# COMPUTATIONAL DESIGN OF A CYCLIC PEPTIDE FOR HRAS

by

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HRAS is a GTPase that functions as a molecular switch in key signaling pathways and is frequently mutated in human cancers. Due to its shallow surface and conformational flexibility, HRAS has long been considered "undruggable." Cyclic peptides offer advantages over linear peptides and small molecules, including increased stability, specificity, and reduced immunogenicity, making them a reasonable approach for targeting HRAS. This research presents a computational pipeline for designing high-affinity cyclic peptides for HRAS. Using a tryptophan anchor residue placed in a binding pocket of the switch region, we generated eight-residue cyclic peptides through backbone design and side chain optimization. Peptides were filtered based on energetic and geometric criteria, including ΔΔG, hydrogen bonding, shape complementarity, and hydrophobic interactions. Stable candidates were folded and passed through molecular dynamics (MD) simulations to assess rigidity and binding persistence under applied force. Top peptides demonstrated stable conformations and strong binding throughout 100 ns MD trajectories. This work illustrates a viable strategy for targeting HRAS with cyclic peptides and advances peptide-based approaches for inhibiting previously undruggable proteins.

## Acknowledgements

I would like to thank Parisa Hosseinzadeh for being my advisor for this thesis and for inviting me into the lab with open arms. Thank you, Ellen Fitzpatrick, for serving on my Thesis Committee. Thank you to Andrew Powers for involving me in this project and providing guidance. Thank you to the rest of the lab for their support throughout my time here. Finally, thank you to my friends and family who have supported me.

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# Introduction

## HRAS

HRAS, a GTPase involved in many complex but essential signaling pathways, has been mostly deemed "undruggable."[1] GTPases, working alongside regulators and effectors, are the control elements of these complex signaling pathways. Within the enzymatic class of GTPases, there is a RAS superfamily of over 170 related proteins.[2] The superfamily contains at least 35 human-related tumor oncoproteins, with HRAS being one of the founding proteins.[2] HRAS, KRAS, and NRAS are the most closely related among oncoproteins, and they are considered the most common drivers in human cancers.[3] The identification and characterization of RAS (rat sarcoma) genes as transduced oncogenes first occurred in the Harvey and Kirsten strains of retroviruses.[2] In subsequent research, mutationally activated isoforms of these oncogenes were found in human tumor cells. Mutations of RAS proteins can have various effects, however, given the roles that RAS proteins play in cell signaling, proliferation, and division, the mutations can be cancer-causing.[2] Some examples of tumor-derived mutations in HRAS include HRASG12V, which disables GTPase function and responsiveness to GTPase-activating proteins (GAPs), and HRASN116H, which enhances the basal activation of the protein by increasing nucleotide exchange activity.[2,4] Although these are only two examples of mutations, Ras mutations are found in more than 20% of all human cancers.[1,5] Particularly problematic mutations at residues G13, Q61, and G12 lead to GAP-insensitivity and disruption of the guanosine triphosphate (GTP)-guanosine diphosphate (GDP) cycle, causing RAS to be constitutively active and the promotion of tumorigenesis.[1,6] The oncogenic properties of RAS proteins have contributed to the importance of their study.

Among KRAS, NRAS, and HRAS, there is 90% similarity in the G-domain, contributing to their conserved structural and enzymatic properties.[1,7] The G-domain is generally defined as residues 1-166 and can be split between the effector and allosteric regions. Enzymatic activity – GTP hydrolysis – is confined to the effector region, but the allosteric region is responsible for interactions with membrane phospholipids.[8] The GTPase function entails the binding and hydrolysis of GTP (Figure 2). Such activity sets up these proteins as molecular switches, bound to GTP or GDP. The ability to interact with downstream effectors depends on which molecule is bound, which gives rise to the protein being "on" or "off." In addition, molecular switches undergo conformation changes, and for GTPases, the switch region is involved in the GTP-GDP conformation change. Thus, the "on" conformation differs from the "off" conformation because of which molecule is bound.[7] HRAS is considered "on" when GTP is bound and "off" when GDP is bound (Figure 1). This conformational distinction is thus tied to the activity of the protein, or the protein's ability to interact with downstream effectors. When GDP is bound, HRAS cannot interact with downstream effectors, meaning it is not activating the subsequent component of the signaling pathway it is involved in.[7] Although KRAS, NRAS, and HRAS share sequence identity at the N-terminal G-domain, their C-termini are variable. This variability leads to specificity in membrane localization and subsequent pathway involvement.[9] Regulating the activity of these signaling pathways is vital given their implications for cell growth and proliferation. As discussed, dysregulation of these pathways can be detrimental to cells. HRAS is a key component in many signaling pathways.

Ras effectors have been identified and categorized into at least 11 catalytically distinct classes.[9] Of these classes, six have been tied to Ras-stimulated cancer growth, including Raf serine/threonine kinases, class I phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3K), GEFs

for Ral (RalGEFs), GEFs for Rac1 (Tiam1), phospholipase C epsilon, and RASSF1A.[9] The Raf and PI3K are strongly validated because of the frequency of mutations leading to oncogenesis in humans and their attractiveness as drug targets.[9,10] The Ras/Raf/MEK/ERK (ERK: extracellular signal-related kinase, MEK: mitogen-activated protein kinase (MAPK)/ERK) pathway leads to the transmission of a signal from the cell membrane to transcription factors that regulate the expression of genes involved in apoptosis and the cell cycle.[9–11] Upon activation by upstream signals such as growth factor receptors, HRAS binds GTP and undergoes a conformational change that allows it to interact with and activate RAF kinases. This leads to a phosphorylation cascade through MEK and ER, ultimately regulating gene expression in the nucleus. Through this pathway, HRAS helps transmit mitogenic signals that drive cell cycle progression. Mutations in HRAS that impair its ability to hydrolyze GTP, such as at residues G12, G13, or Q61, can lead to constitutive activation of this cascade, promoting unregulated cell division and oncogenesis.

In addition to its role in the MAPK pathway, HRAS is also involved in the PI3K/AKT (AKT: serine/threonine kinase) signaling pathway, which regulates cell survival, metabolism, and apoptosis.[9,12] When activated, HRAS can recruit and activate PI3K, leading to the production of phosphatidylinositol (3,4,5)-triphosphate (PIP3) and subsequent activation of AKT. This promotes pro-survival signals and inhibits apoptotic processes, contributing to cellular resilience under stress conditions. Dysregulation of HRAS in this pathway can enhance tumor cell survival and resistance to therapy, further underscoring its importance in cancer biology.[9,12] Given the easily disrupted enzymatic function and contributions to multiple cell proliferation pathways, HRAS is considered an important target. Thus, this work sought to computationally design cyclic peptides to target HRAS.

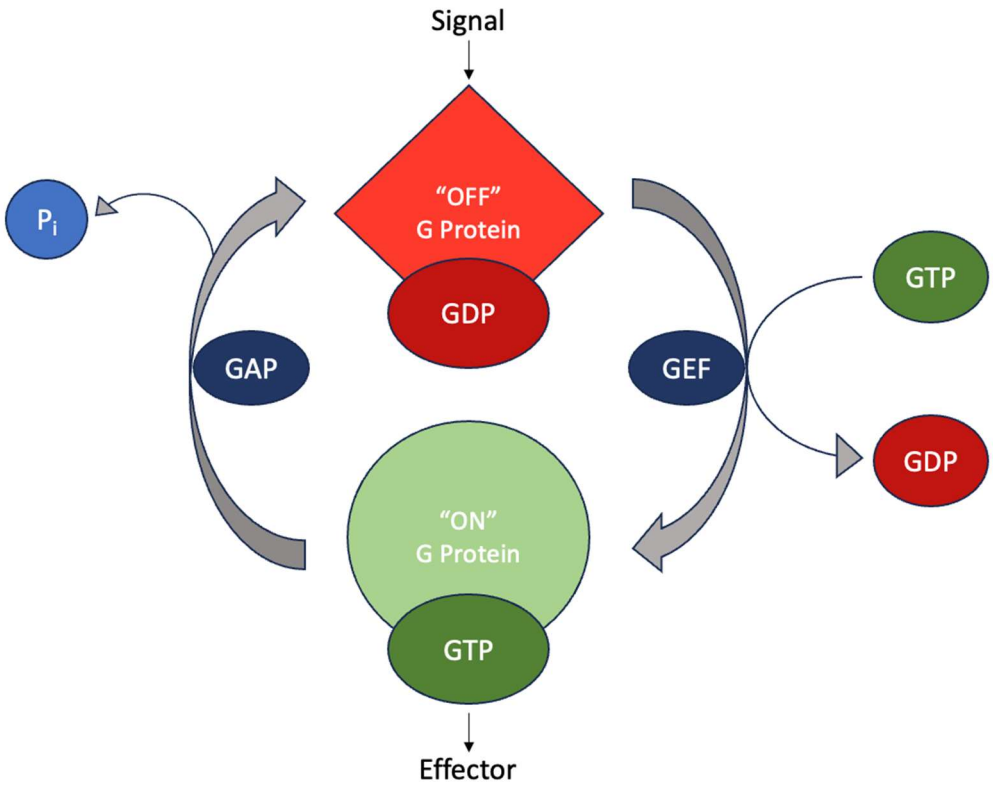



Figure 1: HRAS as a Molecular Switch

HRAS has an "on" and "off" form, depending on whether GDP or GTP is bound. GEFs and GAPs coordinate the exchange of GTP for GDP.

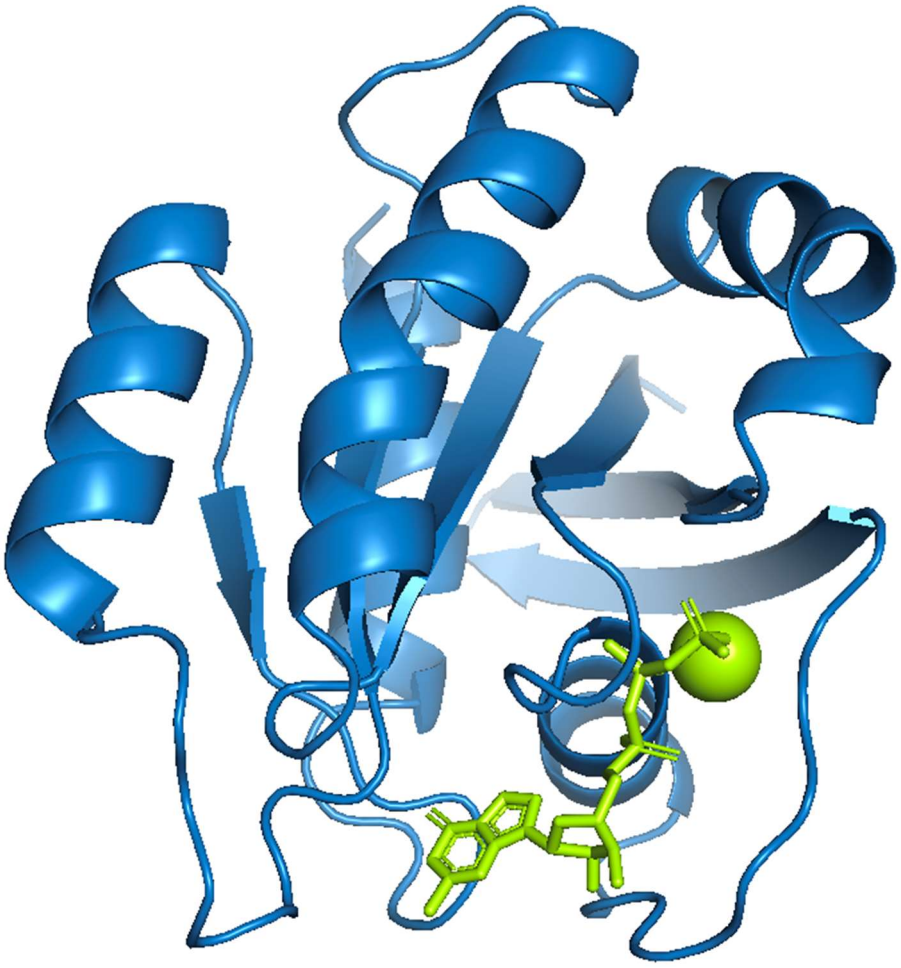


Figure 2: HRAS with GTP Bound

HRAS in blue with GTP and a magnesium ion in green.

## Computational Cyclic Peptide Design

The computational design of peptides has rapidly evolved, establishing itself as a key strategy in drug delivery, altering protein function, material science, and biotechnology. Peptides, short chains of amino acids, exhibit high specificity and potency as therapeutic agents, making them attractive candidates for targeting a wide range of biological processes. Small molecules are commonly used in conventional therapy, but peptides present many advantages over them. Specifically, peptides can have high selectivity, potency, specificity, low accumulation, and fewer side effects. When comparing peptides to proteins or antibodies, the

important benefit of peptides is decreased immunogenicity or response of the immune system to the molecule. Although their widespread benefits prompt their development and usage, their development faces challenges, including rapid degeneration *in vivo*, limited bioavailability, and conformational instability.[13,14]

One solution to some of the problems associated with traditional peptides is cyclization. Cyclization entails linking one end of a peptide to the other, thus forming a cyclic ring structure from a multi-amino acid chain.[14] Though cyclization can be done using different methods, cyclization enhances the advantages of linear peptides while addressing their limitations. For example, by restricting the peptide's spatial vibrations, cyclization reduces conformational changes, which increases binding affinity and selectivity for biological targets. This reduction in conformational changes contributes to the rigidity of the peptide, which is important for the energy barriers involved in peptide-target interactions. The rigidity specifically can reduce the entropy component of Gibb's free energy, which makes the interaction more favorable. Cyclic peptides can hold a more energetically favorable state than linear peptides because the number of possible conformations is significantly restricted by cyclization. This improved Gibbs free energy is crucial to the success of cyclic peptide binding. This is due to higher selectivity, affinity, and the tightness of the binding that occurs.[13,14] Due to the improved performance of cyclic peptides and their strong presence in the peptide drugs approved by the FDA, generation and optimization methods for cyclic peptides are crucial to their continued success.

In generating cyclic peptides, the methodology depends on the starting structure and the intended properties and performance of the peptide. In this research, the goal was to generate a cyclic peptide with high affinity and specificity to a specific site of a target protein. There are many methods used, but two commonly used methods to consider are structure-based design

(SBD) and *de novo* design of cyclic peptides. SBD is widely utilized in peptide design. However, this approach presents several limitations for cyclic peptide design and the target protein. The target protein is a conformational switch, meaning it spends time in two different conformations. This presents a challenge for this design method, as SBD usually relies on a static snapshot of the protein, which can be non-representative of the biologically relevant forms of the protein. Another limitation of SBD is prominent when developing peptides for proteins with shallow surfaces or transient binding sites. These may lack structural features for strong protein-peptide interactions, thus limiting the efficacy of this design method for proteins with these surface characteristics. As for SBD for cyclic peptides, a key limiting factor is the requirement of a co-crystal structure, a structure including both the target protein and the protein binding partner. This is a limitation as many proteins being targeted have not previously been explored and thus do not have a developed binding partner.[15] *De novo* design can overcome many of the limitations associated with SBD.

Computational *de novo* cyclic peptide design is increasingly applied as it can address key limitations of other methods. *De novo* peptide design entails generating a novel protein sequence, allowing for a large exploration of conformational space through the generation of the backbone. The backbone generation process has been improved with a novel model, CyclicCAE. This model reduces macrocycle development and generates energetically stable backbones.[16] Affinity and specificity for the target protein can also be optimized through amino acid selection.[17] These components provide advantages over SBD, but *de novo* design also addresses the limitations of SBD. *De novo* design allows for sampling of a wide range of binding conformations, thus accommodating target proteins that hold more than one conformation. For targeting shallow or transient binding sites, *de novo* design can optimize the binding interface even without a deep

binding pocket.[15] The sequence optimization step in the de novo pipeline is key to overcoming this issue, as key residues can be prioritized to maintain target-peptide interactions. With the benefits of using *de novo* design for cyclic peptides, some methods can be utilized to suit a specific target protein and desired interaction.

One such method is "anchor extension", which was initially applied to designing a peptide that targeted the active site of an enzyme. This method entails the identification of a moiety that is known to bind to or have a strong interaction with a specific site on the target protein. This moiety, likely an amino acid residue, serves as the *anchor* from which the peptide generation stems.[15] The remainder of the computational pipeline is similar to traditional *de novo* design, though the anchor residue will be held constant. The outcome and benefit of this method is a strong, high-affinity, and specific interaction between the generated peptide and the target protein. This is due to an existing interaction with the anchor residue and the binding site being maintained throughout, and more interactions can be optimized further along in the process. This methodology presents a unique opportunity to generate a high-affinity cyclic peptide for the target protein, HRAS.

# Methods

## Protein Visualization and Anchor Identification

The HRAS protein in its GTP-bound form conformation was obtained from the Protein Data Bank (PDB). Additional molecules (GTP) and ions (magnesium) were removed. The protein was placed in context with a tryptophan anchor residue. This placement and selection of the anchor residue were, in part, informed by previous work establishing the Switch I/II pocket and the ability of indole compounds to bind in the pocket.[3] The anchor residue acts as the center of the cyclic peptide, which will have four residues on one side and three on the other to form an eight-residue cyclic peptide. Generating the cyclic peptide involves backbone generation and side chain design, which results in many unique structures. These structures are then filtered, folded, and run through computational simulations to find stable peptides with high affinity and specificity for the binding pocket of the target protein.

## Backbone Generation

With the anchor residue in context with the target protein, the first step is to generate a basic peptide to consider how an eight-residue cyclic peptide will fit with the geometry of HRAS. This step involves extending glycine residues from the anchor residue in different conformations. First, Generalized Kinematic Loop Closure (genKIC) produced backbones to train CyclicCAE. GenKIC adds glycine residues to either side of the anchor and then randomly manipulates the torsion angles of the glycine residues to search a wide conformational landscape (all possible conformations). The PDB containing HRAS and the anchor tryptophan is used as the input for genKIC, and the output is numerous backbones with different conformations containing seven glycine residues and one tryptophan residue. Glycine is used in backbone

generation because it has the simplest side chain of amino acids, allowing for neglect of interactions due to side chain size, charge, and polarity.

Two outputs obtained from genKIC were used to train a superior backbone-generating model, CyclicCAE. The model uses continuous backbone generation instead of stochastic generation, so the input guides the model to a refined conformational landscape. In addition, the model operates as an autoencoder. This model runs independently of the target protein, generating structures with influence from the genKIC backbone. The generated backbones are then placed in context with the target protein. These in-context structures are obtained as PDBs. This model produces energetically favorable backbones, allowing for easier generation of stable peptides.

**Side Chain Design**

With the outputs from the backbone generation model, side chains are designed by exchanging the hydrogen of the glycine to change the identity of the residues in the peptide. This is done using a design script, which completes multiple steps for each backbone input. The steps are MinMover, adding one proline residue, using genKIC to apply small perturbations, designing the sequence, and relaxing the output. MinMover is used to find the lowest energy (most stable) conformation of the backbone and acquaint it with Rosetta's energy function. One proline is added because it has fewer torsional degrees of freedom than other amino acids, giving some rigidity to the peptide. Applying perturbations allows for sampling around the conformation of the input. This results in a refined conformation before designing the remaining side chains. After designing the peptide sequence, the output is relaxed to allow the peptide to energetically adapt to the generated sequence.

The design script was run using three smaller batches of generated backbones. Of 550 backbones, they were separated into folders containing backbones 1 – 250, 251 – 500, and 501 – 550. This was done to increase the number of resultant constructs, increase the efficiency, and improve their scores. Each folder was submitted separately. After running the design script, score files were obtained for each peptide, so the designed peptides can be filtered.

**Filtering and Folding**

The filtering step involves cutoffs for specific metrics for each peptide to remove peptides that do not perform well. Some metrics used in filtering include a low (negative) $\Delta\Delta G$, a low (negative) Rosetta score, at least 70% shape complementarity, at least two hydrophobic interactions between the peptide and target, and at least three backbone–backbone hydrogen bonds. For one round of designs, the filtering resulted in a 25-fold decrease in plausible designs. This step identifies stable peptides that demonstrate ample affinity and specificity within the context of the target protein.

Designs that pass the filtering step are folded. The folding process uses genKIC and the sequence of the peptide to determine whether a peptide with its specific amino acid sequence will spend most of its time in its conformation. This is done by considering the Ramachandran preference of each amino acid. GenKIC applies small perturbations to determine if the phi and psi torsional angles are within the preferred torsional space of the specific amino acids. A metric, PNear, is an in-silico validation of the stability of the generated peptides after folding, and it determines whether the peptides spend much of their time in the given conformation. Peptides with a high PNear ($\geq 0.8$) are considered stable and have passed the folding step.

Stable peptides are then analyzed to determine whether they make sufficient polar interactions with the binding pocket of HRAS. Having substantial polar interactions ensures

binding specificity, indicating that the peptide has some bias towards one specific region of the protein. In addition, there must be sufficient interactions within the peptide because they contribute to the stability of the cyclic structure. Peptides with sufficient internal and external polar interactions are further analyzed via molecular dynamics simulations.

**Molecular Dynamics (MD) Simulations**

MD simulations are a method of *in silico* evaluation of the peptide in more realistic conditions. The trajectories can be used to assess how the position of the peptide changes over time within the context of the target. For the monomer MD simulation, the peptides were run for 100 ns in a neutral water solvent. The root mean square distance (RMSD) was found by comparing the position of the peptide at any time point t to the position at time t = 0 ns. This metric can show whether the peptide is intrinsically disordered or contradicts the PNear. Peptides with a stable RMSD throughout the simulation would be considered rigid and offer a second *in silico* validation of the stability of the peptide's conformation. In contrast, a peptide that has an unstable RMSD, particularly one that starts at one value and jumps up or down to another, would not be considered stable and may be sampling a second conformation. Negative controls for this simulation were a cyclic peptide containing only glycine and the anchor tryptophan and a cyclic peptide with a PNear of 0.

The second MD simulation was an in silico evaluation of the dissolution of the peptide from the target. Here, peptides were run for 100 ns in a neutral solvent and subjected to a constant force. The force pulls the peptide away from the binding pocket of the target. The distance in angstroms between the geometric center of the target protein and the peptide is plotted over time to discern whether the peptide is released from the pocket. Peptides that stay bound will have a minimal fluctuation of distance (between 1.8 and 2.4 Å), whereas peptides that

dissociate will reach greater distances (>2.5 Å). The large distance value is due to the initial distance between the protein and the peptide. The distance can also be found by subtracting the original distance to obtain the movement from the original position in angstroms.

To visualize the results of the monomer MD trajectories, the PDB file and trajectory file were loaded into PyMOL. Once loaded, oxygen molecules and additional ions were removed. The peptide was oriented, and hydrogen bonds within the peptide were visualized. The peptide's state relative to its reference state (state 1, initial) is given as RMSD for each time point of the simulation. The RMSD is then plotted over time to evaluate the conformational stability of the peptide. For complex MD trajectories, the PDB file and trajectory file were loaded into PyMOL. Once loaded, oxygen molecules and additional ions were removed. The absolute distance between the center of HRAS and the peptide was found for each time point. This was then plotted over time to determine whether the peptide becomes unbound during the simulation.

# Results

## Filtering

After completing the design protocol for the peptides, a score file was generated for each subset of inputs (1-250, 251-500, and 501-550). This score file served as the input for Google Colab, which was utilized to filter the outputs and conduct score analysis. Multiple rounds of score files were analyzed; to illustrate the extent of the filtering, one round had an input of 9698 and an output of 371 (Figures 3 and 4).

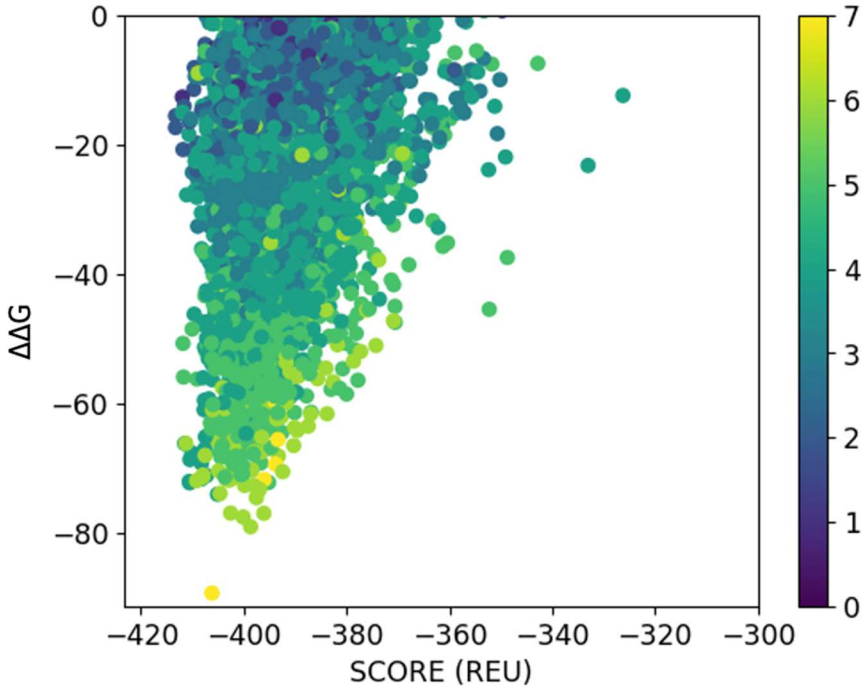



Figure 3: Before Filtering Score (REU) vs ΔΔG

Distribution of peptides based on ΔΔG and score (REU) before filtering. The color bar represents the number of backbone-backbone hydrogen bonds. The input for this round of filtering was 9698 peptides.

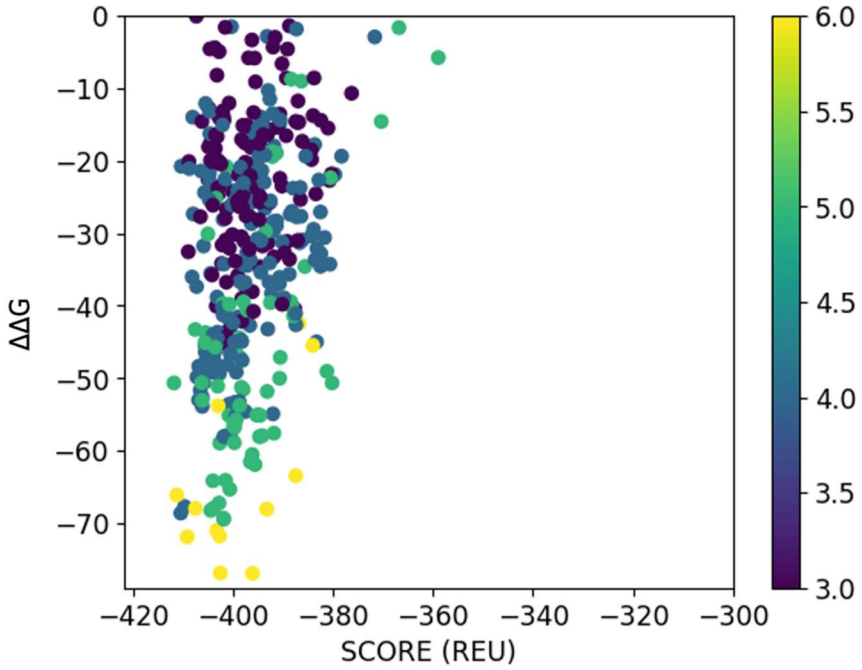



Figure 4: After Filtering Score (REU) versus ΔΔG

Distribution of peptides based on ΔΔG and score (REU) after filtering. The color bar represents the number of backbone-backbone hydrogen bonds. The number of peptides in the distribution is 371.

**Folding**

Peptides that passed through filtering were folded and assessed using a value called PNear. PNear represents how likely the peptide is to spend time in its given conformation. It ranges from 0 to 1, with 0 meaning the peptide is unlikely to spend time in the given conformation and 1 meaning the peptide will spend all its time in the conformation. Thus, peptides with high PNears will spend time in the conformation and can be considered stable by this metric. Of the designed and folded peptides, 14 were found to have PNear greater than 0.8 (PNear was not obtained for some peptides) (Figure 5 and 6). The high PNear peptides then underwent MD simulations as described for further in silico validation of stability and to assess binding character.

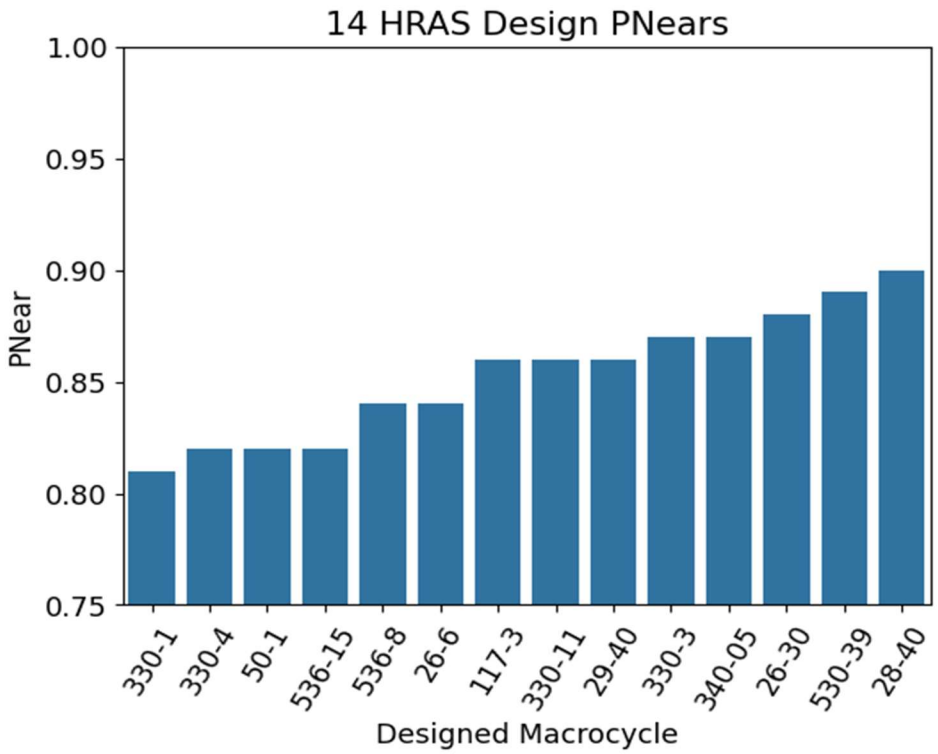



Figure 5: PNear for 14 Designed Macrocycles

Folded peptides with PNear values above 0.8 are displayed here (additional peptides with PNears above 0.8 are not shown).

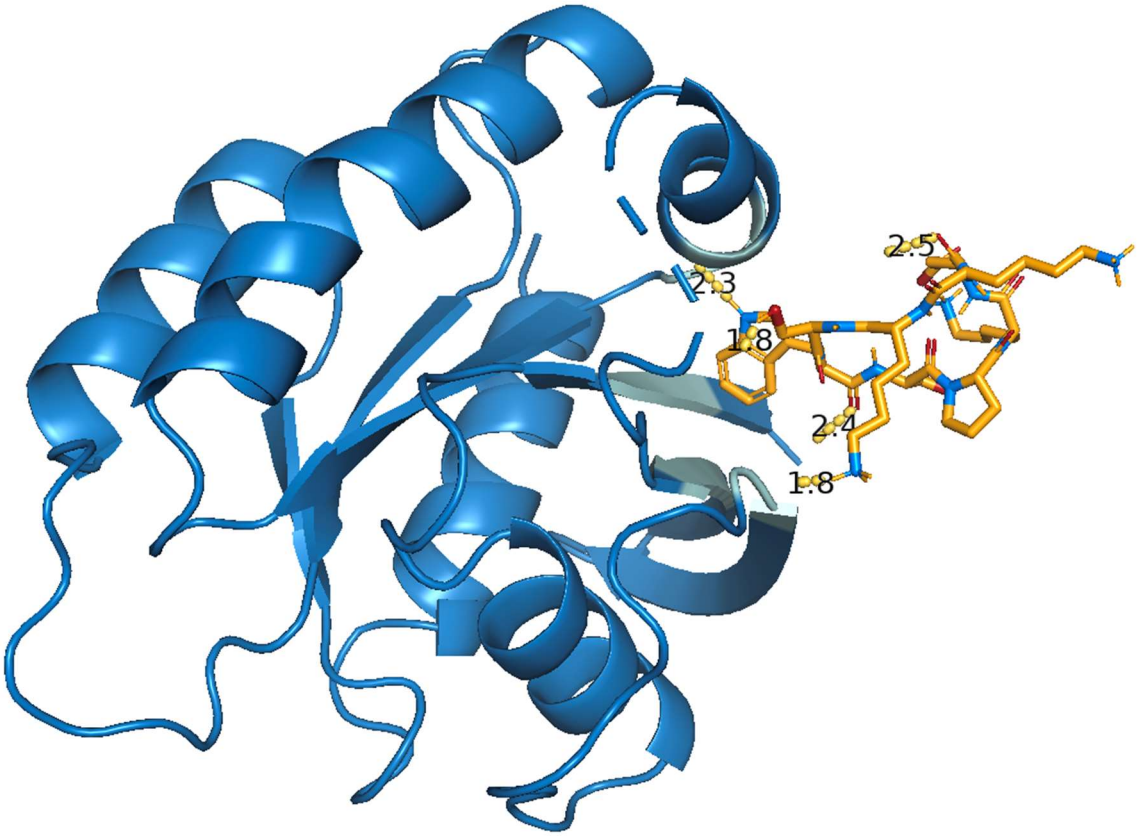



Figure 6: Peptide with a PNear of 0.89

Folded peptide 520-39 with a PNear of 0.89. The peptide is shown in orange, with oxygen atoms in red and nitrogen atoms in blue. HRAS is in blue, and the light blue indicates residues interacting with the peptide. Yellow dashes represent polar hydrogen bonds between the peptide and HRAS.

**MD Simulations**

Monomer MD simulations were run for each peptide with a PNear greater than 0.8. Monomer MD produced RMSDs, allowing for comparison of the conformation of the peptide at each time step to its initial position. The negative controls, a glycine peptide and a peptide with a PNear of 0, displayed poor conformational stability. This is evident as the RMSD was consistently high (>1). Peptides displaying conformational stability have low RMSDs throughout

the simulation. Examples of stable peptides include peptides 330-03 and 330-04 (Figure 7, Supplemental Figure 2).

Complex MD simulations were also run and allowed for assessment of binding strength. Peptides that remained bound during the simulation maintained a relatively steady absolute distance. The absolute distance between the protein and peptide geometric centers should be consistent during the trajectory. Peptides that become unbound display jumps to greater distances. Peptides that remained bound include peptides 530-39 and 330-4 (Figure 8, Supplemental Figure 3).

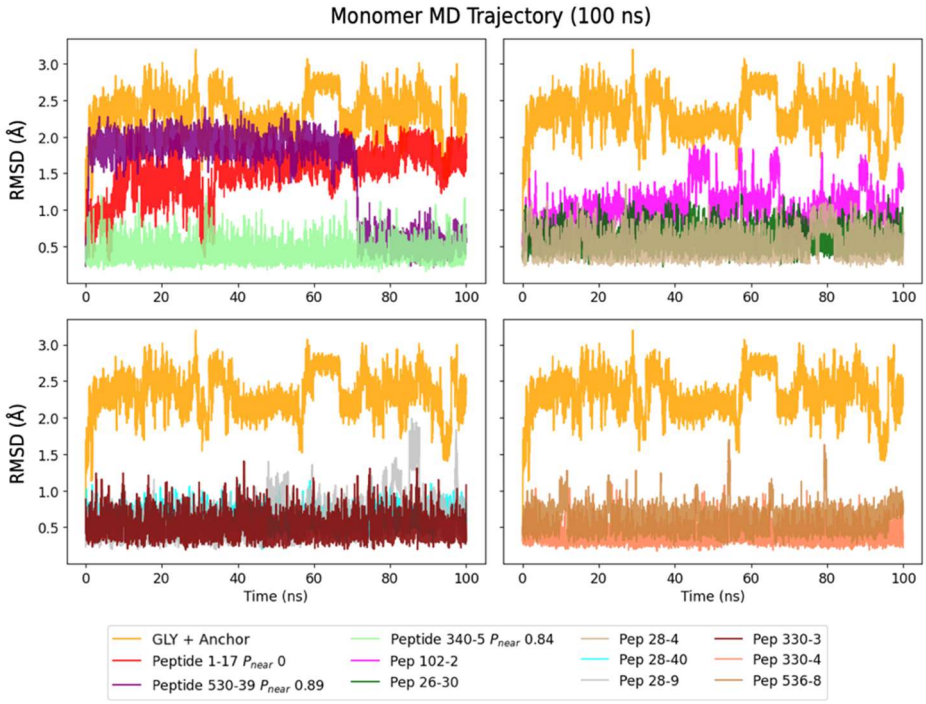



Figure 7: Monomer MD Trajectory

Monomer MD Trajectory run for 100 ns in neutral solvent. Glycine and PNear of 0 controls are included. Conformationally stable peptides are represented by bands remaining near the bottom (~0-1 Å).

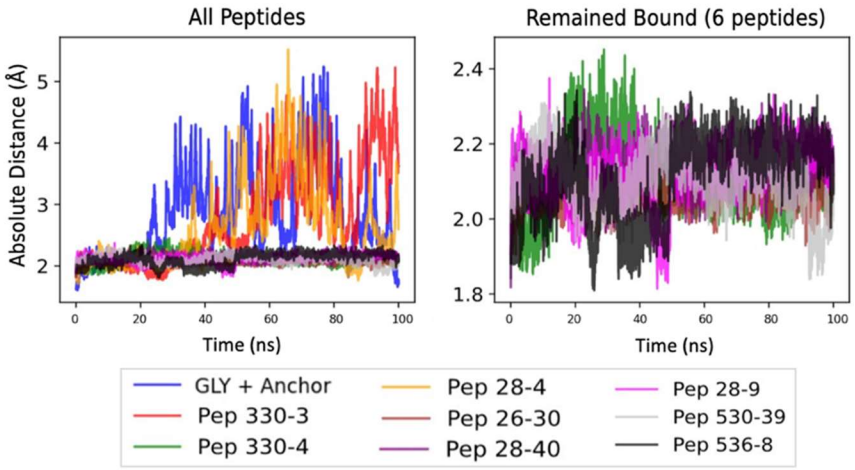



Figure 8: Complex MD Trajectory

Complex MD Trajectory run for 100 ns in neutral solvent. A glycine control is included. All peptides are shown on the left, and peptides that remained bound during the simulation are also shown on the right. Peptides that remained bound had absolute distances between the geometric centers of the peptide and HRAS remaining constant and low (~1.8-2.4 Å).

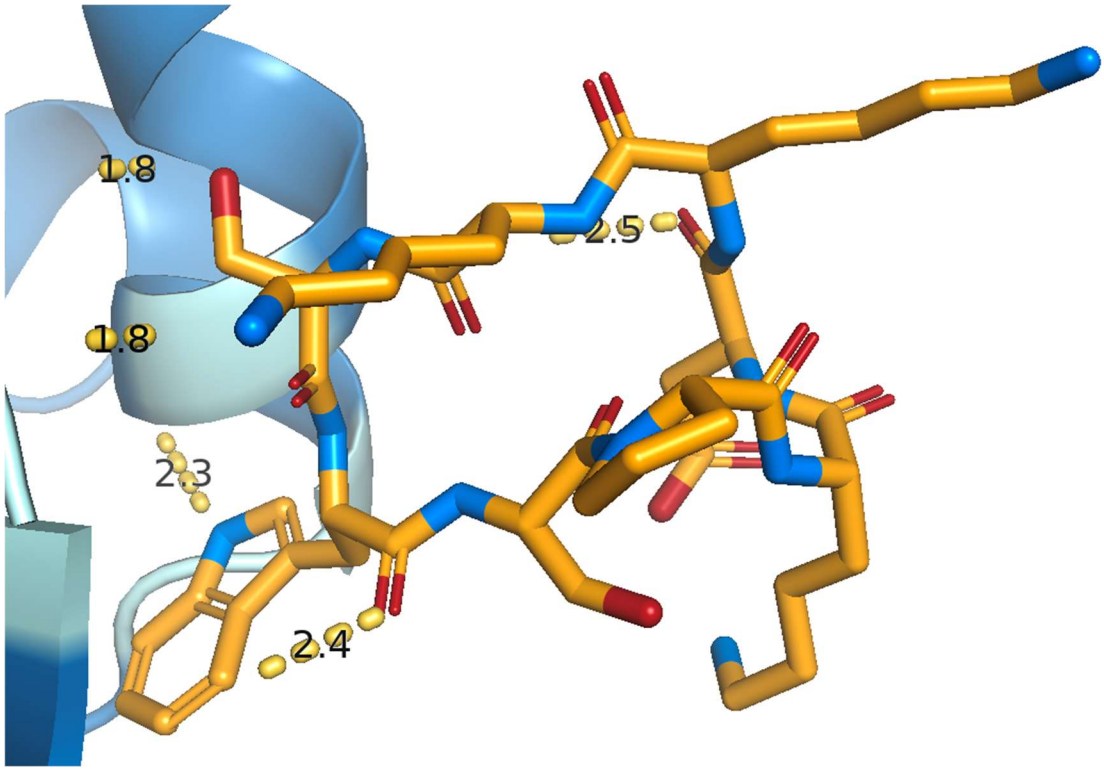
## Discussion/Conclusion

This work illustrates the computationally validated design of multiple peptides for HRAS. These peptides are stable and display high affinity and specificity for HRAS. This *in silico* work allows for future experimental validation to be conducted. To experimentally validate the peptides, they will be tested using biolayer interferometry. This will confirm the peptide's ability to bind HRAS by evaluating the kinetics and affinity of the interaction. An additional direction for this research would be to determine the effect the peptide has on the function of HRAS. While this work has identified potential binding peptides, it does not elucidate the effect this binding will have. In terms of the therapeutic possibilities of cyclic peptides such as these, it is important to understand what function the peptide serves. Identifying cyclic peptides for HRAS is a step towards providing a novel strategy to inhibit its activity, which would be important in cases where it is mutated and constitutively active.

Cyclic peptides offer enhanced stability, binding affinity, and specificity compared to linear peptides and small molecules, making them promising candidates for targeting proteins involved in pathways involving many protein-protein interactions. Developing a framework for computationally designing cyclic peptides with these target proteins could allow for the streamlined design of these challenging targets. Thus, this work indicates progress in targeting challenging proteins and in developing a computational pipeline.

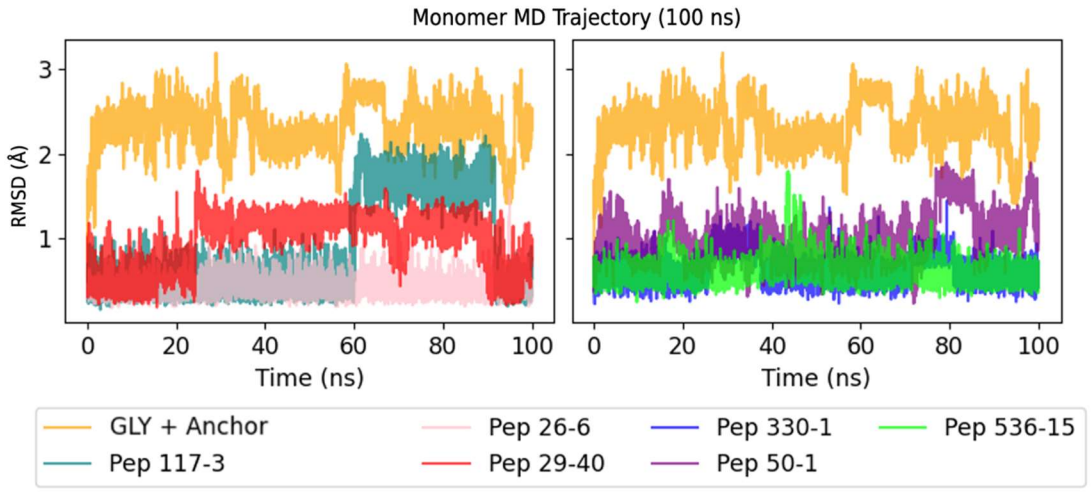
# Appendix

## Supplementary Figures



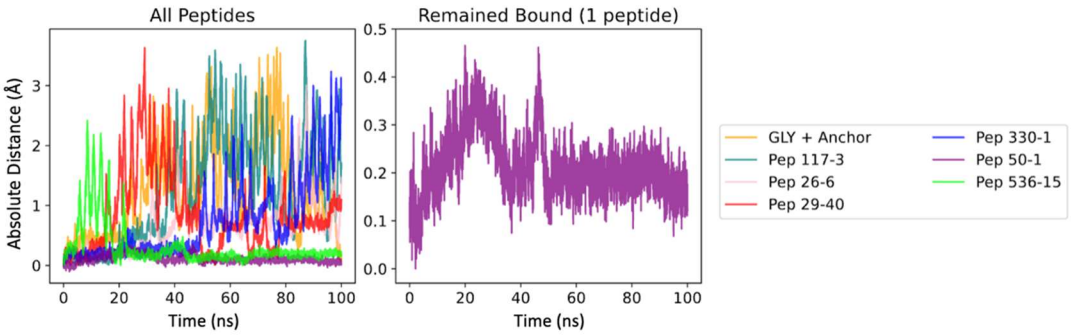

Supplementary Figure 1: Peptide with a PNear of 0.89

Folded peptide 520-39 with a PNear of 0.89. The peptide is shown in orange, with oxygen atoms in red and nitrogen atoms in blue. HRAS is in blue, and the light blue indicates residues interacting with the peptide. Yellow dashes represent polar hydrogen bonds between the peptide and HRAS.




Supplementary Figure 2: Monomer MD Trajectory

Monomer MD Trajectory run for 100 ns in neutral solvent. A glycine control is included. Conformationally stable peptides are represented by bands remaining near the bottom (~0-1 Å).




Supplementary Figure 3: Complex MD Trajectory

Complex MD Trajectory run for 100 ns in neutral solvent. A glycine control is included. All peptides are shown on the left, and the peptide that remained bound during the simulation is also shown on the right. Peptides that remained bound had absolute distances between remaining constant and low (~0-0.5 Å).

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