Computational Design of Agouti Signalling Protein as a Drug to Treat Metastatic Melanoma

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***Abstract*—Our goal is to engineer Agouti Signaling Protein (ASiP) for optimal selectivity and binding affinity to Melanocortin Receptor 1 (MC1R) so that it can be delivered in tandem with cisplatin to increase treatment efficacy for melanoma.**

***Index Terms*—ASiP, Drug, Melanoma, In-Silico, In-Vitro, CADD.**

I. Introduction

CURRENTLY, a diagnosis of metastatic melanoma has a fatal prognosis with a life expectancy of 6 - 12 months [2]. Melanoma has a unique feature in that it is resistant to chemotherapy because melanosomes sequester the treatment agents [1]. For Melanocortin Receptor 1 (MC1R), a cell treated with a-MSH will have greater melanosome production whereas treatment with ASiP decreases melanosome production below basal levels [3]. ASiP would function as a drug, in tandem with cisplatin, by reducing the number of melanosomes present in the cell, which would allow the chemotherapeutic to reach its intended target.

II. Design Constraints

The process of producing a large enough quantity of protein in a pure enough form for biological testing is both time consuming and expensive. Additionally, since there are 20 different amino acids, even the design of a relatively small number of residues presents an enormous number of possible mutants. For example, for a sequence with 5 design residues, there are 20^5, or 3.2 million possible mutants that could be created. It is necessary to drastically reduce the number of possible mutants, not only to produce a mutant sequence to test in a reasonable period of time, but also to reduce the number of necessary syntheses to keep the overall costs reasonable.

There is an additional constraint imposed on the design of any protein, which is the proteins interaction with the solution that it is designed for. In our situation, the goal is to create a protein that will be administered as drug into a cellular environment, which means an aqueous environment. It is therefore essential that, overall, the protein not be too hydrophobic even if we predict that increasing hydrophobicity will increase affinity to the target receptor. In other words, we need to balance affinity with solubility.

III. Background

A. GPCR

G Protein-Coupled Receptors (GPCRs) play a central role in many biological processes and are linked to a wide range of disease areas ranging from feeding disorders to organ failure. GPCRs are expressed in every type of cell in the body where their function is to transmit signals from outside the cell, across the cell membrane, to signalling pathways within the cell. GPCRs also transmit signals between cells, and between organ systems.

GPCRs are characterized by 7 transmembrane alpha helices and are activated by an external signal which could be a ligand or other signal mediator. The binding of the signal causes a conformational change in the receptor, which in turn activates the G protein. The additional downstream effect depends on the class of GPCR. GPCRs are frequently divided into 6 classes (A-F) based on sequence and function similarity [4]. GPCRs function as amplifiers for the signals that they transmit; a small amount of signal reaching the GPCR can result in a large scale downstream effect within the cell.

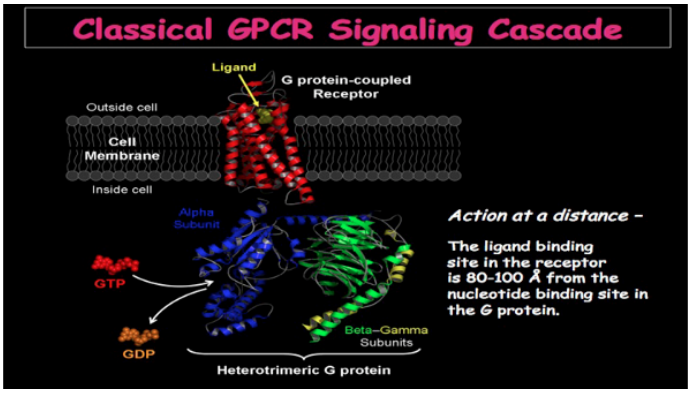

Fig. 1. GPCR Signal Cascade. Image credit cmbi.ru.nl

Formed by over 1000 members and accounting for more than 4% of mammalian genomes, the GPCR superfamily is the largest and single most important family of drug targets in the human body [5] [6]. GPCRs are the site of action of nearly 40% of current drugs. Six of the top 10 and 60 of the top 200 best-selling drugs in the US in 2010 target GPCRs, generating multi-billion dollar sales annually [7]

B. Melanocortin System

The melanocortin system (MC) affects a wide range of mammalian biological activities which range from controls over feeding behavior, sexual behavior, metabolism, and pigmentation. The MC system consists of 5 different G-Protein Coupled Receptors, designated MC1-5, and their ligands.

The ligands belong to three different classifications; agonist, inverse agonist, and neutral antagonist. The agonist ligand for all receptors is Melanocortin Stimulating Hormone (a-MSH), which activate the system. The inverse agonists drop the system below basal levels and are either Agouti Signalling Protein (ASiP) or Agouti Related Protein (AgRP) depending on which system is being studied. There are also neutral antagonists made up of members of the beta-defensin family which prevent the agonist(s) from binding [8].

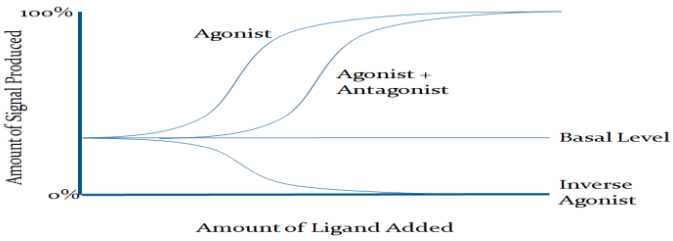

Fig. 2. GPCR Ligand Behavior. Image Credit springerimages.Biomedicine

In the Melanocortin Receptor 1 (MC1R), a cell treated with a-MSH will have greater melanosome production whereas ASiP decreases melanosome production. Melanosomes produce melanin which is often physiologically expressed as dark pigmentation or coat color. Production of melanosomes is controlled through the intermediary of cAMP.

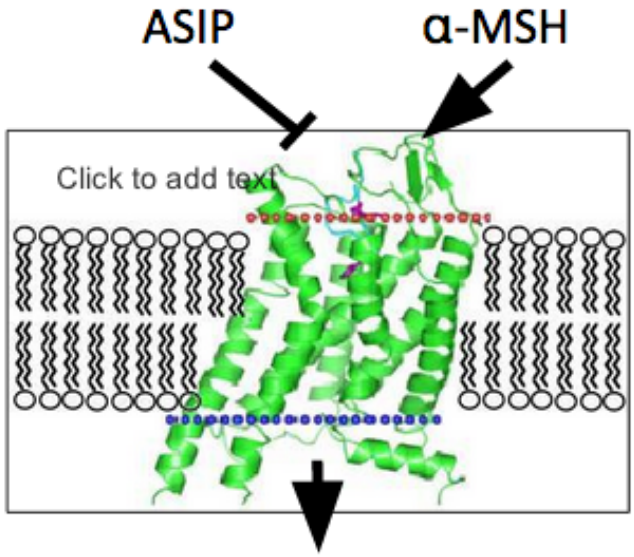

Fig. 3. Signal Cascade at MC1R. Image Credit: Jillian Miller

All these ligands are small and cysteine rich, which means that the majority of of their tertiary conformation is determined by disulfide bonding. ASiP and AgRP have an inhibitor cysteine knot(ick) fold [9], which is a toxin-like fold that had never before been observed in mammals.

ASiP and AgRP are the only known endogenous (naturally occurring as a part of a system) inverse agonists for a G protein-coupled receptor.

This makes the melanocortin system an excellent model for better understanding how GPCRs work, providing insight into what is happening in many other GPCR systems. This research has applications in treating melanoma, metabolic disorders such as diabetes, regulating feeding behavior, vitamin D, increasing UV protection. Additionally, disulfide rich proteins are commonly used as scaffolds for therapeutics (drugs). If we can design drugs that work like inverse agonists many diseases could simply be turned off. For example, research conducted by the Millhauser lab and its collaborators have shown that treatment with ASiP leads to a 2-3 fold increase in the efficacy of chemotherapeutics and that treatment with ASiP prior to chemotherapy would allow metastatic melanomas, which are naturally resistant to chemo, to be treated effectively [3]. Furthermore, it has been shown that altering the potency domain of AgRP by adding positive charge dramatically increases feeding behavior [10], which could be used as a treatment for cachexia, a wasting disease that is a common side effect of both chemo and HIV treatment. Cachexia can also be caused by cancers and HIV themselves as well as by bacteria.

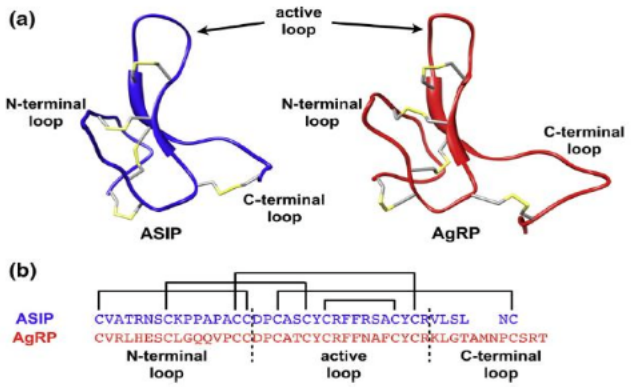

Fig. 4. (a)Tertiary Structures (b)Cystine Bond Sequences [3]

Conformation of wild-type ASiP was determined [8] in order to understand how it binds to MC1R.

C. Melanoma

Cancer of the skin is the most common type of cancer. Melanoma is primarily caused by ultraviolet radiation from the sun [11] and affects people of all skin pigmentations but is most common in lighter skinned people [12]. For 2013, The American Cancer Society estimated that 76,690 new melanomas will be diagnosed in the US and that 9,480 Americans were expected to die of melanoma [13].

While early stage melanomas can be treated very successfully through surgical removal, metastatic melanomas are aggressive and cannot be treated with chemotherapy. Melanoma has a unique feature in that it is resistant to chemotherapy because melanosomes sequester treatment agents [1]. Melanomas resilience to chemo results in a generally fatal prognosis with a five-year survival rate less than 10% [2] and a median life expectancy of 6-12 months after diagnosis.

D. Computational Drug Design

Rational drug design is the inventive process of finding new medications based on the knowledge of a biological target. The phrase “drug design” is somewhat misleading. Fundamentally, drug design is ligand design, or the design of a biomolecule that will bind tightly to its target [14]. In contrast to traditional methods of drug discovery, which rely on trial-and-error testing of chemical substances on cultured cells or animals, rational drug design conducts all preliminary experiments computationally, or in-silico. There are two requirements for a biomolecule to be selected as a drug target. The primary requirement is evidence that modulation of the target will have therapeutic value. This knowledge generally comes from experimental evidence such as disease linkage studies that show an association between mutations in the biological target and certain disease states. The second requirement is that the ligand is ”druggable”, or that it is capable of binding to its target and that its binding activity can produce the desired downstream effect. Typically a drug target is a key molecule involved in a particular metabolic or signaling pathway that is specific to a disease condition or pathology such as a transmembrane receptor. Some approaches attempt to inhibit the functioning of the pathway in the diseased state by causing a key molecule to stop functioning. Drugs may be designed that bind to the active region and inhibit this key molecule. Another approach may be to enhance the normal pathway by promoting specific molecules that may have been affected in the diseased state. Additionally, drugs can be designed to bind to a signalling receptor to either ramp up or turn down a downstream cellular activity. In all cases, it is tremendously important that designed drugs do not affect any other important “off-target” molecules or anti-targets since drug interactions with off-target molecules may lead to undesirable side effects [15].

There are two major types of drug design, ligand-based drug design and structure-based drug design. Ligand-based drug design, or indirect drug design, relies on knowledge of other biomolecules that bind to the biological target of interest. Structure-based drug design, or direct drug design, relies on knowledge of the three dimensional structure of the biological target obtained through methods such as x-ray crystallography, or NMR spectroscopy [16]. If an experimental structure of a target is not available, it may be possible to create a homology model of the target based on the experimental structure of a related protein. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target can be designed.

Computational design uses algorithms that predict 3d structure and molecular dynamics to allow target-ligand interactions and ligand-solvent interactions to be modeled and engineered in-silico.

Ideally, the computational method will be able to predict affinity before a compound is synthesized and hence in theory only one compound needs to be synthesized, saving enormous time and cost. The reality is that present computational methods are imperfect and provide, at best, only qualitatively accurate estimates of affinity. In practice it still takes several iterations of design, synthesis, and testing before an optimal drug is discovered. Computational methods have accelerated discovery by reducing the number of iterations required and have often provided novel structures [17] [18].

IV. Methodology

A. Identify the Design Region

We have strong experimental evidence to suggest that 6 residues within ASiPs C-terminal loop conferred much of the ligands specificity to MC1R [19] through the receptors first extracellular loop [3]. The design focus of this experiment was to optimize the sequence of those 6 residues to maximize affinity between MC1Rs first extracellular loop and ASiPs C-terminal loop, therefore facilitating optimal selectivity.

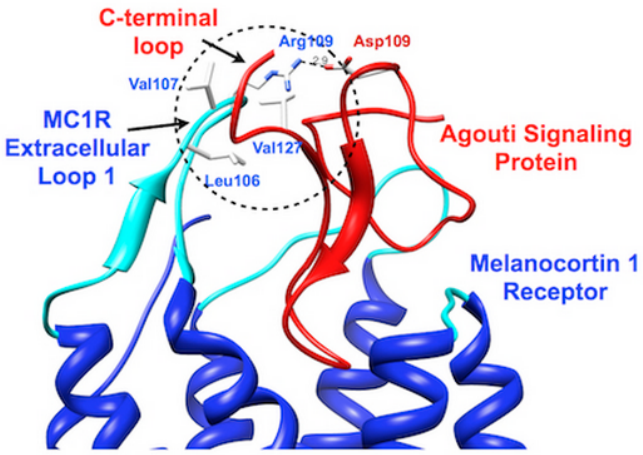

Fig. 5. ASiP C-terminal loop interacting with MC1R EC loop 1 [3]

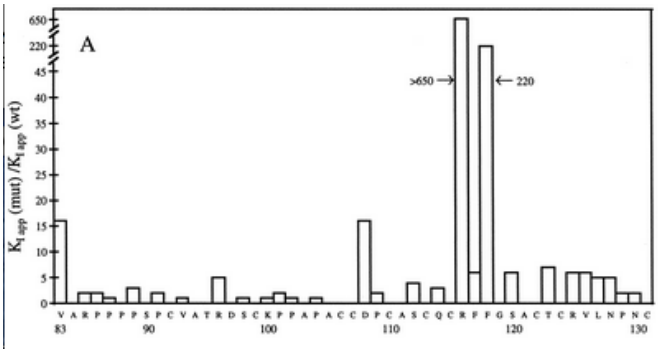

Fig. 6. In-Vitro Alanaine Scan of Design Region [19]

B. Calibration to Known Biochemistry

We sought to calibrate our predicted output by performing a computational alanine binding scan and comparing changes in in-silico binding to the in-vitro alanine scan results. We chose the Docking2 protocol on the ROSIE (Rosetta Online Server that Includes Everyone) server supported by the Gray Lab at Johns Hopkins University as our scoring algorithm for performance, resource allocation, and speed reasons [20] [21]

[22]. Rosetta has repeatedly been shown to produce excellent results in predicting binding between peptides and globular proteins [23] [24]. Having a supported server allowing us to perform our docking runs meant that we did not have to find our own cluster to perform runs on. Finally, Docking2 makes use of Rosettas local docking algorithm which allowed us to use our previous knowledge of the ASiP-MC1R interface and upload a PDB with the peptide already reasonably positioned with regards to the receptor which allowed us to skip global docking and thus save an enormous amount of computational time.

The computational alanine was performed by first using RosettaDock to score Wild Type (WT) ASiP affinity for MC1R. We then generated alanine point mutations for each of the design residues and docked those to MC1R. Finally, we compared the docking scores to the Ratio of Apparent Dissociation Constant reported in the in-vitro Alanine Scan.

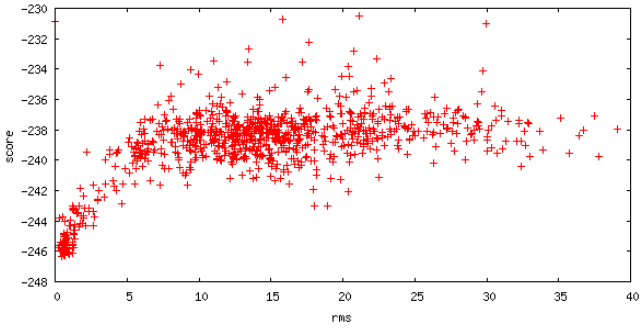

Fig. 7. Ideal RosettaDock Scoring Funnel [20]

The ideal scoring funnel shown above provides a visual reference for the validity of the minimal energy score output. Unfortunately, we did not get an ideal minimization funnel with our first data set. Instead, we got erratic score predictions as can be seen in visual output of one of our runs shown below.

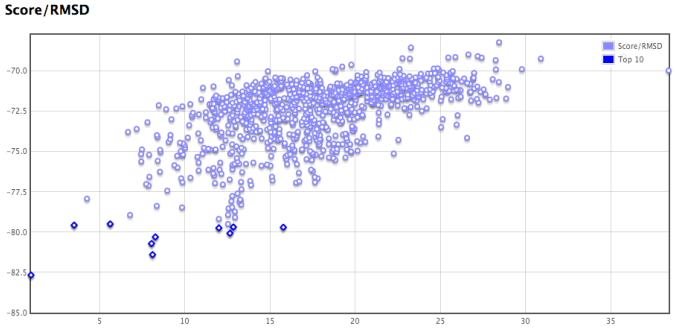

Fig. 8. Scoring Funnel from our first Docking runs [20]

In addition to the visual indication that our output was not funneling well, we observed scores that did not consistently match with known biochemistry. It was determined that our inconsistent results were caused by incomplete sampling. The Docking2 algorithm only provides 1000 decoys and it is not possible to adjust this parameter. We increased our sample size by submitting each docking task ten times for a total of 10,000 decoys. The additional sample size resulted in very consistent scoring results and a minimization funnel that is very close to the ideal.

A large part of our interest in computationally replicating the in-vitro alanine scans was to identify a constant that would allow us to relate changes in docking score to the experimentally observed Kiapp ratios shown in 6

C. Sequence Optimization

To find a sequence for our design region that would optimize affinity for MC1R, we used the Seq-Tol design algorithm through the ROSIE server at Johns Hopkins University. The application uses biological knowledge to predict the relative frequencies with which amino acid residues are predicted to be ”tolerated” without significantly compromising the stability of a given protein-protein interface and the respective interacting partner proteins [25] [26].

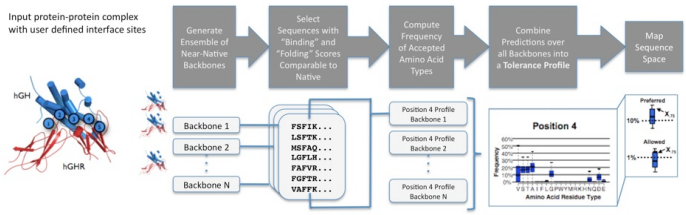

Fig. 9. ROSIE Seq-Tol Application Workflow [25] [26]

We provided a PDB file of Wild Type ASiP docked to MC1R as it input, and specified the 6 residues on ASiP that we wanted to optimize. During the first step, Rosetta scores the native interface. Next, the algorithm removed the 6 native residues and generated a library of biological pseudo-random 6-mers which were then docked to MC1R in the native position and scored. Sequences with folding and binding scores near or significantly better than the starting sequence were saved and considered to be “tolerated”. At the end of a simulation, the frequency with which each amino acid type appeared in the list of tolerated sequences was calculated and used to generate a position-specific ”tolerance profile” for each interface site.

D. Computational Mutation of Native Ligand

ROSIE currently does not return the structure of the ligand with the optimal design sequence, so we manually mutated our ligands PDB file using pyMol. By sampling multiple trials, we determined that the rotameric options that pyMol provides are not relevant to our experiment because Rosetta will determine optimal conformation during the preceding docking runs.

E. Prediction of Mutant Performance

We repeated the Computational Alanine Scan described in Calibration to Known Biochemistry section on our designed mutant to compare its computational binding performance to WT ASiP to predict what the in-vitro biochemical performance of the mutant would be when synthesized. Our hypothesis was that a mutant residue could be considered to be an improvement over the native if it exhibited a larger decrease in score performance than the WT residue when mutated to

alanine and docked to MC1R. A mutant would represent an overall improvement in affinity over the WT if the mutant docking score was better than the WT representing a lower energy state between the mutant ligand and MC1R.

The final step in the computational analysis was to translate the designed mutants Docking Score into an overall prediction of improvement in binding affinity. To do this, we wanted to relate the docking score from the WT ligand to the docking score of the designed ligand using the score-to-affinity constant.

V. Results

A. Calibration

The results of our computational alanine scan are consistent with in - vitro chemical experiments. The scores for alanine substituted positions were worse than the unsubstituted binding score, which qualitatively matches the results of the Keifer in vitro alanine scan.

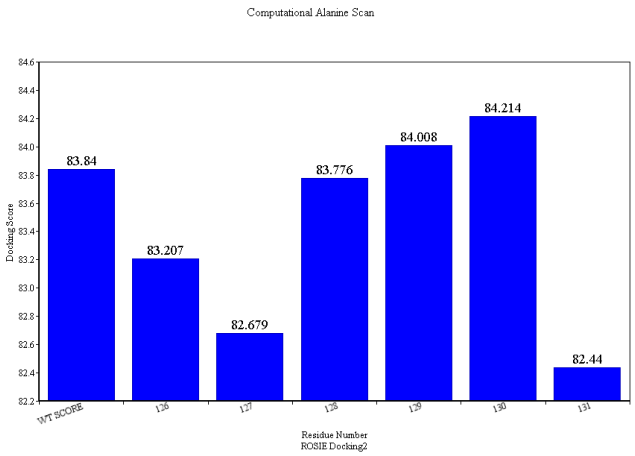

Fig. 10. Computational Alanine Scan Scores

There were 2 exceptions to this rule, the docking scores for residues 129 and 130 show a predicted increase in binding affinity when substituted with an alanine. This was an unexpected outcome since general biochemical knowledge indicates that an alanine substitution should decrease affinity and the in-vitro scan showed a small decrease in binding for alanine substitutions at those positions. Since both the WT and Mutant ligand displayed increased binding for alanine substitutions at these positions, we investigated further. Alanine is a reasonable point-mutation at these residues, in that it is slightly hydrophobic and these positions interface with hydrophobic regions of MC1R EC loop 1. Alanine was also predicted to be tolerated by the Seq-Tol application, though it was not considered to be the most tolerated amino acid for either of those positions.

We were not able to create a consistently viable Score-to-Affinity constant. The in-vitro alanine scan showed decreases in affinity for residues 126 - 129 that were larger than the decreases for residues 130 and 131. Ignoring the results for residues 129 and 130 that we have already discussed, we note that the computational alanine scan shows the largest decrease in affinity for a substitution at 131 and the smallest decrease in affinity for a substitution at 128. While a decrease in affinity is consistent with expectations, it can be noted that the relative magnitude of the computational results at these residues are in direct conflict with the relative magnitudes of the in-vitro results [6, 10].

Our current thoughts are that the Ki ratio for the residues in our chosen design region are too small to detect with consistent docking scoring, that the Ki ratios for these residues are small enough to be lost in scoring noise. (See the Next Steps section about our planned future experiments to identify a docking score to affinity constant by looking at residues outside of our design region.)

B. Sequence Optimization

Repeated trials of the Seq-Tol application returned the same predicted sequence with the same relative tolerances.

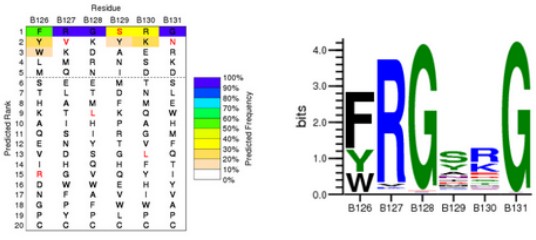

Fig. 11. Relative Tolerance Output per Position

Sequence Comparison:
WT Sequence (126-132): RVLSLNC
MUT Sequence (126-132): FRGSRGC

Examining the changes between the mutant sequence and the WT sequence, we see a single base movement of Arginine. There is an overall drop in hydrophobicity with removal of the valine and leucines, an increase in flexibility with the addition of the two glycines and increase in positive charge provided by R130. These results are not only reasonable from the standpoint of increasing affinity, but the drop in hydrophobicity means that the mutant protein can be expected to be more stable in an aqueous environment which should make the protein both easier to work with in-vitro and more likely to retain functionality in-vivo.

C. Prediction of Mutant Performance

Overall: For all in-silico experiments, our designed mutant has out-performed the WT ligand.
With the exception of residues 129 and 130 which have already been discussed, the computational alanine scan scores showed a decrease in binding affinity for each residue substituted with alanine, indicating that those amino acids contributed significantly to binding. Comparing the computational alanine scan results we see that, in general, at each residue the mutant showed a much larger decrease in predicted affinity than the WT did when the given residue was substituted with alanine. The larger changes in scoring for the mutant indicate that the

amino acid in the mutant that was replaced with an alanine was more for binding than amino acid in the WT.

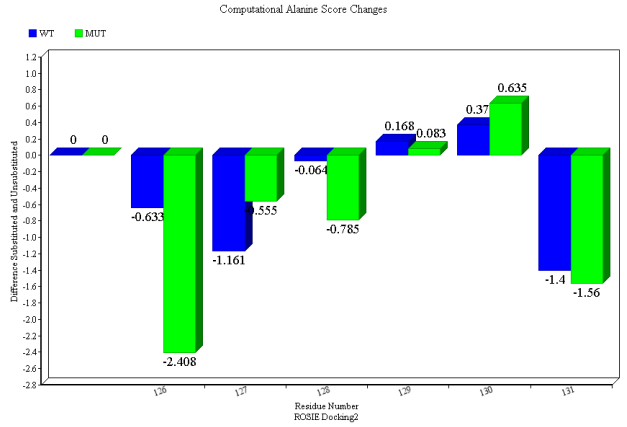

Fig. 12. The Y-axis represents the difference in docking score between the unsubstituted ligand and the alanine-substituted ligand for each residue. Wild Type: Blue. Mutant: Green

The lowest mutant binding score was -84.781 while the lowest WT binding score was -83.840 leading to a prediction that the mutant will demonstrate better overall affinity for MC1R than the WT does.

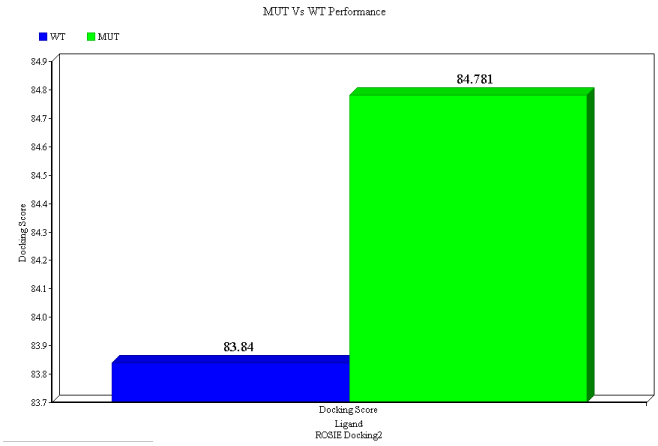

Fig. 13. Scores shown are the lowest docking score obtained for each ligand after 10,000 docks

Fig. 14. Designed ASiP Mutant docked to MC1R

VI. Discussion

In the Calibration Results section we noted that the computational alanine scan showed a much more significant decrease in binding affinity when WT residue 131 was substituted with an alanine than the in-vitro alanine scan reported. It should be noted that the computational alanine scan also showed a relatively large decrease in binding affinity when the mutant residue 131 was substituted with an alanine. Perhaps this is an artifact of the scoring algorithm, but it seems to indicate that perhaps residue 131 plays a more significant role in binding than was previously considered.

At this point we have taken our prediction system as far as is reasonable. We have a mutant sequence that is biochemically promising and reason to believe that the mutant will outperform the WT. Keeping in mind that bioinformatics can only provide us with an informed hypothesis, the next step is to synthesize the mutant sequence and test it in-vitro.

VII. Next Steps

Search for a score to affinity constant: We will run a computational alanine scan on residues where the in-vitro alanine scan showed a very large Ki Mut/WT ratio, specifically residues 118-120 (RFF). Since these residues showed an enormous response to an alanine substitution the comparative score changes should be well outside of the noise range and it is possible that a constant relating score and affinity can be found.

We are interested in the whether the unexpected scores for both the WT and mutant for alanine substitutions at residues 129 and 130 are the result of an artifact or indeed significant. However, since the primary goal of this project is to produce a mutant of ASiP with optimal affinity for MC1R to be used as a drug for metastatic melanoma, we will focus our current efforts on whether the results can be used to produce a mutant sequence with stronger affinity to MC1R than the

mutant sequence (126-132: FRGSRGC). To this end, we will replace both 129S and 130R with alanines and dock the new mutant. We will repeat this substitution with leucines instead of alanines to test whether an increase in hydrophobicity is beneficial. Based on the results of the first two experiments, we may choose to computationally experiment with different combinations of A and L at mutant residues 129 and 130. If the AA or LL mutants produce better affinity score predictions we will consider synthesizing mutants with these amino acids replacing the R and G.

Once we have a sequence that is agreed upon by both the in-vitro and in-silica researchers we will proceed to synthesis. The Millhauser lab has a well-established solid state protocol for synthesizing ASiP. We will synthesize our computationally-designed mutant and test its affinity for MC1R using our already-established competition assay. In-vitro competition performance will inform our computational model and provide possible changes to our design and prediction methods so that further iterations are even more successful.

VIII. Concluding Thoughts

Designing drugs is a complicated and time-consuming practice. While no computational design method is able to produce a perfect drug on its first iteration, computational design has been shown to speed up discovery time, predict novel structures, and significantly reduce costs Proteins-as-a-drug are a cutting edge technology that can offer multiple advantages over small molecule drugs. For example, proteins bind more selectively to their targets (less promiscuous) which results in less “off target” hits, aka side effects. Being physically larger than small molecule drugs, proteins are also less likely to cause side effects by diffusing into unintended parts of the body. Additionally, proteins are the typical signals for most biological systems. Using proteins as drugs allows us to use the systems’ own signals to get the cellular response that we desire (this probably needs to be cited). However, the design of proteins as drugs is even more costly and time consuming than that of small molecules, due both the size of the design regions and the complicated nature of protein tertiary structure and its interactions with its solvent environment. So, while using computational methods to assist in the design of a peptide as a drug is much more difficult than using similar methods to design small molecule drugs, the reduction of time and costs associated with the use of computational methods to aid the design of proteins is of even greater value. Using in-silico predictions to guide melanocortin in-vitro chemistry represents a rare meeting of rich biochemical knowledge and decades of experimental results, with a new technique in computational design. The combination will allow for both practices (in-silico and in-vitro) to inform each other, providing rapid feedback and improvement of methodology.

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