De novo design of passively permeable cyclic peptides and incorporation of noncanonical amino acids to improve predicted binding affinity

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Abstract

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Cyclic peptides fill an intermediate niche between traditional small molecule therapeutics and larger biologics. In ideal cases they combine the passive permeability and oral availability of small molecules with the binding specificity of larger biologics. However, achieving both of these features in a single designed cyclic peptide has remained elusive, with the majority of clinically approved cyclic peptides being derived from natural products. In order to address this problem my doctoral research focuses on developing new methods for the de novo computational design of cyclic peptides to have improved permeability and binding properties. Collaborators and I designed over 70 cyclic peptides that have apparent passive membrane permeabilities > 1*10^-6 cm/s that are of a broader size and conformational range than have been previously reported. I also worked to expand computational methods for the incorporation

of noncanonical amino acids at cyclic peptide binder interfaces to increase predicted binding affinity.

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1.INTRODUCTION

Over the last fifty years the pharmaceutical industry in the United States has experienced decreasing efficiency of drug discovery as R&D costs rise but the number of new approved drug each year holds steady.[1] This decrease seems to have stabilized and slightly rebounded in the last decade, but the underlying problems still persist.[2] One of the proposed solutions to this problem is improved selection of disease-associated targets, aided by the extensive efforts that have been made to map the human genome and proteome. Despite the identification of many new potentially useful targets, most new drugs are not directed at novel targets.[3] An analysis of clinically relevant drug targets has shown this may be because the majority of these targets are out of reach for traditional small-molecule drugs,[4] those that adhere to the 'rule of five'. The rule of five is a set of principles for the design of orally available small molecules that includes a maximum size of 500-600 Daltons as well as principles of minimum hydrophobicity and maximum hydrogen bond donors and acceptors.[5] These rules make selectively targeting large, hydrophobic, flat or grooved shaped targets, such as those found at protein-protein interfaces, extremely challenging.[6]

One way around this problem is to abandon the rule of five entirely and turn to newer 'biologics', large biologically derived therapeutics such as antibodies.[7] The larger size of biologics allows them to tightly and specifically bind to targets, often resulting in fewer off-target effects than are seen with small molecule therapeutics.[8] Biologics have become a rapidly growing segment of the pharmaceutical market,[9] but they are not without drawbacks. Often biologics suffer from complicated quality control during production, low stability that requires cold chains for distribution, and poor oral bioavailability that necessitates administration via

injection.[7,10] Their general membrane impermeability is especially relevant, as it restricts the types of novel targets biologics can affect to those that are extracellular.

As such, there is growing interest in identifying membrane permeable and orally-available molecules that bridge the gap between traditional small molecules and large biologics.[11] One of the most promising candidates to fill this intermediate space is peptides, which range in size from about 5 to 50 amino acids.[12] Peptides are promising drug leads due to the large structural and chemical space they can access using a set of relatively simple and well characterized building blocks.[13] Most proteins have evolved to interact with other proteins or peptides, so peptide drugs can frequently use natural ligands as leads for binding to targets of interest.[14] This, combined with their increased size relative to small molecules, gives peptide drugs the potential to bind difficult targets with high affinity, leading to higher potency and fewer off-target effects compared to small molecules. Despite these strengths, peptides are not commonly used as therapeutics due to their poor oral availability, membrane permeability, and short plasma half-lives.

Many of the problems with linear peptide therapeutics can be addressed through cyclization, which may be why over half of the beyond-rule-of-five drugs that target flat or grooved interfaces are macrocycles.[14,15] Linear peptides often suffer from poor half-lives because they are readily degraded by plasma proteases. Cyclization has been shown to confer protease resistance and increase serum stability by reducing proteolytic degradation,[16–18] improve membrane permeability,[19,20] and increase binding affinity by providing a degree of structural preorganization that minimizes the entropic cost of receptor binding relative to linear counterparts.[21,22]

Despite the potential for cyclic peptides to help bridge the gap between high affinity biologics and orally available small molecules, achieving that potential has proven difficult. To date, there are only 20 cyclic peptide therapeutics available in the clinic, only five of which were rationally designed and all of which are derived from or based on natural products.[23–25] Several groups have developed library based methods to address this problem by synthesizing and screening large numbers of peptide sequences for desired activity. One class of these methods focus on chemical synthesis, and generally produce libraries that contain thousands of different sequences focused around a motif of interest.[26] The other class of methods utilize natural expression systems, trading flexibility in the inclusion of noncanonical amino acids and exotic cyclization for higher throughput of up to billions of sequences.[27] While library methods have been effective in identifying peptides that bind to therapeutically interesting targets[28,29] and are useful in exploring the features of peptides of interest, they are insufficient to explore the full available sequence space. For example, for a ten-residue cyclic peptide there are over a trillion possible combinations of the canonical twenty amino acids and the number continues to grow when including noncanonical amino acids, chemically conjugated groups, and different cyclization patterns or chemistries. Computational methods can serve as a way to rapidly and inexpensively sample this vast conformational space to guide libraries and design efforts.[30]

Our lab has recently reported the extension of Rosetta protein structure prediction and design methods to peptide macrocycles.[31,32] These methods have had some success in being extended to the design of peptides capable of binding therapeutically interesting targets.[33,34] However there remain significant challenges that must be overcome to perform de novo design of peptide macrocycle therapeutics. I report here progress on two major challenges. Firstly, I describe the de novo design of passively permeable cyclic peptides (Chapter 2). Secondly I

describe new methods for the increase of predicted binding affinity by the incorporation of noncanonical amino acids (Chapter 3),

2. DE NOVO DESIGN OF PASSIVELY MEMBRANE PERMEABLE CYCLIC PEPTIDES

2.1. INTRODUCTION

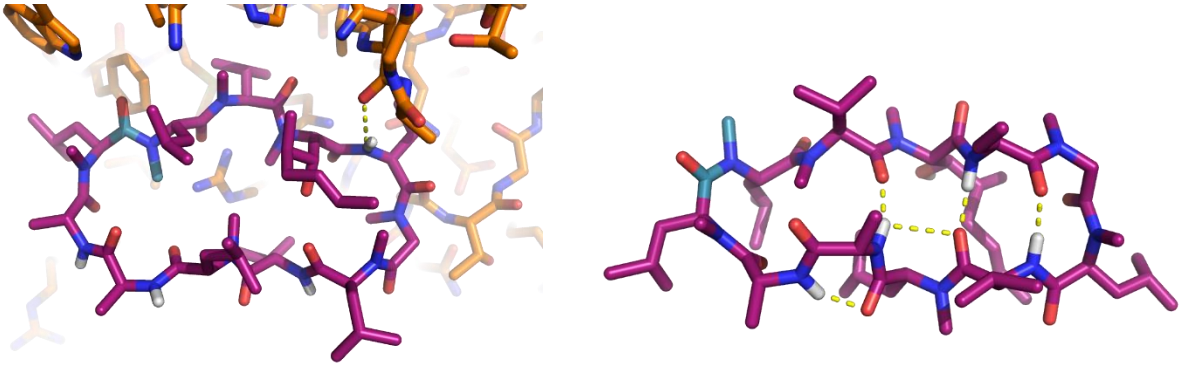
Given the size and chemical composition of an amino acid, a cyclic peptide of six residues or greater will likely break every one of the rule of five. Nevertheless, there are many examples of natural products and synthetic peptides that demonstrate membrane permeability and oral availability,[35] suggesting the need for a new set of design principles for cyclic peptides. Some peptides are known to achieve permeability and availability through active transport mechanisms like endocytocis and direct translocation.[36] However the primary application for these peptides is as carriers for other cargo, which has been stymied by difficulty in having the attached cargo escape the endosome.[37] For now, we are choosing to focus on the other class of cyclic peptides that achieve permeability through passive diffusion.

Studies of passively permeable cyclic peptides have identified different features important to their permeability, which mostly relate to their ability to reduce their polar surface area that needs to be desolvated to enter the membrane. Some peptides seem to achieve this by creating hydrophobic patches through organization of sidechains or chemical modifications.[38,39] Others reduce the number of hydrogen bond donors that need to be desolated by adopting conformations that promote intramolecular backbone hydrogen bonding,[40,41] occluding backbone amides through bulky side chains,[13,42,43] or completely eliminating potential hydrogen bonding

by methylating backbone amides[44–46] or incorporating peptoid residues.[47] These hypotheses all help to explain the behavior of passively permeable peptides after their discovery, but they have been difficult for researchers to translate to design rules because they require knowledge of the conformation a peptide sequence will adopt in solution. In many cases, cyclic peptides do not adopt a single low energy conformation and are structurally flexible.[48,49] This previously made it difficult to implement these principles, because it required multiple rounds of structural characterization to direct modifications and to assess their impact on the final structure.[50] We reasoned that with Rosetta's capability to designs structured peptide macrocycles we should be able to implement these principles in the design process. Using Rosetta, we searched for peptide backbone conformations that favored intramolecular backbone hydrogen bonding, methylated amides predicted to not form intramolecular hydrogen bonds, and stabilized the desired backbone conformation through sequence design. Using this approach, we were able to successfully design over 70 cyclic peptides with apparent passive permeabilities greater than $1*10^{-6}$ cm/s, a cutoff used in the field as a rough prediction of oral availability.[11,51]

While permeable, the resulting peptides often have poor water solubility and the limited number of polar interactions limits their potential selectivity as a binder.[52] In order to address these shortcomings, we also explored a second principle previously identified as being a factor in cyclic peptide permeability: chameleonic behavior. Cyclosporine A, a passively permeable cyclic peptide therapeutic, has been shown to adopt multiple conformations under different solvent conditions (Figure 2.1).[53,54] Cyclosporine has four backbone amides that are capable of forming hydrogen bonds, the remaining amide positions all are methylated. It has been shown that when bound to its target cyclosporine adopts a conformation where none of the backbone amides are forming intramolecular hydrogen bonds, instead forming interactions with the target or

solution.[55] However under hydrophobic conditions it adopts a conformation that results in all of its backbone amides are engaging in intramolecular hydrogen bonds. Cyclosporin seems to



achieve both binding selectivity and permeability by being able to switch between the two confirmations which each achieve only one of the functions. This chameleonic behavior has also been implicated in the permeability of cyclic peptides identified through library screens and four non peptidic beyond-rule-of-five macrocycle drugs.[56,57]

Figure 2.1 Conformations of cyclosporine. A) PDB 1CWA. Crystal structure of cyclosporine (shown in magenta) bound to its native target (shown in orange). In this conformation the peptide's amide bonds are all in the *trans* conformation and its backbone amides are making hydrogen bond interactions with the target (shown in yellow) or the solvent. B) CCSD DEKSAN. Crystal structure of cyclosporine (shown in magenta) grown from acetone. In this conformation one of the peptide's amide bonds has isomerized to the *cis* state (shown in cyan) and all amides are engaged in intramolecular hydrogen bonds (shown in yellow).

Rosetta design methods are optimized for generating sequences that have one low energy conformation, often resulting in highly stable structures. When there is dynamism in Rosetta designed proteins, it usually comes from tuning the thermodynamics of binding events, such as in pH dependent antibody binding,[58] or the LOCKR system.[59] However Rosetta has been successfully used previously to design multistate protein systems.[60,61] In order to extend multistate design methods to cyclic peptides we needed a discrete change in backbone conformation that has an energy barrier high enough that the discrete states can be isolated and

observed, but low enough that they can interconvert on a timescale relevant for permeability in a therapeutic context.

In cyclosporine, chameleonic behavior has been linked to isomerization around peptide bonds.[62] In most peptide bonds, steric repulsion from the Cα atom of the i-1 residue makes the *trans* conformation more energetically favorable than the *cis* conformation, causing them to be much more commonly observed in protein structures.[63] However at proline residues or N-methylated residues the replacement of the amide hydrogen with a carbon introduces a similar level of repulsion in the *cis* conformation.[64] This repulsion, in concert with changes in electrostatic interactions and the entropy loss upon isomerization, results in the *trans* state only being ~4 kcal/mol higher in energy than the *trans* state.[65] However, the double-bond character of the peptide bond makes the activation energy of this transition relatively large (~20 kcal/mol), so the interconversion happens on a timescale of seconds allowing for separation of the *cis*/*trans* conformers in NMR structural characterization.[66]

Given these features we decided to attempt to use multistate design methods to design chameleonic peptides that take on two roughly isoenergetic conformations separated by isomerization around a peptide bond. One of these conformations would follow our design rules for permeability and one of which would expose backbone amides to solution. We designed several peptides using this method that were membrane permeable and by using NMR we were able to show that some of these peptides do exhibit the designed chameleonic behavior.

2.2. Design of membrane permeable structured cyclic peptides

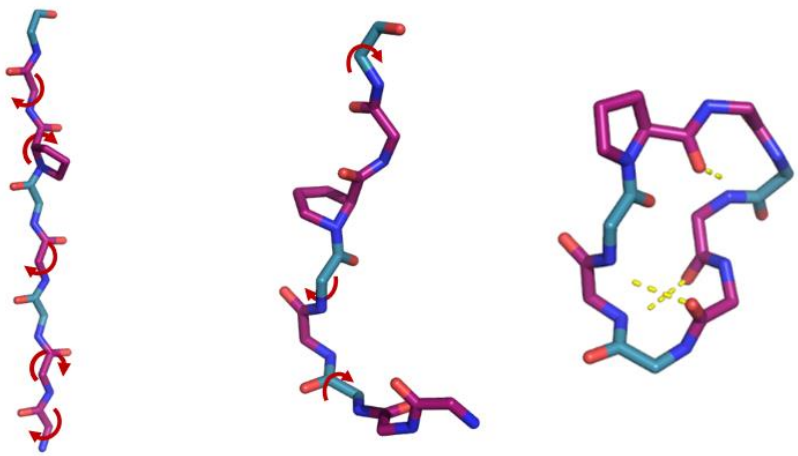
2.2.1 Design protocol

We began our design process by using Rosetta to search for peptide backbone conformations that position all non-methylated backbone amides to form intramolecular hydrogen bonds (Figure 2.2). To do this we first built linear chains of patterned glycine, N-methyl glycine, and L or D-proline residues ranging from six to twelve amino acids in length. We then utilized the generalized kinematic closure algorithm previously utilized in the design of structured cyclic peptides to create closed conformations of these chains.[32] During this process all but three of the phi and psi dihedrals along the chain are randomly perturbed, chosen from tables of values biased by the Ramachandran preferences of the proline or for combined preferences of L and D alanine acids. Additionally, we allowed the omega dihedrals at N-methyl or proline amides to sample the *cis*-conformation at a 50% rate during this perturbation step. The three remaining dihedral pairs are then assigned values chosen analytically to achieve loop closure. After a closed loop conformation is generated, it is filtered on the basis of any clashes along the backbone and to ensure there is at least one intramolecular hydrogen bond for every three residues in the peptide. This process was implemented in RosettaScripts and by using the

simple_cycpep_predict Rosetta application for distributed computing through the Rosetta@home project.

After generating millions of cyclic backbones with sufficient intramolecular hydrogen bonds, the backbones with the same sequences were clustered based on RMSD using the energy_based_clustering app (Figure 2.3). This allows us to reduce the number of conformationally similar backbones that are likely to have the same optimal sequence before performing more computationally intensive steps, usually from the millions to the tens of thousands. We then used Rosetta FastDesign to search for sequences that would stabilize the backbone conformations at the center of each cluster. During this step design was restricted to nonpolar and noncharged amino acids and any amides not engaged in an intramolecular hydrogen bond were methylated (Figure 2.4). This required the implementation of a N-methylation patch in Rosetta and modified rotamer libraries for the altered amino acids.[23]

From these larger set of tens of thousands of designs, subsets of hundreds of designs were selected based on their scoring on Rosetta metrics, the number of N-methylated amides in the final design, and to achieve a range of conformational diversity. Then in order to evaluate if a given sequence was likely to fold into the desired design conformation, we utilized the simple_cycpep_predict app to generate hundreds of thousands of conformations of the sequence, score them, and plot their backbone RMSD from the original design conformation (Figure 2.5). This allows us to visualize a prediction of the conformational landscape the sequence can access



and ensure that the design state is the lowest energy conformation for the design sequence. Based on these structure prediction analyses we selected ~200 designs to synthesize and assay. An example of the code used for this process is available in Appendix I.

Figure 2.2 Visualization of cyclic backbone conformer generation. In the first step the torsions of the magenta residues are selected based on Ramachandran preferences. In the second step the torsions of the cyan residues are assigned values that will connect the two termini in a peptide bond. The final cyclic conformer is evaluated for intramolecular hydrogen bonds (shown in yellow) and backbone clashes and either accepted or rejected.

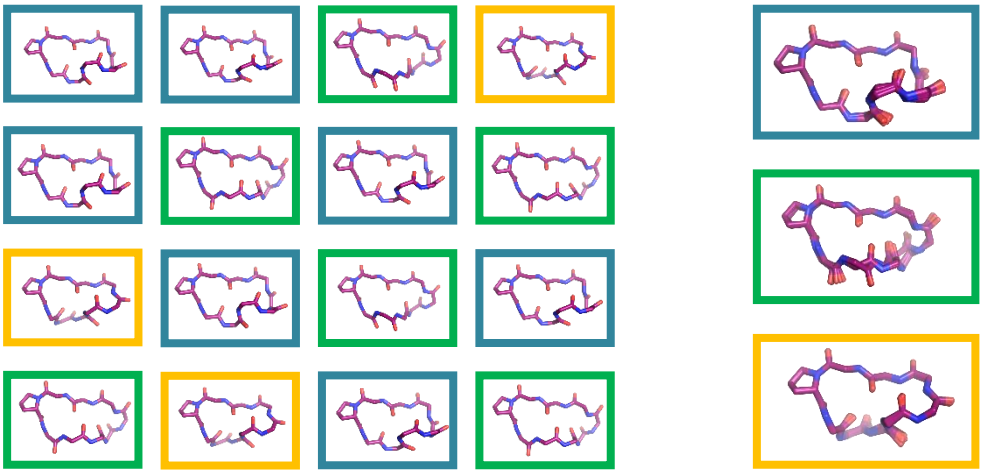


Figure 2.3. Visualization of the conformational clustering. Backbones with the same sequences are aligned and then RMSD between the backbone atoms are calculated. The backbones are then sorted into clusters of conformations that differ by 1.0 A or less.

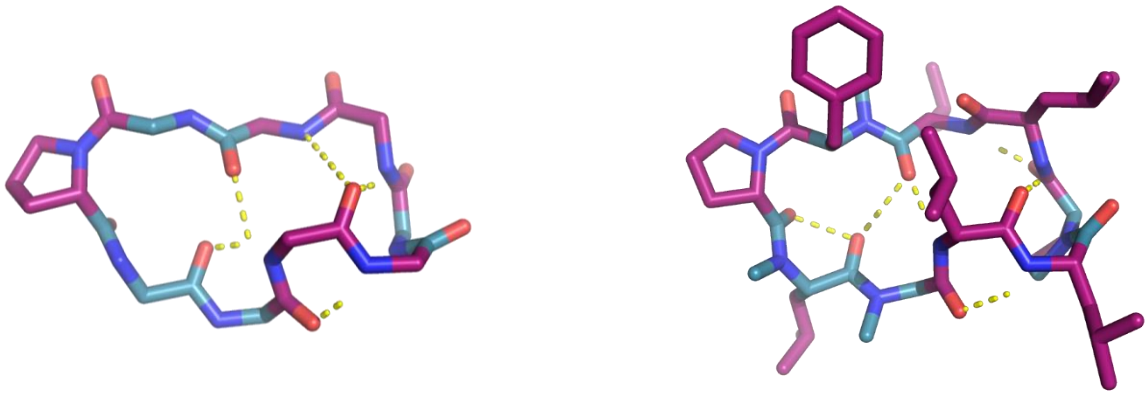


Figure 2.4. Rosetta sequence design. Rosetta FastDesign is performed on the lowest energy conformation from each structure, adding L and D nonpolar amino acids to stabilize the conformation. Additionally, backbone amides not engaged in intramolecular hydrogen bonds are methylated (shown in cyan).

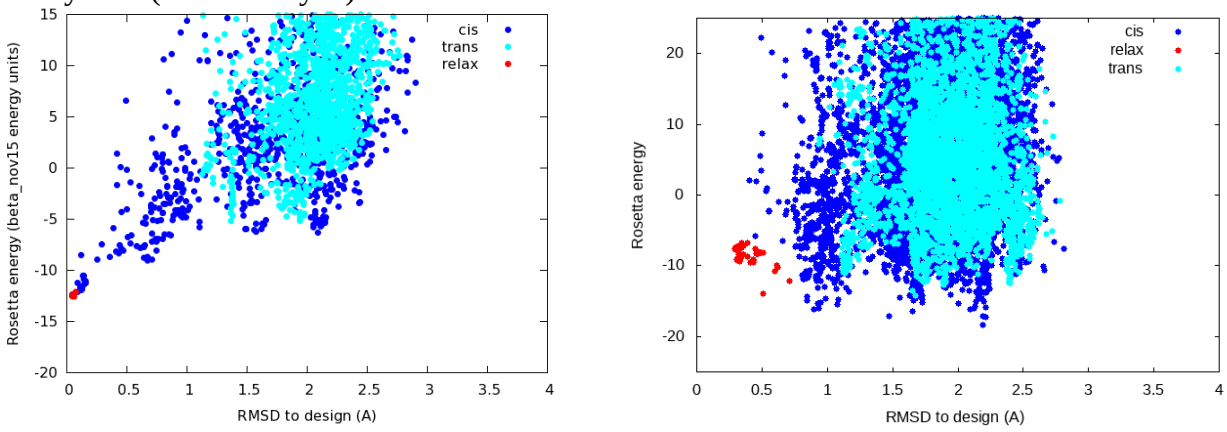


Figure 2.5. Rosetta sequence-based structure prediction of cyclic peptides. Each point on the chart represents a different backbone conformation of the specific sequence, plotted based on its predicted energy and RMSD relative to the design model. Each point is also colored based on the presence of *cis* peptide bonds in the conformation or whether the conformation was based on a unique structure prediction trajectory of from a FastRelax trajectory of the original design. Final sequences are selected if predicted to have a single low energy conformation near the design state (left plot) and not if the landscape is flat or has a secondary minimum (right plot).

2.2.2 Permeability of the designed peptides across artificial membranes

Once designs were selected, synthesized, and purified, their passive permeability was assessed using a parallel artificial membrane permeability assay (PAMPA). Using this method, of the ~220 peptides assayed we identified 76 that showed apparent passive permeability (P_{app}) greater than $1*10^{-6}$ cm/s (Figure 2.6). This is a high success rate, indicating this is a successful method for the design of membrane permeable cyclic peptides. For comparison, in a 1,152 member library of cyclic 6mers, using hydrophobic D and L residues and N-methylation, only identified 108 members that showed any permeability at all.[13]

The permeable peptides range in size from 6-12 amino acids, a marked expansion in size compared to most previous studies utilizing library-based methods to generate permeable cyclic peptides and that dramatically increases the number of scaffolds in the beyond-rule-of-five space

for known permeable cyclic peptides.[35] These peptides also display increased permeability based on their size relative to libraries of hydrophobic cyclic peptides where every amide position is methylated.[67] Perhaps this is due to the engagement of hydrogen bond acceptors in intramolecular hydrogen bonds in our designs that is not possible without backbone amide donors.

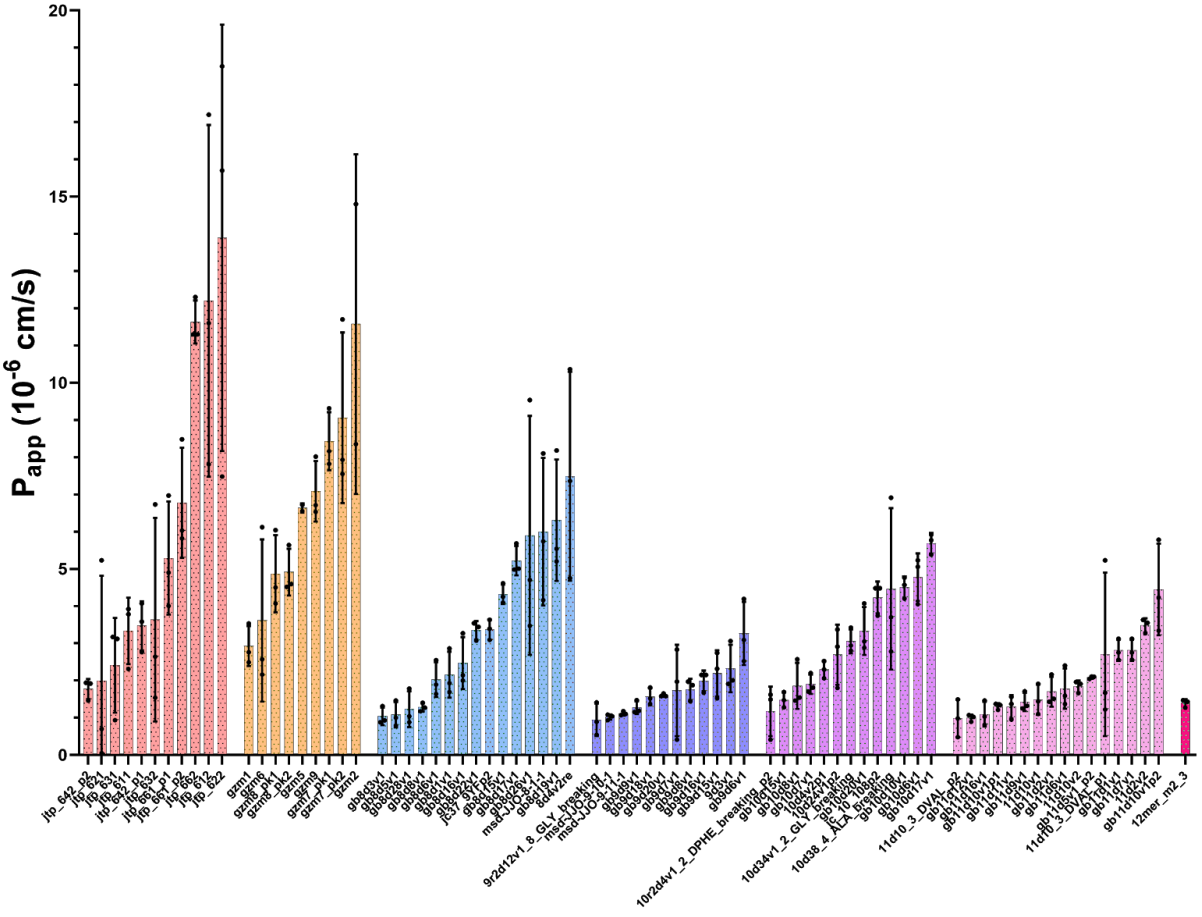


Figure 2.6. Apparent permeability values from PAMPA assays of designed cyclic peptides. Peptides are grouped by sequence length from 6 residues (left) to 12 (right).

2.2.3 Structural characterization of the designed peptides

It is possible that the peptides are not crossing because of our design criteria and it is merely their hydrophobic nature or the adoption of an alternate conformation that allows them to

be permeable. In order to further support our hypothesis, we pursued structural characterization of the peptides we synthesized using peptide X-ray crystallography. We were able to obtain crystal structures of XX of the peptides we assayed. The majority of the crystal structures we obtained match extremely closely to the designed states (Figures 2.8 and 2.9). This supports our lab's previous work showing that we can extend the control of peptide structure we've previously demonstrated[32] to cases that only utilize nonpolar amino acids. This also suggests that our design criteria are effective in achieving membrane permeability for cyclic peptides.

There were a few cases where the crystal structures did not match the design conformations well (Figure 2.10). One common cause for this problem came arose from racemization at the cyclization point during synthesis, and when the structure was re-predicted based on the actual sequence. The other major cause for this problem was the introduction of a water molecule in a position that disrupted the designed hydrogen bonding pattern. Because Rosetta uses an implicit solvent model, we are unable to fully account for its directional impact on peptide structures. There has been work incorporating explicit water into Rosetta in order to better describe and predict binding interactions at protein surfaces.[68] These methods are currently not implemented in the peptide structure prediction process, but may serve as a way in the future to refine our predictions and prevent similar errors.

Two of the peptides with mismatches between the crystal structure and the design to not exhibit membrane permeability, likely due to the energetic cost to desolvating the amides in order to enter the membrane. This also may suggest that other designs that were not membrane permeable may be because of inaccuracies in our structure prediction methods and the peptide sequences preferring conformers that break the designed intramolecular hydrogen bonding pattern. Interestingly, the peptide that differed from the design state due to racemization was

permeable even though the crystal structure doesn't have the designed hydrogen bonding pattern and exposes an amide to solution. We hypothesize this peptide is acting as a chameleon and that in the membrane it isomerizes around the *cis*-peptide bond it has in the crystal structure to adopt a conformation that shields the amides. However, we have been not yet been able to obtain clear structural data on this peptide to support or disprove this hypothesis.

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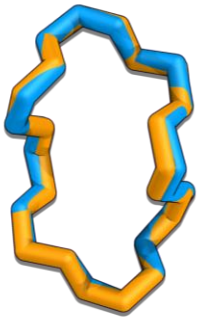

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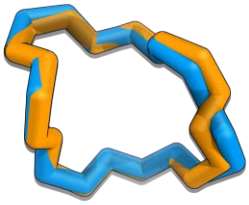

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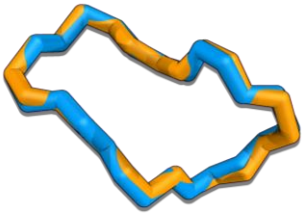

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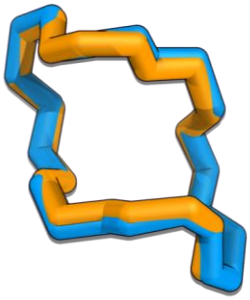

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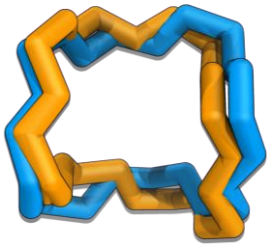

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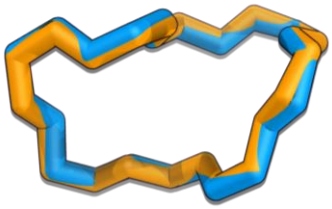

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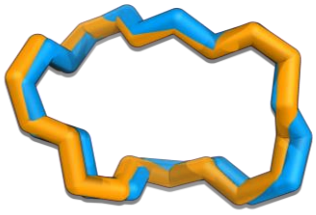

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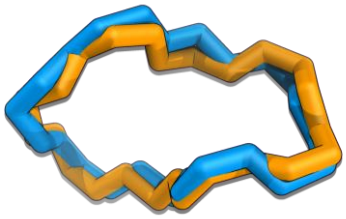

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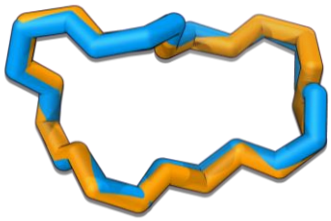


Figure 2.8 Structural characterization of de novo designed passively permeable cyclic peptides 6-8 residues long. For each peptide the backbone coordinates of the design model are shown in cyan and the coordinates of the backbone atoms from the crystal structure are shown in orange.

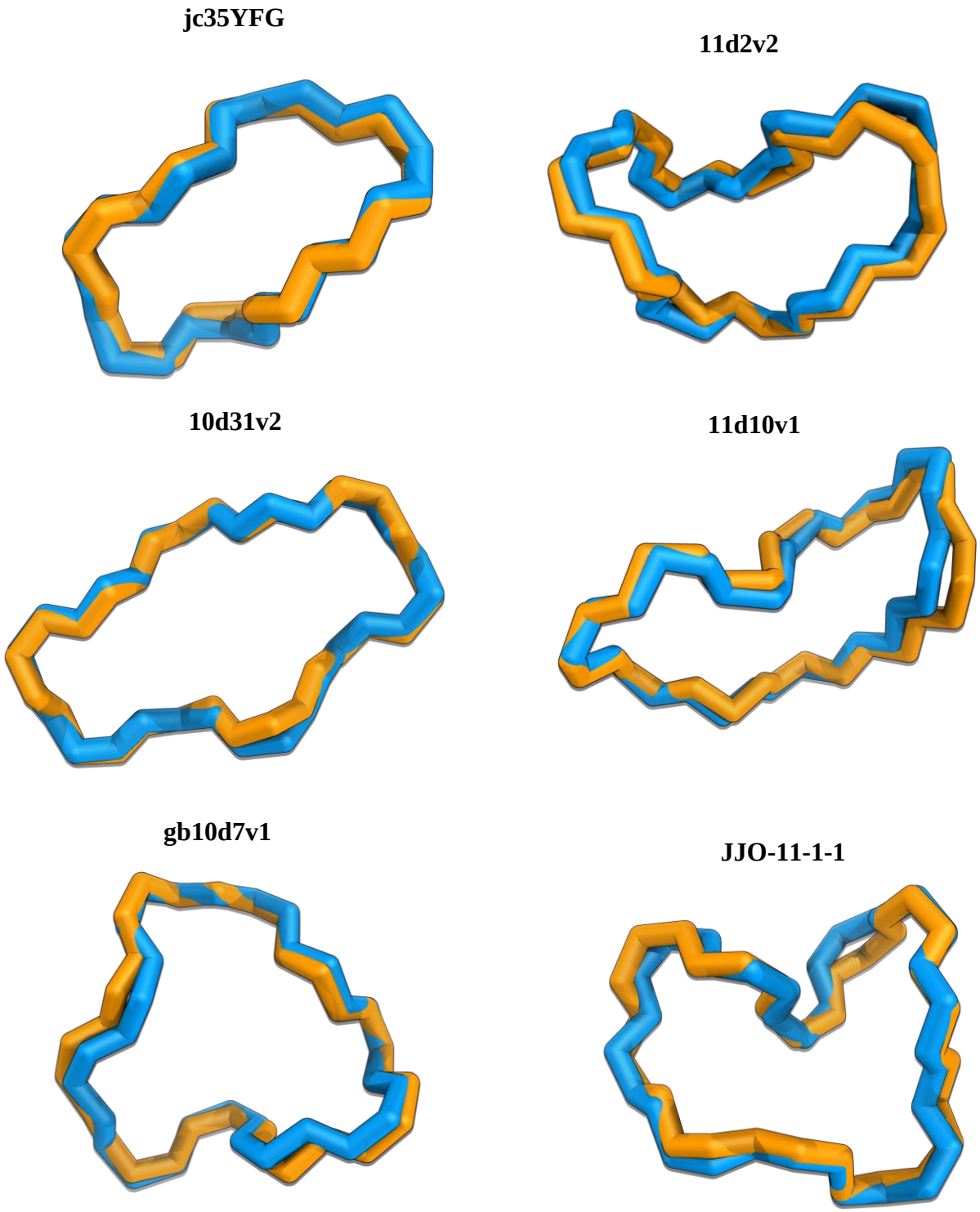


Figure 2.9 Structural characterization of de novo designed passively permeable cyclic

peptides 9-11 residues long. For each peptide the backbone coordinates of the design model are shown in cyan and the coordinates of the backbone atoms from the crystal structure are shown in orange.

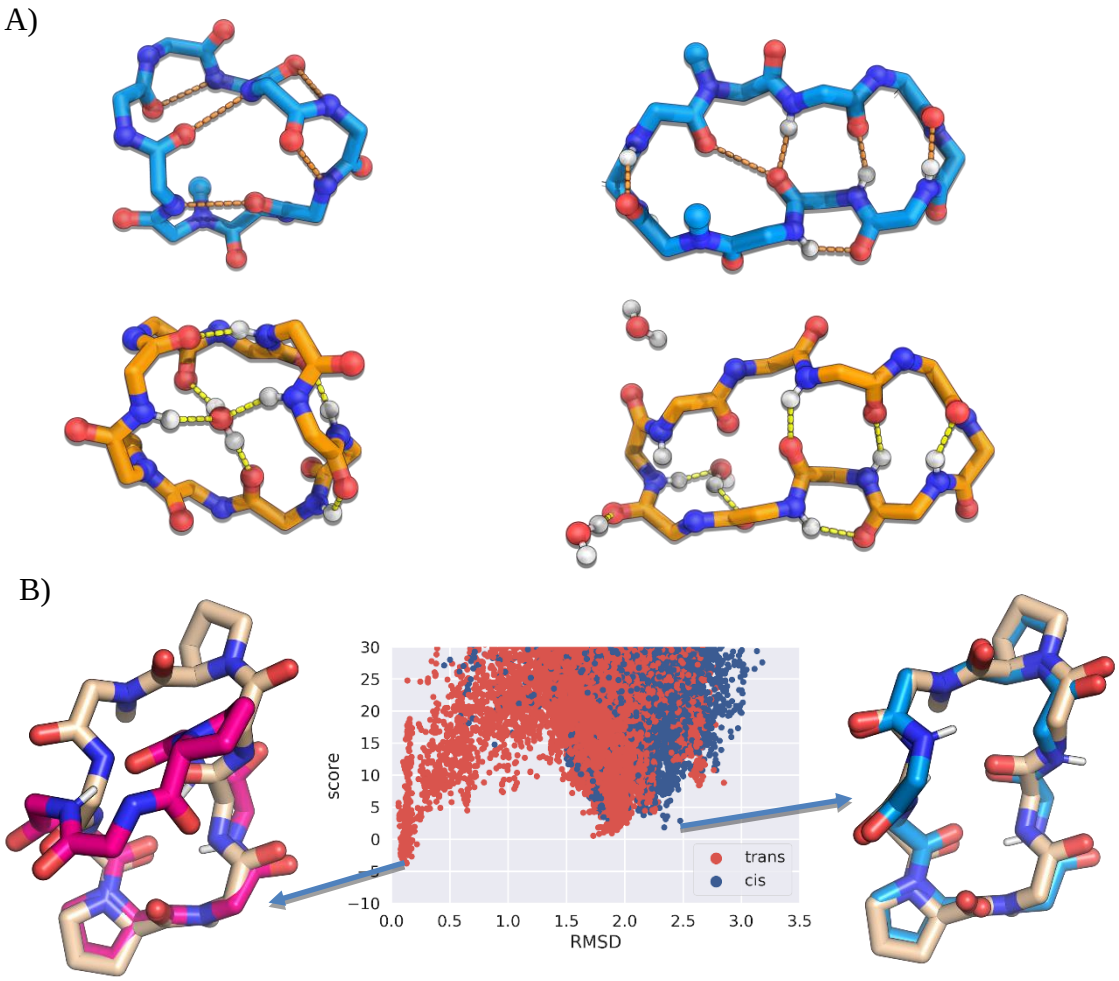


Figure 2.10. Crystal structures of designed peptides that do not match their designed conformations. A) The crystal structure of the peptides are shown in orange while the designed models are shown in cyan. The presence of a water molecule disrupts the designed hydrogen bonding pattern, likely leading to the lack of permeability in these structures. B) The conformational landscape for the design is shown. On the left is the overlay of the design state (magenta) with the crystal structure (wheat) and on the right is the overlay of a secondary low energy conformation (cyan) and the crystal structure. The crystal structure differs from the original design conformation due to isomerization around a peptide bond. Interestingly this peptide is permeable even though there is an amide exposed to solution, suggesting the peptide may exhibit chameleonic behavior.

2.2.4 Permeability of the designed peptides in living systems

As the end goal for these design methods is to aid in the design of peptide therapeutics we decided to explore if the permeability values shown across artificial membranes translated to permeability in living systems. Peptides that showed high levels of passive permeability were selected for evaluation in an in vitro transwell assay, similar to PAMPA, in which the two wells are separated by a monolayer of human colorectal adenocarcinoma (Caco-2) cells. This assay is commonly used as a model for intestinal permeability and oral absorption, as the cells form tight junctions and have efflux transporters that mimic the barriers in the gut an oral drug must pass through to enter the circulatory system.[69,70] High levels of permeability on PAMPA generally correlate with permeability in Caco-2 assays, although compounds that undergo active transport or efflux may show important differences.[71,72] For our selected peptides we did see permeablility across the Caco-2 monolayer, indicating that the passive permeability of these peptides is sufficient to overcome whatever efflux may occur (Figure 2.11).

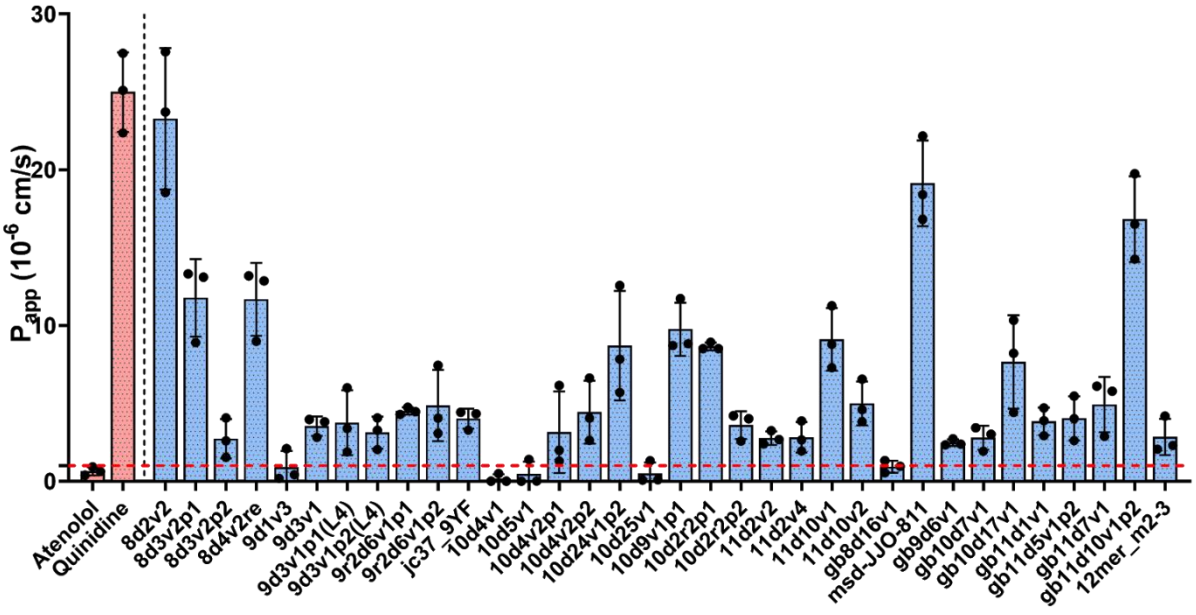


Figure 2.11 Apparent permeability (P_{app}) of selected designed peptides that showed high permeability in PAMPA assays.

In order to assess if these Caco-2 permeabilities do correspond to oral availability we performed pharmacokinetic studies of three of the peptides that showed good Caco-2 permeability. The peptides showed oral availability (calculated as a ratio of the dose-corrected plasma concentration of the compounds delivered orally relative to those delivered intravenously) ranging from 10% to 40%. These place the peptides in the "low" range of oral availability when compared to small molecule therapeutics, but at a comparable range of availability to other beyond-rule-of-five peptides and existing peptide therapeutics.

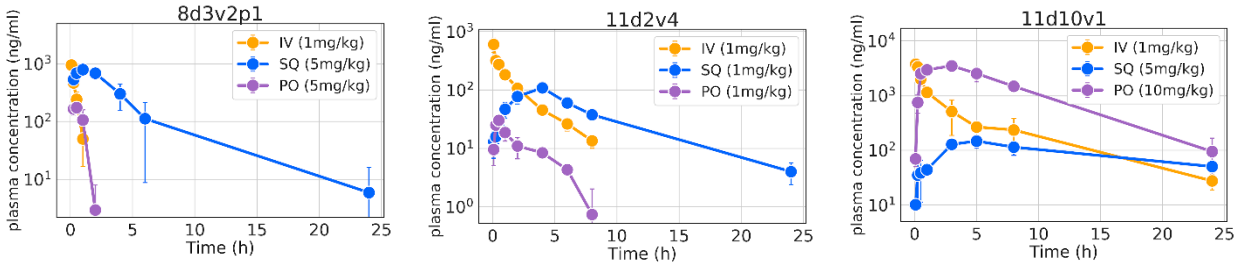


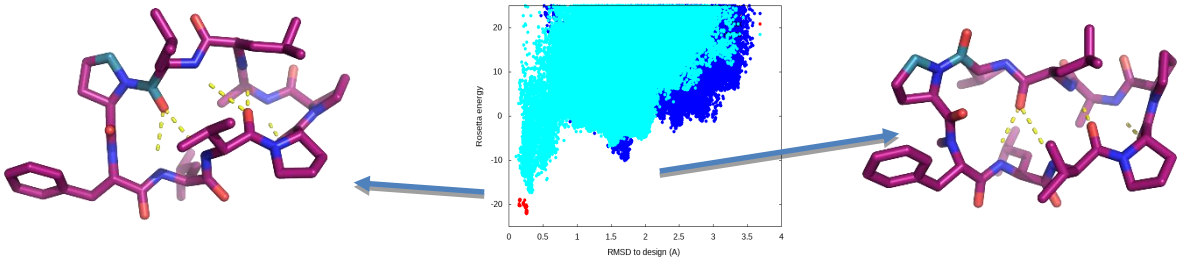
Figure 2.12. Pharmacokinetic studies of three membrane permeable cyclic peptides. Plasma concentrations were measured in three mice over the course of 24 hours after introduction of the peptides intravenously, subcutaneously, and orally. The peptides have oral availability (from left to right)of 10%, 11%, and 40%..

2.3. DESIGNING PASSIVELY PERMEABLE CHAMELEONIC PEPTIDES

2.3.1 Design protocol

In order to design peptides that exhibit the desired chameleonic behavior we began by reviewing the structure prediction landscapes for permeable peptides we had previously designed, but not carried forward to synthesis because they lacked a predicted single low energy state that aligned with the design. We instead searched for cases where the conformational landscapes suggested the presence of two low energy conformations, one with a *cis*-peptide bond and one with only *trans* peptide bonds (Figure 2.13). We then reviewed the predicted structures of those conformations to ensure that one state had the designed intramolecular hydrogen

bonding pattern and the other exposes at least one backbone amide to solution. We were able to find a few cases in our structure prediction landscapes that matched these criteria, but oftentimes there was an energy gap between the two predicted conformers of a few Rosetta energy units. In order to maximize the opportunity for the designed peptide to access both states we employed a genetic algorithm to search for sequences that equally stabilize both backbone conformations as low energy states (Figure 2.14). Then we performed structure prediction calculations to ensure that the new sequence didn't introduce a new low energy state not observed in the original



prediction. Using these methods, we designed 30 peptides to have this chameleonic behavior. We assayed their permeability and selected the permeable peptides for further analysis.

Figure 2.13 Selecting peptides with chameleonic potential. We searched for cases where there were two low energy conformations separated by peptide bond isomerization (shown in cyan), one of which lacked the designed intramolecular hydrogen bonding pattern (right conformation).

2.3.2 Assessing chameleonic behavior by NMR

In order to determine if the permeable designed peptides were exhibiting chameleonic behavior, we utilized NMR to assess the conformational states of the peptides in different solvent conditions. Because the peptides have poor water solubility, we were unable to sufficiently concentrate them in D2O for NMR studies, so instead we used DMSO and water mixtures as proxies for an aqueous environment, and CDCL3 as a proxy for the membrane environment. We first utilized 1D 1H NMR to assess the structural stability of these peptides and the presence or

absence of multiple conformations by looking for the number of defined peaks in the amide region of the spectra (Figure 2.15).

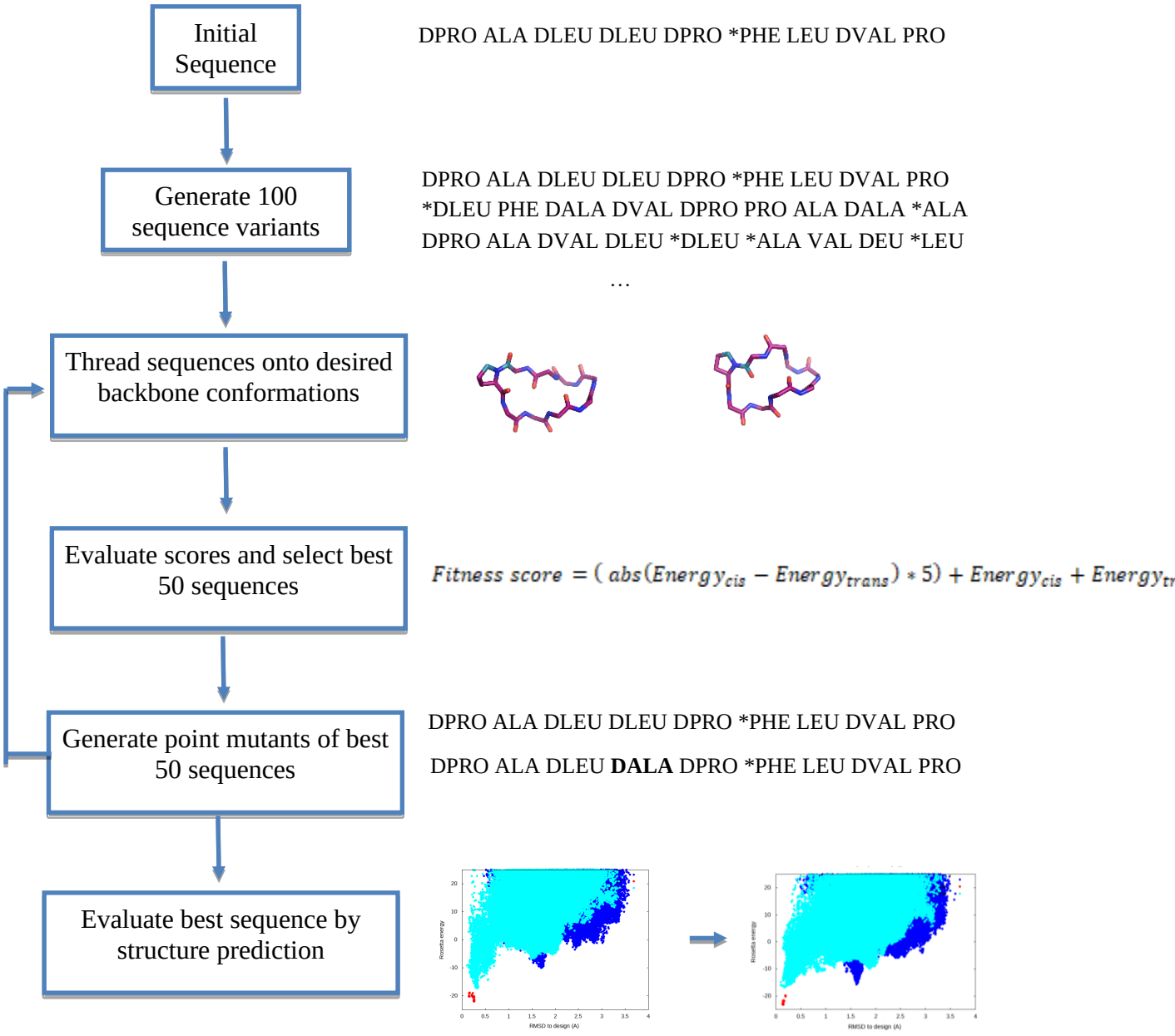


Figure 2.14 The genetic algorithm used to perform multistate design to select sequences that would equally favor the two desired conformations. Starting from an initial seed sequence a list of 100 variants of that sequence are made. Each list member mutates the original sequence, allowing for changing between different hydrophobic residues but maintaining chirality and N-methylation at each position. Each mutant sequence is then threaded onto the original backbone conformations and scored by Rosetta. The sequence is given a final score defined by the difference in energies of the two conformations, as well as their combined energy, in order to select for sequences that stabilize both conformations equally. The best 500 sequences based on this metric are carried on to the next cycle of evaluation, where a point mutant of each

sequence is added to the list and the process repeats. Over 1,000 cycles this generates sequences that optimize the selection criteria. At the last cycle the best sequence is chosen and then structure prediction is performed to ensure both desired conformational states are unique minima in the conformational landscape.

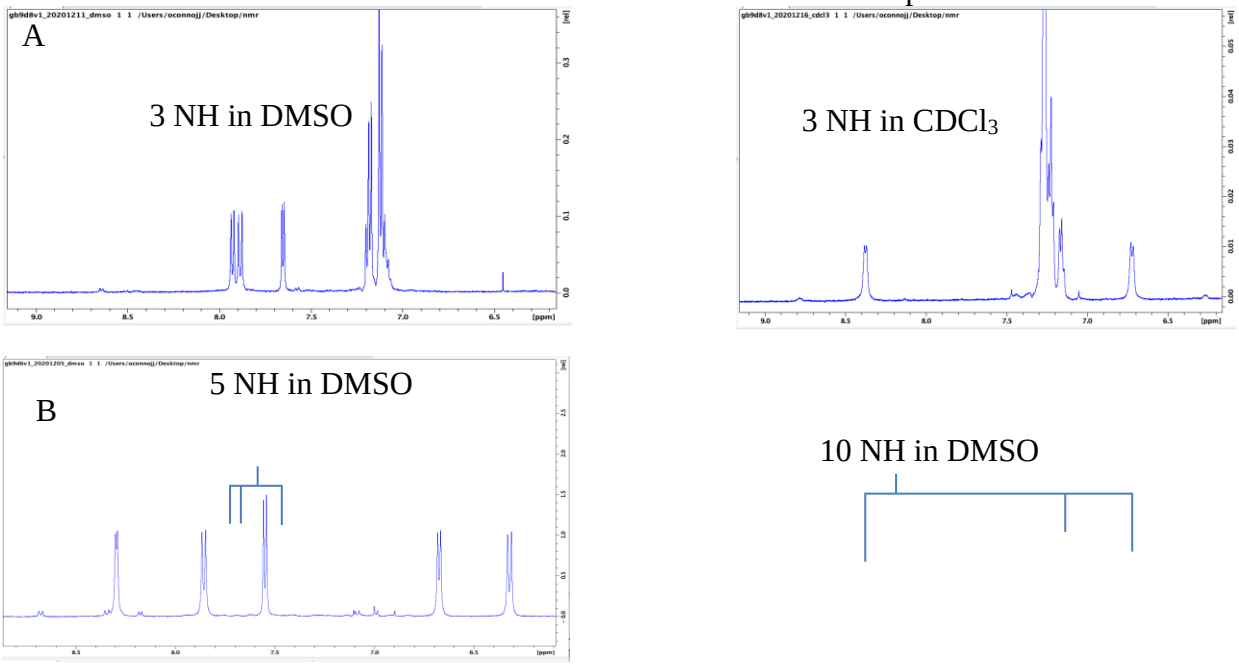
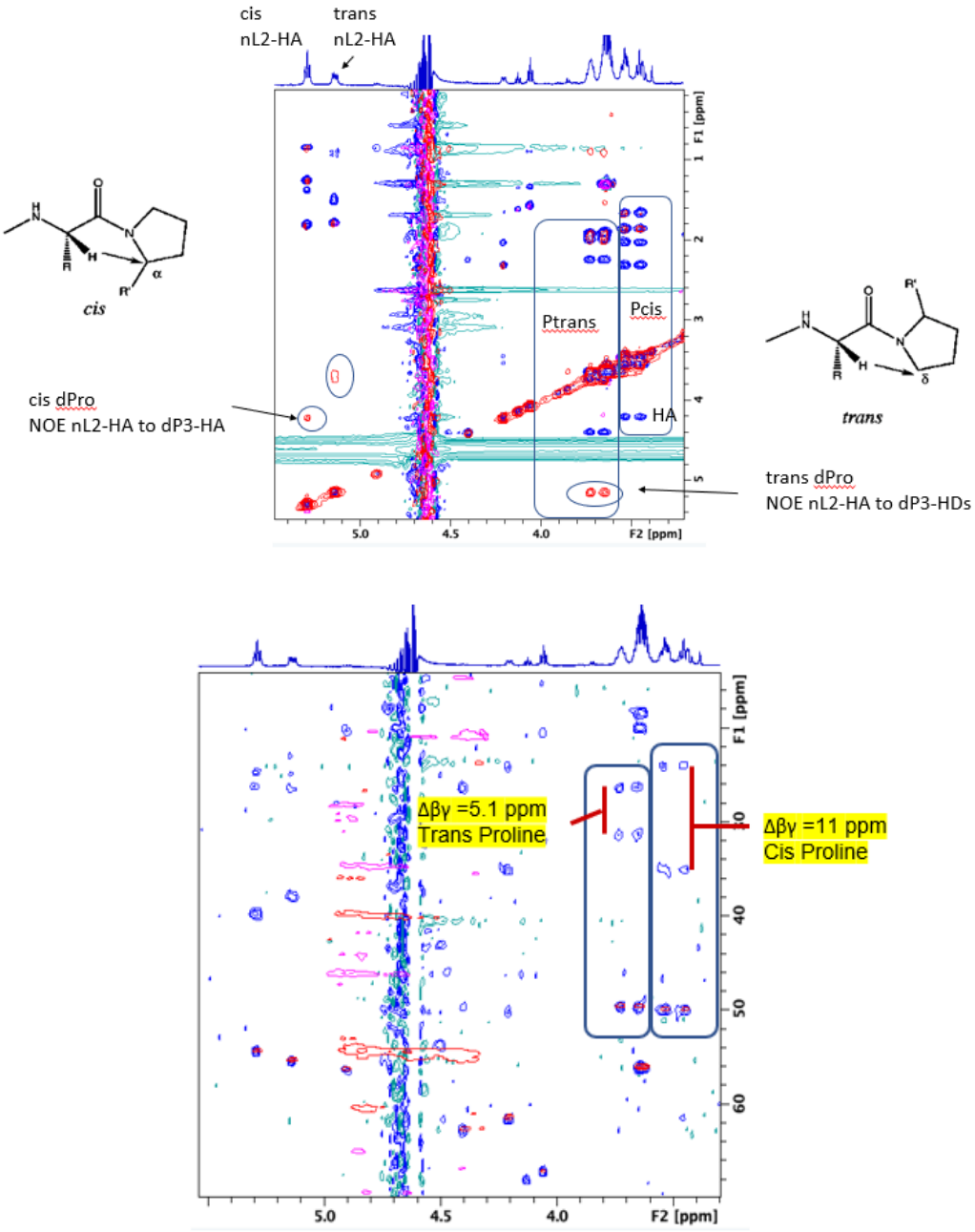


Figure 2.15. Screening for chameleonic behavior by 1D 1H NMR. P... change in the number of amide peaks in different solver... intramolecular hydrogen bonding conformation and do ... Peptides that do appear to have a novel secondary popul... be exhibiting the designed chameleonic behavior and were...

Once we had identified peptides that appeared to be sampling multiple conformations in a solvent-dependent manner, we pursued further structural validation through TOCSY, NOSEY, ROESY, and 1H-13C HSQC experiments. In particular we used these experiments to assess the isomerization of the peptide bonds at the positions we had designed to isomerize during the chameleonic switching. By looking for NOEs Hα of the previous residue and either the proline

Hα or Hδs we can assign the proline as the *cis* or *trans* conformer respectively. In cases where there are no clear NOEs, the chemical shift between the proline Cβ and Cγ in the HSQC can also



be used to classify the isomerization state of the proline. In combination with the TOCSY and NOESY/ROESY data we were able to assign the peaks to the different conformations and monitor them in changing solvent conditions (Figure 2.16).

Figure 2.16. NMR features used to determine the peptide bond conformation at proline residues in the chameleonic designs. (Top) Overlay of NOSEY (red) and TOCSY (blue) data for a chameleonic peptide with characteristic NOEs circled. (Bottom) HSQC data with characteristic peak distances highlighted in

Using these methods, we identified two peptides that seem to exhibit the designed chameleonic switching behavior. One of these peptides is an 8-residue peptide with a measured permeability in PAMPA of $2*10^{-6}$. The peptide has a symmetric sequence, which means half of the residues experience the same chemical environment as the other half, appearing as combined signals in NMR. In the designed conformation the proline residues are all in the *trans* conformation and all amides are making intramolecular hydrogen bonds. In the alternate state both prolines isomerize to the *cis* conformation, maintaining symmetry, and breaking two of the amides' hydrogen bonds (Figure 2.17). In NMR we observed the presence of one symmetric conformation in DMSO, where both prolines are in the *trans* conformation. But in 50% DMSO/H2O mixtures a second population of a symmetric conformation appears where both prolines are in the *cis* conformation, as designed. This equilibrium can be influenced by changing the solvent mixture and by modifying the temperature.

The second peptide to exhibit chameleonic behavior is a 9 residue peptide with an asymmetric sequence and a PAMPA permeability of $3*10^{-6}$. In the original designed state every peptide bond is in the *trans* conformation and all amides are engaged in intramolecular hydrogen bonds, but in the alternate conformation one of the prolines isomerizes to the *cis* conformer and disrupts three amide hydrogen bonding interactions. In this peptide we observe only one conformation in DMSO, which has one proline in the *cis* conformation and one in the *trans* conformation, suggesting the peptide is in the alternate conformation. In $CDCl_3$ we observe two

populations, one of which has a *cis* and a *trans* proline, and the other has only has prolines in the *trans* conformation.

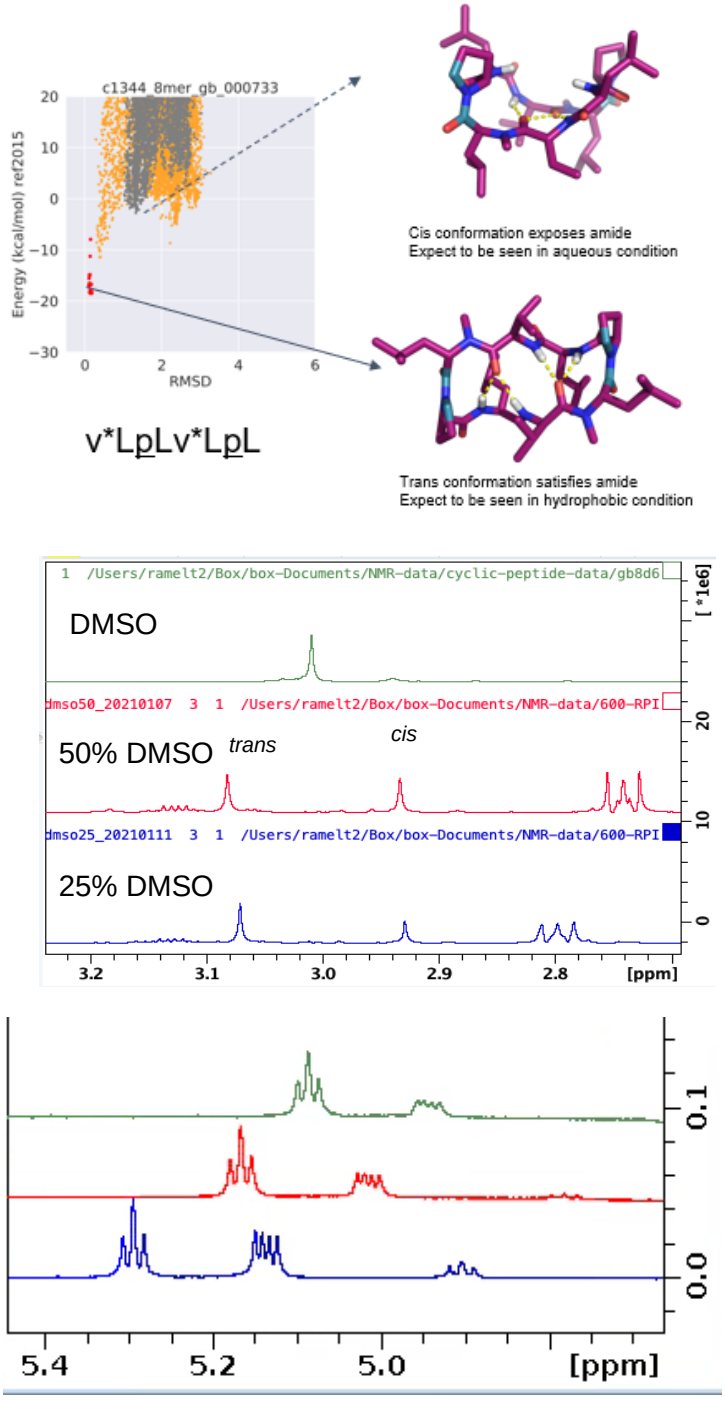
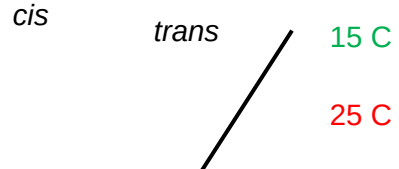


Figure 2.17. Characterization of the chameleonic symmetric 8mer peptide. (Top) The predicted conformational landscape for the peptide and the conformations of the two states of



interest, separated by peptide bond isomerization (cyan). (Middle) 1D 1H NMR data of proline peaks in DMSO/H_2O mixtures. *Cis*/*trans* state was assigned based on HSQC data. (Bottom) 1D 1H NMR data of proline peaks collected at different temperatures.

2.4. CONCLUSIONS AND FUTURE DIRECTIONS

Using Rosetta we designed a large number of cyclic peptide ranging from 6-12 amino acids that showed permeability across artificial membranes. Selected peptides were also shown to have permeability across gut endothelial cells and oral availability in rodents. We were able to do this by designing the peptides so that they adopt a conformation where all backbone amides are engaged in intramolecular hydrogen bonds, reducing the number of polar interactions that need to be desolvated when crossing the membrane. We were also able to utilize multistate design methods to design peptides that exhibit chameleonic behavior, switching between two structured conformations, one of which is favored under more aqueous conditions and one of which is favored under hydrophobic conditions.

In the future the peptides described here can be investigated further by library methods to probe the limits of their permeability. For example, we designed the peptides to have no exposed backbone amides, but there are peptides that do not shield every amide that are known to achieve permeability. One future experiment of interest would be to synthesize variants of the peptides that introduce polar groups by removing N-methylations or by incorporating side chains with polar groups, perhaps at locations where they can make hydrogen bonds with backbone atoms to mitigate their impact. These types of experiments can help us further map out a rulebook for designing permeable peptides.

Additionally, these methods can be directly applied during the process of designing new peptide therapeutics. The ability to design chameleonic peptides will be particularly useful when trying to incorporate binding specificity and permeability in the same scaffold.

3.INCREASING PREDICTED BINDING AFFINITY OF CYCLIC PEPTIDES BY INCORPERATION OF NONCANONICAL AMINO ACIDS AT THE BINDING INTERFACE

3.1. INTRODUCTION

In order for a cyclic peptide to work as a therapeutic in needs to be able to bind tightly and selectively to its target. There has been success in using high-throughput library screens to identify peptides that bind with high affinity to therapeutic targets.[73–75] However the hits from library screens can be difficult to optimize, due to the lack of structural models of the interaction of a lead with a target. Our lab has previously demonstrated the utility of Rosetta in designing high affinity protein binders to therapeutic targets.[76] We've had some success in extending these design principles to design cyclic peptides that bind tightly to histone deacetylases[34] and beta lactamases,[33] but in both of these cases the initial hits did not actually adopt the originally designed binding conformation.

This gap between our ability to design protein binders and cyclic peptide binders suggests that designing cyclic peptide binders is currently a more difficult challenge. In part this is because the number of interactions a peptide could potentially make with a target to increase affinity are limited compared to proteins by the small sequence length of most cyclic peptides. One way around this limitation is to capitalize on the synthetic nature of most cyclic peptides and incorporate noncanonical amino acids into the design process. The increase in potential

conformational and chemical space would allow designs to access stronger interactions or better shape complementarity than a protein binder.

The utility of noncanonical amino acid in improving binding affinity of peptides has been shown in several cases,[77,78] but faces some important practical limitations. Firstly, Fmoc-protected noncanonical amino acids can be expensive, and more exotic amino acids may not be commercially available at all. Secondly, inclusion of noncanonical amino acids vastly increases the sequence space that needs to be explored to find an optimal sequence. For both of these reasons, methods for computational evaluation of incorporation of noncanonical amino acids would be valuable.

Efforts to incorporate noncanonical into Rosetta were first performed by members of the Kuhlman lab in 2012.[79] They were able to successfully add 114 noncanonical amino acids to Rosetta. However, in order to do this they needed modify the score function to include custom molecular mechanics based terms for intra-residue torsion and Lennard Jones potentials. It was also necessary for each noncanonical amino acid to generate a custom reference energy, used to weight amino acids relative to one another, and rotamer library, a set of conformations the amino acid can access during the design process. These steps make incorporation of new amino acids a time-intensive project and we wished to develop more generalizable methods that could potentially allow for the incorporation of many arbitrary amino acids into our pipelines.

This goal was made possible by the creation of the new generalized Rosetta score function (‘genpot’) by Hahnbeom Park and Frank Dimaio. This score function was originally developed for the evaluation of small molecule ligands at protein interfaces and has two key advantages for noncanonical incorporation. Firstly, it parameterizes a much broader set of atom types than was previously available in Rosetta, allowing us to consider more exotic noncanonical

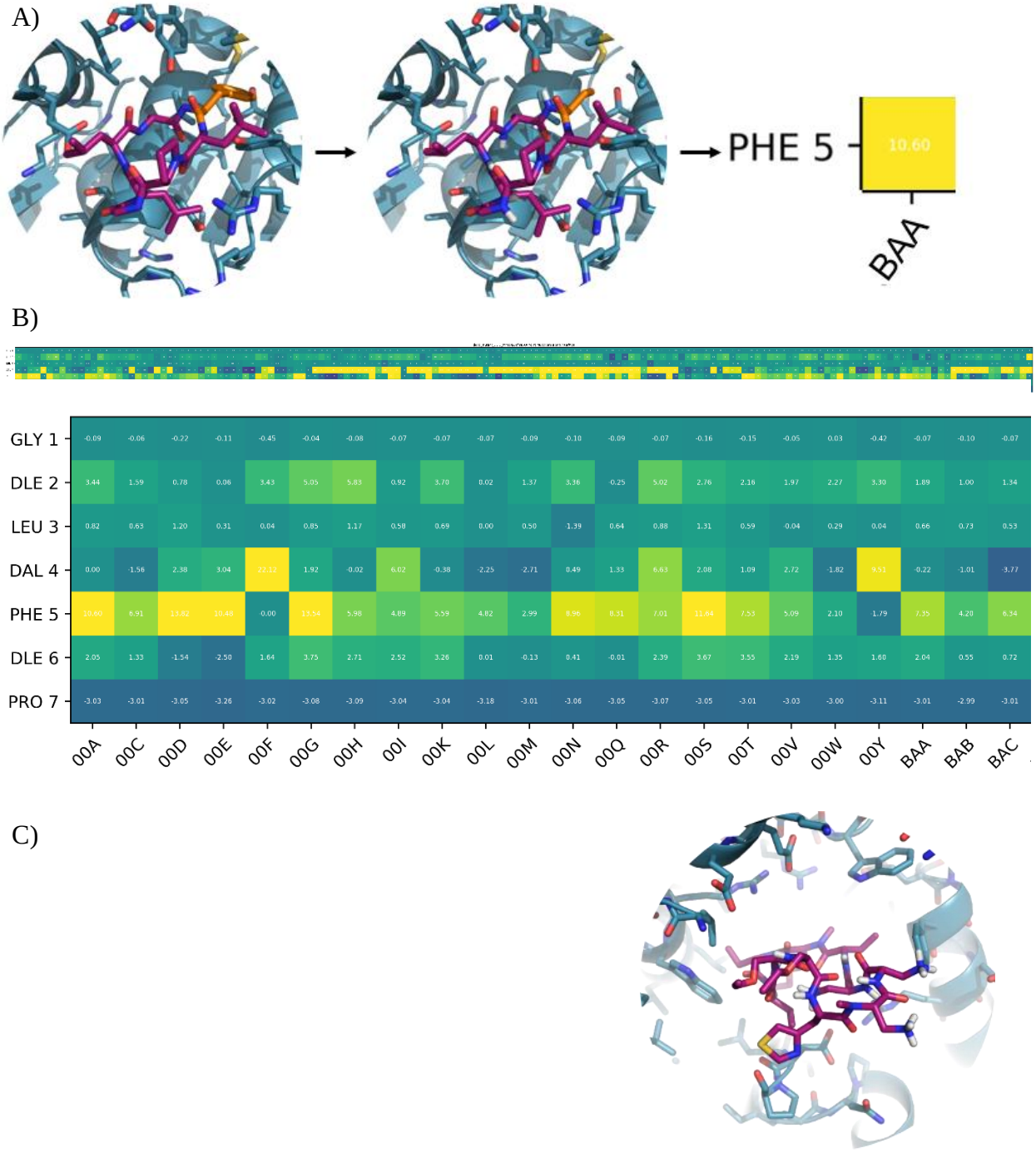
amino acids than was previously possible. Secondly, it implements a generalized torsion potential that allows for sampling and scoring of a generic set of bond types, circumventing the need to generate custom backbone torsion preferences for each noncanonical amino acid. With this new score function, we were able to develop new methods for the evaluation of a large number of noncanonical residues at interfaces, with the goal of increasing predicted binding affinity of cyclic peptides.

3.2. DESIGN PROTOCOL

We first needed a simple way to incorporate a new noncanonical into Rosetta's software, so it can be used during design. To do this we utilized the antechamber python package, which is a package generated to build files describing molecules for calculations with the AMBER force field.[80] Using the bond and atom recognition tools in the package, we developed a script that can take a generic SMILES code for any noncanonical amino acids and generate an appropriate parameter file with correct atom typing. This allows for easy incorporation of any potential noncanonical amino acid into our pipeline, as long as it consists of the atoms currently included in the 'genpot' score function (C, N, O, H, S, P, F, Cl, Br, and I). Utilizing this method, we generated parameter files for over 200 noncanonical amino acids (See Appendix I).

Once we had the set of noncanonical amino acids in a Rosetta format, we then needed a way to select the best amino acids at an interface. The ideal method would be to use the Rosetta FastDesign protocol to perform a Monte Carlo search through all of the combinations of amino acids at the interface to find the optimal sequence. However, with so many amino acids the potential combinations are too numerous to explore without prohibitive amounts of computational time and memory. So instead, we first decided to narrow our search by selecting the amino acids that make the best predicted single mutations at the interface (Figure 3.1).

Starting from a model of a cyclic peptide at the interface of interest, the residues of interest were defined. Then for each noncanonical amino acid we



wanted to test, each of these positions was

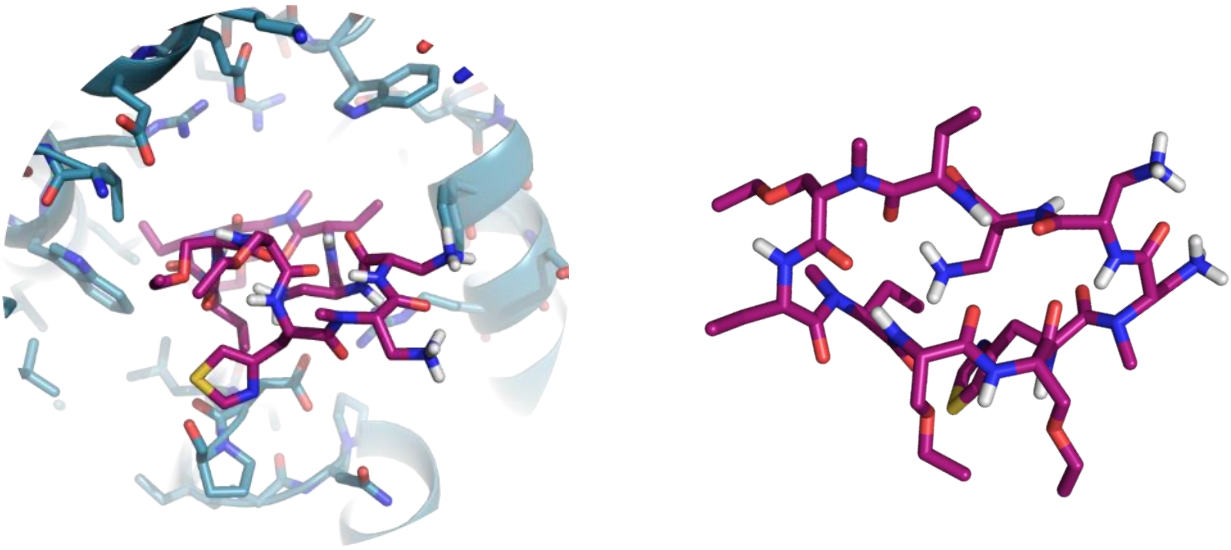
Figure 3.1. Protocol for evaluating noncanonical residues at peptide-protein interfaces. A) A position of interest is chosen and mutated to a noncanonical amino acid, packed and minimized, and the change in binding energy caused by the mutation is scored using Rosetta

metrics. B) This process is repeated for each noncanonical being considered, for each position of interest at the interface, generating a table of predicted changes in binding energy for each mutation. Here a portion of the table is expanded for visibility. C) From this table the best 7 noncanonical amino acids for a given position are selected and stored in a Rosetta resfile, which is then used to guide the final FastDesign protocol that considers the combinatorial design of all of the positions of interest and the best noncanonical residues to generate the final design. mutated to the D or L stereoisomer of that noncanonical amino acid, depending on the torsional space the backbone occupies. After being mutated, the sidechain orientations are optimized through Rosetta's packing algorithm, and then minimized in cartesian space. The resulting mutated peptide can then be evaluated using various Rosetta metrics. In particular we chose to focus on the change in predicted binding energy of peptide upon mutation, as well as the percent of the mutated residue that is buried at the interface. Based on these metrics a variable number, usually 7, of the best scoring mutations for each position was selected and stored in a residue file. This file can be used directly as a seed for libraries to screen for increased binding. Additionally, the file can be used to perform Rosetta FastDesign, allowing for exploration of combinations of noncanonical sequences that are likely to enhance binding affinity.

3.3. GENERATING REFERENCE ENERGIES

When we first implemented tested this design protocol, we noticed that some amino acids seemed to be selected disproportionately often, even if they did not seem to making particularly beneficial contacts with the target (Figure 3.2). This seemed to be an extension of a known issue with the Rosetta score function, which often favors larger residues because of their ability to create more interactions which generate better scores, but fails to account that these additional interactions are often not unique to the positioning of the amino acid. In the standard Rosetta score function this problem is solved through the implementation of "Reference Energies" which account for the difference between the unfolded state of the sequence and the designed conformation.[81] Rather than calculating these scores by explicit calculations of the unfolded

states, which are often error prone and computationally extensive, the values are optimized by searching for values that maximize native sequence recovery. Such a method is not applicable to

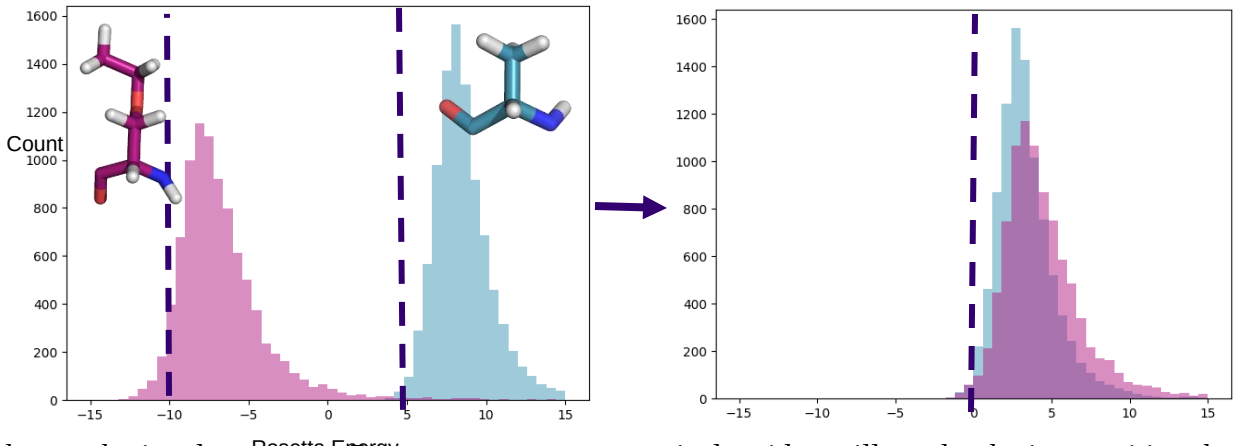


the generation of reference energies for noncanonical amino acids, as there are not large numbers of high-quality structures that incorporated amino acid under evolutionary pressures. Previously, the Kuhlman group used molecular mechanics methods to perform explicit calculations of the unfolded state of non canonical amino acids to generate the reference energies for Rosetta, but as already stated, such methods are time and computationally intensive.

Figure 3.2. A common example of a design produced from the protocol before the implementation of reference energies. Note the high number of 2,3-Diaminopropionic acid (KAA) residues and ethoxyserine residues (SAA) that are not making particularly beneficial contacts with the target or other residues within the peptide.

Because we are interested in the evaluation of noncanonical amino acids specifically for improving binding affinity of designed peptides we opted for a slightly different approach. We selected a set of high-quality PDB structures, the same set used for the generation of the first canonical reference energies.[82] Then we mutated each residue in the structure, one at a time, to

the noncanonical amino acid of interest, packed and minimized the surrounding side chains, and stored the Rosetta score for the noncanonical amino acid. From this large distribution of scores we assigned a reference energy equal to the 10th percentile of the distribution. This makes it so



that to obtain a low-energy Rosetta score a noncanonical residue will need to be in a position that scores 90% better than any other position it could be at in a protein (Figure 3.3). In order to assess if this would have the desired effect we selected a high quality set of protein-protein interface structures, and packed the set of noncanonical at positions along the interface. We then plotted how often a noncanonical was identified as the lowest scoring residue at any position (Figure 3.4). Without the reference energies some residues are largely overrepresented, but with the reference energies implemented we see a much more even distribution of residues.

Figure 3.3. Developing reference energies for noncanonical amino acids. On the left is plotted the overlay of the distribution of scores for alanine (00A, shown in cyan) and ethoxy serine (SAA, shown in magenta) when mutated at every position in a set of pdbs, packed, minimized, and scored. The large difference in the distributions shows that at almost any position Rosetta will evaluate ethoxyserine as scoring better than alanine, even if it is not a uniquely good position for ethoxyserine. By taking the Rosetta energy of the 10th percentile and apply a reference energy the overall distributions become shifted, so that only unusually good placements of either residue should be selected during the design process.

3.4. ASSESSING DESIGN IMPROVEMENTS

Using this new design method and reference energies, we redesigned several de novo cyclic peptide binders to therapeutically relevant targets and assessed the results, visually and through Rosetta scoring metrics.

One such target is the tuberculosis DNA sliding clamp protein, DNAN, which has been identified as a potential target for the treatment of antibiotic resistant tuberculosis. The natural product cyclic peptide griselimycin has been found to have antibacterial activity against

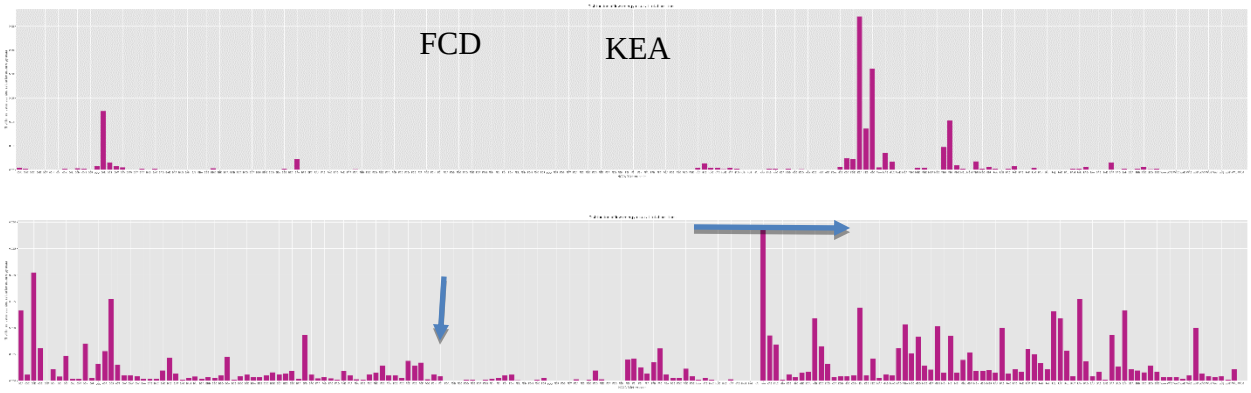


Figure 3.4 Assessing the effect of reference energies on the evaluation of the best noncanonical amino acid to place at a position. Each bar represents the number of times a specific noncanonical amino acid was evaluated by Rosetta as being the best noncanonical amino acid mutation (compared to all other noncanonicals on the chart) at a position in a set of pdbs. Before reference energies are applied (top) some noncanonical amino acids (KEA for example) are far more frequently chosen as the best residue, while others (FCD and others) are never selected as such. When the reference energies are applied (bottom) the frequency of being evaluated as the best noncanonical becomes much more evenly distributed, with almost every residue being found to be the best at least once.

mycobacterium species, but has poor pharmacokinetic properties, so there has been interest in developing variants that avoid these shortcomings[83] We have been pursuing multiple different design strategies to design de novo cyclic peptides that mimic griselimycin's binding mode and achieve antimicrobial activity. We have also been pursing the creation of cyclic peptide binders to the bacterial serine protease ClpP, an antibacterial target.[84] While we have been successful in

designing a few binders to these targets, their affinity is quite poor, making them ideal candidates for this new design protocol.

When thus new design protocol is applied to DNAN and ClpP designs the results are promising. We see increases in predicted delta-G of binding on the order of 10s of Rosetta energy units, improvements in shape complementarity, and improvements or maintaining of ligand docking predictions (Figure 3.5). Rosetta is able to find noncanonical that can reach deeper into pockets than canonical amino acids, and create new hydrogen bonding interactions that could confer selectivity.

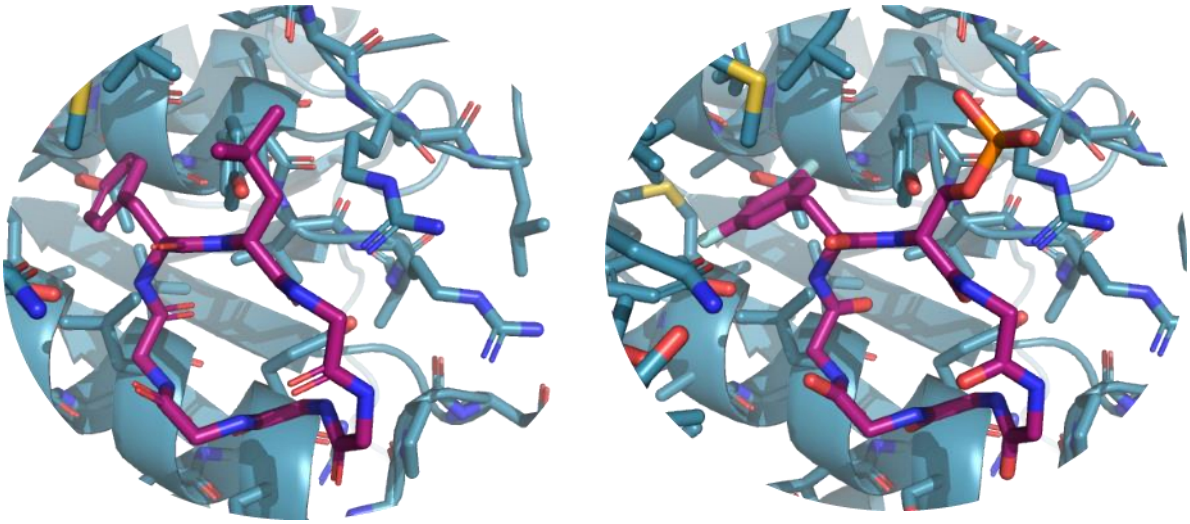


Figure 3.5 Comparison of the original *de novo* designed binder to ClpP and the design after undergoing noncanonical amino acid design. The design protocol identified several mutations that were able to create new contacts inside the binding pocket.On the left is shown the backbone of the original binder along with two residues at the interface mutated by the design protocol. One the left is the design after the mutations were made, introducing a noncanonical phenylalanine variant that has halogen substituents that better fill the pocket and a phosphoserine residue that forms a complementary charge interaction with a target arginine residue. These new contacts increase the predicted binding affinity of the complex by ~10 Rosetta Energy Units.

3.5. CONCLUSIONS AND FUTURE DIRECTIONS

We have developed a method for the incorporation and evaluation of arbitrary sets of noncanonical amino acids at peptide-protein interfaces. When applied to designs at therapeutically relevant interfaces it increases the predicted binding energy and shape complementarity. We are working on evaluating and improving the efficacy of this method by using its predictions to guide library optimizations of low affinity cyclic binders to therapeutically relevant targets. In the future, the ability to design with noncanonical will be an important step in the optimization of cyclic peptide therapeutics.

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APPENDIX A – MATERIALS AND METHODS

Peptide Synthesis

All peptides were synthesized using standard Fmoc solid phase peptide synthesis (SPPS) either in-house, or by Wuxi AppTec. Linear, protected peptides were synthesized using standard Fmoc solid phase peptide synthesis protocols using a CEM Liberty Blue peptide synthesizer with microwave-assisted coupling and deprotection steps. Peptides were synthesized with the C-terminus tethered to Cl-TCP(Cl) ProTide resin from CEM, cleaved from the resin with 1% (v/v) TFA in dichloromethane (DCM), and cyclized by a solution-phase coupling reaction prior to the final total deprotection. Crude peptides were purified based on mass via reverse phase HPLC using a Waters AutoPurify HPLC/MS system in line with a SQD2 mass spectrometer and then lyophilized. Peptides were typically purified via a water (0.1% formic acid) and acetonitrile (0.085% formic acid) gradient at 2%/min on an XBridge Prep C18 10um, 19x150mm column. Masses and purities were assessed via electrospray ionization mass spectrometry during and subsequent to purification on an SQD2 mass spectrometer.

PAMPA Assay

Passive permeability was assayed using a Corning® BioCoat™ Pre-coated PAMPA Plate System. Starting donor solutions of the peptide were prepared by adding 2mg peptide/ml DMSO solutions to 1X PPS buffer to create 5% DMSO solutions. 300 uL of peptide solution was added to the donor well and 205 uL of 5% DMSO 1X PBS was added to the acceptor well. Plates were combined overnight (~16 hours) and then separated and transferred to 96-well plates for concentration analysis using an Agilent 6230 LC/TOF. Samples were separated on a water (0.1% formic acid) and acetonitrile (0.1% formic acid) 20%/min on an Acquity UPLC BEH C18 1.7 um column.

Design Scripts

Example computational design scripts used to generate backbone and designs of membrane permeable peptides, along with README files that explain how to run them, can be found at the public github repository: github.com/JacobOConnor/OConnor_thesis_2021. Also included in the repository are the parameter files for the noncanonical amino acids used in the methods reported here, along with the design scripts and descriptions of how to run them.

VITA

Jacob O'Connor grew up in Yakima, WA, and graduated from La Salle High School in 2012. Jacob first became interested in structural biology and biochemistry when given the opportunity to visit the Fred Hutchinson Cancer Care Center as part of a high school biology field trip and shadow some researchers for a day. Seeing the crystal structure of native hemoglobin and the sickle-cell mutant excited them because of their own condition with sickle cell trait. Jacob pursued a Bachelor of Arts in Biochemistry, Biophysics, and Molecular Biology at Whitman College in Walla Walla, Washington. During their studies they had the opportunity to work with Professor Dalia Biswas to research the use of QM-MM studies to guide the development synthetic mimics of the active site of a Mo-Cu carbon monoxide dehydrogenase. This experience of performing computational and experimental research, along with coursework on protein design, inspired Jacob to search out further research experience in the field of protein design after graduating from Whitman in 2016. Jacob joined the Biological Physics Structure and Design program at the University of Washington in fall of 2016, and joined the Baker Lab in 2017. As they researched the *de novo* design of permeable and binding cyclic peptides, Jacob also engaged with science communication and science policy opportunities outside the lab. After graduating with their PhD in Biochemsitry in 2021 Jacob will apply that experience in their work as a California Council of Science and Technology Science Policy Fellow, advising the California state government on science policy topics.