 2.8 The secondary structure of tRNA\textsuperscript{Phe} (left) and how it folds into the three dimensional structure (right).
2.1.3.4 HIV-1 Trans-Activation Response Region RNA

As an initial target for therapeutic intervention, a metallopeptide targeting the trans-activation response region (TAR) RNA of the human immunodeficiency virus (HIV) was designed based on the Tat peptide. The Tat peptide, RKKRQRRRPPQ, is a small arginine rich peptide derived from the larger Tat protein that is well characterized as binding to TAR RNA and is based on the nuclear regulatory protein Tat. TAR RNA is a structured RNA present at the 5’-end of HIV-1 transcripts and has been shown to produce microRNAs that prevent apoptosis in infected cells. It is well established that compounds that interfere with the Tat-TAR interaction can disrupt replication of the virus and therefore TAR represents an attractive target for a catalytic metallodrug. Therefore, the peptide Cu-GGHGGGRKKRRQRRRPPQ-amide was evaluated for *in vitro* activity against HIV-1 TAR RNA.

2.2 Methods and Materials

2.2.1 Materials. Yeast tRNA$^\text{Phe}$ and diethyl pyrocarbonate were purchased from Sigma. ATCUN motifs were purchased from Bachem (CA), or from Genemed Synthesis Inc (South San Francisco, CA). TAR RNA was purchased from Dharmaco, part of Thermo Fisher Scientific (Lafayette, CO). All solutions were prepared in water treated for RNases with 0.1% by volume diethyl pyrocarbonate (DEPC) and tubes and pipette tips were autoclaved prior to use. The sequence of the Tat-binding catalytic metallodrug used
was GGHGGGRKKRRQRRRPPQ-amide. The sequence of the TAR RNA used was 5’-fluorescein-GGCAGAUCUGAGCCUGGGAGCUCUCUGCC-3’. Copper complexes were made by mixing peptide with CuCl$_2$ in a 1.1:1 peptide: copper ratio and allowed to sit for at least 5 minutes.

2.2.2 *Determination of Peptide Concentrations.* Peptide concentrations were determined by serial additions of CuCl$_2$ to a solution containing an estimated peptide concentration of 1 mM. UV-visible spectroscopy was used to monitor the formation of the band at 520 nm corresponding to the d-d absorption and the concentration of CuCl$_2$ at the turning point used to determine the peptide concentration.

2.2.3 *HPLC Analysis.* Samples were loaded onto a VYDAC monomeric C-18 reverse phase column equilibrated with 0.1 M ammonium acetate (NH$_4$OAc), pH = 6.8, with a flow rate of 0.500 mL/min. The products were eluted with acetonitrile according to the gradient described in Table 2.1 and monitored at 254 nm.

| Table 2.1 Solvent elution profile used for HPLC analysis. |
|---|---|---|---|---|---|---|---|
| Time (min) | 0 | 10 | 25 | 40 | 50 | 60 | 74 |
| % Acetonitrile | 0 | 0 | 0.6 | 2.0 | 10.0 | 30.0 | stopped |

The column was calibrated for the elution of adenine, guanine, uracil, and cytosine, as well as the two copper-ATCUN complexes used in these experiments, [Cu$^{2+}$-KGHK-COOH]$^+$ carrying a free C-terminal carboxyl, and [Cu$^{2+}$-KGHK-amide]$^{2+}$ with C-
terminal amidation. To monitor base release from the cleavage of tRNA\textsuperscript{Phe}, reaction samples containing tRNA\textsuperscript{Phe}, copper-ATCUN complex, and ascorbate (5 mM) in 50 mM Tris buffer, pH = 7.4, were loaded onto the HPLC column following incubation at various times.

2.2.4 Reaction Kinetics. The kinetics of metallopeptide-promoted scission of tRNA\textsuperscript{Phe} were followed by monitoring the rate of disappearance of tRNA\textsuperscript{Phe} substrate for each cleavage reaction. The working solution contained tRNA\textsuperscript{Phe} (200 µM), copper-ATCUN complex (200 µM), MgCl\textsubscript{2} (10 mM), and ascorbate (5 mM) in 50 mM Tris buffer (pH = 7.4). EDTA (5 mM) and urea (6 mM) were added to stop the reaction and were added immediately before loading and denatured by heating at 90 °C for 5 minutes. Residual tRNA\textsuperscript{Phe} was determined by calculating the area of the peak for tRNA\textsuperscript{Phe} following reaction. The same amount of EDTA and urea were added to controls according to the same procedure.

2.2.5 Binding Experiments. The binding affinities of [Cu\textsuperscript{2+}-KGHK-COOH]\textsuperscript{+} and [Cu\textsuperscript{2+}-KGHK-amide]\textsuperscript{2+} toward tRNA\textsuperscript{Phe} were determined fluorimetrically by following the second order scattering of tRNA\textsuperscript{Phe} on a Perkin Elmer LS 50B Luminescence Spectrometer. The resulting binding curves were fit by use of a standard equation (2.1) that accommodates two distinct classes of binding site (strong and weak) to yield apparent binding constants (K\textsubscript{d1} and K\textsubscript{d2}) using Origin 7.0 software, where I is the observed emission intensity at the concentration of binding species C (copper ion or
copper complexes), $I_B$ is the emission intensity in the fully bound state, $I_F$ is the emission intensity in the fully free state, and $I_I$ is the emission for the intermediate bound state.

$$I = \left( K_{d1} K_{d2} I_F + K_{d2} C I_I + C^2 I_B \right) / \left( K_{d1} K_{d2} + K_{d1} K_{d2} C + K_{d1} K_{d2} C^2 \right) I_B \quad (2.1)$$

A working solution containing tRNA$^{\text{Phe}}$ (20 nM) in 50 mM Tris buffer (pH = 7.4) and 10 mM MgCl$_2$ was prepared and placed in a quartz cuvette. Fluorescence was monitored using an excitation wavelength of 260 nm and monitored at 520 nm. The influence of serial addition of aliquots of copper-peptide or copper ion on intensity was recorded, and no correction was required for inner filter effects at the wavelengths used.

### 2.2.6 Mass Spectrometry

Reactions for mass spectrometry were carried out in 40 µL reaction volumes with the following concentrations: 100 µM tRNA$^{\text{Phe}}$, 50 mM Tris buffer (pH = 7.4), 10 mM MgCl$_2$, 100 µM copper-ATCUN complex, 5 mM ascorbate. Reactions were run for 24 hours and then submitted for mass spectrometric analysis by the Ohio State University Campus Chemical Instrumentation Center. The samples were dialyzed and desalted using a ZipTip (Millipore, Bedford MA) prior to analysis via matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. MALDI experiments were performed on a Bruker Reflex III (Bruker, Breman, Germany) mass spectrometer operated in linear, positive ion mode with a N$_2$ laser. Laser power was used at the threshold level required to generate signal. The accelerating voltage was set to 28 kV.
2.3 Results

2.3.1 Determination of Peptide Concentrations

In order to determine the concentration of the peptides used, titration of CuCl$_2$ into a solution of peptide was used to generate a curve as shown in Figure 2.9. Binding of copper to peptides containing the ATCUN sequence is very fast and tight and the complex can be assumed to be formed in a 1:1 ratio where the turning point is used to determine the peptide concentration.

![Graph showing the titration of CuCl$_2$ into a solution of peptide. The x-axis represents [CuCl$_2$] (mM) and the y-axis represents Abs (520 nm). The graph shows a turning point where [CuCl$_2$] = Peptide concentration.](image)

**Figure 2.9** Example of a copper titration showing the formation of the d-d band at 520 nm. From this, the concentration of peptide can be determined.
2.3.2 tRNA\textsuperscript{Phe} Cleavage

2.3.2.1 Binding of Cu\textsuperscript{2+}-ATCUN to tRNA\textsuperscript{Phe}.

Binding of [Cu\textsuperscript{2+}-KGHK-COOH]\textsuperscript{+} and [Cu\textsuperscript{2+}-KGHK-amide]\textsuperscript{2+} to tRNA\textsuperscript{Phe} is shown in Figure 2.10. The RNA was not labeled with a fluorophore, but binding was monitored by following the second order scattering.\textsuperscript{64} It is clear that both complexes bind similarly, but that the peptide control (no copper) does not show significant binding under these solution conditions. Optimal fitting of both copper peptides required a weaker and higher affinity site, while free Cu\textsuperscript{2+} (aq) revealed only one class of site. The amidated [Cu\textsuperscript{2+}-KGHK-amide]\textsuperscript{2+} complex showed apparent affinities of $K_{d1}^{\text{app}} \sim 71$ µM and $K_{d2}^{\text{app}} \sim 211$ µM, while the carboxylated form showed $K_{d1}^{\text{app}} \sim 34$ µM and $K_{d2}^{\text{app}} \sim 240$ µM. Binding of CuCl\textsubscript{2} to the tRNA\textsuperscript{Phe} under the same conditions was also observed, yielding $K_{d1}^{\text{app}} \sim K_{d2}^{\text{app}} \sim 108$ µM.

![Graph showing binding curves for tRNA\textsuperscript{Phe}. Triangles = KGHK-COOH, circles = [Cu\textsuperscript{2+}-KGHK-amide]\textsuperscript{2+}, squares = [Cu\textsuperscript{2+}-KGHK-COOH]\textsuperscript{+}, and diamonds = Cu\textsuperscript{2+} (aq).]
2.3.2.2 Cu\(^{2+}\)-ATCUN Mediated Cleavage of tRNA\(^{\text{Phe}}\) and HPLC Characterization of Products.

The time-dependence of the reaction of [Cu\(^{2+}\)-KGHK-COOH]\(^+\) and [Cu\(^{2+}\)-KGHK-amide]\(^2+\) with tRNA\(^{\text{Phe}}\) was monitored by HPLC and the disappearance of the RNA peak over time was used to monitor the kinetics of the reaction (Figure 2.11). A \(k_{\text{obs}} \sim 0.075\) h\(^{-1}\) was determined for both complexes by monitoring the loss of peak intensity as a function of time. No activity was observed for free copper ion under comparable reaction conditions and concentrations of tRNA\(^{\text{Phe}}\) and catalyst.

**Figure 2.11** Degradation of tRNA\(^{\text{Phe}}\) by Cu-KGKH-COOH as monitored by HPLC (left); data was fit to a first order exponential decay to obtain \(k_{\text{obs}}\).

HPLC Analysis of tRNA\(^{\text{Phe}}\) cleavage by [Cu\(^{2+}\)-KGHK-COOH]\(^+\) (Figure 2.12) shows a peak corresponding to guanine (11.35 min) that increases as the reaction proceeds. This experiment was performed without the addition of urea so that the early peaks, which correspond to where free bases occur, could be easily seen. No other free bases were
observed.

![HPLC analysis of tRNA<sup>Phe</sup> cleavage by [Cu<sup>2+</sup>-KGHK-COOH]<sup>+</sup>. A peak corresponding to guanine (11.35 min) increases as the reaction progresses.](image)

**Figure 2.12** HPLC analysis of tRNA<sup>Phe</sup> cleavage by [Cu<sup>2+</sup>-KGHK-COOH]<sup>+</sup>. A peak corresponding to guanine (11.35 min) increases as the reaction progresses.

2.3.2.3 Mass Spectrometric Characterization of Reaction Products.

Mass spectrometric data for a tRNA<sup>Phe</sup> control, a 24 hour reaction with [Cu<sup>2+</sup>-KGHK-COOH]<sup>+</sup>, and a 24 hour reaction with [Cu<sup>2+</sup>-KGHK-amide]<sup>2+</sup> (Figure 2.13), show 2 peaks in the tRNA<sup>Phe</sup> control corresponding to the singly and doubly charged species. For the reaction with the amidated complex, [Cu<sup>2+</sup>-KGHK-amide]<sup>2+</sup>, it is seen that these 2 peaks are still present (m/z = 24,550.336 and 12,299.710), but that they are diminished. Additional peaks, corresponding to smaller fragments of the RNA, the largest peak at m/z = 6460.344 and smaller peaks at 7151.455, 6331.242, and 5927.019, were observed. The reaction with [Cu<sup>2+</sup>-KGHK-COOH]<sup>+</sup> also shows mass peaks corresponding to product fragments but the parent peaks are no longer present. The major peak is at 6478.172 with peaks also observed at 7172.008, 6316.278, and 5940.528. The smaller fragments that
are observed correspond to products from tRNA\textsuperscript{Phe} cleavage.

2.3.3 TAR RNA Cleavage

Studies of the reactivity of Tat-based metallodrugs with TAR RNA did not show observable reactivity. As an example, the reaction of Cu-GGHGGGRKKRRQRRPPQ-amide with TAR RNA as monitored by HPLC is shown in Figure 2.14. By monitoring the peak area over time, it can be seen that there was no significant change in the concentration of the parent RNA. Reactivity was also monitored by PAGE and in vitro fluorescence assays in the presence of different combinations of ascorbic acid and
hydrogen peroxide but no clear reactivity could be established.

**Figure 2.14** Monitoring of TAR cleavage by HPLC did not show any significant changes.

2.4 Discussion

2.4.1 tRNA$\text{Phe}^*$ Cleavage

2.4.1.1 Binding and Cleavage Efficiency.

Figure 2.10 shows that both $[\text{Cu}^{2+}\text{-KGHK-COOH}]^{+}$ and $[\text{Cu}^{2+}\text{-KGHK-amide}]^{2+}$ bind to tRNA$\text{Phe}^*$ with similar affinity (34 $\mu$M versus 71 $\mu$M at the higher affinity sites).

Amidation of the C-terminus increases the overall positive charge of the complex and would be expected to significantly impact binding to negatively-charged RNA if binding was largely dependent on electrostatics. In fact, the carboxylate form binds more tightly, suggesting that factors other than electrostatics control binding. The importance of
structural recognition, rather than simple electrostatics, as a driving force for binding is underscored by the fact that the copper-free peptides do not show any observable binding to tRNA\(^{\text{Phe}}\) under these solution conditions. While binding to tRNA\(^{\text{Phe}}\) is observed for free Cu\(^{2+}\)\textsubscript{(aq)}, no reactivity with RNA was observed under these conditions.

Both copper peptides appear to bind to tRNA\(^{\text{Phe}}\) where the D and \(\Psi\)X loops form pockets in a structurally complex fold that has previously been associated with magnesium binding.\textsuperscript{65,66} These pockets, and other loop domains, display unpaired bases that might favor copper binding. Free copper ion tends to bind to base heteroatoms rather than the phosphates, and the coordination chemistry with tRNA has previously been examined.\textsuperscript{67} It is therefore plausible that free copper could bind to a select few sites, consistent with the relative simplicity of the binding behavior for the free ion.

To avoid complications through competitive binding, the absence of solvated copper in solutions of copper peptide was insured by the use of a slight excess of peptide ligand, and the high affinity of the peptide for divalent copper.\textsuperscript{68} The absence of significant levels of free copper under the conditions used for these experiments has been previously documented,\textsuperscript{41,69} although for both complexes it is likely that the metal center contributes to binding through direct coordination to functional groups on RNA, while also defining the structure of the peptide motif that optimizes contact with the RNA target. No reaction chemistry was observed for the free copper, and the free peptide alone does not show any binding.

The similarity between the two copper complexes in binding to tRNA\(^{\text{Phe}}\), and RNA cleavage activity, reflects other similarities in the electronic properties of each complex.
In particular, the Cu\textsuperscript{2+/3+} redox couples for the [Cu\textsuperscript{2+}-KGHK-COOH]\textsuperscript{+} and [Cu\textsuperscript{2+}-KGHK-amide]\textsuperscript{2+} complexes have previously been determined as 1.049 V and 1.050 V (vs. NHE), respectively.\textsuperscript{70} There is also likely to be a similarity in the orientation of metal-associated reactive oxygen species relative to scissile bonds. This is consistent with the work by Long et al.,\textsuperscript{40} described in section 2.4.1.3, where the overall charge also did not have a significant effect.

2.4.1.2 Site Selectivity.

The mass spectrometric data show a limited number of cleavage fragments, consistent with the observed chemistry occurring at a discrete site or sites. Using the observed product peak masses as a selection criterion, the potential cleavage sites map to two loops that are structurally juxtaposed (not the acceptor region), as illustrated in Figure 2.15. The peak for the tRNA\textsuperscript{Phe} is seen at or near 25 kDa. The sites that give rise to the largest product peak (~ 6460 Da) are marked with arrows. Other peaks were observed at 5927.0, 6272.8, and 7151.5. Structurally, these two loops lie close together in space (Figure 2.15, left) and the data are consistent with the presence of one binding site with cleavage occurring within this proximity via C-1’H or C-4’H oxidative cleavage paths as previously described for reactivity with DNA. The observed release of guanine is also consistent with these mechanisms.
2.4.1.3 Comparison to Ni$^{2+}$-ATCUN

The reaction of nickel complexes of a series of C-terminally amidated peptides (GGH-amide, KGH-amide, and RGH-amide) with tRNA$^{Phe}$ has previously been reported by Eric Long and coworkers.$^{40}$ For the case of the nickel complex, the reactions were carried out for only 1 minute, however, cleavage was observed only with oxone as a coreactant and following treatment with aniline acetate. In the present studies, no reaction was observed for the nickel complexes when ascorbate and dioxygen were used as coreagents. However, reactivity was confirmed for [Ni$^{2+}$-GGH-amide] when using oxone as a reactive oxygen precursor. Reactivity was observed both with and without treatment with aniline acetate, although the method for monitoring reaction progress was different [PAGE gels (previous work) versus HPLC (this work)]. This is consistent with prior

![Figure 2.15](image)

**Figure 2.15** The cleavage sites as determined by mass spectrometry map to the same location in three dimensional space. These sites of cleavage are shown highlighted in the three dimensional structure (left).
observations that nickel peptide derivatives appear to be less capable of efficiently mediating formation of reactive oxygen species from ascorbate and dioxygen, demonstrating enhanced activity only when the peroxide is provided as a preformed activated species.\textsuperscript{70}

\textbf{Figure 2.16} Secondary structure of tRNA\textsuperscript{Phe}. The arrows denote the sites of cleavage induced by Ni-bound peptides, where the length of the arrow is proportional to the extent of cleavage chemistry. The dashed lines indicate positions of intraloop hydrogen bonding [from I.J. Brittain, X. Huang, E.C. Long, Biochemistry. (1998), 12113].

The conditions described herein for the copper complexes utilized ascorbate and dioxygen as reagents and the observed cleavage sites are more limited relative to the cleavage sites observed for the nickel analogs (Figure 2.15 compared to Figure 2.16). The sites suggested by the mass spectrometric data for the copper complexes do, however, correspond to a region where chemistry was also observed for the [Ni\textsuperscript{2+}-GGH-
amide] complex. It is likely that the lower selectivity demonstrated by the nickel peptide derivatives reflects the use of a shorter tripeptide motif, relative to the copper tetrapeptides used in the current studies, although one of those sites could also correspond to a non-productive secondary binding site for the copper peptides reported here, consistent with the double filter mechanism described in chapter 1.

Studies of the reactivity of the non-amidated form \([\text{Ni}^{2+}\text{KGHK-COOH}]^+\) showed no reaction, while reactivity was observed for both the amidated and the carboxylated forms of the copper peptide. These subtle differences in reactivity emphasize the fact that small changes in catalyst configuration can and do have significant effects on the reactivity of the metallopeptide complexes. This observation is consistent with a double filter mechanism where both binding and chemistry need to be tuned in order to show activity against the target. Variation in the peptide composition, therefore, provides the opportunity for fine-tuning of properties and the design of systems that react more efficiently with RNA.

2.4.2 Lack of Reactivity with TAR RNA

The lack of observed reactivity of Cu-GGHGGGRKKRRQRRRPPQ-amide with TAR RNA as shown in Figure 2.14 emphasizes the double filter mechanism where both the RNA binding domain and the metal binding domain have to be optimized for the given target. Other methods such as polyacrylamide gel electrophoresis and \textit{in vitro} fluorescence assays did not show reactivity either. Binding alone is not sufficient to
achieve an active catalyst. The TAR system, therefore, represents an experimental demonstration of this double filter effect.

2.5 Conclusions

Oxidative cleavage of tRNA\textsuperscript{Phe} mediated by [Cu\textsuperscript{2+}-KGHK-COOH]\textsuperscript{+} and [Cu\textsuperscript{2+}-KGHK-amide]\textsuperscript{2+} complexes has been described. The fragments observed by mass spectrometry lie close together in three-dimensional space and suggest the presence of one major reaction site. Amidation of the peptide did not result in a significant change of binding or reactivity of the copper peptide complex, although an impact on relative reactivity for copper versus nickel peptide complexes was observed. Variations in reactivity for copper and nickel peptides could reflect either the large gain in LFSE accompanying the transition from d\textsuperscript{9} (Cu\textsuperscript{2+}) to d\textsuperscript{8} (Cu\textsuperscript{3+}), relative to the smaller gain in LFSE for the transition from d\textsuperscript{8} (Ni\textsuperscript{2+}) to d\textsuperscript{7} (Ni\textsuperscript{3+}), or relative changes in coordination geometry, where the geometry for copper in both the d\textsuperscript{8} and d\textsuperscript{9} configurations is expected to be similar (square planar), while for nickel derivatives a distinct coordination preference would be expected for each of the d\textsuperscript{7} and d\textsuperscript{8} configurations. Such differences in activity most likely reflect subtle variations in the orientation of the metal-associated reactive oxygen species relative to the RNA,\textsuperscript{71, 72} and emphasize optimal positioning of the metal-associated reactive oxygen species, relative to scissile bonds, as a major criterion for development of efficient catalytic nucleases or therapeutics.

The metallopeptide targeting TAR presents an example of a system where simply
attaching a metal binding domain to an RNA binding domain does not result in an active catalytic metallodrug.

A deeper understanding of the mechanism and how to optimize catalytic metallodrugs is required in order to design active metallodrugs. This requirement should help to reduce any reactivity with molecules other than the target of interest and lower toxicity. A more detailed understanding in the future could allow for the modification of this peptide to generate an active catalytic metallodrug. Part of understanding this optimization is also a more detailed and effective way to assay and characterize this class of compounds. The next chapter describes a catalytic metallodrug targeting hepatitis C RNA. These compounds represent potentially useful therapeutics but also provide a system for probing the mechanism and exploring the best ways to characterize and optimize catalytic metallodrugs in general.
3.1 Introduction

3.1.1 Hepatitis C Virus General Information

Hepatitis C is a chronic viral disease that affects approximately 170 million people worldwide.\(^73\) This is approximately five times the number of people worldwide infected by human immunodeficiency virus (HIV). After identification of the viruses that cause hepatitis A and hepatitis B, it was discovered that most cases of hepatitis could not be attributed to either one of these. It was because of this that hepatitis C was originally called non-A, non-B hepatitis (NANBH). It wasn’t until 1989 that the active agent for hepatitis C was actually identified. There are seven different genotypes with genotype 1 causing ~70% of cases in the United States and genotype 2 about 20%. This varies significantly, however, depending on location. For example, genotype 3 is the most common form in Pakistan where hepatitis C infection is estimated to be as high as 6%.\(^74\) The symptoms for hepatitis C infection are nonspecific and include fatigue, nausea, and joint pains. On top of this, a person can often be infected for years or decades before the symptoms become apparent. It can also develop into a spectrum of diseases including fibrosis, cirrhosis and hepatocellular carcinoma, and is a major factor in liver disease.
The most common modes of transmission are via needles in intravenous drug use or from blood transfusion. Hepatitis C is usually diagnosed by the detection of antibodies or enzymes in the blood or by a liver biopsy. The spread of hepatitis C is usually prevented simply by careful use of sterilized needles and following the appropriate protocol in a healthcare environment such as the proper screening of blood samples.

3.1.1.1 Current Treatment for Hepatitis C

No effective vaccine is available for hepatitis C infection and the current treatment involves a combination of pegylated interferon-alpha-2b (rIFNα-2b) and ribavirin (Figure 3.1), neither of which is selective for hepatitis C virus (HCV) or viral disease in general. Interferon α helps to protect from viral infection by a variety of mechanisms including boosting the immune response, and the addition of polyethylene glycol (PEG) increases its half life in vivo. For an RNA virus such as hepatitis C, ribavirin acts as a nucleoside analog mimicking adenine or guanine and is incorporated into viral RNA and inducing mutations. Drawbacks to this combination therapy include a variety of side effects including flu-like symptoms, cardiovascular toxicity, and anemia. In fact, it has been estimated that 70-90% of patients that go to the clinic seeking treatment end up refusing it. Drugs against an HCV target molecule would have the potential for greater specificity and, therefore, lower toxicity. Two HCV NS3 serine protease inhibitors, boceprevir and telaprevir (shown in Figure 3.2), were approved by the Food and Drug Administration (FDA) in 2011 and represent the first class of compounds with a
mechanism of action specific to hepatitis C.\textsuperscript{77, 78} Inhibition of this protease prevents the formation of proteins important for processing of the HCV genome and it is known to be essential for the virus. These protease inhibitors show great promise but are currently limited to HCV genotype 1 and involve a complicated regimen which can negatively impact patient compliance even further.

![Figure 3.1 Structure of ribavirin, one of the current treatments for hepatitis C.](image)

![Figure 3.2 Structures of the recently approved NS3 protease inhibitors boceprevir and telaprevir.](image)
3.1.1.2 Future Directions for HCV Treatment

There are currently a large number of compounds being tested in clinical trials that target a variety of enzymes important to HCV.\textsuperscript{79} There are four enzymes that are well known to be required for replication of the virus. These are NS3/4A serine protease, NS2/3 autoprotease, NS3 helicase, and NS5B RNA dependent RNA polymerase (RdRp). These are some of the most common targets for drug design and include the serine protease inhibitors mentioned previously and similar compounds, as well as NS5B inhibitors and HCV RdRp inhibitors. Another interesting drug target is NS5A. Interfering with this protein seems to be especially potent despite the fact that its function is still unknown.\textsuperscript{76} Other classes of compounds being studied include entry inhibitors and inhibitors of cyclophilin, another protein important for HCV replication and infection.

3.1.1.3 Application of Catalytic Metallo drugs to HCV Treatment

HCV treatment is currently moving towards multidrug therapy where mixtures of drugs with different mechanisms of action attack the disease from different angles. One advantage of this is to help combat resistance wherein it is difficult for the virus to develop resistance to multiple drugs at the same time. Catalytic metallo drugs fit perfectly into this paradigm by virtue of the fact that they act by a unique mechanism that nicely complements current treatments. The design of these catalytic metallo drugs is also such that mutations reducing drug binding could potentially not affect, or could even enhance,
activity on the RNA target due to improved catalyst release and turnover. Aside from combating resistance, catalytic metallodrugs are expected to have lower required doses and higher specificity, addressing current issues with side effects. Toxicity due to side effects also causes problems with patient compliance and catalytic metallodrugs will help in this area as well.

3.1.1.4 HCV Internal Ribosomal Entry Site RNA

Viruses commonly contain RNA motifs that are both structurally complex and functionally important, but are not found in humans. These structured RNAs are attractive as potential targets for therapeutic agents. HCV is an enveloped, positive-strand RNA virus that belongs to the genus Hepacivirus in the Flaviviridae family, and contains ~9600 ribonucleotides in its genome. The presence of a highly conserved sequence in the 5’-untranslated region (5’-UTR), termed the internal ribosomal entry site (IRES), directs translation of the viral RNA to viral polyprotein in a manner that is independent of capping initiation, which is typically required by the eukaryotic translation system of a host. The IRES initiates translation by recruiting proteins to form a complex that binds to the ribosome. Translation results in the synthesis of a single polyprotein precursor that is processed by the host and its viral proteases to form mature viral proteins for virus assembly. Structurally, the HCV IRES consists of 4 domains (Figure 3.3, left), with distinct functions that result in initiation of translation. Another feature that makes the HCV IRES an attractive drug target is that it is highly conserved
(~90% identity) across genotypes.\textsuperscript{81} The HCV protease inhibitors mentioned earlier, boceprevir and telaprevir, only work for genotype 1. There is a large amount of variation in NS3 proteases across genotypes and, in fact, it has been shown that only a small number of mutations are required to infer resistance in genotype 1.\textsuperscript{82}

**Figure 3.3** Secondary structures of the full length HCV IRES (left), SLIIb (middle), and SLIV (right).

3.1.1.4.1 Stem Loop II

The stem loop IIb (SLIIb, Figure 3.3, middle) of IRES has been shown to be important for the initiation of translation for HCV and represents a promising target for therapeutic intervention. Domain II stabilizes changes in the conformation of the 40S subunit of the ribosome that are important for the initiation of translation. Mutation studies have shown that stem loop II is essential for IRES activity.\textsuperscript{83} Other studies have also shown that the structure of domain II remains intact in the absence of the other subunits, making the
isolated stem loop a suitable model for study in vitro. Interference with the normal activity of the stem loop II domain will inhibit the virus’s ability to pass through its life cycle and is a potential target for the design of new anti-HCV drugs. A SLIIa binding agent based on a benzimidazole scaffold has been previously reported. This compound causes a structural change in SLIIa and showed activity in an HCV cellular replicon assay. Catalytic metallodrugs targeting SLIIb will be the focus of this chapter and chapter 4.

3.1.1.4.2 Stem Loop IV

Several recent studies on the HCV IRES have shown that SLIIb and stem loop IV (SLIV, Figure 3.3, right) lie close together in the 40S bound structure and, in fact, it was shown that mutations of SLIIb lead to structural perturbations in the bound structure of SLIV. The IRES SLIV also binds to the human La protein of which the NMR structure is already known. SLIV contains the AUG start codon and previous work has looked at the importance of SLIV and its potential as a target for therapeutic intervention, including the use of short hairpin RNA targeting the GCAC sequence near the AUG start codon as well as the use of the LaR2C peptide, derived from the La protein, to block binding of the full length protein. Catalytic metallodrugs targeting SLIV will be the focus of chapter 5.
3.1.1.4.3 Other Stem Loop Domains

Domain III of the HCV IRES is the largest domain of the IRES and consists of the six subdomains a-f. It is important for the recruitment of the 40S subunit of the ribosome as well as binding to the eukaryotic initiation factor 3 (eIF-3) which is important for 60S subunit binding. NMR and x-ray crystal structures are now available for many of these individual subdomains. While both domains II and III are necessary for ribosome assembly, domain I is not required for translation but instead is important for replication.

3.1.2 Design of a Catalytic Metallodrug Targeting HCV IRES SLIIb

The first compound used to demonstrate catalytic degradation of HCV IRES SLIIb is shown in Figure 3.4. Selective recognition of the HCV IRES stem-loop IIb domain derives from incorporation of a C-terminal tetrapeptide (YrFK-amide) targeting domain into the metallodrug design, which includes a D-configuration arginine. This sequence has been found to bind to stem loop IIb of the HCV IRES and has also been implicated as a potential uptake sequence for the delivery of other molecules to cells. This tetrapeptide is a dermorphin analog known as DALDA and is a highly specific μ-opioid receptor agonist. It has been studied as an analgesic with potentially lower side effects relative to current nonspecific opioid analgesics such as morphine. These peptides have been shown to cross a cell membrane by an energy-independent mechanism. Cu-GGHYrFK-amide is evaluated both in vitro and in a HCV cellular replicon assay. It
incorporates a copper ATCUN metal binding domain and the modular design provides flexibility for the future studies and optimization described in chapter 4.

![Figure 3.4](image-url) Structure of Cu-GGHYrFK-amide, containing a D-configuration arginine, a catalytic metallodrug targeting HCV IRES SLIIb.

3.2 Methods and Materials

3.2.1 Materials. RNA was purchased from Dharmacon, part of Thermo Fisher Scientific (Lafayette, CO). Peptides were purchased from Genemed Synthesis Inc. (South San Francisco, CA). The sequence for the IRES SLIIb RNA used was 5’-fluorescein-GGCAGAAAGCGUCUAGCCAUGGCUUAGUAUGCC-3’, for the IRES SLIV RNA was 5’-fluorescein-GGACCGUGCACCAUGAGCACGAAUCC-3’, and for HIV Rev Response Element (RRE) RNA was 5’-fluorescein-UUGGUCUUGGGCGACGCACGUGACCGGUACAGGCC-3’. The sequence of the 5mer RNA used for calibration in the MALDI-TOF experiments was 5’-fluorescein(T)-UGUG-3’. All RNA was annealed by heating to 95 °C and then cooled slowly to room
temperature before use. Sodium chloride, sodium hydroxide, and acetonitrile were purchased from Fisher, and HEPES, ammonium citrate, and 3-hydroxypicolinic acid were purchased from Sigma. C_{18} ZipTips were obtained from Millipore. All experiments were performed using diethyl pyrocarbonate (DEPC) treated water and autoclaved pipette tips and tubes. Copper complexes form quickly and were made by mixing in a 1.1:1 peptide:CuCl\(_2\) ratio and waiting at least five minutes for complex to form. Structures were rendered in the Visualization Applet for RNA (VARNA) for RNA secondary structures\(^93\) and the PyMOL Molecular Graphics System, Version 1.5.0.4 (Schrödinger, LLC) or AutoDockTools, as indicated, for three dimensional structures.

3.2.2 RNA Binding Experiments. For GGHYrFK-amide, RNA binding experiments were performed in the presence of 84 nM GGHYrFK-amide (1) in 20 mM HEPES (pH 7.4), 100 mM NaCl. Aliquots of unlabeled IRES SLIIb RNA were added and tyrosine emission was monitored (\(\lambda_{cx} = 280\) nm, \(\lambda_{em} = 313\) nm). Binding data for GGHYrFKGGGYGRKRRQRRR-amide (1-Tat) and GGHYrFKGGGGKDEL-amide (1-KDEL) were obtained by adding serial additions of peptide to 5'-fluorescein labeled IRES SLIIb and monitoring the emission at 518 nm (Ex = 485 nm). Data were then fit to a one-site binding model using Origin 7.0 software.

3.2.3 Polyacrylamide Gel Electrophoresis. All reactions were run in 20 mM HEPES, 100 mM NaCl, pH 7.4 and in the presence of 1 mM ascorbic acid and 1 mM H\(_2\)O\(_2\) and reacted for varying times with 1 \(\mu\)M RNA and 20 \(\mu\)M Cu-GGHYrFK-amide. The tubes
were then heated to 90 °C in the presence of EDTA and 8 M urea for 5 min and loaded onto a 16% polyacrylamide 8 M urea gel. Gel electrophoresis was performed at 200-250 V for 3-4 h. Gels were analyzed on a Bio-Rad Gel Doc 1000.

3.2.4 Reaction Kinetics via Fluorescence. HCV IRES RNA cleavage was monitored in vitro by fluorescence using 5’-fluorescein end-labeled RNA with excitation and emission wavelengths of 485 nm and 518 nm, respectively. Reactions were carried out in reaction volumes of 100 µL in the presence of 1 mM ascorbate and 1 mM H₂O₂ in 20 mM HEPES buffer (pH = 7.4), 100 mM NaCl with 100 nM of the catalyst and analyzed according to the change in fluorescence observed as the reaction occurred. Both a time dependence and a concentration dependence of loss of RNA substrate were observed. The initial velocities were fit to a line using Origin 7.0 software and kₘₜₜ obtained and plotted as a function of RNA concentration. These values were then used to obtain the Michaelis-Menten parameters.

3.2.5 Mass Spectrometry. Reactions for MALDI-TOF analysis were run as above but using 10 µM fluorescein labeled IRES SLIIb and 10 µM copper-peptide and incubated for two hours. Reactions were then quenched by being placed on ice and desalted. Desalting was performed using C₁₈ ZipTips from Millipore Co. in order to desalt reaction mixtures prior to mass spectrometric analysis. ZipTips were wetted with a 50:50 mixture of acetonitrile:water and equilibrated with 2 M triethylammonium acetate (TEAA), pH 7.0. The reaction mixture was then bound to the ZipTip, washed with nanopure water,
and eluted with 50:50 acetonitrile:water. These samples were spotted onto a Bruker ground steel 96 target microScout plate by first spotting with 1 µL of the 0.3 M 4-hydroxypicolinic acid (HPA), 30 mM ammonium citrate matrix solution in 30% acetonitrile, drying, then spotting with 2.5 µL of a 1:1 RNA: matrix mixture, and allowed to dry. A calibration mixture containing 3 RNAs covering a range of molecular weights (Fl-5mer, Fl-IRES SLIV, and Fl-RRE, with molecular weights of 2057.5, 8861.5, and 12172.5 amu, respectively) was used to calibrate the instrument. All MALDI-TOF MS analysis was performed on a Bruker MicroFlex LRF instrument, equipped with a gridless reflectron, using negative ion mode and reflectron mode. The pulsed ion extraction time was 1200 ns. At least 1000 shots were summed per spectrum. Data analysis was performed using Bruker flexAnalysis software. Only m/z values > 1000 amu were considered, since excessive spectral crowding occurred at lower m/z ranges. Reaction peaks were compared to controls containing SLIIb RNA alone and SLIIb RNA in the presence of 1 mM ascorbate, 1 mM H₂O₂ and new peaks were identified as products of cleavage by the metallopeptide.

3.2.6 Conformational Analysis. All molecular modeling experiments were performed in collaboration with Martin James Ross from the Cowan lab. A mixed torsional/low-mode sampling conformational search utilizing Schrodinger’s MacroModel v9.8 (Schrödinger, LLC, New York, NY, 2011) was used to determine the global minimum of the peptides in aqueous solution. The starting structures were modified to avoid getting trapped in a local minimum by linearizing the backbone of the peptide. The amino terminal nitrogen,
the guanidine group on arginine, and the amino side chain nitrogen on lysine were simulated in the neutral form due to the limitations of the program’s ability to handle charges. The energy window used in the searches was 100.0 kJ/mol. The conformational search\textsuperscript{94} was done with 10,000 MC steps with water as the solvent while using an Amber* force field. For YrfK-amide, a total of 6,253 conformers were found out of 10,000 conformers processed with the global minimum being -786.036 kJ/mol which was converged to twice. The top five conformations were within 7 kJ/mol and found eleven times.

3.2.7 Docking. The NMR structure of SL1Ib is readily available from the protein data bank (PDB: 1P5N). The top NMR structure (out of 20) was used for docking simulations in Autodock version 4.2. The free RNA targeting domain and the targeting domain containing Cu-GGH were both simulated. The docking area considered the full length SL1Ib. This was done to investigate the site of localization of the peptide/complex on the RNA. In all cases, the peptide/complex was made flexible except for aromatic carbons and peptide bonds. As a result of the high number of flexible bonds already in the complex, the RNA was restricted to its initial state. The top structure from conformational analysis for each peptide was used for docking. Autodock does not have parameters for copper so an iron atom was substituted as a place holder to mimic the geometry of the copper with a charge of +1.5. A value of +1.5 is used to simulate the +2 charge in order to compensate for the tendency of Autodock to overestimate electrostatic interactions.\textsuperscript{95} The geometry around the metal was based on the x-ray crystal structure of
Cu-GGH and was restricted to a square planar configuration.\textsuperscript{42} A Lamarckian Genetic Algorithm was used with a docking sample of 60 sites.

### 3.2.8 HCV Cellular Replicon Assay
All HCV cellular replicon assays were performed by Dr. Zhuhui Huang and staff at Southern Research Institute. A stable cell line ET (luc-ubi-neo/ET) was employed in the assay. The ET is a Huh7 human hepatoma cell line that contains an HCV RNA replicon with a stable luciferase (Luc) reporter and three cell culture-adaptive mutations. The HCV RNA replicon antiviral evaluation assay examined the effects of compounds at six half-log concentrations each. Human interferon alpha-2b was included in each run as a positive control compound. Sub-confluent cultures of the ET line were plated out into 96-well plates that were dedicated for the analysis of cell numbers (cytotoxicity) or antiviral activity, and various concentrations of metallodrugs and controls were added to the appropriate wells the following day. Cells were processed 72 hours later when the cells were still sub-confluent. Six half-log serial dilutions of the compound were performed, and values derived for IC\textsubscript{50} (the concentration that inhibited virus replication by 50%), TC\textsubscript{50} (the concentration that lowered cell viability by 50%) and TI (the selectivity index: TC\textsubscript{50}/IC\textsubscript{50}). HCV RNA replicon levels were assessed as the replicon-derived Luc activity. The toxic concentration of drug that reduced cell numbers (cytotoxicity) was assessed by the CytoTox-1 cell proliferation colorimetric assay (Promega).
3.2.9 *Real-time Polymerase Chain Reaction.* From the above replicon assays, medium was replenished and compound was added every 3 days for a total of 9 days. The cells were collected at 0, 3, 6 and 9 days for RNA extraction and measurement of HCV RNA copies by qRT-PCR (TaqMan).\(^6\) Ribosomal RNA determined simultaneously using qRT-PCR was used for calibration of HCV RNA reduction.

3.3 Results

3.3.1 GGHYrFK-amide Binding to SLIIb

The $K_d$ for binding of Cu-GGHYrFK-amide to SLIIb was determined to be 44 nM. This is consistent with the value that was expected based on previous results (unpublished data, H. D. Robertson, Cornell University). The plot for determination of the $K_d$ is shown in Figure 3.5.

![Figure 3.5](image_url)  

*Figure 3.5* Binding of GGHYrFK-amide to 5'-fluorescein labeled HCV IRES SLIIb.
3.3.2 Monitoring Cleavage by Polyacrylamide Gel Electrophoresis

Reaction kinetics were initially monitored by PAGE analysis of reaction mixtures at different time points. An example is shown in Figure 3.6. The data obtained from PAGE was limited and loss of the starting material could be measured but product bands were not observed. *In vitro* fluorescence assays were the primary method used for measuring reaction kinetics.

![Figure 3.6 Analysis of reaction kinetics by polyacrylamide gel electrophoresis.](image)

3.3.3 *In Vitro* Fluorescence Assays

\[
E + S \xrightleftharpoons[k_-]{k_1} ES \xrightarrow{k_2} E + P
\]  

(3.1)

\[
v = V_{\text{max}} \frac{[S]}{K_m + [S]} = k_{\text{cat}}*[E]_0 \frac{[S]}{K_m + [S]}
\]  

(3.2)

Isolated HCV IRES SLIIb was purchased with a 5’-fluorescein label and kinetics was monitored by following the fluorescence as a function of time (Figure 3.7, left). Initial
velocities were measured as a function of substrate (RNA) concentration to generate a standard Michaelis-Menten plot (Figure 3.7, right) in the presence of 100 nM complex, 1 mM ascorbate, 1 mM H₂O₂, and 100 mM NaCl in 20 mM HEPES (pH 7.4). The catalytic metallodrug is treated as the enzyme and the RNA as the substrate according to equation 3.1 and parameters calculated according to equation 3.2. No reactivity was observed for 1-Cu with other small RNAs (shown in Figure 3.7, right) represented by the Rev Response Element (RRE) from HIV and the isolated SLIV from IRES. Also, free CuCl₂ does not react with the SLIIb RNA, consistent with a model where specific binding is necessary to achieve activity.

**Figure 3.7** Sample kinetic trace for degradation of SLIIb RNA (left). Michaelis-Menten plot (right). [Catalyst] = 100 nM, [H₂O₂] = 1 mM, [Ascorbic acid] = 1 mM, [HEPES] = 20 mM, [NaCl] = 100 mM, pH = 7.4. 1-Cu, SLIIb (filled squares); CuCl₂, SLIIb (open circles); 1-Cu, SLIV (filled triangles); 1-Cu, RRE (open diamonds).
3.3.4 MALDI-TOF Mass Spectrometry

MALDI-TOF analysis of RNA cleavage products is complicated by the presence of a large number of fragmentation peaks that are present even in the absence of catalyst (Figure 3.8, left). This has been reported for other systems and cleavage products were determined by considering only those peaks that were new and not contained in controls of RNA alone or RNA in the presence of the coreagents, ascorbate and H$_2$O$_2$, but no catalyst. It can be seen in Figure 3.8 (right) that the major peaks are centered around m/z = 5000. Exact assignments could not be made for most of the new peaks based on MALDI alone but general trends in terms of sites of reaction become clear. The new peaks are listed in Table 3.1.

![Figure 3.8](image_url) Mass spectrometric analysis of RNA cleavage by Cu-GGHYrFK-amide. RNA alone is shown in black and a reaction with I-Cu is shown in red.
Table 3.1 List of new mass spectrometric peaks observed after reaction with 1-Cu.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Intensity</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5117.8</td>
<td>21670</td>
<td>5’ fragment at 14</td>
</tr>
<tr>
<td>6412.3</td>
<td>8245</td>
<td>5’ fragment at 18</td>
</tr>
<tr>
<td>4879.2</td>
<td>7853</td>
<td>5’ fragment at 13</td>
</tr>
<tr>
<td>2547.13</td>
<td>6202</td>
<td>5’ fragment at 6</td>
</tr>
<tr>
<td>5150.9</td>
<td>3004.7</td>
<td>3’ fragment at 19</td>
</tr>
<tr>
<td>2530.49</td>
<td>1849</td>
<td>5’ fragment at 6</td>
</tr>
<tr>
<td>5171.2</td>
<td>1408</td>
<td>3’ fragment at 19</td>
</tr>
<tr>
<td>5044.8</td>
<td>1394</td>
<td>5’ fragment at 14</td>
</tr>
<tr>
<td>4960.7</td>
<td>1343</td>
<td>5’ fragment at 13</td>
</tr>
<tr>
<td>5189.4</td>
<td>1305</td>
<td>5’ fragment at 14</td>
</tr>
<tr>
<td>6429.3</td>
<td>1199</td>
<td>5’ fragment at 18</td>
</tr>
<tr>
<td>6380.14</td>
<td>1140</td>
<td>3’ fragment at 15</td>
</tr>
</tbody>
</table>

3.3.5 Docking to the NMR Structure of Stem Loop IIb

In order to probe the binding of YrFK-amide and Cu-GGHYrFK-amide to SLIIb, docking to the NMR structure of the isolated SLIIb was performed. The top 60 YrFK-amide bound structures are shown in Figure 3.9 (top left) with spheres representing the center of the peptide in each of the 60 structures. It can be seen that the peptide bound preferentially to the upper loop. The lowest energy structure of YrFK-amide bound to SLIIb is shown on the top right. Docking of the copper complex Cu-GGHYrFK-amide was also performed. The site of localization of the copper center for the top 60 structures is shown on the bottom left as cyan spheres and the top structure for Cu-GGHYrFK-amide bound to SLIIb is shown on the bottom right.
3.3.6 HCV Replicon Assay Results

Metallodrug 1-Cu was submitted to Southern Research Institute for evaluation in cell culture by use of a cellular HCV replicon assay that mimics the native HCV replication system, containing virus-like RNA that can replicate autonomously using...
HCV specific replicase. A stable cell line ET (luc-ubi-neo/ET) was employed in the assay, specifically, a Huh7 human hepatoma cell line that contains three cell culture-adaptive mutations. The composition of the HCV RNA replicon with a stable luciferase (Luc) reporter and replicon is shown diagrammatically in Figure 3.10, wherein the Luc reporter is used as an indirect measure of HCV replication. The activity of the Luc reporter is directly proportional to HCV RNA levels and positive control antiviral compounds behave comparably using either Luc or RNA endpoints. The toxic concentration of drug that reduces cell numbers was assessed by use of the CytoTox-1 cell proliferation assay (Promega), a colorimetric assay of cell numbers (and cytotoxicity).

Table 3.2 summarizes the results of antiviral activity, as well as selectivity and toxicity indices. Cu-GGHYrFK-amide showed good antiviral activity ($IC_{50} = 0.58 \mu M$) and so did derivatives containing uptake sequences (Tat and KDEL), though the uptake sequences did not improve efficacy. No toxicity was observed up to 100 $\mu M$ and controls of free copper, metal free peptide, RNA binding domain alone, and Cu-GGH alone did not show activity. The current treatment rIFNα-2b was used as a positive control.

![Figure 3.10](image_url) Composition of HCV RNA replicon used in the cellular assays by Southern Research Institute.
In order to further understand the trend in the IC$_{50}$ values upon addition of the uptake sequences Tat and KDEL, their *in vitro* properties were determined as shown in Table 3.3. It can be seen that the binding is progressively weaker from the original peptide to the peptide with the Tat sequence to that with the KDEL sequence. The *in vitro* activity and IC$_{50}$ in replicon assays, however, do not vary significantly.

**Table 3.2** Anti-viral parameters for peptides and copper derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral IC$_{50}$ (μM)</th>
<th>Cytotoxicity TC$_{50}$ (μM)</th>
<th>Selectivity Index TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Cu</td>
<td>0.58</td>
<td>&gt; 100</td>
<td>&gt; 172</td>
</tr>
<tr>
<td>1-Tat-Cu, 2-Cu</td>
<td>1.09</td>
<td>&gt; 100</td>
<td>&gt; 91</td>
</tr>
<tr>
<td>1-KDEL-Cu, 3-Cu</td>
<td>0.67</td>
<td>&gt; 100</td>
<td>&gt; 149</td>
</tr>
<tr>
<td>YrFK-amide</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na $^a$</td>
</tr>
<tr>
<td>[GGH]-Cu$^{2+}$</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
<tr>
<td>Cu$^{2+}$ (aq)</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
</tbody>
</table>

$^a$ na- not applicable

**Table 3.3** Effect of uptake sequences on binding and reactivities of metallodrugs targeting IRES SLIIf.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM)</th>
<th>K$_d$ (nM)$^a$</th>
<th>Slope (RFU*min$^{-1}$*μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Cu</td>
<td>0.58</td>
<td>44</td>
<td>0.111</td>
</tr>
<tr>
<td>1-Cu-Tat</td>
<td>1.09</td>
<td>174</td>
<td>0.09</td>
</tr>
<tr>
<td>1-Cu-KDEL</td>
<td>0.67</td>
<td>656</td>
<td>0.143</td>
</tr>
</tbody>
</table>

$^a$ K$_d$ values listed are for the free peptide.
3.3.7 Combination Therapy

The ability of Cu-GGHYrFK-amide to act in combination with rIFNa-2b was also evaluated and is shown in Figure 3.11. It can be seen that additive and possibly slightly positive synergic behavior was observed. Concentrations of 1-Cu and rIFNα-2b were used that generated approximately the same antiviral activity.

![Figure 3.11](image)

**Figure 3.11** Combination treatment of 1-Cu with rIFN alpha-2b showing additive-synergistic effects (I, 0.2 μM 1-Cu; II, 0.2 IU/mL rIFN alpha-2b; III, 0.2 μM 1-Cu + 0.2 IU/mL rIFN alpha-2b).

3.3.8 RT-PCR

To confirm that the metallodrug is working in the cellular replicon assays according to the mechanism described for *in vitro* experiments, RT-PCR experiments were performed to look at RNA levels for both HCV RNA and rRNA (as a control) in the replicon assays.

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It can be seen in Figure 3.12 that there is a preference for HCV RNA and that there is still activity up to 9 days. A small reduction in levels of rRNA are also observed.

**Figure 3.12** RT-PCR data showing the preferential cleavage of HCV RNA (left) over ribosomal RNA (right) at increasing dosages of 1-Cu. 0 µM 1-Cu (black), 2.5 µM (blue), 5 µM (green), 10 µM (orange), 20 µM (red). Traces on top are normalized to the RNA levels in the absence of metallodrug.
3.4 Discussion

3.4.1 Binding to SLIIb

The binding of peptide sequence YrFK-amide to HCV IRES SLIIb has not been previously reported in the literature and was discovered by H. D. Robertson of Cornell University. There is little to no information available on this peptide and how it binds to SLIIb. It was found, however, that it binds very tightly with a $K_d$ of 44 nM (Figure 3.5). Initially, the binding constant determination was attempted by a titration of GGHYrFK-amide into 5'-fluorescein labeled SLIIb but a significant change in fluorescence was not observed. The binding data shown were obtained by titrating unlabeled SLIIb into peptide and monitoring the change in tyrosine fluorescence from the YrFK-amide. This would suggest that the tyrosine might be interacting with the bases in the RNA and, as will be described in chapter 4, other systems with varied stereochemistry did not show the same response. Based on the sequence, it might be expected that binding is a combination of electrostatic interactions involving the lysine and arginine with intercalation of the phenylalanine and tyrosine into the bases of the RNA. The proposed mechanism of binding will be discussed in more detail in chapter 4 in the context of molecular modeling and studies of derivatives of Cu-GGHYrFK-amide.
3.4.2 Michaelis-Menten Reactivity

Part of the challenge of studying catalytic metallodrugs is the development of the best methodologies to study them. Original studies looked at the activity by HPLC as described in chapter 2 for tRNA$^{\text{Phe}}$ as well as by polyacrylamide gel electrophoresis (PAGE), an example of which is shown in Figure 3.6. Reactivity could be established such as shown in the gel but only the loss of the starting material was observed, not cleavage products. In order to develop a method for monitoring kinetics which was more amenable to screening multiple compounds at multiple concentrations, \textit{in vitro} fluorescence assays using 5’-fluorescein labeled RNA were developed. The catalytic metallodrug is treated as an enzyme with the RNA as the substrate and characterized according to Michaelis Menten kinetics. A Michaelis Menten plot is then generated by plotting the initial velocity as a function of the substrate (RNA) concentration. These assays are also done under oxidizing conditions in the presence of ascorbic acid and H$_2$O$_2$. For these systems, reactivity was observed only when both coreagents were present. This contrasts with the reactivity described in chapter 2 for tRNA$^{\text{Phe}}$ where H$_2$O$_2$ was not required and emphasizes the potential for variation of the mechanism from metallodrug to metallodrug. Both ascorbic acid (vitamin C) and H$_2$O$_2$ are present physiologically and are expected to be available for any chemistry that occurs \textit{in vivo}, although other oxidants or reductants could also be involved.
Table 3.4  Michaelis-Menten parameters for degradation of SLIIb.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (µM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Cu</td>
<td>0.53</td>
<td>0.85</td>
<td>0.62</td>
</tr>
<tr>
<td>Eu$_2^a$</td>
<td>0.0009</td>
<td>25</td>
<td>0.36 x 10$^{-4}$</td>
</tr>
<tr>
<td>Hammerhead ribozyme$^b$</td>
<td>2.31</td>
<td>0.154</td>
<td>15</td>
</tr>
<tr>
<td>RNase A$^c$</td>
<td>108,000</td>
<td>670</td>
<td>161.2</td>
</tr>
</tbody>
</table>

$^a$Bis-europium complex of 1,3-bis[1-(4,7,10-tris (carbamoylmethyl)-1,4,7,10-tetraaza-cyclododecane]-m-xylene; UpU substrate, 25$^\circ$ C, 20 mM ammonium acetate (pH = 7.6), 100 mM NaNO$_3$. $^b$Oligoribonucleotide substrate (see ref. x), 25$^\circ$ C, 50 mM Tris·HCl (pH = 7.5), 10 mM MgCl$_2$. $^c$ CpA substrate, 25$^\circ$ C, 200 mM sodium acetate (pH = 5.5).

Figure 3.13  Structure of the artificial nuclease 1,3-bis[1-(4,7,10-tris (carbamoylmethyl)-1,4,7,10-tetraaza-cyclododecane]-m-xylene (Eu$_2$).
It can be seen in Figure 3.7 (right) that complex 1-Cu reacting with SLIIb exhibits saturation kinetics at high substrate concentrations, yielding a $K_m$ of 0.85 μM, a $V_{max}$ of 0.053 μM RNA/min, and a $k_{cat}$ of 0.53 min$^{-1}$. The value observed for the $K_m$ is ~19 times the value of the $K_d$. Consequently, $k_2$ is significant relative to $k_{-1}$ and it can be estimated that $k_2 \sim 18 \cdot k_{-1}$. This means that the chemistry is fast relative to the dissociation of the RNA from the peptide, consistent with a system that exhibits such tight binding. The $k_{cat}$ value observed compares favorably to nuclease mimics (such as the di-europium complex shown in Figure 3.13, $k_{cat} = 0.0009$ min$^{-1}$),$^{57}$ and natural ribozymes (hammerhead ribozyme, $k_{cat} = 0.09$ min$^{-1}$),$^{99}$ while still being significantly less than that for RNase enzymes (Table 3.4). The catalytic efficiency, $k_{cat}/K_m$, of 1-Cu is comparable to that of the hammerhead ribozyme, while still significantly less than that for RNase A,$^{100}$ which is not unexpected. It is important to point out that those systems likely operate by a distinct hydrolytic mechanism, relative to the oxidative path described here. Nevertheless, the demonstration of Michaelis-Menten behavior and the high levels of reactivity are almost unprecedented for cleavage catalysts mediating chemistry on natural RNA sequences. Prior studies have been carried out almost exclusively with activated mono-or dinucleotides.

A turnover number of 32 for 1-Cu was estimated, based on the limiting amount of RNA consumed by a specific amount of catalytic drug, and is consistent with one metallodrug having the capacity to react with multiple RNA targets, validating the catalytic metallodrug approach described earlier. This mechanism of catalytic inactivation is expected to result in low toxicity due to the ability to administer lower
dosages. Higher levels of specificity are also expected as a result of a double filter selection mechanism, where nonspecific binding to other biomolecules does not lead to productive chemistry due to the fact that the mechanism described also requires the metal-binding domain to be positioned optimally for chemistry to occur. Selectivity should also be improved due to the fact that the target of the metallopeptides is an RNA specific to hepatitis C. The absence of significant cellular toxicity was confirmed in the cellular replicon studies as discussed in section 3.4.4.

3.4.3 Mass Spectrometry and Modeling

The interaction of this class of peptides and even the target recognition domain itself with HCV IRES SLIIb is not well characterized and computational studies were performed in order to further probe the interaction of 1 and 1-Cu with SLIIb. The NMR structure of the isolated SLIIb is available (PDB: 1P5N) and docking of 1 and 1-Cu to the top NMR structure was performed. Figure 3.9 (top left) shows the center of YrFK-amide for each of the sixty structures obtained with the coloring showing the calculated binding energy. It can be seen that binding is predominantly at the top of SLIIb. The structure of the lowest energy structure is shown in the top right of Figure 3.9. The positively charged residues are involved in interactions with the phosphate backbone while the aromatic groups are positioned near the bases and the tyrosine is hydrogen bonded to the U14 residue, one of the primary sites of proposed reaction. One limitation of the docking studies is that the RNA is not flexible during the computation and so any
potential intercalation of the phenylalanine or tyrosine residues into the bases of the RNA will not be observed. Docking of the copper complex was also performed and is shown at the bottom of Figure 3.9. The bottom left shows where the copper center localizes for the sixty structures obtained for the docking of 1-Cu to the RNA. The lowest energy structure for 1-Cu bound to SLIIb is shown on the bottom right.

The mechanism and site of cleavage for 1-Cu was further probed by MALDI-TOF mass spectrometry to identify cleavage products (Figure 3.8). It was observed that RNA alone, in the absence of reaction with catalytic metallodrug, showed fragmentation during the MALDI process. This has been shown previously for other RNA systems. Cleavage products from reaction with 1-Cu were analyzed by considering those peaks which did not appear in controls of SLIIb RNA alone or SLIIb in the presence of both 1 mM ascorbate and 1 mM H₂O₂ then compared to the predicted masses for expected products based on hydrolysis and known pathways for oxidation of DNA. It should be noted that pathways for the oxidative cleavage of DNA are well characterized, but those for RNA oxidation are not. Correlations to the pathways for DNA can be made, however, even if hydrogen abstraction is expected to be more difficult for RNA than for DNA. Reaction of 1-Cu with isolated SLIIb showed reaction products primarily centered around m/z = 5000 (Figure 3.8, right). The major new peaks (Table 3.1) do not generally give exact matches to predicted oxidation products but the assignments shown are the closest matches. Exact mechanisms can’t be determined with confidence but overall trends show common residues for reaction and these sites are mapped to the secondary structure of SLIIb and shown in Figure 3.14 (left) and shaded according to the intensity of the
MALDI peak. The location of these residues on the three dimensional structure are shown on the right in Figure 3.14. These proposed sites of reactivity are consistent with the modeling data shown in Figure 3.9 where binding is primarily at the top loop but with a small amount of peptides docked to the bulge region below. These sites of cleavage also are consistent with the localization of copper ions for the docked structures of 1-Cu. A recent paper performed selective 2’ hydroxyl acylation analyzed by primer extension (SHAPE) on the full length HCV IRES and showed high accessibility at U14 and A15, consistent with the reactivity observed above. Therefore, the observed preference for reaction in this area could be due to the placement and orientation of the metal but also due to the intrinsic reactivity and accessibility as well. Previous work by Kalliampakou et al. looked at mutations of the apical region of SLIIb and showed that single substitutions at the proposed residues (13, 14, 18, and 19) cause dramatic reductions in IRES mediated translation, down to ~30-60% for a single residue. This indicates the importance of these residues but also shows that activity for 1-Cu would be expected even if dissociation of the RNA does not occur. It might be expected that cleavage at the top loop, especially in the context of the full length IRES and under physiological conditions, would not necessarily result in dissociation of the two fragments because most of the hydrogen bonding interactions should remain intact.
3.4.4 HCV Cellular Replicon Assay

One of the major challenges in studying hepatitis C is the lack of a proven animal model. Standard small animal models such as mice and rats do not develop hepatitis C upon exposure to the virus. The only natural animal model susceptible to HCV infection is the chimpanzee but even in this model there are significant differences relative to humans.\textsuperscript{104} For both financial and ethical reasons, experiments with chimpanzees are not practical. Probably the most promising animal model is the chimeric mouse where immune deficient mice have had their hepatocytes replaced with human hepatocytes. These mice are then able to maintain infection by HCV and this has been shown for all of the HCV genotypes.\textsuperscript{105} Nevertheless, these animal models and their usefulness are still
under investigation and because of this, the current standard for testing the activity of HCV compounds is the HCV cellular replicon assay described below which is accepted by the FDA as a measure of drug efficacy.

In order to test the efficacy of Cu-GGHYrFK-amide *in vivo*, it was sent to Southern Research Institute and tested in this replicon assay, shown diagrammatically in Figure 3.10. A luciferase reporter is used as an indirect measure of HCV replication and is known to correlate well with RNA levels. Cytotoxicity is also measured and the results are shown in Table 3.2. It can be seen that Cu-GGHYrFK-amide showed good activity with an IC\textsubscript{50} of 0.58 μM and no toxicity was observed up to 100 μM (the highest concentration tested). Significantly, neither the targeting peptide YrFK-amide, lacking the metal binding ATCUN motif, nor the metal binding domain Cu-GGH show any cellular activity. Moreover, the inclusion of a cellular uptake sequence, represented by both Tat arginine rich motif (1-Cu-Tat) and KDEL (1-Cu-KDEL), provides no obvious enhancement of cellular efficacy, and so there is either no barrier to uptake, or the targeting sequence YrFK-amide facilitates cellular uptake as well as the added sequences. As shown in Table 3.3, the addition of the C-terminal uptake sequences reduces the binding to HCV IRES SLIIb, but does not have a significant effect on the rate of RNA cleavage under saturating conditions (slope ~ k\textsubscript{cat}[complex]), a factor that could prove useful in the design of derivatives to optimize the pharmacokinetic profile of this class of molecule. The binding of 1-Cu-Tat and 1-Cu-KDEL was monitored by the fluorescence change of fluorescein-labeled IRES RNA upon addition of peptide whereas binding of 1 was only detected by monitoring emission of the peptide tyrosine with serial addition of
RNA. Tyrosine emission did not significantly change following binding of 1-Cu-Tat and 1-Cu-KDEL, most likely reflecting minor changes in the binding mode for the modified versus unmodified peptides. While 1-Cu-Tat and 1-Cu-KDEL show weaker binding, the chemistry for the copper complex did not significantly change. This is consistent with a system where catalyst release, rather than binding and subsequent chemistry, constitutes the rate-determining step and supports direct cellular uptake of 1-Cu. The targeting sequence YrFK-amide is derived from a neuroactive peptide found in the skin of South American frogs and has previously been implicated with facilitating cellular uptake though the mechanism is unclear.90

Current treatment involves a combination of ribavirin and pegylated interferon-alpha, neither of which has a mechanism of action specific to hepatitis C. Ribavirin is a nucleoside analog, while pegylated interferon-alpha stimulates the immune response. A drug with a new mechanism of action that is specific to hepatitis C has great potential, especially if given in combination with one or both of the above treatments. The recently approved NS3 protease inhibitors represent the first class of drugs that specifically target HCV. Metallodrug 1-Cu was therefore further evaluated in a simple combination assay with pegylated-interferon α-2b. Results are summarized in Figure 3.11, clearly demonstrate at least additive to slightly positive synergic behavior over a range of concentrations, and certainly support the idea that metallodrugs such as 1-Cu hold some promise for therapeutic use in combination with pegylated interferon α-2b.

To further confirm that the mechanism of action in the cellular replicon assays is consistent with that proposed for catalytic metallodrugs, real-time polymerase chain
reaction (RT-PCR) was performed to measure the RNA levels for both HCV RNA and ribosomal RNA (rRNA). The results can be seen in Figure 3.12 (top) and show a clear preference for HCV RNA over rRNA. The top data are shown as the amount of RNA normalized to the control without any metallopeptide. Significantly, 1-Cu still showed activity up to 9 days and reduced RNA levels to close to zero. In contrast, the rRNA levels decreased much less although there was still a decrease relative to the control. These data are further complicated by the fact that it will be a reflection of both the amount of RNA being made and the amount of RNA being consumed. Also, the rate of signal amplification can be different for the two RNAs which further complicates any quantitative comparison. The data for copies of RNA (not normalized) are shown on the bottom of Figure 3.12 and it can be seen that the amount of rRNA being made over time increases both with and without 1-Cu but that the rRNA levels are slightly higher in the absence of catalyst. This could be due to small levels of background cleavage of the rRNA but also is consistent with the possibility that higher levels of rRNA are being made to compensate for the presence of the IRES and the amount of rRNA that is being used for viral protein synthesis, thereby reducing the concentration of rRNA available for use by the cell. In the presence of catalytic metallodrug, however, the reduction in the effective concentration of IRES means that more rRNA is available for the cell to use and, therefore, it does not need to make as much. Regardless, there is a clear preference for the HCV RNA and no significant overall cellular toxicity was observed as evidenced by the TC$_{50}$ values. This is consistent with the metallodrug behaving in the cellular replicon assays according to the mechanism that is proposed for catalytic metallodrugs.
and shown for the \textit{in vitro} experiments.

3.5 Conclusions

It should be noted that while the parameters used to define activity are those that are typical for the classical inhibitory profiles exhibited by most drugs, the catalytic metallodrugs defined here function by irreversible inactivation and it is likely that a distinct parameterized model will be required to describe this pathway. Nevertheless, even in this form the apparent inhibition is both insightful and impressive. Cellular assay results are also consistent with binding and cleavage by metallodrug 1-Cu and catalytic metallodrugs such as the one presented here represent a new approach to drug design. In the absence of an accepted animal model, activity in the cellular replicon assay meets FDA standards for demonstration of efficacy and these results validate the potential of this approach. The novel mechanism of action holds promise for development of drugs with high specificity and low toxicity, and for use in combination with other currently used therapeutics such as rIFN-2b, in particular where standard combination therapy has either already proved ineffective or failed due to resistance development. The irreversible nature of the chemistry promoted by such a therapeutic approach, combined with the intrinsic selectivity for an HCV target molecule, has the potential to inhibit resistance development by the virus. This study reveals catalytic metallodrugs to be promising candidates for development as therapeutics, especially in combination with current treatments. This compound also represents only the first metallodrug based on the YrFK-
amide binding motif and therefore there is significant room for enhancement of efficacy both \textit{in vitro} and \textit{in vivo}. Chapter 4 details preliminary efforts to look at derivatives in order to optimize both the \textit{in vitro} chemistry and the \textit{in vivo} properties.
Chapter 4- Optimization of Catalytic Metallodrugs

4.1 Introduction

4.1.1 General Strategies and Considerations

Traditional drugs are often developed by taking a lead compound that has shown activity and looking at how activity varies with structure to develop a structure activity relationship (SAR). This allows for the screening of different compounds to find those that are the most active but also provides information on the mechanism of that class of compounds. Cu-GGHYrFK-amide is the first catalytic metallodrug demonstrated to react with HCV IRES SLIIb. As such, it can be viewed as a lead compound that can be improved upon by a second generation of metallopeptides. As a drug, this compound can potentially be improved by varying different parameters, and which ones are most important for determining catalytic activity is still unclear. The modular design of 1-Cu, as shown in Figure 4.1, allows for the systematic variation of different components to see how each contributes to activity and how this activity can be optimized.
For an ATCUN motif, the requirement for copper and nickel binding is the presence of the N-terminal amine plus a histidine in the third position. Therefore, the R groups $R^1$ and $R^2$ can be varied without interfering with metal binding such as to alter the properties of the metal, add additional contacts to the target RNA and therefore alter binding, or by tailoring physical properties such as solubility or partition coefficient. The identity of the metal used can also be varied or the identity of the metal binding domain itself changed. The linker $X$, much like the R groups, can be used to tailor physical properties and to vary the flexibility between the metal binding domain and the RNA binding domain. The sequence of the RNA binding domain itself can be altered to probe the mode of binding and also to find the optimal RNA binding properties while still maintaining good catalyst release and turnover. Finally, the peptide could also be modified at the C-terminus to alter binding or to tailor the properties of the metallodrug as described for the other modifications or to alter in vivo properties such as uptake, targeting, or stability. *In vivo*
properties must also be considered because the compound that is most active in an *in vitro* assay may not be the best compound for use *in vivo*.

A common method to enhance the activity of a lead drug compound is to enhance its binding. For a catalytic metallodrug, depending on the rate determining step, tighter binding could lead to better or worse (or the same) chemistry. It is possible that a metallopeptide that binds tightly to the RNA may also bind tightly to the cleavage product and therefore the *k*\textsubscript{on} may increase but catalyst release may be poor. The mode of binding could also have an impact, such as in the possibility of two metallopeptides with similar *K*\textsubscript{d} values but that have different binding orientations. This would potentially change the location of the metal binding domain so that its reactivity with the RNA changes, such as by moving it near less reactive residues or by changing the orientation of the reactive species relative to the scissile bond in the RNA.

Another way to potentially enhance the activity of a catalytic metallodrug is to improve the reactivity of the metal center. Changing the metal binding domain from an ATCUN motif to another metal binding domain can change the placement of the metal as mentioned above and can also alter the intrinsic reactivity of the metal center, such as by altering the reduction potential. For example, changing the metal binding domain from Cu-GGH to something like Cu-NTA would have a dramatic effect on the reduction potential, changing it from \textasciitilde1 V to \textasciitilde0.2 V. This change also would alter the preferred oxidation state for the metal, by changing it from Cu\textsuperscript{3+/2+} to Cu\textsuperscript{2+/+}. Depending on the mechanism and what the reaction intermediates are, one may be preferred over the other. The reactivity of the metal center can also be varied by changing the identity of the metal.
itself. The possible metals available that bind tightly to the ATCUN motif are limited to copper and nickel, but inclusion of a different binding domain also increases the number of available metals that can be tested.

4.1.2 Use of Peptides as Drugs

The use of peptides as drugs has a variety of advantages including high activity and specificity, low toxicity, and low tissue accumulation. This is in addition to the advantages already described for catalytic metallo drugs. Peptides also allow access to a diverse array of compounds through chemistry that is already well established. There are, however, a variety of drawbacks as well.¹⁰⁶,¹⁰⁷ These include sometimes poor bioavailability and the potential for an unwanted immune response. Peptides will often have trouble crossing membranes and being taken up into cells unless an appropriate uptake sequence is included. They also have a tendency to be cleared from the body too quickly to be useful therapeutically. Also, peptides are susceptible to degradation by proteases and, because of this, are not stable in the stomach and generally cannot be administered orally. Many of these disadvantages can be addressed by modification of the peptide or by the application of drug delivery strategies.

4.1.3 Peptide Sequences Tested

In order to probe these factors, several variations on the sequence of the RNA binding
domain were studied as well as a small number of variations in the metal binding domain and metal. One potential issue with using peptides as drugs is their susceptibility to cleavage by proteases \textit{in vivo}. One strategy to increase the stability of a peptide to proteases is the use of D-amino acids which are not recognized by proteases.\textsuperscript{90,108} To this end, the all D-amino acid form of the peptide, Cu-GCyryfK-amide (2-Cu), is studied in terms of its reactivity \textit{in vitro} and its efficacy in the HCV cellular replicon assay. The tetrapeptide YrFK-amide was the compound found to bind to HCV IRES SLIIb but the role and importance of having the arginine in the D-form is not clear so the reactivity of the all L-amino acid form Cu-GCHYRFK-amide (3) is also studied. Other derivatives to look at the importance of amidation (Cu-GCHYrFK-COOH), as well as the effect of adding a short spacer (Cu-GCHGYrFK-amide) and two lysines onto the ATCUN motif (Cu-KKHYrFK-amide) are also presented. Finally, initial attempts to study variation of the metal binding domain and metal are described, as exemplified by 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and diethylene triamine pentaacetic acid (DTPA) derivatives.

4.2 Methods and Materials

4.2.1 Materials. RNA was purchased from Dhharmacon, part of Thermo Fisher Scientific (Lafayette, CO). Peptides were purchased from Genemed Synthesis, Inc. (South San Francisco, CA). The sequence for the IRES SLIIb RNA used was 5’-fluorescein-GGCAGAAAGCGUCUAGCCAUGGCGUAGUAUGCC-3’, for the IRES SLIV RNA...
was 5’-fluorescein-GGACCGUGCACCAUGAGCACGAAUCC-3’, and for HIV Rev response element (RRE) RNA was 5’-fluorescein-UUGGUCUGGGCGCAGCGCAAGCUGACGGUACAGGCC-3’. The sequence of the 5mer RNA used for calibration in the MALDI-TOF experiments was 5’-fluorescein(T)-UGUG-3’. All RNA was annealed by heating to 95 °C and then cooled slowly to room temperature before use. Sodium chloride, sodium hydroxide, and acetonitrile were purchased from Fisher, and HEPES, ammonium citrate, and 3-hydroxypicolinic acid were purchased from Sigma. DOTA-NEM and DTPA-NEM derivatives were purchased from Macrocyclics. C18 ZipTips were obtained from Millipore. All experiments were performed using diethyl pyrocarbonate (DEPC) treated water and autoclaved pipette tips and tubes. Copper complexes form quickly and were made by mixing in a 1.1:1 peptide:CuCl₂ ratio and waiting at least five minutes for complex to form.

4.2.2 RNA Binding Experiments. Binding data for GGHYRFK-amide (3) was obtained by adding serial additions of peptide to 5'-fluorescein labeled IRES SLIIb and monitoring at 518 nm (Ex = 485 nm). Data were then fit to a one-site binding model using Origin 7.0 software. A binding constant for GGhyrfk-amide (2) was determined by fitting the change in T_m2 as a function of peptide concentration and fit to a one site binding model.

4.2.3 Melting Temperatures. Melting temperatures were measured by monitoring fluorescein emission (Ex = 485 nm, Em = 518 nm) with increasing temperature (15 to 95 degrees Celsius at a rate of 3 degrees Celsius per minute) in the presence or absence of
metal-free peptide and varying [NaCl]. Melting temperatures were determined under conditions of 20 mM HEPES, 2 mM NaCl, pH 7.4, and 1 μM fluorescein-labeled SLIIb IRES. Melting profiles were fit to a three state consecutive melting model using Origin 7.0 software to obtain $T_{m1}$ and $T_{m2}$. The values shown are an average of at least 3 trials.

A binding constant for GGhyrfk-amide was determined by fitting the change in $T_m$ as a function of peptide concentration and fit to a one site binding model.

4.2.4 Conformational Analysis. A mixed torsional/low-mode sampling conformational search utilizing Schrodinger’s MacroModel v9.8 (Schrödinger, LLC, New York, NY, 2011) was used to determine the global minimum of the peptides in aqueous solution. The starting structures were modified to avoid getting trapped in a local minimum by linearizing the backbone of the peptide. The amino terminal nitrogen, the guanidine group on arginine, and the amino side chain nitrogen on lysine were simulated in the neutral form due to the limitations of the program’s ability to handle charges. The energy window used in the searches was 100.0 kJ/mol. The conformational search was done with 10,000 MC steps with water as the solvent while using an Amber* force field. The minimization parameters for YrFK-amide are as described in chapter 3. The yrfk-amide had a total of 6,237 conformers with the global minimum being -776.192 kJ/mol which was converged to twice. The top five conformations were found a combined eleven times and their potential energies were within 5 kJ/mol.

4.2.5 Molecular Dynamics. The top five peptide structures from each conformational
analysis were then optimized with Gaussian 09 vA01* using a DFT/B3LYP/6-311++G** force field. Solvent interactions, water in this case, were considered by utilizing the recent universal solvation model, SMD, by Truhlar and co-workers. Prior to submission for calculations, the arginine, lysine, and N-terminal amine were changed to reflect their positive charges at neutral pH.

4.2.6 Docking. The NMR structure of SLIIb is readily available from the protein data bank (PDB: 1P5N). The top NMR structure (out of 20) was used for docking simulations in Autodock version 4.2. The free targeting domain and the Cu-ATCUN targeting domain were both simulated. The docking area considered the full length SLIIb. This was done to investigate the site of localization of the peptide/complex on the RNA. In all cases, the peptide/complex was made flexible except for aromatic carbons and peptide bonds. As a result of the high number of flexible bonds already in the complex, the RNA was restricted to its initial state. The top structure from conformational analysis for each peptide was used for docking and the Cu-ATCUN domain modeled according to the x-ray crystal structure. Autodock does not have parameters for copper so an iron atom was substituted as a place holder to mimic the geometry of the copper with a charge of +1.5. A value of +1.5 is used to simulate the +2 charge in order to compensate for the tendency of Autodock to overestimate electrostatic interactions. The geometry around the metal was based on the x-ray crystal structure of Cu-GGH and was restricted to a square planar configuration. A Lamarckian Genetic Algorithm was used with a docking sample of 60 sites.
4.2.7 Synthesis of DOTA and DTPA Derivatives. Synthesis of DOTA and DTPA derivatives was performed by Martin James Ross of the Cowan lab. The cysteine-containing peptide (1.5 equivalents) was added to the NEM-chelator (1 equivalent) in 1 mL of 100 mM sodium bicarbonate at pH 6.75 for 2 hours. The product was then diluted with deionized water prior to separation using a Vydac C18 monomeric reverse phase column and a gradient of 0-100% acetonitrile over 35 minutes. The product was confirmed by ESI-MS with m/z of 1230 corresponding to DTPA-Peptide and m/z of 1241 for DOTA-Peptide. Products were dried and stored at -20 °C until used.

4.2.8 Reaction Kinetics via Fluorescence. HCV IRES RNA cleavage was monitored in vitro by fluorescence using 5’ fluorescein end-labeled RNA with excitation and emission wavelengths of 485 nm and 518 nm, respectively. Reactions were carried out at 25 °C in reaction volumes of 100 µL in the presence of 1 mM ascorbate and 1 mM H$_2$O$_2$ in HEPES buffer (pH = 7.4, 100 mM NaCl) with 1 µM fluorescein labeled IRES SLIIb and analyzed according to the change in fluorescence observed as the reaction occurred. A dependence on both a time and catalyst concentration was observed. The initial velocity of the time dependence plot was fit to a line and used to generate the pseudo Michaelis-Menten plots which were then fit to the Michaelis Menten equation. All fits were performed using Origin 7.0 software. The values shown are an average of at least three trials. A turnover number was estimated based on the limiting amount of peptide catalyst consumed by a specific amount of RNA.
4.2.9 Mass Spectrometry. Reactions for MALDI-TOF analysis were run as described in 4.2.8 but using 10 µM fluorescein labeled IRES SLIIb and 10 µM copper-peptide and incubated for two hours. Reactions were then quenched by being placed on ice and desalted. Desalting was performed using C\textsubscript{18} ZipTips from Millipore Co. in order to desalt reaction mixtures prior to mass spectrometric analysis. ZipTips were wetted with a 50:50 mixture of acetonitrile:water and equilibrated with 2 M triethylammonium acetate (TEAA), pH 7.0. The reaction mixture was then bound to the ZipTip, washed with nanopure water, and eluted with 50:50 acetonitrile:water. These samples were spotted onto a Bruker ground steel 96 target microScout plate by first spotting with 1 µL of the 0.3 M 4-hydroxypicolinic acid (HPA), 30 mM ammonium citrate matrix solution in 30% acetonitrile, drying, then spotting with 2.5 µL of a 1:1 RNA:matrix mixture, and allowed to dry. A calibration mixture containing 3 RNAs covering a range of molecular weights (Fl-5mer, Fl-IRES SLIV, and Fl-RRE, with molecular weights of 2057.5, 8861.5, and 12172.5 amu, respectively) was used to calibrate the instrument. All MALDI-TOF MS analysis was performed on a Bruker MicroFlex LRF instrument, equipped with a gridless reflectron, using negative ion mode and reflectron mode. The pulsed ion extraction time was 1200 ns. At least 1000 shots were summed per spectrum. Data analysis was performed using Bruker flexAnalysis software. Only m/z values > 1000 amu were considered, since excessive spectral crowding occurred at lower m/z ranges. Reaction peaks were compared to controls containing SLIIb RNA alone and SLIIb RNA in the presence of 1 mM ascorbate, 1 mM H\textsubscript{2}O\textsubscript{2} and new peaks were identified as products of
cleavage by the metallopeptide.

4.2.10 HCV Cellular Replicon Assay. Samples were submitted to Southern Research Institute for evaluation in an HCV replicon assay as described in chapter 3. A stable cell line ET (luc-ubi-neo/ET) was employed in the assay. The ET is a Huh7 human hepatoma cell line that contains an HCV RNA replicon with a stable luciferase (Luc) reporter and three cell culture-adaptive mutations. The HCV RNA replicon antiviral evaluation assay examined the effects of compounds at six half-log concentrations each. Human interferon alpha-2b was included in each run as a positive control compound. Sub-confluent cultures of the ET line were plated out into 96-well plates that were dedicated for the analysis of cell numbers (cytotoxicity) or antiviral activity, and various concentrations of metallodrugs and controls were added to the appropriate wells the following day. Cells were processed 72 hours later when the cells were still sub-confluent. Six half-log serial dilutions of the compound were performed, and values derived for IC\textsubscript{50} (the concentration that inhibited virus replication by 50%), TC\textsubscript{50} (the concentration that lowered cell viability by 50%) and TI (the selectivity index: TC\textsubscript{50}/IC\textsubscript{50}). HCV RNA replicon levels were assessed as the replicon-derived Luc activity. The toxic concentration of drug that reduced cell numbers (cytotoxicity) was assessed by the CytoTox-1 cell proliferation colorimetric assay (Promega).
4.3 Results

4.3.1 All D-Amino Acid Derivative Binding and Thermal Melts

The results from the melting temperature analysis are shown below. Melting profiles were fit to a three state consecutive model and the fits are shown in Figure 4.2 (left) of a sample curve for conditions of RNA alone, in the presence of 50 μM GGHYrFK-amide, and in the presence of 50 μM GGhyrfK-amide. In order to determine a dissociation constant for GGhyrfk-amide, the shift in the melting temperature was monitored as a function of peptide concentration under conditions of 2 mM NaCl as shown in Figure 4.2 (right) and a $K_d$ of 11.3 μM was determined. To further compare the binding of the two forms of the peptide, the salt dependence with and without 50 μM peptide was studied (Figure 4.3). At high to intermediate salt concentrations, a noticeable shift in the melting temperature was not observed. At lower salt concentrations, however, both of the peptides increase $T_{m1}$ but only the D-amino acid form causes a significant shift in $T_{m2}$. 
Figure 4.2 (Left) Variation of fluorescein emission as a function of temperature (Just SLIIb (black); SLIIb, 50 μM GGHYrFK-amide (blue); SLIIb, 50 μM GGHyrFK-amide (red)). Fits shown are for a three state consecutive melt. The determination of $K_d$ for 2 via changes in melting temperature (right). $K_d = 11.26 \mu M + 1.18 \mu M$. [RNA] = 1 μM, [HEPES] = 20 mM, [NaCl] = 2 mM, pH = 7.4.

Figure 4.3 Variation of shifts in IRES SLIIb melting temperature in the presence and absence of peptides. SLIIb only (black); SLIIb, 50 μM GGHYrFK-amide (blue); SLIIb, 50 μM GGHyrFK-amide (red)). [RNA] = 1 μM; [peptide] = 50 μM, [HEPES] = 20 mM, pH = 7.4.
4.3.2 Molecular Modeling

Aside from the dissociation constant reported in chapter 3, there is nothing known about the binding of these peptides to SLIIb but there is an NMR structure of the isolated SLIIb. Therefore, molecular modeling was used to probe the effect of the amino acid conformations on the overall peptide solution structure and to dock the peptide to the SLIIb NMR structure. The lowest energy conformations for YrFK-amide and yrfk-amide are shown in Figure 4.4 and show the general trends for the lowest energy conformations obtained.

![Figure 4.4](image)

*Figure 4.4* The effect of incorporation of D-amino acids on the calculated structures for YrFK-amide (left), and yrfk-amide (right). Distances are shown in Angstroms. Peptide structures were prepared in Gaussian.

Docking of each of the peptides to the RNA was performed and is shown in Figure 4.5 (right) for the all D-amino acid form. It can be seen that most of the top structures docked the peptide at the top of loop as was observed for GGHYrFK-amide in chapter 3. The structure of the lowest energy conformation bound to SLIIb is also shown. The data
for YrFK-amide from chapter 3 is also shown on the left for comparison.

**Figure 4.5** (Top) Summary of the sites of localization for YrFK-amide (left) and yrfk-amide (right), showing the center of each peptide as a sphere. (Bottom) Structures of the lowest energy conformations for YrFK-amide (left) and yrfk-amide (right). The structures were prepared in Autodock Tools.
4.3.3 Synthesis

Variation of the metal binding domain was achieved by taking advantage of the coupling chemistry of thiols with N-ethylmaleimide. DOTA and DTPA derivatives were purchased with an NEM group already attached to one of the carboxylic acids and coupled to cysteine containing peptides as shown in Figure 4.6 to generate compounds with the metal binding domain on either the C- or the N-terminus (using YrFKC-amide and CYrFK-amide, respectively). Compounds were purified by reverse phase HPLC and characterized by electrospray ionization (ESI) mass spectrometry as well as UV-vis spectroscopy.

![DOTA and DTPA structures](image)

**Figure 4.6** Synthetic strategy for DOTA and DTPA derivatives of 1-Cu.
4.3.4 Reactivity

The reactivity of the metal complexes was determined by following the degradation of 5'-fluorescein labeled SLIIb by fluorescence as a function of time. Initial velocities as a function of catalyst concentration were plotted to generate a pseudo Michaelis Menten plot and obtain $K_m$ and $k_{cat}$ values (Figure 4.7). For 2-Cu, a turnover number was estimated based on the limiting amount of peptide catalyst consumed by a specific amount of RNA and an exact value for the turnover number for the all D-amino acid peptide could not be directly determined except that it was greater than 40.

![Figure 4.7 Pseudo Michaelis-Menten profile for reactivity of Cu-GGhyrfk-amide with fluorescein labeled SLIIb RNA.](image)

$[\text{Cu-GGhyrfk-amide}] (\mu\text{M})$

Initial Velocity (µM RNA/min)

$V_{max} = 0.14 \mu\text{M RNA/min}, k_{cat} = 0.14 \text{ min}^{-1}.$

The dissociation constant determination and pseudo Michaelis-Menten plot for the all L-amino acid version of Cu-GGHYrFK-amide are shown in Figure 4.8. This peptide showed activity similar to that of the all D-amino acid analog and the overall kinetics are
summarized in Table 4.3.

Data for the initial screening of further derivatives of 1-Cu, varying the metal and metal-binding domain, is shown in Figure 4.9. It can be seen that activity was observed only in systems containing both copper and an ATCUN motif.

**Figure 4.8** Determination of the dissociation constant for the all L-amino acid analog 3 binding to HCV IRES SLIIb (left) and pseudo Michaelis Menten plot for degradation of SLIIb by 3-Cu (right).

**Figure 4.9** The effect of variation of the metal and metal binding domain on kinetics of RNA cleavage.
4.3.5 MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry was performed as described in chapter 3. SLIIb RNA alone showed a large number of fragmentations and analysis of cleavage data was performed by considering those peaks that did not appear in controls of RNA alone and RNA in the presence of coreagents ascorbic acid and hydrogen peroxide. These new peaks are listed in Table 4.1.

Table 4.1 List of new mass spectrometric peaks observed after reaction with 2-Cu.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Intensity</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6428.3</td>
<td>1298</td>
<td>5’ fragment at 18</td>
</tr>
<tr>
<td>5160.6</td>
<td>1033</td>
<td>3’ fragment at 19</td>
</tr>
<tr>
<td>1162.2</td>
<td>867.4</td>
<td>5’ fragment at 2</td>
</tr>
<tr>
<td>3195.6</td>
<td>763</td>
<td>5’ fragment at 8</td>
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<tr>
<td>2548.5</td>
<td>668</td>
<td>5’ fragment at 6</td>
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<tr>
<td>7254.9</td>
<td>603</td>
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<td>532</td>
<td>5’ fragment at 13</td>
</tr>
<tr>
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<td>532</td>
<td>3’ fragment at 19</td>
</tr>
<tr>
<td>5189.6</td>
<td>501</td>
<td>5’ fragment at 14</td>
</tr>
</tbody>
</table>
4.3.6 HCV Cellular Replicon Assay

The activity of metallodrugs 2-Cu and 3-Cu were evaluated in cell culture by use of a cellular HCV replicon assay that mimics the native HCV replication system as described in chapter 3. The results are shown in Table 4.2 and are compared to the data for 1-Cu presented in chapter 3. Both showed activity similar to 1-Cu and, as for 1-Cu, no activity was observed for controls of the RNA binding domain alone, Cu-GGH alone, free copper, or the peptide without copper. Recombinant IFNα-2b was used as a positive control.

Table 4.2 HCV cellular replicon data for all D-amino acid and all L-amino acid analogs of 1-Cu.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral IC₅₀ (μM)</th>
<th>Cytotoxicity TC₅₀ (μM)</th>
<th>Selectivity Index (TI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[GGHYrFK-amide]-Cu, 1-Cu</td>
<td>0.58</td>
<td>&gt; 100</td>
<td>&gt; 172</td>
</tr>
<tr>
<td>[GHyrfk-amide]-Cu, 2-Cu</td>
<td>1.92</td>
<td>&gt; 100</td>
<td>&gt;52</td>
</tr>
<tr>
<td>[GHHYRFK-amide]-Cu, 3-Cu</td>
<td>1.08</td>
<td>&gt; 50</td>
<td>&gt;46</td>
</tr>
<tr>
<td>YrFK-amide</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
<tr>
<td>yrfk-amide</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
<tr>
<td>YRFK-amide</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
<tr>
<td>[GGH]-Cu²⁺</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
<tr>
<td>Cu²⁺(aq)</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
</tbody>
</table>

*na = not applicable*
4.4 Discussion

4.4.1 D-Amino Acids in Nature

The incorporation of D-amino acids into a peptide sequence is a common approach to improve the stability of the peptide to proteases, which is often a problem for the use of peptides \textit{in vivo}. It also presents a fundamentally interesting question with regard to how inversion of stereochemistry might influence binding, while the reactivity of the D-form, relative to the parent peptide, can provide additional insights on the mechanism of action and binding of this class of compounds. Changes in stereochemistry at the \( \alpha \)-carbon can potentially change the conformation of the peptide and therefore affect its ability to interact with its target. A complex set of factors, including binding affinity, the off-rate for drug release from the inactivated target, the orientation of the metal relative to the site of reaction, the reduction potential of the metal center, uptake, and stability, will all influence the efficacy of the peptide-metal complex. If conversion of the amino acids to the D-form negatively impacts one of these factors, it could be detrimental to its effectiveness as a therapeutic. Precedent exists, however, for proteins and peptides that retain their activity upon conversion to the D-amino acid form. Examples include HIV-1 protease where the D-form of the enzyme retained activity but with inverted specificity,\textsuperscript{111} a peptide derived from the N-terminus of HIV-1 gp41,\textsuperscript{112} and D-amino acid forms of channel-forming peptides used as antibiotics.\textsuperscript{113} In the case of an amyloid beta assembly inhibitor peptide, the activity actually increased.\textsuperscript{114} The binding of the
sequence YrFK-amide to SLIIB of the IRES was only recently reported and there is no structural information available on how it binds. The impact of converting the peptide sequence GGHYrFK-amide to the corresponding sequence containing all D-amino acids, G Gh yr fk-amide (2), is investigated and addresses the influence of affinity and release rates on catalyst efficacy and turnover. Structural modeling also suggests a model for how this class of peptides interacts with and cleaves its target RNA, which is supported by mass spectrometric analysis of product fragments.

4.4.1.1 All D-Amino Acid Form of Cu-GGHYrFK-amide

The catalytic metallodrugs described herein incorporate well characterized metal-binding domains with high stability combined with a targeting domain to provide specificity. The stability and mechanism of action of these compounds is consistent with the previously described replicon assay data in which activity was observed only in the presence of all of the components of the metallodrug: copper, the metal-binding domain, and the RNA targeting tetrapeptide. Toxicity was not observed up to the highest concentration tested (100 μM) and neither the individual components nor the peptide without metal show activity. Previous work (chapter 3) has also shown that incorporation of uptake sequences into the parent peptide does not significantly enhance activity, suggesting that these peptides are readily taken up by cells. One common approach to improving the stability of a peptide \textit{in vivo} is the incorporation of D-amino acids which are not recognized by proteases. Depending on the mechanism of action of the peptide,
however, this inversion of chirality can be detrimental to its efficacy. The stereoisomer can also be used to probe structure activity relationships and study how these changes can impact chemistry. For example, while an enhancement in binding might be expected to improve activity it is also possible that a reduction in binding could enhance release from the cleaved RNA and therefore, if release of the catalyst is rate determining, actually improve activity. A comparison of the sites of cleavage for the two peptides will also provide insight on the mechanism.

4.4.1.2 Binding and Thermal Melts

![Diagram](image)

**Figure 4.10** Expected model for melting of HCV IRES SLIib RNA.

It has been shown that the isolated stem loop domains of the HCV IRES maintain their structures adopted in the full length RNA and are a useful probe for *in vitro* assays. Binding and cleavage assays were performed using 5'-fluorescein labeled SLIib. Titration of the all D-amino acid version of GGHYrFK-amide, 2, into RNA to monitor
binding did not yield significant fluorescent changes and so binding was monitored by following the variation of melting temperature as a function of peptide concentration. Sample melting profiles are shown in Figure 4.2. Melting temperatures were determined by measuring the change in fluorescence emission as a function of temperature and the melting profiles were fit to a three state consecutive model. A consecutive model for melting of the SLIIB secondary structure would be expected with initial melting of the double stranded region at the 3’- and 5’-ends prior to melting of the double stranded region near the upper loop as shown in Figure 4.10. This model also provided the best and most consistent fit to the data. A dissociation constant of 11.3 μM ± 1.18 μM was observed for 2 binding to SLIIB (Figure 4.2, right). It can also be seen that addition of the peptide to the RNA caused the melting temperature to increase. This shows that the peptide-RNA complex is more stable than free RNA, and is consistent with the observation that these assays had to be performed at lower salt concentrations in order to see a noticeable shift in melting temperature (2 mM NaCl versus 100 mM NaCl for reactions) where higher salt concentrations would compete with the peptide for binding to the negatively charged RNA and suggests a mode of binding for 2 that is dominated by electrostatic interactions.

The original binding motif YrFK-amide exhibits surprisingly tight binding ($K_d = 44$ nM) for such a small peptide. Therefore, it might be expected that each of the amino acid residues are important for binding. There is not currently a structure of the peptide bound to the IRES SLIIB but it would be expected that the lysine and the arginine make electrostatic contacts with the negatively charged RNA backbone. The tyrosine,
phenylalanine, and/or arginine could also be involved in stacking interactions with
the bases. The peptide backbone would generally be flexible but tighter binding could also
be achieved by preordering the side chains, for example by π-stacking between the
tyrosine and phenylalanine (and/or arginine) or by hydrogen bonding between functional
groups. Upon inversion of chirality at the α-carbon, it might be expected that the
positioning of the amino acid side chain R groups would change relative to each other
and that some contacts to the RNA may be lost or diminished. This is consistent with the
large change in $K_d$ upon conversion to the all D-amino acid peptide (44 nM for 1 versus
11.3 μM for 2). The side chains of the lysine and arginine are flexible and would be more
tolerant of these changes but some of the hydrophobic/base interactions may be lost more
readily. This is consistent with the effect of [NaCl] on the melting temperature shifts for
the IRES SLIIb in the presence and absence of the two peptides as shown in Figure 4.3.
As expected, both $T_m1$ and $T_m2$ for RNA alone increase with increasing [NaCl] due to
stabilization of the negatively charged phosphate backbone by sodium binding. The
original peptide GGHYrFK-amide does not show a significant effect on $T_m2$, presumably
due to a balance between stabilizing electrostatic interactions and destabilizing
interactions with the bases. If the binding for GGhyrfk-amide is dominated more by
electrostatics, it would be expected that little or no shift would be observed at high
[NaCl] due to the loss of electrostatic interactions with the target RNA and at lower
[NaCl] there would be a stabilizing effect, consistent with the data shown in Figure 4.3.
For both 1 and 2, there is an increase in $T_m1$ at low salt concentrations. These differences
are a reflection of subtle differences in binding for 1 versus 2. For the original peptide,
GGHYrFK-amide, binding was monitored according to the change in fluorescence emission of the tyrosine in the peptide upon addition of RNA. This approach did not yield an observable change for 2 and is a reflection of the different modes of binding, consistent with a model where the tyrosine, phenylalanine and/or arginine are involved in base interactions in 1 but not 2, or at least not to the same extent.

4.4.1.3 Molecular Modeling of Binding

In order to further probe the binding model proposed above, computational studies were performed to determine the global energy minimized structures for YrFK-amide and yrfk-amide (Figure 4.4) using a mixed torsional/low mode sampling conformational search in Schrodinger’s MacroModel v.9.8 using an Amber* force field to vary the structures and then optimization of the top 5 structures in Gaussian 09. If the tyrosine and phenylalanine are important for RNA binding they would be expected to be involved in interactions with bases, whereas the arginine could be involved in electrostatic interactions and/or pi-interactions, and the lysine would be expected to engage in electrostatic interactions with the phosphate backbone. Figure 4.4 shows only the top structure for each peptide but the general trends discussed were present in all five of the optimized structures. The predicted structures show a difference in the arrangement of the side chains relative to one another for these two peptides. YrFK-amide shows the tyrosine, arginine, and phenylalanine all arranged on the same side of the peptide backbone potentially engaging in pi-stacking interactions. The spacing between these

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residues matches closely with that expected for pi-stacking. It can be seen from the structure for yrfk-amide that the phenylalanine is flipped out, potentially reducing the number of residues available for pi-stacking interactions in the groove of the RNA. This is consistent with the model above and illustrates the potential impact of changing the configuration of the amino acids in this class of peptides. As can be seen in Figure 4.11, this spacing between residues is similar to the spacing between the bases in the NMR structure of SLIIb in the proposed area of binding. Therefore, these peptides may be arranged in a way to promote intercalation into the RNA. The alternate spacing of aromatic residues and positive charges in YrFK-amide could provide the appropriate spacing to optimize both base interactions and electrostatic interactions.

![Image](image-url)

**Figure 4.11** NMR structure of HCV IRES SLIIb showing the spacing between residues (in Angstroms) in the area of expected binding by YrFK-amide peptides.

To further test this model, these peptides were docked to the NMR structure of SLIIb. Initial docking was performed using Autodock 4.2. Sixty structures were simulated and
the localization of the peptides can be seen in Figure 4.5 (left), where the center of each of the sixty peptide structures is shown as a sphere. It can be seen that the most common docking sites for both YrFK-amide and yrfk-amide localize to the top of the RNA with a smaller number of docking sites along the major groove down to the bulge in the middle of the RNA. The top structure for each peptide bound to the RNA is also shown at the bottom of Figure 4.5. Once again, while the figure only shows the top structures the general trends described are consistent among the top 5 structures. The structure for YrFK-amide shows both aromatic residues oriented in the same direction pointing toward the bases of the RNA and the tyrosine hydrogen bonding with the base on U14 whereas the yrfk-amide shows the tyrosine pointed in a different direction. This mode of interaction with the RNA is consistent with the binding model described in the previous section and the free peptide structures shown in Figure 4.4. This once again emphasizes the effect that conversion to D-amino acids can have not only on the conformation of the peptide, but also how it interacts with the target RNA.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (μM$^{-1}$ min$^{-1}$)</th>
<th>$K_d^*$ (μM)</th>
<th>Turnover Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Cu</td>
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<td>0.85</td>
<td>0.62</td>
<td>0.044</td>
<td>32</td>
</tr>
<tr>
<td>2-Cu</td>
<td>0.14</td>
<td>7.9</td>
<td>0.018</td>
<td>11.3</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>3-Cu</td>
<td>0.17</td>
<td>6.87</td>
<td>0.025</td>
<td>19.8</td>
<td>nd</td>
</tr>
</tbody>
</table>

*$K_d$ values listed are for the free peptide.
nd = not determined
4.4.1.4 Reactivity

Reaction kinetics were followed by monitoring the change in fluorescein emission over the course of the reaction as previously described for 1-Cu. Initial velocities were plotted as a function of CuGGHyrfk-amide concentration to generate a pseudo Michaelis-Menten plot (Figure 4.7). A $k_{\text{cat}}$ value of $0.14 \text{ min}^{-1}$ and a $K_m$ of $7.9 \text{ µM}$ were determined (Table 4.3). The $K_m$ value is consistent with the $K_d$ determined under the assumption that the chemistry is fast relative to the binding. It should be noted that the $K_d$ for 2 was determined at a lower salt concentration than that for 1 and the [NaCl] used for cleavage reactions, but the $K_d$ is already equal to the $K_m$ and would not be expected to improve with increasing [NaCl]. The parent peptide 1 demonstrated a $K_d$ much lower than its $K_m$, which is consistent with a higher affinity binding peptide where release of the RNA from the peptide catalyst is most likely rate determining. For 1-Cu, it was estimated by comparing the $K_m$ and $K_d$ that $k_2 \sim 18*k_1$ but for 2-Cu the $K_d$ is close to the $K_m$ and $k_{-1}$ is significant relative to $k_2$. Therefore, for 2-Cu release of the metallopeptide is significant relative to the chemistry. The previously reported data for 1-Cu was obtained under conditions of fixed catalyst concentration while varying [RNA], whereas 2-Cu showed inconsistent behavior at high [RNA] and so was studied under pseudo Michaelis-Menten conditions although the values are directly comparable. The $k_{\text{cat}}$ value for 1-Cu is slightly greater than 2-Cu ($0.53 \text{ min}^{-1}$ versus $0.14 \text{ min}^{-1}$) and the $K_m$ is about an order of magnitude lower ($0.85 \text{ µM}$ versus $7.9 \text{ µM}$), but they are closer than might be expected based on the $> 200$ fold difference in the $K_d$ values. This is consistent with a system.
where binding is not rate-determining. The catalytic efficiency, $k_{\text{cat}}/K_m$, is still
significantly better for 1-Cu, which is not unexpected. An exact turnover could not be
determined for 2-Cu except to say that it is greater than 40. This is higher than the
turnover number of 32 for 1-Cu and provides an example of a catalytic metallodrug
where weaker binding promotes catalyst release, leading to a more efficient turnover of
the metallopeptide. A classical reversible inhibitor would show reduced efficacy with
reduced binding, such as in the case of a mutation of the drug target that would promote
resistance by the virus. The catalytic metallodrug approach, however, provides the
possibility of the retention, or enhancement, of activity upon mutation of the target
binding site. This emphasizes the fact that standard predictors for the efficacy of a drug,
such as binding affinity, are not necessarily the best predictors for the efficacy of a
catalytic metallodrug. As long as specificity can be achieved, the catalyst can still be
effective. Along with the targeting domain, specificity is also achieved by a double filter
mechanism where both binding and positioning of the metal binding domain must be
optimized for a given target.
An analysis of the binding energies obtained from docking experiments is shown in Figure 4.12. It can be seen that the differences in binding are due to differences in electrostatics rather than differences in other factors such as hydrogen bonding (HB), van der Waals interactions (vdW), or desolvation energy. The calculations provide only the combination of these three factors, however, so it is possible that there is a change in one or more of these properties without changing the overall sum of them. It seems, however,
that the differences for the copper complexes are due to electrostatics. There is not a large difference between the RNA binding domains alone but the electrostatic energy shifts by \(~2-3\) kcal/mol for Cu-GGhyrfk-amide and \(~6\) kcal/mol for Cu-GGHYrFK-amide. The addition of the Cu-GGH provides additional binding energy presumably through additional interactions with the RNA. Differences between 1-Cu and 2-Cu may reflect differences in the binding of 1 and 2 that affect the placement of the metal binding domain, where the metal binding domain in 1-Cu is better positioned to interact with the RNA. Also, this trend could have an impact on the trend mentioned for the turnover numbers where 2-Cu showed better turnover than 1-Cu. If the binding has a larger electrostatic component then those interactions may not be lost even after RNA cleavage and any resulting structural changes. Any significant structural changes would be expected to have a significant effect on hydrophobic interactions, however. Therefore, for 1-Cu, which has a higher electrostatic component, it may be more difficult to release from the cleaved RNA and then turn over. This may also suggest another way to optimize future metallodrugs by tuning the properties to obtain better catalyst release.
4.4.1.6 Mass Spectrometry

The site of cleavage by 2-Cu was further probed by MALDI-TOF mass spectrometry as described in chapter 3 for 1-Cu (Table 4.1). It was observed that RNA alone, in the absence of reaction with catalytic metallo-drug, showed fragmentation during the MALDI process. This has been shown previously for other RNAs. Cleavage products from reaction with 2-Cu were analyzed by considering those peaks which did not appear in controls of SLIIb RNA alone, as well as SLIIb in the presence of both 1 mM ascorbate and 1 mM H$_2$O$_2$, and then compared to the predicted masses for expected products based on hydrolysis and known pathways for oxidation of DNA. It should be noted that pathways for oxidative cleavage of DNA are well characterized, but those for RNA oxidation are not. Reactions of 2-Cu with SLIIb showed similar trends to those of 1-Cu

![Figure 4.13](image) MALDI assignments for reaction of Cu-GHyrf-k-amide with SLIIb (left); how these assignments map to the three dimensional structure are shown on the right.
but spread out over a larger range (Figure 4.13). Once again, exact products could not be assigned with confidence but sites of reactivity could be determined. Consistent with the results previously described for 1-Cu, the proposed sites of cleavage at the top loop have been shown to be important for translation. The sites of reaction shown are consistent with a system that binds in a location similar to 1-Cu but in a different orientation as previously suggested based on the modeling results. Once again, peaks are also observed that correspond to an area towards the middle of the RNA that match with the second small cluster of docking sites in the middle of SLIIb as shown in Figure 4.5. Figure 4.14 (left) shows where docking calculations place the copper centers for the top 60 structures and this is consistent with this model as well where it is similar to that observed for 1-Cu (chapter 3).

**Figure 4.14** Localization of the copper centers for 2-Cu (left). The structure of the lowest energy conformation for 2-Cu bound to SLII is shown on the right. Structures were prepared in AutoDockTools.
4.4.1.7 Replicon Data

Complex 2-Cu was then tested in the HCV cellular replicon assay described in chapter 3 to assess activity *in vivo*. This cellular replicon assay is accepted by the FDA as a measure of drug efficacy for HCV treatment. The copper complex of 2 was found to have an IC$_{50}$ of 1.92 μM, which is comparable to the previously reported data for 1-Cu (IC$_{50}$ = 0.58 μM), and it exhibited no cytotoxicity up to 100 μM (Table 4.2). Despite significantly worse binding (~250 fold higher K$_{d}$) and a ~10 fold higher K$_{m}$, the observed cellular activity was approximately the same. This is a reflection of the complex combination of factors that determine the activity of these metallodrugs. The all D-amino acid analog also seems to maintain the ability to be taken up by cells and the lack of toxicity shows the specificity for the virus.

Overall, the chiral analog of 1-Cu shows potential for use *in vivo*. The assays described show that the chiral analog maintains activity but the expected enhancement of efficacy due to increased stability to proteases is not reflected in these experiments. Therefore, it might be expected that the all D-amino acid version would perform better in an animal model. It still binds to the target RNA, though significantly worse than 1-Cu, possibly due to the loss of interactions with the RNA. Since binding does not seem to be rate-determining, however, the effect on the chemistry is not as pronounced and, in fact, is comparable in the replicon assay. This similar efficacy, combined with potentially higher *in vivo* stability, should lead to a more effective catalytic metallodrug in more complex systems such as animal models or humans.

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4.4.2 Other Peptides

The results from the all D-amino acid peptide suggest future directions for variation of the peptide structure. Based on the comparison between 1-Cu and 2-Cu, enhancing the binding does not have a significant effect on the activity especially in the replicon assays and therefore the best activity may be achieved by varying other parameters. For example, a reduction in RNA binding could also result in a reduction in binding to the cleaved RNA product and, therefore, enhanced catalyst release and turnover. Also, changing the flexibility of the metal binding domain relative to the RNA binding domain could improve its ability to access sites on the RNA. Another way to enhance catalysis could be to improve the chemistry itself, such as by varying the properties or identity of the metal and metal binding domain.

4.4.2.1 All L-Amino Acid Form

The sequence of the RNA targeting domain, YrFK-amide, contains an arginine residue in the D-configuration but it is not clear what role this plays or even if it is necessary for activity. To study this and test another potential candidate for catalytic inactivation of the HCV IRES, the all L-amino acid form Cu-GGHYRFK-amide was evaluated. This compound showed activity similar to the all D-amino acid form, with a $K_m$ of 6.87 μM and a $k_{cat}$ of 0.17 min$^{-1}$ (Table 4.3). Much like for Cu-GGhyrfk-amide, the binding is significantly worse ($K_d = 19.8$ μM) relative to the lead compound Cu-GGHYrFK-amide.
but the chemistry is only slightly worse. Unlike 2, binding for 3 could be measured by looking at the fluorescence of the tyrosine in the same way that binding was measured for 1, emphasizing the subtle changes in binding upon changes in the configuration of the peptide. It makes sense that the mode of binding doesn’t change as much on going from 1 to 3, where only the configuration of the arginine is changed, but the change is more drastic for 2, where the stereochemistry at four different residues is changed. Much like the all D-amino acid form, 3-Cu shows comparable activity to the parent compound in vivo (Table 4.2). The all L-amino acid derivative may be a cheaper form of the metallodrug due to price and ease of access to the L-form amino acids over the D-form. This metallopeptide also provides mechanistic information on RNA binding by YrFK-amide. The D-configuration arginine is important for binding but, as has already been established, binding is not the only determinant of activity. The all D-amino acid form may still have an advantage, though, in terms of in vivo stability to proteases.

4.4.2.2 Further Variation of the Peptide Sequence

The mechanism by which the ATCUN motif binds to copper only requires that the sequence contains a histidine in the third position and so the identity of the first two residues can be varied to look for an effect on the binding or chemistry. As a first study to look at if varying the composition of the ATCUN motif would affect the chemistry, the peptide KKHYrFK-amide was tested. A $K_m$ of 1.14 $\mu$M and a $k_{cat}$ of 0.58 min$^{-1}$ (Table 4.3) were found for the copper complex of this peptide, values that are similar to those
found for Cu-GGHYrFK-amide. The addition of a lysine to the ATCUN motif could potentially affect the chemistry, such as by affecting the reduction potential, but would be more likely to simply affect the binding by adding an additional electrostatic interaction with the RNA. Overall, it did not seem to have a significant effect. This is just one example, though, and many other R group variations on the ATCUN motif are possible.

Another peptide that was tested was GGHGYrFK-amide, where a glycine was added between the two domains thereby altering the distance between them but also adding additional flexibility. The correct positioning of the metal binding domain could be essential for optimizing the activity. Also, additional flexibility could allow it to access more sites on the RNA or to access a site that has a higher intrinsic reactivity. From the kinetic data, however, this change did not cause a significant effect on the chemistry, resulting in a $K_m$ of 1.2 $\mu$M and a $k_{cat}$ of 0.54 min$^{-1}$. Once again, further changes may increase (or decrease) the activity but the spacing of the parent peptide seems to already be effective.

The sequence of the original binding domain YrFK-amide contains a D-configuration arginine, the effect of which has already been discussed with the all L-amino acid peptide but it also contains an amidated C-terminus. The effect of changing this functional group was studied by looking at the metallopeptide Cu-GGHYrFK-COOH where the C-terminus is now in the carboxylic acid form. The largest effect from this modification is the addition of a negative charge which might reduce binding to the negatively charged RNA. It could also potentially enhance selectivity by reducing some of the potential for nonspecific binding based on the overall positive charge. From the table it can be seen
that the $K_m$ increases from 0.85 μM to 8.87 μM and that the $k_{cat}$ decreases slightly from 0.53 min$^{-1}$ to 0.12 min$^{-1}$. The modification in the peptide sequence interferes with the RNA binding domain itself and, like for the all D-amino acid and all L-amino acid variants, this peptide shows reduced binding and reactivity. This contrasts with the results obtained for the reactions of Cu-KGHK with tRNA$_{Phe}$ (chapter 2), where amidation did not affect reactivity, and emphasizes that the optimization of catalytic metallodrugs is likely to differ from system to system. This modification could also have an effect on the *in vivo* properties such as by altering the overall charge and affecting the uptake, distribution, or solubility.

Overall, these sequence variations represent only the beginning of what can be studied to enhance the efficacy of this class of catalytic metallodrugs, but some general conclusions can be made. Any sequence that deviates from YrFK-amide shows a reduction in binding and a small reduction in activity but the overall chemistry isn’t affected as significantly. Therefore, optimization of the metallodrug might not be achieved by enhancement of the binding as much as by achieving a proper balance between tight binding to the target and weak binding to the cleaved product. If a large component of the binding is through electrostatic interactions, then it may be expected that these interactions between the catalyst and the cleaved RNA would still be relatively strong. Hydrophobic interactions, however, would be more likely to be lost upon structural changes in, or cleavage of, the RNA. It also may be more effective to optimize the selectivity, even at the cost of reduced binding, to obtain the most effective metallodrug.
4.4.3 Variation of the Metal-Binding Domain and Metal

Another way to improve catalyst activity is to change the chemistry at the metal binding domain itself, such as by altering the metal binding domain and therefore its properties or by changing the identity of the metal ion. Preliminary screening of several compounds toward this end is shown in Figure 4.9. The copper and nickel complexes were tested for all of the metallodrugs shown, and for the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and diethylene triamine pentaacetic acid (DTPA) derivatives, the iron and cobalt derivatives were tested as well. The synthesis of the DOTA and DTPA derivatives was performed by taking advantage of the reactivity of a thiol with N-ethylmaleimide (NEM) as shown in Figure 4.6. Metal-binding domains DOTA and DTPA were purchased with one NEM group already attached and were reacted with cysteine containing peptides with either a cysteine on the N-terminus, CYrFK-amide, or on the C-terminus, YrFKC-amide.

Since these experiments were performed as an initial screening, not enough information is available for all of them to convert the fluorescence change to a concentration change. Therefore, the data shown are the initial velocities in units of fluorescence change per minute so that they are all shown in the same units. As can be seen from the figure, none of the DOTA or DTPA containing peptides showed significant activity in the in vitro fluorescence assay. Also, varying the metal to something other than copper did not result in activity. The effect of positioning of the metal binding domain at the N-terminus versus the C-terminus could not be determined due to the
overall lack of reactivity for these systems. These results, while not yielding a new form of catalyst to study, further confirm that the GGHYrFK-amide scaffold is already relatively well optimized to perform chemistry. These systems also present another example of experimental confirmation of the double filter mechanism, where changing the metal binding domain turns off catalytic activity because it is no longer optimized for the target. Overall, activity is observed only when both copper and an ATCUN motif are used. This is likely a reflection of the importance of the Cu$^{2+/3+}$ redox chemistry to the mechanism, where both DOTA and DTPA are expected to undergo redox chemistry via a Cu$^+$ intermediate. Future library design should incorporate metal binding domains that can generate metal ions in higher oxidation states, such as ones that are known to also undergo redox chemistry through a Cu$^{3+}$ intermediate, ones which span a range of reduction potentials, or binuclear metal centers which could simulate a high oxidation state by having two metal ions in close proximity. A larger assortment of metals could also be tested. For the Rev metallopeptides targeting the HIV RRE described in chapter 2, it was found that the Cu-DOTA-Rev system was one of the most active. Once again, this emphasizes the variation depending on the system and illustrates the need to develop structure activity relationships for each system under consideration.
Table 4.4  Overall kinetics for derivatives of 1-Cu.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (μM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-GGHYrFK-amide</td>
<td>0.85</td>
<td>0.53</td>
<td>0.62</td>
</tr>
<tr>
<td>Cu-GGHYrFK-COOH</td>
<td>8.87</td>
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<td>0.51</td>
</tr>
<tr>
<td>Cu-GGHGYrFK-amide</td>
<td>1.12</td>
<td>0.54</td>
<td>0.48</td>
</tr>
</tbody>
</table>

4.4.4 Overall Summary of Kinetics

Table 4.4 shows a summary of the overall kinetics for 1-Cu and the active derivatives described in this chapter. The most detrimental effects on \textit{in vitro} activity are observed when the YrFK-amide RNA binding domain is changed. Modification of the ATCUN motif does not seem to have a significant effect and neither does changing the space and flexibility between the metal binding domain and the RNA binding domain. Future studies can explore these trends in more detail. Also, while the rates may not change, the observed cleavage sites and products may change when studied by mass spectrometry. Current results suggest that these trends do not directly translate to \textit{in vivo} activity in the cellular replicon assay, where 1-Cu, 2-Cu, and 3-Cu all show comparable activity. This
suggests that, in a more complicated biological system, other factors contribute to ultimately determining efficacy.

4.5 Conclusions

These studies lay the foundation for future improvement of this class of metallodrugs targeting HCV IRES SLIIb. Overall, the chemistry occurs according to the general pathway shown in Figure 4.15. The first step is binding to the target, followed by chemistry. After chemistry occurs, the metallodrug must be released from the target in order to be regenerated in its active form. Therefore, there are several steps that can be important for catalysis and this can vary depending on the system and which step is rate determining (Figure 4.15). For the peptides described based on the YrFK-amide scaffold, significantly enhanced binding resulted only in a small increase in activity. The other two steps, therefore, may represent better options for future structure activity studies. The first steps in varying the metal binding domain and the metal are described, but future studies focused on other Cu$^{2+/3+}$ metal binding domains or systems with more oxidizing...
metals may provide a more effective way to tune this step. The final step, catalyst release, represents another interesting dynamic involved in the design of catalytic metallodrugs that is not present in traditional drugs. Optimization of this step by reducing product binding while maintaining tight binding to the target will be challenging to do rationally. It may be that for these metallopeptides the important factor isn’t tight binding as much as selectivity, such that a weaker binding catalyst with better turnover and specificity may ultimately be most effective.
Chapter 5- Catalytic Metallodrugs Targeting HCV IRES Stem Loop IV

5.1 Introduction

After the demonstration of the effectiveness of catalytic metallodrugs in degrading HCV IRES SLIib RNA, the approach was extended to stem loop IV (SLIV, Figure 5.1) of the HCV IRES. The whole IRES represents an attractive target for therapeutic intervention and there are a variety of stem loops that can be targeted. HCV IRES SLIV is important because it contains the AUG start codon and it has been shown that blocking SLIV leads to an inhibition of translation. Previous work with short hairpin RNA targeting the GCAC sequence near the start codon has shown the potential of this type of approach.\textsuperscript{86} It has also been shown that peptides based on the La protein which binds to SLIV inhibit viral translation.\textsuperscript{87} Therefore, the HCV IRES SLIV is an attractive target for the design of catalytic metallodrugs.

LaR2C: KYKETDLLILFKDDYFAKKNEERK

\textbf{Figure 5.1} Secondary structure of HCV IRES stem loop IV (left); the sequence of the LaR2C peptide known to bind to stem loop IV (right).
5.1.1 IRES Structural Information

![Diagram of IRES structure](image)

**Figure 5.2** Cryo-electron microscopy data showing the structure of the 40S-IRES complex. Adapted from Berry et al. 2011.

As mentioned in chapter 3, the HCV IRES consists of several stem loop structures each of which has a specific function and interacts with a known protein partner, one of which is the 40S subunit of the ribosome. In 2005, the low resolution cryo-electron microscopy (cryo-EM) structure of the 40S-IRES complex was published (Figure 5.2).\(^{116}\) This structure provided insight into the overall positioning of the domains of the IRES and how this positioning facilitates the initiation of translation. More recent work has taken the high resolution structures of isolated HCV IRES stem loops and superimposed them on the cryo-EM data to generate a more detailed model of 40S binding.\(^{102}\) One important result of these studies is the positioning of SLIV, and therefore the start codon, into the mRNA binding cleft. This provides an overall model for the structure and how it relates to the function. It also provides information on how the domains are positioned relative to one another in three dimensions. This could be important for catalytic metallodrug design because interactions through space could
affect binding and chemistry relative to that observed with individual domains.

5.1.2 Catalytic Metallo-drugs Targeting IRES SLIV

The human La protein is part of a class of proteins containing RNA recognition motifs (RRMs) and is known to interact with a variety of RNAs including viral RNAs as well as RNA polymerase III transcripts.\textsuperscript{117} It is also necessary for HCV IRES mediated translation.\textsuperscript{118} LaR2C is a peptide derived from the RRM2 of La protein (sequence shown in Figure 5.1, right) and is responsible for most of the binding to SLIV.\textsuperscript{119} Further truncation of the LaR2C provides a 7mer (sequence shown in Figure 5.1 in red) that still maintains most of the binding.\textsuperscript{120} These two peptides, therefore, represent a promising starting point for catalytic metallo-drug design. The design strategy described herein consists of the incorporation of a metal-binding ATCUN motif into the LaR2C peptide (4) as well as the 7mer (5), as shown in Figure 5.3. Taking advantage of the modular design of the original metallo-drug targeting SLIIb, Cu-GGHYrFK-amide, the target recognition domain is changed from one binding SLIIb to the LaR2C peptide. As demonstrated by the metallo-peptide targeting HIV TAR RNA described in chapter 2, however, this binding alone is not sufficient to guarantee activity and therefore the potential of these metallo-drugs is assessed.
5.2 Methods and Materials

5.2.1 Materials. RNA was purchased from Dharmacon, part of Thermo Fisher Scientific (Lafayette, CO). Peptides were purchased from Genemed Synthesis Inc. (South San Francisco, CA). The sequence for the IRES SLIIb RNA used was 5’-fluorescein-GGCAGAAAGCGUCUAGCCAUGGCGUUAAGUAUGCC-3’, for the IRES SLIV RNA was 5’-fluorescein-GGACCGUGCACCAUGAGCACGAAUCC-3’, and for HIV Rev Response Element (RRE) RNA was 5’-fluorescein-UUGGUCUGGGCGCGAGCAAGUGACGGUACAGGCC-3’. The sequence of the 5mer RNA used for calibration in the MALDI-TOF experiments was 5’-fluorescein(T)-UGUG-3’. All RNA was annealed by heating to 95 °C and then cooled slowly to room temperature before use. Sodium chloride, sodium hydroxide, and acetonitrile were purchased from Fisher and HEPES, ammonium citrate, and 3-hydroxypicolinic acid were purchased from Sigma. C18 ZipTips were obtained from Millipore. All experiments were
performed using diethyl pyrocarbonate (DEPC) treated water and autoclaved pipette tips and tubes. Copper complexes form quickly and were made by mixing in a 1.1: 1 peptide: CuCl$_2$ ratio and waiting at least five minutes for complex to form.

5.2.2 Binding via Fluorescence. Binding data for GGHKYKETDLLILFKDDYFAKKNEERK-amide and GGHKYKETDL-amide were obtained by adding serial aliquots of IRES SLIib or SLIV RNA to 500 nM of the peptide and monitoring tyrosine emission ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 313$ nm). Data was then fit to a one-site binding model using Origin 7.0 software.

5.2.3 SHAPE Analysis. SHAPE analysis studies were performed by Kasia Purzycka in Stuart LeGrice’s lab (National Institutes of Health). 72 picomoles of RNA were heated at 90 °C for 1 minute in 60 μL of equilibration buffer (10 mM Tris–HCl pH 8.0, 100 mM KCl, 0.1 mM EDTA) and slowly cooled to 4 °C. Then, 287.4 μL of water and 87.6 μL of refolding buffer (200 mM Tris–HCl pH 8.0, 650 mM KCl, 2.5 mM EDTA, 25 mM MgCl$_2$ and 40 U RNase inhibitor) were added. The solution was divided into six 72.5 μL aliquots (12 picomoles of RNA each) and then the aliquots were incubated at 37°C for 10 minutes. Peptides were dissolved in 20 mM HEPES pH 7.5, 100 mM NaCl to a concentration of 48.8 μM. One μL of peptide (48.8 picomoles - sample) or 1 μL of buffer (control with no peptide) was added to each one of the RNA aliquots and allowed to bind at 37 °C for 1 hour. For 1M7 modification, the RNA-peptide complexes were treated with 7.3 μL of 35 mM 1M7 in anhydrous DMSO (+) or DMSO alone (−) and allowed to
proceed at 37 °C for 5 minutes. The RNA was precipitated and resuspended in 10 µL of water. Modified sites were detected by primer extension, followed by electrophoretic fragment separation. The primer extension reactions were performed using 4.8 picomoles of RNA, 5 picomoles of primer and SuperScript Reverse Transcriptase III (Life Technologies) following manufacturer recommended conditions. After reverse transcription, RNA was degraded using 1 µL 4 M NaOH by heating to 90 °C for 3 minutes. Then solutions were neutralized using 2 µL 2M HCl. cDNA products were precipitated. Dideoxy sequencing markers were generated using unmodified RNA. Two 5’-end-labelled DNA primers were used for the analysis. cDNA band intensities for the (+) and (−) reactions were integrated using ImageQuant TL.

5.2.4 Reaction Kinetics via Fluorescence. HCV IRES RNA cleavage was monitored in vitro by fluorescence using 5’ fluorescein end-labeled RNA with excitation and emission wavelengths of 485 nm and 518 nm, respectively. Reactions were carried out at 25 °C in reaction volumes of 100 µL in the presence of 1 mM ascorbate and 1 mM H₂O₂ in HEPES buffer (pH = 7.4, 100 mM NaCl) with 1 µM fluorescein labeled IRES SLIIb and analyzed according to the change in fluorescence emission observed as the reaction occurred. Both a time-dependence and a concentration-dependence of catalyst were observed. For reactions with SLIIb, the initial velocity of the time dependence plot was fit to a line and used to generate the pseudo Michaelis-Menten plots which were then fit to the Michaelis Menten equation as described in previous chapters. Reactions with SLIV showed two phases and were fit to a consecutive reaction model. All fits were
5.2.4 Mass Spectrometry. Reactions for MALDI-TOF analysis were run as described in 5.2.4 but using 10 µM fluorescein labeled IRES SLIIb and 10 µM copper-peptide and incubated for two hours. Reactions were then quenched by being placed on ice and desalted. Desalting was performed using C\textsubscript{18} ZipTips from Millipore Co. in order to desalt reaction mixtures prior to mass spectrometric analysis. ZipTips were wetted with a 50:50 mixture of acetonitrile:water and equilibrated with 2 M triethylammonium acetate (TEAA), pH 7.0. The reaction mixture was then bound to the ZipTip, washed with nanopure water, and eluted with 50:50 acetonitrile:water. Samples were spotted onto a Bruker ground steel 96 target microScout plate by first spotting with 1 µL of the 0.3 M 4-hydroxypicolinic acid (HPA), 30 mM ammonium citrate matrix solution in 30% acetonitrile, drying, then spotting with 2.5 µL of a 1:1 RNA:matrix mixture, and allowed to dry. A calibration mixture containing 3 RNAs covering a range of molecular weights (Fl-5mer, Fl-IRES SLIV, and Fl-RRE, with molecular weights of 2057.5, 8861.5, and 12172.5 amu, respectively) was used to calibrate the instrument. All MALDI-TOF MS analysis was performed on a Bruker MicroFlex LRF instrument, equipped with a gridless reflectron, using negative ion mode and reflectron mode. The pulsed ion extraction time was 1200 ns. At least 1000 shots were summed per spectrum. Data analysis was performed using Bruker flexAnalysis software. Only m/z values > 1000 amu were considered, since excessive spectral crowding occurred at lower m/z ranges. Reaction

performed using Origin 7.0 software. The initial velocity values shown are an average of at least three trials.
peaks were compared to controls containing RNA alone and RNA in the presence of 1 mM ascorbate, 1 mM H₂O₂ and new peaks were identified as products of cleavage by the metallopeptide.

5.2.6 HCV Cellular Replicon Assay. A stable cell line ET (luc-ubi-neo/ET) was employed in the assay. The ET is a Huh7 human hepatoma cell line that contains an HCV RNA replicon with a stable luciferase (Luc) reporter and three cell culture-adaptive mutations. The HCV RNA replicon antiviral evaluation assay examined the effects of compounds at six half-log concentrations each. Human interferon alpha-2b was included in each run as a positive control compound. Sub-confluent cultures of the ET line were plated out into 96-well plates that were dedicated for the analysis of cell numbers (cytotoxicity) or antiviral activity, and various concentrations of metallodrugs and controls were added to the appropriate wells the following day. Cells were processed 72 hours later when the cells were still sub-confluent. Six half-log serial dilutions of the compound were performed, and values derived for IC₅₀ (the concentration that inhibited virus replication by 50%), TC₅₀ (the concentration that lowered cell viability by 50%) and TI (the selectivity index: TC₅₀/IC₅₀). HCV RNA replicon levels were assessed as the replicon-derived Luc activity. The toxic concentration of drug that reduced cell numbers (cytotoxicity) was assessed by the CytoTox-1 cell proliferation colorimetric assay (Promega).
5.3 Results

5.3.1 RNA Binding

Dissociation constants for binding of 4 and 5 to SLIIb and SLIV RNA were obtained by serial addition of RNA to peptide and following tyrosine emission as previously described for 1 binding to SLIIb (chapter 3). A sample plot is shown in Figure 5.4 and the data are summarized in Table 5.1. All peptides showed similar $K_d$ values for RNA binding.

![Sample curve for binding of 5 to HCV IRES SLIV.](image)

**Figure 5.4** Sample curve for binding of 5 to HCV IRES SLIV.

**Table 5.1** Dissociation constants for binding to HCV IRES SLIV and SLIIb for peptides based on LaR2C.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>SLIV $K_d$ (μM)</th>
<th>SLIIb $K_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[GGHKYKETDLLILFKDDYFAKKNEERK-amide] (4)</td>
<td>4.56</td>
<td>4.72</td>
</tr>
<tr>
<td>[GGHKYKETDL-amide] (5)</td>
<td>9.68</td>
<td>4.33</td>
</tr>
</tbody>
</table>
5.3.2 Selective 2’-Hydroxyl Acylation Analyzed by Primer Extension

To further evaluate binding of 4 to the HCV IRES, selective 2’-hydroxyl acylation analyzed by primer extension (SHAPE) experiments were performed by Stuart LeGrice’s lab (National Institutes of Health). These experiments confirmed binding of 4 to HCV IRES SLIIb (Figure 5.5) but the RNA construct used did not extend out far enough to probe SLIV binding.

![SHAPE Data showing binding of 4 to HCV IRES stem loop II; RNA alone (blue), and RNA plus 4 (red). The sequence corresponding to SLIIb is underlined in red.](image)

**Figure 5.5** SHAPE Data showing binding of 4 to HCV IRES stem loop II; RNA alone (blue), and RNA plus 4 (red). The sequence corresponding to SLIIb is underlined in red.

5.3.3 Kinetics

Upon confirmation of binding to SLIV, kinetics were monitored by following the fluorescence change over time as previously described. A sample trace is shown in Figure 5.6. These data were fit to a consecutive reaction model to obtain \( k_1 \) and \( k_2 \) and the variation of these rates with catalyst concentration is shown in Figure 5.7. Peptide 4-Cu showed poor solubility and inconsistent behavior above 20 µM and so data were only
fit up to 20 µM. Each of these plots was then fit to obtain $k_{cat}$ and $K_m$ values as summarized in Table 5.2.

**Figure 5.6** Sample kinetic trace for reactions with HCV IRES SLIV showing the presence of two phases. The fit (shown in red) is for a consecutive reaction model.

**Figure 5.7** Pseudo Michaelis-Menten plots for reaction of 4-Cu (filled squares) and 5-Cu (open circles) with HCV IRES SLIIb.

**Table 5.2** Michaelis-Menten parameters for degradation of SLIV.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{cat1}$ (min$^{-1}$)</th>
<th>$K_{m1}$ (µM)</th>
<th>$k_{cat1}/K_{m1}$ (µM$^{-1}$ min$^{-1}$)</th>
<th>$k_{cat2}$ (min$^{-1}$)</th>
<th>$K_{m2}$ (µM)</th>
<th>$k_{cat2}/K_{m2}$ (µM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Cu</td>
<td>0.93</td>
<td>6.07</td>
<td>0.15</td>
<td>0.38</td>
<td>24.3</td>
<td>0.016</td>
</tr>
<tr>
<td>5-Cu</td>
<td>0.99</td>
<td>5.76</td>
<td>0.17</td>
<td>0.17</td>
<td>8.32</td>
<td>0.02</td>
</tr>
</tbody>
</table>
After establishing binding of 4 and 5 to SLIIb as well, reactivity with this RNA was also monitored. These profiles showed normal monophasic behavior and the pseudo Michaelis Menten plots are shown in Figure 5.8. Results are summarized in Table 5.3.

![Figure 5.8](image_url) Pseudo Michaelis Menten plots for reaction of 4-Cu (filled squares) and 5-Cu (open circles) with HCV IRES SLIIb.

**Table 5.3** Michaelis-Menten parameters for degradation of SLIIb.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (μM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[GGHKYKETDLIILFKDDYFAKKNEERK-amide]-Cu, 4-Cu</td>
<td>0.14</td>
<td>20.1</td>
<td>0.007</td>
</tr>
<tr>
<td>[GGHKYKETDL-amide]-Cu, 5-Cu</td>
<td>0.14</td>
<td>10.1</td>
<td>0.014</td>
</tr>
<tr>
<td>[GGHYrFK-amide]-Cu, 1-Cu</td>
<td>0.53</td>
<td>0.85</td>
<td>0.62</td>
</tr>
</tbody>
</table>

To further investigate the binding and mechanism of reaction for 5-Cu with SLIV RNA, the salt dependence of $k_1$ and $k_2$ on [NaCl] was determined (Figure 5.9). It can be seen that $k_1$ showed a significant dependence on [NaCl] but $k_2$ did not.
As mentioned in previous chapters, MALDI-TOF analysis of RNA cleavage products is complicated by the presence of a large number of fragmentation peaks that are present even in the absence of catalyst. This has been reported for other systems and cleavage products were determined by considering only those peaks that were new and not contained in controls of RNA alone or RNA in the presence of coreagents ascorbate and H$_2$O$_2$ but no catalyst. The mass spectra are shown in Figure 5.10 and assignments listed in Tables 5.4 and 5.5.
Table 5.4  List of new mass spectrometric peaks observed after reaction with SLIV.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Intensity</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2569.7</td>
<td>1304.8</td>
<td>3’ fragment at 19</td>
</tr>
<tr>
<td>1093.1</td>
<td>614.7</td>
<td>5’ fragment at 2</td>
</tr>
<tr>
<td>2490.1</td>
<td>397.3</td>
<td>3’ fragment at 19</td>
</tr>
<tr>
<td>3217.1</td>
<td>355.5</td>
<td>5’ fragment at 8</td>
</tr>
<tr>
<td>1210.5</td>
<td>254.5</td>
<td>3’ fragment at 2</td>
</tr>
<tr>
<td>3544.9</td>
<td>247.6</td>
<td>5’ fragment at 9</td>
</tr>
<tr>
<td>4442.6</td>
<td>215.9</td>
<td>3’ fragment 13</td>
</tr>
</tbody>
</table>
Table 5.5  List of new mass spectrometric peaks observed after reaction with SLIIB.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Intensity</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3851.5</td>
<td>63.2</td>
<td>5’ fragment at 10</td>
</tr>
<tr>
<td>4196.5</td>
<td>38.1</td>
<td>3’ fragment at 22</td>
</tr>
<tr>
<td>2590.4</td>
<td>22.7</td>
<td>3’ fragment at 27</td>
</tr>
<tr>
<td>4847.6</td>
<td>19.1</td>
<td>3’ fragment at 20</td>
</tr>
<tr>
<td>2511</td>
<td>16.38</td>
<td>3’ fragment at 27</td>
</tr>
<tr>
<td>3868.2</td>
<td>12.5</td>
<td>5’ fragment at 10</td>
</tr>
<tr>
<td>2606.2</td>
<td>12.4</td>
<td>5’ fragment at 6</td>
</tr>
<tr>
<td>5176.4</td>
<td>11.3</td>
<td>3’ fragment at 19</td>
</tr>
</tbody>
</table>
5.3.5 Replicon Assay

The activity of metallodrugs 4-Cu and 5-Cu were evaluated in cell culture by use of a cellular HCV replicon assay that mimics the native HCV replication system as described in chapter 3. The results are shown in Table 5.6 and compared to the data for 1-Cu. Both metallopeptides showed activity similar to 1-Cu and, as for 1-Cu, no activity was observed for controls of the RNA binding domain alone, Cu-GGH alone, free copper, or the peptide without copper. Recombinant IFNα-2b was used as a positive control.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral IC_{50} (μM)</th>
<th>Cytotoxicity TC_{50} (μM)</th>
<th>Selectivity Index TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>[GGHKYKETDLLILFKDDYFAKKNEERK-amide]-Cu, 4-Cu</td>
<td>0.75</td>
<td>&gt; 50</td>
<td>&gt; 67</td>
</tr>
<tr>
<td>[GGHKYKETDL-amide]-Cu, 5-Cu</td>
<td>2.17</td>
<td>&gt; 50</td>
<td>&gt; 23</td>
</tr>
<tr>
<td>[GGHYrFK-amide]-Cu, 1-Cu</td>
<td>0.58</td>
<td>&gt; 100</td>
<td>&gt; 172</td>
</tr>
<tr>
<td>[KYKETDLLILFKDDYFAKKNEERK-amide] (4)</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na (^a)</td>
</tr>
<tr>
<td>[GGH]-Cu(^{2+})</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
<tr>
<td>Cu(^{2+}) (aq)</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>na</td>
</tr>
</tbody>
</table>

\(^a\) na- not applicable
5.4 Discussion

5.4.1 Binding to IRES SLIV and SLIIb

As can be seen in Table 5.1, binding for both peptides to SLIV was confirmed. It can also be seen, however, that binding to the isolated SLIIb was observed. This binding of LaR2C to SLIIb was not previously reported and was further confirmed by selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) studies (Figure 5.5). The basic principle of SHAPE is shown in Figure 5.11 where reaction of the 2'-hydroxyl with N-methylisotoic anhydride (NMIA) is dependent on the local flexibility of that residue. The presence of this modification then impedes the ability of the reverse transcriptase to read through this position and make cDNA. Similar to DNA footprinting, when a

\[
\text{NMIA} \xrightarrow{\text{H}_2\text{O}, \text{CO}_2} \text{2-methylaminobenzoate (emission at 440 nm)}
\]

**Figure 5.11** Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE).
compound binds to a region of the RNA it can protect that area from reaction with NMIA. It is also possible that the flexibility in this region could change due to a conformational change rather than due to binding there. SHAPE data of the full length IRES was obtained in the presence and absence of 4. In the presence of 4, the area corresponding to SLIIb was protected, suggesting binding in this region (Figure 5.5). Binding to SLIV was not observed in the SHAPE analysis but this is due to the limitations of the RNA construct which extended only out to residue 249 which maps to SLIII. These SHAPE experiments also confirm binding of 4 in the context of the full length IRES. Binding of LaR2C to SLIV is already well established in the literature and further confirmed by the in vitro fluorescence assays described above. It was previously mentioned that recent studies combining NMR structural data for individual stem loop domains with the cryo-EM structure of the full length HCV IRES bound to the 40S ribosomal subunit have shown SLIIb and SLIV to be close together in three dimensional space, approximately 30 angstroms apart (Figure 5.1). The NMR structure of the human La protein is available and shown in Figure 5.13, with the region corresponding to the LaR2C peptide highlighted. The region corresponding to the 7mer KYKETDL-amide is shown in red. It can be seen that the length of the region corresponding to the LaR2C peptide is also roughly 30 angstroms long and is consistent with a model where this peptide could bind to both domain IIb and IV of the IRES.

Previous papers establishing the binding of human La protein, and LaR2C, to SLIV have been done using full length IRES. Mutations of SLIV showed greatly decreased binding to the La protein. These experiments, however, do not preclude binding to SLIIb.
as well. In fact, a recent paper has shown that mutations of SLIib lead to a
misconfiguration in the decoding groove and a reduction in the ability to initiate
translation. This demonstrates the potential for interactions between the two domains.

**Figure 5.12** Overall structural model of the HCV IRES bound to the 40S ribosomal subunit showing the close proximity of SLIib and SLIV (contains AUG start codon) in three dimensional space. Adapted from Filbin et al. 2011.

**Figure 5.13** NMR structure the human La protein with the portion corresponding to LaR2C highlighted. The portion in red corresponds to the truncated 7mer. The length of the peptide roughly corresponds to distance between SLIib and SLIV in the cryo-EM structure.
5.4.2 Reactivity

In order to determine the ability of these compounds to catalytically degrade RNA, activity in an *in vitro* fluorescence assay was determined as reported for Cu-GGHYrFK-amide. Reactions were studied using both the isolated SLIIb and SLIV containing 5’-fluorescein labels. For reactions with isolated IRES SLIV, the time dependence plots show two phases (Figure 5.6) and were fit to a consecutive reaction model to determine $k_1$ and $k_2$. These values were then plotted as a function of catalyst concentration (Figure 5.7) and the pseudo Michaelis-Menten parameters are shown in Table 5.2. The $K_m$ values obtained are similar and consistent with a system containing one binding site where two consecutive reactions occur. The first reaction could generate a new site in the same area where a second reaction can occur. To test this model, the salt dependence of $k_1$ and $k_2$ was studied for the reaction of 5-Cu with SLIV (Figure 5.9). It can be seen that increasing the salt concentration has a significant effect on $k_1$ but does not affect $k_2$. The higher salt concentrations would be expected to interfere with any steps that involve binding to the negatively charged RNA substrate. Therefore, the observed data are consistent with the above model where the first step is dependent on [NaCl] but the second step, which is intramolecular, is not. The first modification/cleavage event could cause a structural change in the RNA altering the accessibility of a second site.

Once binding to SLIIb was established, reactivity with 4-Cu and 5-Cu was also studied. Unlike reactions with SLIV, reactions with SLIIb showed a simple monophasic profile much like those described in chapters 3 and 4. Initial velocities at varying catalyst
concentrations were determined for SLIIb to generate pseudo Michaelis-Menten plots which were then fit to the Michaelis Menten equation to determine $K_m$, $V_{max}$, and $k_{cat}$ values (Table 5.3, Figure 5.8). Both metallopeptides showed similar binding to, and reactivity with, SLIIb. 4-Cu had a $K_m$ of 20.1 µM and 5-Cu had a $K_m$ of 10.1 µM. These $K_m$ values approximate the previously described $K_d$ values, consistent with a system where $k_1 \gg k_2$.

5.4.3 Mass Spectrometry

![Diagram](image.png)

**Figure 5.14** Mass spectrometric analysis of cleavage products for reactions with HCV IRES SLIIb (left) and SLIV (right).

To further probe the mechanism of reaction and map potential cleavage sites, MALDI-TOF mass spectrometry was performed as described in chapters 3 and 4 for the reaction of 4-Cu with both SLIIb and SLIV. Most of the observed products did not match closely to predicted products based on hydrolysis or DNA oxidation pathways (Tables 5.4 and
but some overall trends are evident in terms of the general sites of reactivity (Figure 5.14). For reaction with SLIIb, it is notable that the sites of reaction differ from those previously described for 1-Cu and 2-Cu. The most significant difference is the lack of reactivity in the area corresponding to C13 to A15 which was one of the major sites of reactivity for 1-Cu and also showed high accessibility in SHAPE experiments. For reaction with SLIV, the most significant site of reaction is at the A19 which is the A residue of the previously mentioned GCAC sequence which is known to be important for ribosome assembly. Reaction is also observed at the G of this sequence. Once again, the importance of these specific residues suggests that reactivity at these sites could be effective even if in the context of the full length IRES if dissociation of the cleavage products does not occur due to the fact that most of the hydrogen bonding would remain intact.

5.4.4 Replicon Data

After binding and reactivity were established in vitro, the activity of 4-Cu and 5-Cu were tested in a cellular HCV replicon assay as described in chapter 3. Due to the absence of a proven animal model for HCV, this replicon assay is accepted by the FDA as a measure of the efficacy of potential HCV treatments. The effectiveness of Cu-GGHYrFK-amide in this assay was previously reported and is included in Table 5.6, along with 4-Cu and 5-Cu. Both of these metallopeptides had similar activity and were also comparable to Cu-GGHYrFK-amide. Despite the absence of a known uptake
sequence, these peptides still seemed to be effective. As was observed for Cu-GGHYrFK-amide, neither the peptides (no metal bound), the Cu-GGH metal binding domain, nor the RNA binding domain showed activity on their own. This is consistent with these compounds reacting in the cellular assays according to how they were designed as opposed to some other mechanism such as nonspecific reaction or inhibition simply due to binding. The lack of activity of 4 (no copper) in replicon assays contrasts with a previous report of inhibition of translation in vivo by the LaR2C peptide. The HCV cellular replicon assays used herein were the standard assays accepted by the FDA whereas the previous studies were performed using a simpler, less robust assay. The differences reflect the differences between these assays and the lack of activity of free peptide in the more robust assay reflects the complexity of analyzing these systems and the importance of using the more established assay for testing efficacy. This validates 4-Cu and 5-Cu as potential treatments for HCV infection and further demonstrates the general applicability of the catalytic metallodrug approach to drug design.

The metallopeptides described herein expand the available pool of potential catalytic metallodrugs for the treatment of HCV infection. Previous work with Cu-GGHYrFK-amide demonstrated its effectiveness in combination with rIFNα-2b. It may be that the most effective way to administer these compounds is as a mixture targeting different stem loops. The novel mechanism of action of these compounds also makes them ideal for use in combination with current treatments. They represent only the first generation of catalytic metallodrugs targeting HCV infection and have great potential for further optimization by variation of the different components of the metallodrug. This may
require the optimization of parameters different from those used to screen and optimize traditional drugs.

5.5 Conclusions

Demonstration of binding to, and reactivity with, HCV IRES SLIIb by these peptides based on the human La protein provides several more examples to explore the design and optimization of catalytic metallo drugs targeting SLIIb. A summary of the overall data is presented in Table 5.7. In terms of the *in vitro* kinetics, the highest activity is observed for metallopeptides containing the YrFK-amide targeting domain. Modifying the domain or changing it to a different domain that still binds SLIIb results in a reduction of activity. As discussed in chapters 3 and 4, one question that still remains is how to best optimize this class of compounds. As shown in Figure 5.15, enhancement of binding has a variable effect on the overall activity. The best catalysts do correspond to those with the lowest $K_d$ values but the graph quickly levels off, possibly due to a tradeoff between target binding and product release. Future work should probe this in more detail but also look for ways to enhance catalyst release and the intrinsic reactivity of the metal binding domain itself. These trends, however, do not extend to the activity in HCV cellular replicon assays. This is a reflection of the complex combination of factors that determine the activity of a catalytic metallo drug, particularly *in vivo*.
Compounds targeting the HCV IRES represent some of the most promising catalytic metallodrugs to date. They demonstrate the potential of this approach and future work will look at the mechanism and optimization in more detail. These systems will ultimately need to be tested and optimized in animal models to look at not only efficacy but toxicity, stability, excretion, and distribution.

**Table 5.7** Overall summary of binding, kinetics, and *in vivo* activity for peptides targeting HCV IRES SLIIb.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_d ) (µM)*</th>
<th>( K_m ) (µM)</th>
<th>( k_{cat} ) (min(^{-1}))</th>
<th>( k_{cat}/K_m ) (µM(^{-1})min(^{-1}))</th>
<th>IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Cu, Cu-GGHYrFK-amide</td>
<td>0.044</td>
<td>0.85</td>
<td>0.53</td>
<td>0.62</td>
<td>0.58</td>
</tr>
<tr>
<td>2-Cu (D-amino acid form)</td>
<td>11.3</td>
<td>7.9</td>
<td>0.14</td>
<td>0.018</td>
<td>1.92</td>
</tr>
<tr>
<td>3-Cu (all L-amino acid form)</td>
<td>19.8</td>
<td>6.87</td>
<td>0.17</td>
<td>0.025</td>
<td>1.08</td>
</tr>
<tr>
<td>4-Cu, Cu-GGH-LaR2C-amide</td>
<td>4.72</td>
<td>20.1</td>
<td>0.14</td>
<td>0.007</td>
<td>0.75</td>
</tr>
<tr>
<td>5-Cu (truncated form of 4-Cu)</td>
<td>4.33</td>
<td>10.1</td>
<td>0.14</td>
<td>0.014</td>
<td>2.17</td>
</tr>
</tbody>
</table>

*\( K_d \) values shown are for metal free peptide.*

**Figure 5.15** Variation of catalytic efficiency \( (k_{cat}/K_m) \) with dissociation constant.
References


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