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Analysis of Structure-Activity Relationships Based on the HCV SLIIB IRES RNA-Targeting GGHYRFK-Cu Complex

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Abstract

New therapeutics for the targeting of the hepatitis C virus have been released in recent years. Although these therapies are less prone to resistance, they are still administered in cocktails as a combination of drugs targeting various aspects of the viral life cycle. Herein, we aim to contribute to an arsenal of new HCV therapeutics by targeting HCV internal ribosomal entry sequence (IRES) RNA via development of catalytic metallodrugs that function to degrade rather than inhibit the target molecule. Based on a previously characterized HCV IRES stem-loop IIb RNA-targeting metallopeptide Cu-GGHYrFK (1-Cu), an all *L*-analogue (3-Cu) and a series of additional complexes with single alanine substitutions in the targeting domain were prepared and screened to determine the influence each amino acid side-chain on RNA localization and recognition, and catalytic reactivity toward the RNA. Additional substitutions of the tyrosine position of complex 3-Cu were also investigated. Good agreement of calculated and measured binding affinities provided support for *in silico* modeling of the SLIIB RNA binding site and correlations with RNA cleavage sites. Examination of cleavage productions from reaction of the Cu-complexes with the SLIIB provided mechanistic insights with the first observation of the 5'-geminal diol and 5'-phosphopropenal as products through use of a Cu-ATCUN catalytic motif. Together, the data yielded insights on structure-function relationships that will guide future optimization efforts.

TOC image

Based on a previously characterized HCV IRES stem-loop IIb RNA-targeting metallopeptide Cu-GGHYrFK, a series of substitution derivatives of the targeting domain (YrFK) have been

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Supplementary Information.

MALDI-TOF peak assignments for each time point for reactions of 3-Cu through 8-Cu with SLIIB in the presence of co-reagents, first order exponential fits, initial fits of distinct reaction products, gels, and illustrative figures are available as supplementary information.

characterized for binding and reactivity toward SLIIb RNA, and structurally modeled to yield insights on structure-function relationships that will guide future optimization efforts.

Keywords

HCV; IRES; ATCUN motif; RNA cleavage; copper

INTRODUCTION

According to estimates from the Centers for Disease Control, 30,500 cases of acute HCV were reported in 2014 for the United States alone, with an estimated 2.7-3.9 million individuals living with chronic HCV and approximately 75-85% of the acute cases expected to develop into chronic HCV. Recent advances in HCV drug development have resulted in therapeutics with high activity and minimal side effects in the form of sofosbuvir and ledipasvir, marketed as Sovaldi® (sofosbuvir) and Harvoni® (a mixture of sofosbuvir and ledipasvir), with a combined total revenue exceeding \$12 billion in 2014 and \$19 billion in 2015.^[1] These two drugs target RNA polymerase and phosphoprotein NS5A, respectively,^[1] both of which are involved in viral replication. In 2016, Epcalsa® (a mixture of sofosbuvir and velpatasvir) was released, which enable treatment for further genotypes via the alternative NS5A inhibitor Velpatasvir.^[1] These current therapies are far superior to prior treatments that used non-specific ribavirin and interferon drugs^[2] that had numerous side effects and resulted in problems with patient compliance. However these drugs, together with newer protease-targeting drugs, are facing challenges in equitable usage as a result of the high cost of treatment.^[3] Moreover, while such drugs appear less prone to resistance development than older therapies, there is an ongoing need to identify new drugs, drug targets, and treatment options, to fuel the multi-drug cocktail mixtures that currently underpin most viral treatment regimens. This cocktail approach overwhelms the virus and prevents it from forming resistant mutants.

Motivated by a desire to add to the arsenal of new HCV therapeutics, we have explored the internal ribosomal entry sequence (IRES) RNA as a novel drug target, and the development of new families of catalytic metallodrugs that function via a novel mode of action.^[4] In principle, such a pathway allows one catalytic molecule to irreversibly destroy or inactivate multiple therapeutic targets. The design and scope of such a strategy has been described in recent publications,^[4b-d, 5] including preliminary studies of HCV-targeting molecules.^[4b, 4d, 6] Such metallodrugs utilize the ATCUN (GGH) motif that has proven to be particularly useful as a high-affinity metal chelate that promotes facile formation of reactive oxygen species (ROS) that remain closely associated with the metal and the target molecule

(in contrast to Fenton-like formation of diffusible ROS). Combining the catalytic ATCUN motif with a recognition domain has yielded a diverse array of potential catalytic metallodrugs as antimicrobial agents,^[7] cancer therapeutics,^[8] cardiovascular disease,^[9] and applications to saccharide targets.^[10]

Previously we have reported the results of catalytic and antiviral activity of a variety of complexes that target HCV IRES RNA. Some have demonstrated activity against stem-loop IV IRES RNA by use of a specific RNA targeting peptide,^[6] while two additional complexes, GGHYrFK-Cu (1-Cu) and GGHyrfk-Cu (2-Cu) were shown to target stem loop IIb (SLIIb) RNA.^[4d] Both of these complexes displayed activity in an FDA-approved cellular replicon assay, with complex 1-Cu displaying an additive or synergistic effect towards HCV when dosed with interferon- α ,^[4b] while the 2-Cu complex demonstrated the importance of stereochemistry in controlling the reactivity and recognition of metallopeptides toward target HCV RNA. In this paper, we sought to systematically investigate the role of each amino acid in the targeting domain (YrFK) on binding, kinetic reactivity, and reaction mechanism, to provide insights for future optimization efforts.

RESULTS and DISCUSSION

GGHYrFK (1-Cu) and its derivative GGHyrfk (2-Cu) have been previously characterized for their ability to bind and cleave stem loop IIb (SLIIb) of the HCV IRES RNA. Herein, we have synthesized additional peptides in order to analyze and understand the contributions of each residue in the YrFK targeting domain to binding and reactivity, and elucidate potential avenues for optimization of the peptide. The peptides are listed in Table 1. Peptide 3 analyzes a stereochemical effect by replacement of the D-Arg with the L-conformer. Peptides 4-7 maintain the all L-configuration but utilize replacement of each individual residue with alanine to examine the contribution from each amino acid. Peptide 8 has the targeting domain inverted to analyze the impact of directionality. Finally, peptides 9-12 explore the importance of the tyrosine residue at position 4, since replacement with alanine was not as pronounced at this position as it was for the other residues.

Metallopeptide Binding to Structured Stem-Loop IIb RNA

It has been shown that the isolated stem loop IIb (SLIIb) domain of HCV IRES RNA maintains the structure adopted in the full length sequence and is a useful probe for *in vitro* assays.^[11] Binding affinities for metallopeptides 1-Cu and 2-Cu were previously measured from changes in Tyr emission following RNA binding. Because some of the peptides in this study lacked Tyr, a different and more sensitive approach was used for the determination of binding constants. Specifically, the conformational changes in SLIIb RNA associated with the binding of copper complexes were monitored by circular dichroism (CD) to yield binding constants. CD is a sensitive technique because the RNA possesses a distinct spectrum in the near UV region (200 – 300 nm) demonstrating that the RNA adopts an A-form conformation, with a strong positive band at 266 nm, a strong negative band at 208 nm and a weaker negative band at ~ 240 nm (data not shown). A moderate decrease in the ellipticity of the 266 nm band was observed for all complexes following titration with the Cu-complexes, most likely reflecting a disruption of base-stacking interactions. Dissociation

constants for metallopeptides (excluding the metal chelate motif, 13-Cu) were observed in the range from < 10 to 980 nM (Table 1) determined by monitoring the change in the CD signal at two different wavelengths. A representative binding curve is shown in Figure 1 (the remainder are available as Figure SM1 in the supplementary). The binding constants determined in this manner are comparable to those obtained by use of a fluorescence-based tyrosine emission assay,^[4b] and the previously reported value of 44 nM determined for the free peptide alone (GGHYrFK) agrees with the value reported in this study (Table 1).

The parent compound in this report, 3-Cu, has a binding affinity of the same order of magnitude as the previously reported, 1-Cu (73 ± 8 nM compared to 19 ± 1 nM).^[4b, 4d] The decrease in binding affinity reflects the change in stereochemistry in the arginine residue from the D-configuration (1-Cu) to L-configuration (3-Cu), demonstrating the stereochemistry of this residue to be important for recognition. Following examination of the stereochemistry of the residue at position 5, the role of charge and size of the fourth position amino acid was investigated with complexes 4-Cu and 9-Cu through 12-Cu. Substitution with a positively charged residue (11-Cu) yielded a binding affinity was found to be close to that of the parent compound (69 ± 12 nM), despite the amino acid being in the L-configuration. Alternatively, changing the tyrosine to a negatively-charged aspartic acid leads to a decrease in binding affinity (485 ± 12 nM for 9-Cu), most likely due to electrostatic repulsion with the negatively charged RNA backbone. Of more interest was the impact of changing the tyrosine to either alanine (4-Cu), asparagine (10-Cu), or phenylalanine (12-Cu). In the case of 4-Cu, the binding constant did not change considerably from 3-Cu (94 ± 17 nM). However, 10-Cu and 12-Cu show a dramatic decrease in affinity (980 ± 25 nM and 530 ± 50 nM, respectively). These trends in binding affinity indicate that the fourth position is most likely involved in H-bonding, such that the larger non-charged sidechains of asparagine and phenylalanine cause steric inhibition, while the alanine derivative (4-Cu) is small enough to avoid interference with binding.

Additional determinants of complex binding can be observed when comparing the series 3-Cu through 8-Cu. Both 4-Cu and 6-Cu represent changes from aromatic residues to alanine, relative to the parent compound 3-Cu, and show a negligible and a slightly favorable increase in binding affinity (94 ± 17 nM and <10 nM, respectively). The increase in affinity associated with 6-Cu is the result of steric strand relaxation. Complexes 5-Cu and 7-Cu represent a change from positively charged residues, arginine (5-Cu) and lysine (7-Cu) to alanine. Complex 7-Cu did not electrostatic contact, while complex 5-Cu showed the greater change in binding affinity (189 ± 54 nM). Given this change in affinity, the arginine residue is either involved in interacting with the phosphate backbone or in π -cation interaction with a nucleotide base. Finally, 8-Cu which contains the targeting domain in the reverse order, showed comparable binding to 3-Cu (38 ± 2 nM), suggesting that this compound could bind in the same fashion as 3-Cu without disrupting contacts with the RNA, but with the metal binding domain on the opposite side of the complex.

In Silico Docking and Inhibition Constants

Since all of the examined peptides demonstrated some measure of binding to SLIIB RNA, we investigated the possible binding sites by computational methods. Previously we have

reported the geometric optimization and simulated docking of 1-Cu and 2-Cu to SLIIb.^[4d] This process was carried out for the copper complexes 3-Cu through 12-Cu, and the inhibition constants were calculated by taking the weighted average of the K_I 's of the first structure of the lowest energy clusters to cover the majority of the 200 docked structures (Table SM5). This K_I is compared to the K_D determined from the CD experiments (Table 1). Since the K_D represents the total sum of microstates of the bound- versus the unbound-form of the SLIIb RNA, it is not surprising that the weighted K_I 's obtained are similar to the experimentally determined K_D values. Further, weighted averages were used to calculate binding energies, non-electrostatic and electrostatic contributions (Table 2).

Figure 2 shows the docking of the top clusters with respect to the structure of SLIIb. The excellent correlation with experimental binding constants suggests that these models provide useful structural information for the complex with the target RNA, and in particular the location of the metal binding domain and the mode of complex binding.

Reactivity of Metallopeptides Toward Target Stem-Loop IIb RNA

In earlier studies we have characterized the reactivity of the Cu-ATCUN motif as deriving from formation of metal-associated reactive oxygen species via transient formation of Cu(III).^[4d, 12] The hard ligand environment and square planar geometry does not support Cu(I). Formation of Cu(III) is attributed to Fenton-type chemistry with H_2O_2 from the ATCUN-bound Cu(II) where ascorbate serves as an efficient one-electron donor to reduce Cu(III) to Cu(II). While ascorbate/ O_2 can promote formation of peroxide, reactivity is improved, as is ease of experimentation, by the direct use of peroxide, essentially shunting the reaction forward.^[9b, 13]

The reactivity of the metal complexes under such oxidative conditions was determined by following the degradation of 5'-fluorescein-labeled SLIIb via time-dependent fluorescence spectrophotometry, as previously described for complex 1-Cu.^[4b] Initial velocities were plotted as a function of catalyst concentration to generate a pseudo-Michaelis–Menten plot to generate K_M and k_{cat} values (Figure 3) (additional data for the remaining complexes is available in the supplemental material as Figure SM2). Table 3 summarizes the results. The traces and values for the turnover values of 3-Cu, 4-Cu, 6-Cu, and 7-Cu are available in Figure SM3. On average, the complexes displayed turnover numbers in the range of 30-60 and agree with the previously reported values for 1-Cu^[4b] and 2-Cu,^[4d] implying that the system is limited by the Cu-GGH catalyst and not the binding domain.

Although all of the compounds illustrate similar turnover values, trends were observed for the other pseudo-Michaelis–Menten parameters. Conversion of the D-arginine in 1-Cu to the L-arginine for 3-Cu results in a ~400 fold decrease in catalytic efficiency (k_{cat}/K_M), due to a combination of a decrease in the k_{cat} by ~6 fold as well as an increase in K_M by ~64 fold (Table 3). This decrease in efficiency is surprising, given the relative dissociation constants determined for these compounds (19 nM and 72 nM for 1-Cu and 3-Cu, respectively, Table 1). Focusing on the Tyr, with 3-Cu there is a steric clash between the tyrosine and arginine, which can be relieved by changing the tyrosine to alanine (4-Cu) to restore the k_{cat} to that of 1-Cu. The K_M decreases and the k_{cat}/K_M increases from 1.4×10^{-3} to $38 \times 10^{-3} \mu M^{-1} \text{ min}^{-1}$ (Table 3). Complex 4-Cu also has the largest k_{cat}/K_M for the amino acids examined at the

fourth position, despite having the smallest sidechain. Analysis of all of the residues varied at this site demonstrates that the smaller the sidechain at this position, the larger the corresponding k_{cat}/K_M (Table 3).

Comparison of the alanine substitution data from other residue positions (3-Cu through 7-Cu) shows an increase in k_{cat} for the positively-charged residues of arginine (5-Cu) and lysine (7-Cu). Although the k_{cat} 's for these are relatively close (Table 3), the K_M for the 7-Cu is approximately two-fold better than 5-Cu: $36 \pm 2 \mu\text{M}$ and $78 \pm 7 \mu\text{M}$, respectively, which is consistent with the experimental binding affinities of $56.6 \pm 4.6 \text{ nM}$ and $189.2 \pm 54.2 \text{ nM}$ for 5-Cu and 7-Cu, respectively.

Likewise, alanine substitution of the phenylalanine at position 6 has a pronounced influence on the catalytic properties for 6-Cu compared to the parent compound 3-Cu. The K_M for the complex is now greater than $100 \mu\text{M}$ (greater than 2 fold for 3-Cu) and the catalytic efficiency is the lowest of all the complexes screened (Table 3), indicating that the phenylalanine is an important residue for recognition of SLIIB. For all of the compounds tested, K_M is larger than the K_D , which indicates that the catalytic rate constant is significant relative to the release rate constant for the peptide, and is consistent with a system in which the compound exhibits tight binding to the target.

Reactivity was also measured by an alternative method, in which fluorescein-labeled SLIIB was monitored via a 4% agarose gel. The concentrations of the Cu-complexes were Gel data for all reactions is summarized in Figure SM4, which yielded time-dependence plots such as shown in Figures 4 and SM5. Comparison of the initial rates in Table 4 reveals that complex 4-Cu has the fastest initial rate followed by 8-Cu, 6-Cu, and 5-Cu. There is a general correlation between K_D and the initial rate of reactivity. The outliers were 10-Cu and 11-Cu. However, in the case of 10-Cu, only ~ 33% of the RNA was consumed, relative to ~ 100% for the other cases, while the addition of 11-Cu to SLIIB resulted in significant precipitation from solution.

Mass Spectrometric Studies as a Probe of Reactivity, Site Selectivity, and Mechanism

Since all of the peptides were reactive towards SLIIB RNA, we sought to determine the relative sites of reactivity and understand the mechanism of action. As previously reported for 1-Cu and 2-Cu,^[4b, 4d] time-dependent reactivity studies were performed by MALDI-TOF mass spectrometry. The mass spectra for each time point was screened by use of the script Mass Daddy^[9b] for possible products based upon previously reported work with DNA (the mass list is available in the supplemental material as Table SM1). The output of this data is available in the supplemental material (Table SM3 through SM8 and Figure SM6 through SM11). This data was filtered to ensure the product appeared across the time range and most importantly showed a time-dependence in the peak intensity. A summary of reactivity with overlap of the predicted Cu positions from Figure 2 is available in Figure 5 and the individual initial cleavage rates from the mass spectrometric analysis of each position are also summarized in Table 5.

Comparison of the mass spectral results with the calculated binding model allows an additional level of scrutiny and also provides insights into the binding motif (cluster) that is

most responsible for reactivity. In general the reactivity of the copper complexes corresponds well with the proposed thermodynamically favorable binding sites. In the case of 6-Cu and 8-Cu, it is likely that a less thermodynamically favorable, but more catalytically active site is reflected in the reactivity data, a phenomenon previously documented for the parent complex 1-Cu,^[4d] consistent with the substantially lower affinity and reactivity ($K_M \sim 335 \mu M$, Table 3) displayed by 8-Cu. We have previously reported in detail on possible mechanisms and sites of cleavage for both, 1-Cu and 2-Cu complexes.^[4d] For 3-Cu and 4-Cu, the majority of the reactivity is centered toward the top of the SLIIb motif. It is worth noting that the second and third clusters also have a larger population than observed for the first cluster for 3-Cu (Figure 2 and Table SM2).

Although the majority of the reactivity is directed towards the top of SLIIb, a shift in the predicted binding and reactivity towards the middle bulge of SLIIb RNA was observed for 4-Cu. The increase in reactivity associated with 4-Cu and 3-Cu is the result of a more clearly defined binding site associated with cluster 2, compared to 3-Cu, which had three clusters with similar populations. This better populated binding site allows greater accumulation of products localized to a narrower region of the RNA, relative to 3-Cu.

In addition to determining sites of reactivity, the mass spectrometric analysis also allowed for characterization of the RNA cleavage products following reaction with the Cu-complexes. With high throughput accessibility by use of an expected product list, in combination with Mass Daddy, the relative abundance of each overhang could be determined. A summation of the 3' predicted products (2', 3'-cyclic phosphates, 3'-phosphates, 3'-hydroxyl, 3'-phosphoglycaldehyde, 3'-phosphoglycolate, 3'-aldehyde) as well as the 5' predicted products (5'-hydroxyl, 5'-aldehyde, 5'-diol, 5'-phosphate, and 5'-phosphopropenal) was performed at each time point and was normalized over each overhang position to determine the fraction of that overhang for each time point. Figure 6 shows the percent of each 3' product overhang at a particular time point, whereas Figure 7 shows the respective percent of 5' products. The complete summary of the possible mechanism of action for all the complexes is summarized in Table 6. Furthermore, relative initial rates for the different overhangs obtained from time-dependence experiments monitored by MALDI-TOF mass spectrometry are summarized in Table 7 for complexes 3-Cu through 8-Cu.

As seen in Figures 6 and 7, each of the various overhangs are observed and all products are known to arise through a combination of hydrolytic and H'-abstraction, primarily at the 3', 4' and 5'-H sites, as shown for 1-Cu and 2-Cu.^[4d] 3'-H abstraction yields 3'-phosphoglycaldehydes (3'-PGA) and 5'-base phosphopropene, which can degrade into 5'-phosphates (5'-PO₄) and base propenates (Figure SM 12). Alternatively, 3'-aldehydes and 5'-PO₄'s can also derive from 3'-H abstraction (Figure SM 14). Following 4'-H abstraction, 3'-phosphoglycolate (3'-PG) and 5'-phosphopropenal (5'-PPA) can form (Figure SM 13). This 5'-PPA product can then yield 5'-PO₄'s and 3'-hydroxypropenal. Finally, 5'-H abstraction may lead to 5'-aldehyde (5'-AL), which can form a 5'-geminal diol along with a 3'-PO₄ if it occurs through an intramolecular mechanism. However, if it occurs through an intermolecular process then the 5'-geminal diol forms without the 5'-AL (Figure SM 15).

CONCLUSIONS

A series of complexes were synthesized based upon the reported complexes 1-Cu and 2-Cu and characterized for binding and reactivity towards HCV SLIIB IRES RNA. Complexes 3-Cu through 12-Cu were shown to have decreased catalytic efficiency compared to the previously reported complexes 1-Cu and 2-Cu, which primarily stemmed from a decrease in K_M .

Reaction sites and mechanisms underlying the reactivity of complexes 3-Cu through 8-Cu were evaluated by time-dependent MALDI-TOF MS, and primary products were observed to arise from a hydrogen abstraction pathway. Products detected included 3'-phosphoglycolates, 3'-phosphoglycolaldehydes, 3'-aldehydes, 5'-aldehyde, 5'-geminal diols and 5'-phosphopropenals. This is the first time that the 5'-geminal diol and 5'-phosphopropenal have been reported as products using a Cu-ATCUN motif as the catalyst. Although 3-Cu and 4-Cu did not demonstrate tight binding to the level of the parent compound, 1-Cu, they were the most similar across all forms of characterization to 1-Cu, confirming that the stereochemistry at position 5 and the size and hydrogen bonding ability of the residue at position 4 do contribute to binding, and did not show a loss of reactivity (relative to 1-Cu) as observed for the other sites in the targeting domain. Additionally, the sites of reactivity displayed good agreement with the docking locations from *in silico* simulation, suggesting these structural models to be a valid indicator of metalloprotein binding to the target RNA.

EXPERIMENTAL SECTION

Peptide Synthesis and complex formation

Peptides were synthesized by standard solid phase peptide synthesis utilizing Fmoc chemistry on a PS3 synthesizer (Protein Technologies). All peptides were purified by HPLC using reverse-phase Axia packed C18 Gemini 5 μm 100 $\times$ 21.20 mm column (Phenomenex) and characterized by ESI-MS. A gradient of 2%/min from water with 0.1% TFA (100%) to acetonitrile with 0.1% TFA (100%) was employed. Product was determined by ESI-MS and stock of peptides were determined by either tyrosine determination ($\epsilon_{270\text{nm}} = 1400 \text{ M}^{-1} \text{ cm}^{-1}$) and/or serial titration with a standardized CuCl_2 solution monitoring at 250 nm and 520 nm. Complexes were made with a 1.1:1 ratio of peptide to copper solution.

Binding Constant Determination

For the complexes, RNA binding experiments were performed in the presence of 2.5 μM SLIIB in 20 mM phosphate buffer (pH 7.4) containing 100 mM NaCl. Serial aliquots of the peptides were added and the CD signal was monitored at selected wavelengths of 263 nm, another in the range 230-240 nm, and at 283 nm. Data was fit to a one-site binding model using Origin software.

$$F = \frac{F_0 + \left((KD + R_0 + P_0 - \left(((KD + R_0 + P_0)^2 - (4 \times R_0 \times P_0))^{\frac{1}{2}} \right) \right)}{2(R_0 \times (F_1 - F_0) + m \times P_0)} \quad (1)$$

A quadratic one-site binding equation, equation (1), was used, which is based upon two lines intersecting. F0 is the y-intercept of the first line and F1 is the y-intercept of the second line. KD and R0 correspond to the dissociation constant and inflection point. To account for any additional phases (a non-zero slope of the second line) an m term, or slope, is used to define this. P0 is the independent variable in concentration.

Complex Optimization

The peptide structures were optimized with Gaussian 09 vA01* using an Amber MM/MD force field in an aqueous solvent, followed by a more thorough refinement with a DFT/B3LYP/3-21G force field.^[15] Solvent interactions, water in this case, were considered by using the universal solvation model, SMD, by Truhlar and co-workers.^[16] The structures of the metal complexes were optimized by attaching the crystallographically determined structure for CuGGH^[17] to the peptide and submitting the complexes using the above-mentioned force field and solvation model. The charge for the complex structures was calculated based on the overall charge of the complex. For example, for the complex (3-Cu), the charge was set to +3 with a multiplicity of 1, because this represents the overall charge of the complex (Arg, Lys, and the N terminus each contribute +1, the copper contributes +2, and the two deprotonated backbone amides each have a -1 charge). The Cu-GGH atoms in the complexes were held fixed during the geometry optimization of the complexes by freezing the atoms in Gaussian 09.

In Silico Complex Docking

The solution state NMR structure of stem loop IIb (SLIIb) of the HCV IRES RNA is readily available from the protein data bank (PDB: 1P5N). The top solution state NMR structure (out of the 20 available) was used for docking simulations using AutoDock 4.2. The entire SLIIb domain was considered for docking. In all cases, the copper complex was made flexible except for aromatic carbons, peptide bonds, and the metal binding domain (restricted to a square planar configuration^[17]). The Lamarckian Genetic Algorithm allows a large degree of flexibility in the selection of the initial starting position: essentially searching a local area, identifying the thermodynamically most favorable orientation, and then “mutating” to a different spot on the RNA. Comparison of the docking in this manner provides a measure of how readily the complex can be positioned in a manner to perform effective chemistry.

Autodock does not have parameters for either Cu or Ni, and so Fe, which is of similar atomic radius and charge, is used as a substitute, but requires a change of charge to +0.8 in order to compensate for the tendency of Autodock to overestimate electrostatic interactions.^[18] We have previously demonstrated this approach.^[4d] a Lamarckian Genetic Algorithm and the following parameters were used for docking: population size of 150, a random starting position and conformation, a maximal mutation of 2 Å in translation and 50° in rotations (elitism of 5), iterations of Solis and Wets local search of 300, torsional degrees of freedom of 23 for the peptide complex, an external grid energy of 1000 kcal/mol, a mutation rate of 0.02 and a crossover rate of 0.8, and local search rate of 0.06. Simulations were performed with a maximum of 10⁷ energy evaluations and a maximum of 27000 generations. The total number of hybrid GA-LS runs was set to 200. Docking analyses were

performed on the UNIX system at the Ohio Supercomputer Center using the OAKLEY cluster platform.

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 491 nm and 518 nm, respectively. Reactions were carried out at 25 °C in reaction volumes of 150 µL in the presence of 1 mM ascorbic acid and 1 mM H₂O₂ in 20 mM HEPES buffer (pH = 7.4, 100 mM NaCl) with 333 nM 5'-fluorescein labeled HCV SLIib and analyzed according to the change in fluorescence observed as the reaction occurred. A calibration curve of concentration of SLIib versus fluorescence intensity was constructed to convert the RFI to intensity. Both a time-dependence and a concentration-dependence of catalyst activity were observed. The initial velocity of the time dependence plot was used to generate the pseudo Michaelis-Menten plots which were then fit to the Michaelis-Menten equation. All fits were performed using Origin software. The values shown are an average of at least three trials. A turnover number was determined based on the limiting amount of complex and determining the amount consumed by a specific amount of RNA by measuring the change in intensity at 518 nm.

Reaction Rates Monitored by Gel Electrophoresis

Reactions were carried out in 10 µL containing 1 µM FI-SLIib and 1 µM of the appropriate copper peptide complex in addition to the co-reagents of 1 mM ascorbate and 1 mM hydrogen peroxide. Reactions were staggered to have them end at the same time. Time points were 180, 120, 90, 60, 40, 20, and 10 min. A control lane of 1 µM FI-SLIib was also included. Five microliters of 20% sterile glycerol was added to each reaction before 12 µL of the reaction mixture was loaded onto a 4% agarose gel. The gel was run at 70 mV for approximately 20 min and bands subsequently quantified by use of a Gel Doc (Bio-Rad) in UV light mode and Quantity One software. Data was then fit by use of Origin software to an exponential decay function to yield kinetic parameters.

MALDI-TOF Mass Spectrometry

Reactions for MALDI-TOF analysis were run as described above, but using 10 µM fluorescein-labeled IRES SLIib and 10 µM copper-peptide incubated for up to 180 min. Reactions were then quenched by being placed on ice and Zip Tipped. Zip Tip was performed using C₁₈ Zip Tips from Millipore Co. in order to desalt the reaction mixtures prior to mass spectrometric analysis. Zip Tips 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 Zip Tip, 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 a matrix solution containing 0.3 M 4-hydroxypicolinic acid (HPA) and 30 mM ammonium citrate in 30% acetonitrile, drying, spotting with 1 µL of a 2:1 RNA:matrix mixture, and allowed to dry. A calibration mixture containing 4 RNAs covering a range of molecular weights, namely (GU)₃, (GU)₉, (GU)₁₄, (GU)₂₀, with molecular weights of 1,892.1, 5,800.4, 9,057.3, 12,965.6 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. Typically, at least 1000 shots were summed per spectrum to acquire an accurate representation of the reaction. Data analysis was performed using Bruker flexAnalysis software. Assignment of peaks was performed by comparison of each peak list with the expected masses for possible cleavage products (Table SM1). Only m/z values > 1500 amu and those with a signal to noise ratio greater than five were considered, since excessive spectral crowding occurred at lower m/z ranges. Time dependence was used as a determination of peak validity.

Reactions for the time dependent assay were collected at: 2, 10, 20, 40, 60, 90, 120, and 180 min. Heat maps and initial rates were generated by first summing the total change in intensity at a position within the RNA sequence and determining the fraction of associated change at each time point. An apparent initial rate was then determined from the linear region of the data (first ~40 min).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Binding affinity of **3**-Cu to 2.5 μ M SLIIb. Additional binding curves are available in the supplementary material as Figure SM1.

Figure 2.

Positions of the top three copper atoms in the representative clusters for complexes 3-Cu through 12-Cu (spheres). The spheres represent the thermodynamically favored sites in decreasing order of (red, green, blue), where the red spheres are more thermodynamically favored compared to blue. 3-Cu (red) cluster 1, 46 structures; (green) cluster 2, 65 structures; (blue) cluster 3, 55 structures. 4-Cu (red) cluster 1, 29 structures; (green) cluster 2, 63 structures; (blue) cluster 3, 15 structures; (orange) cluster 4, 57 structures. 5-Cu (red) cluster 1, 69 structures; (green) cluster 2, 67 structures. 6-Cu (red) cluster 1, 80 structures; (green) cluster 2, 62 structures. 7-Cu (red) cluster 1, 35 structures; (green) cluster 2, 90 structures; (blue) cluster 3, 25 structures. 8-Cu (red) cluster 1, 104 structures; (green) cluster 2, 57 structures. 9-Cu (red) cluster 1, 15 structures; (green) cluster 2, 27 structures; (blue) cluster 3, 45 structures. 10-Cu (red) cluster 1, 14 structures; (green) cluster 2, 74 structures; (blue) cluster 4, 57 structures. 11-Cu (red) cluster 1, 102 structures; (green) cluster 2, 34 structures; (blue) cluster 3, 13 structures. 12-Cu (red) cluster 1, 44 structures; (green) cluster 2, 91 structures; (blue) cluster 3, 13 structures. These spheres appear also in Figure 5, but with revised colors (red = white, green = grey, and blue = black) to avoid conflict with the reactivity data.

Figure 3.

Concentration of 3-Cu versus initial rate of cleavage with 333 μM SLIIB, 1 mM ascorbic acid and 1 mM hydrogen peroxide at pH 7.4. Error bars represent the standard deviation of at least three trials.

Figure 4. Consumption of 5'-fluorescein-labelled SLIIB mediated by 3-Cu, showing the time-dependent loss of SLIIB RNA by use of $1\ \mu\text{M}$ 3-Cu and starting with $1\ \mu\text{M}$ 5'-fluorescein-labelled SLIIB and 1 mM co-reagents. Additional plots of the remaining complexes are available in a supplemental Figure SM5.

Figure 5.

Heat maps for complexes 3-Cu through 8-Cu that depict the relative initial rate per minute at each position in relation to the simulated docking of Cu atoms from the copper complexes (white to black spheres), previously defined in Figure 2. The spheres represent the thermodynamically favored sites in decreasing order of (white, grey, black; refer to the legend to Figure 2), where the white spheres are more thermodynamically favored compared to the black. The reactivity scale is as follows: red > 3.0, orange 2.5 – 3.0, yellow 2.0 – 2.5, green 1.5 – 2.0, blue 1.0 – 1.5, purple 0.5 – 1.0. A numerical representation is available in Table 5.

Figure 6.

Relative percentages observed for 3'-overhangs (left to right: 3'-hydroxyl (3'-OH), 2',3'-cyclic phosphates (2',3'-cPO4), 3'-phosphates (3'-PO4), 3'-phosphoglycaldehyde (3'-PGA), 3'-phosphoglycolate (3'-PG), 3'-aldehyde (3'-AL)) with time.

Figure 7. Relative percentages observed for 5'-overhangs (left to right: 5'-hydroxyl (5'-OH), 5'-phosphate (5'-PO₄), 5'-aldehyde (5'-AL), 5'-geminol diol (5'-GemDiol), 5'-phosphopropenal (5'-PPA)).

Table 1

Summary of binding constant for peptides binding SLIIB.

	Complex	K _D (nM) Determined by CD	K _I (nM) Docking Simulation
1	GGHYrFK	66 ± 2 ^a	39.1 ^b
1-Cu	GGHYrFK-Cu	19 ± 1	7.7
2-Cu	GGhyrfk-Cu	76 ± 3 ^c	38
3-Cu	GGHYRfK-Cu	73 ± 8	69
4-Cu	GGHARfK-Cu	94 ± 17	108
5-Cu	GGHYAfK-Cu	189 ± 54	159
6-Cu	GGHYRAK-Cu	<10 ^d	9.4
7-Cu	GGHYRfA-Cu	57 ± 5	61.7
8-Cu	GGHKFRY-Cu	38 ± 2	69.6
9-Cu	GGHDRfK-Cu	485 ± 12	31700
10-Cu	GGHNRfK-Cu	980 ± 25	3363
11-Cu	GGHKRfK-Cu	69 ± 12	46.4
12-Cu	GGHFRfK-Cu	530 ± 50	1048
13-Cu	GGH-Cu	3840 ± 1230	718

^aPreviously reported value of 44 nM determine by fluorescence based tyrosine emission assay for free peptide alone.^[4b] This shows values of this assay are comparable in this study to that of the previously reported values.

^bDocking is for the tetrapeptide YrFK

^cPreviously reported value as determined by fluorescence based tyrosine emission assay.^[4d]

^dA lower limit of 10 nM was used in the regression analysis fitting in Origin.

Table 2

Summary of in silico screening of complexes.

	# of Docked Structures <i>a</i>	K _i (nM)	Binding Energy (kcal/mol)	Intermolecular Energy (kcal/mol)	Non- electrostatic Energy <i>b</i> (kcal/mol)	Electrostatic Energy (kcal/mol)	Total Internal Energy (kcal/ mol)	Torsional Energy (kcal/mol)
1 <i>c</i>	144	39.1	-10.24	-16.81	-9.03	-7.78	-2.63	6.56
1-Cu	137	7.7	-11.15	-18.01	-9.21	-8.80	-1.58	6.86
2 <i>c</i>	173	38	-10.89	-17.75	-9.14	-8.07	-4.81	6.86
3-Cu	166	69.0	-10.36	-17.22	-10.76	-6.45	-4.09	6.86
4-Cu	164	108.5	-9.53	-15.93	-8.60	-7.37	-2.64	6.56
5-Cu	136	159.1	-9.38	-15.05	-9.01	-6.04	-3.20	5.67
6-Cu	142	9.4	-11.11	-17.38	-10.01	-7.37	-1.92	6.26
7-Cu	150	61.7	-10.12	-15.49	-9.94	-5.19	-4.11	5.37
8-Cu	161	69.6	-9.83	-16.69	-9.03	-7.67	-3.80	6.86
9-Cu	107	3.2×10^4	-6.19	-13.65	-10.50	-3.15	-2.48	7.46
10-Cu	156	3.4×10^3	-7.70	-14.85	-9.75	-5.10	-3.20	7.16
11-Cu	149	46.4	-10.17	-18.22	-9.19	-9.02	-3.64	8.05
12-Cu	148	1048	-8.35	-15.51	-9.17	-6.34	-3.94	7.16
13-Cu	138	718	-8.38	-8.68	-4.07	-4.61	-0.53	0.3

*a*This is the total number of docked states in the clusters used out of a maximum total of 200.*b*This represents the sum of van der Waal's, hydrogen bonding, and desolvation energy*c*This is for the tetrapeptide YrFK or yrfk.

Table 3

Pseudo Michaelis-Menten parameters determined by fluorescence.

Cu-Peptide	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\mu\text{M}^{-1} \text{min}^{-1}$)
1-Cu ^[4b] GGHYrFK	0.53 ± 0.02	0.8 ± 0.1	663×10^{-3}
2-Cu ^[4d] GGhyrfk	0.14 ± 0.01	7.9 ± 1.2	18×10^{-3}
3-Cu GGHYRfK	0.08 ± 0.01	55 ± 3	1.4×10^{-3}
4-Cu GGHARFK	0.51 ± 0.03	13 ± 2	38×10^{-3}
5-Cu GGHYAFK	1.4 ± 0.1	78 ± 7	18×10^{-3}
6-Cu GGHYRAK	0.11 ± 0.01	117 ± 3	1×10^{-3}
7-Cu GGHYRFA	0.9 ± 0.1	36 ± 2	25×10^{-3}
8-Cu GGHKFRY	2.3 ± 0.1	335 ± 17	7×10^{-3}
9-Cu GGHDRFK	1.5 ± 0.1	45 ± 4	33×10^{-3}
10-Cu GGHNRfK	0.06 ± 0.01	14 ± 2	4×10^{-3}
11-Cu GGHKRfK	0.01 ± 0.01	0.5 ± 0.4	22×10^{-3}
12-Cu GGHFRfK	0.5 ± 0.3	49 ± 2	10×10^{-3}

Table 4

Initial rates as determined by fluorescein-labelled SLIib in agarose gel experiments.

Complex	Initial Rate (nM/min)	% SLIib consumed
3-Cu	24 ± 2	100
4-Cu	135 ± 4	88
5-Cu	28 ± 2	49
6-Cu	42 ± 2	97
7-Cu	20 ± 2	100
8-Cu	95 ± 3	98
9-Cu	14 ± 2	98
10-Cu	25 ± 2	33
11-Cu	4 ± 1	90
12-Cu	8 ± 2	80

Table 5

Truncated summary of initial rates based on MALDI-TOF mass spectrometry for complexes 3-Cu through 8-Cu. Relative initial rates greater than 1 Intensity/min⁻¹ × 1000 listed. A complete list is provided in supplemental material (Table SM 9).

	Relative Initial Rate (Normalized Intensity/min × 1000)						
Position	3-Cu	4-Cu	5-Cu	6-Cu	7-Cu	8-Cu	
C3	.a	.a	.a	.a	1.0 ± 0.3	.a	
G5	.a	.a	<1	.a	.a	3.7 ± 1.8	
A6	.a	.a	.a	1.7 ± 0.5	.a	.a	
G9	1.4 ± 0.3	2.0 ± 0.5	.a	.a	<1	.a	
C10	.a	.a	1.0 ± 0.2	.a	.a	.a	
G11	.a	.a	.a	<1	1.6 ± 0.7	0.6 ± 0.2	
U12	1.2 ± 0.7	.a	<1	.a	<1	.a	
U14	1.2 ± 0.1	.a	.a	<1	.a	.a	
A15	.a	14.2 ± 4.6	.a	<1	.a	.a	
A19	.a	2.9 ± 1.4	1.0 ± 0.7	.a	0.8 ± 0.4	.a	
U25	.a	.a	3.1 ± 0.6	.a	<1	<1	
U26	.a	1.9 ± 0.4	.a	.a	<1	<1	
A27	.a	2.1 ± 0.3	1.1 ± 0.3	.a	.a	1.1 ± 0.3	
G28	1.0 ± 0.5	.a	.a	2.3 ± 1.6	.a	<1	
U29	.a	.a	.a	.a	2.9 ± 0.6	<1	
A30	.a	<1	.a	2.4 ± 0.3	1.0 ± 0.4	<1	
U31	.a	1.4 ± 0.4	.a	.a	.a	.a	
C33	.a	.a	<1	1.4 ± 0.5	.a	.a	

^aNo measurable response.

Table 6

Description of overhangs and possible mechanisms associated with them.

Overhang		mass (amu)	Possible Mechanism ^a
3'-hydroxyl		0.00	Hydrolysis ^b
2',3'-cyclic phosphates		+61.96	Hydrolysis
3'-phosphates		+79.98	Hydrolysis, 1', 2', 3', 4', or 5'-H abstraction ^c
3'-phosphoglycoldehyde		+106.98	3'-H abstraction ^d
3'-phosphoglycolates		+138.02	4'-H abstraction ^d
3'-aldehyde		-2.016	3'-H abstraction ^d
5'-hydroxyl		0.00	Hydrolysis
5'-aldehyde		-2.016	5'-H ^d
5'-geminal diol		+16.00	5'-H, followed by hydration of 5'-aldehyde ^d
5'-phosphates		+79.98	Hydrolysis, 1', 2', 3', 4', or 5'-H abstraction ^c

Overhang		mass (amu)	Possible Mechanism ^a
5'-phosphopropenal		+133.02	4'-H abstraction Intermediate to 5'-PO ₄ ^d

^aProposed mechanisms are based upon the equivalent DNA mechanism.[13–14]

^bThere is a closely related oxidative product resulting for 3'-H abstraction with mass difference of 2 amu which may inflate this value.

^cAlthough 1' through 5' H-abstraction is possible, 4' and 5'-H abstraction are expected to be the most prevalent as these positions are more solvent exposed.

^dProposed mechanisms are shown in supplementary materials (Figures SM 12-15)

Table 7

Relative initial rates for the different overhangs with time on MALDI-TOF mass spectrometry for complexes 3-Cu through 8-Cu.

Overhang	Initial Rate (%Overhang/min)							
	3-Cu	4-Cu	5-Cu	6-Cu	7-Cu	8-Cu		
3'-OH	1.1 ± 0.5	1.2 ± 0.4	1.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.4 ± 0.1		
2',3'-cPO4	0.7 ± 0.3	0.5 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	0.1 ± 0.1	0.1 ± 0.1		
3'-PO4	0.4 ± 0.1	0.1 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.1 ± 0.1	1.3 ± 0.2		
3'-PGA	0.3 ± 0.1	1.1 ± 0.4	0.1 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.3 ± 0.1		
3'-PG	0.2 ± 0.1	1.5 ± 0.4	0.1 ± 0.1	1.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1		
3'-AL	0.8 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.2 ± 0.1		
5'-OH	2.0 ± 0.6	1.1 ± 0.4	0.6 ± 0.1	1.2 ± 0.3	1.1 ± 0.2	1.1 ± 0.4		
5'-PO4	0.4 ± 0.1	0.4 ± 0.1	1.5 ± 0.6	1.2 ± 0.2	0.5 ± 0.2	0.7 ± 0.1		
5'-AL	0.7 ± 0.1	0.2 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.5 ± 0.5	0.9 ± 0.1		
5'-GemDiol	0.3 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.9 ± 0.3	0.2 ± 0.1	0.5 ± 0.1		
5'-PPA	0.3 ± 0.1	1.4 ± 0.3	0.2 ± 0.1	1.9 ± 0.1	0.5 ± 0.1	0.7 ± 0.1		