Impact of Leucine 193 to Serine 193 Mutation and FR0 Ligand Binding on Adenosine Deaminase Function in Adenosine Deaminase Deficiency

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ABSTRACT

Adenosine deaminase (ADA) deficiency is a critical metabolic disorder and a leading cause of severe combined immunodeficiency (SCID), significantly impairing immune function. While gene therapy and enzyme replacement therapy have shown promise, their accessibility and long-term efficacy remain limited. This study explores the molecular environment of ADA deficiency through computational modeling, focusing on the mutation of leucine 193 to serine 193 and substituting the ligand 9-Deazainosine (9DI) with N''-(4-(5-((1H-BENZIMIDAZOL-2-YLAMINO)METHYL)-2-THIENYL)-1,3-THIAZOL-2-YL)GUANIDINE (FR0). Using protein-ligand docking protocols, we assessed the impact of these changes on the structure of ADA and binding affinity, as related to potential functionality. The mutation from leucine to serine at position 193 destabilized the binding of 9DI, suggesting a potential for restoring ADA function. In contrast, replacing 9DI with FR0 resulted in stronger binding, which could exacerbate ADA deficiency by further inhibiting enzyme activity. These findings provide new insights into the potential for molecular modifications to restore ADA function and contribute to the development of targeted therapies for ADA-deficient SCID and related diseases. Further research is needed to confirm the long-term effects and therapeutic potential of these interventions.

INTRODUCTION

Adenosine deaminase (ADA) is a vital enzyme for purine degradation, specifically adenosine and deoxyadenosine catalysis into inosine and deoxyinosine in purine salvage pathways.[1] ADA influences proliferation and differentiation of leukocytes, especially T-lymphocytes, macrophages, monocytes, and erythrocytes.[2] Accordingly, inherited ADA dysfunction can have devastating effects,

including non-immunological manifestations ranging from sensorineural deafness[3] to skeletal abnormalities. Impaired or absent ADA function, although rare with an estimated incidence in 1:375,000 to 1:660,000 live births in Europe, leads to the accumulation of toxic metabolites adenosine, 2'deoxyadenosine, and deoxyadenosine triphosphate (dATP).[1] Moreover, over 90% of ADA mutations that lead to ADA deficiency result in autosomal recessive severe combined immunodeficiency (SCID),[4] accounting for approximately 10-20% of SCID cases.[5] Without treatment, this fatal form of SCID is characterized by severe lymphocytopenia, dysfunction, and destruction of T-lymphocytes, B-lymphocytes, and NK Cells.[6]

Despite advances in medical interventions, early diagnosis and treatment programs, which are critical to preventing the irreversible accumulation of toxic metabolites and life-threatening infections,[7] are lacking in many regions. Notable areas subject to this include rural locations and South Africa. Specific populations also possess pathogenic markers that leave them at increased risk for ADA deficiency and its resulting effects—severe illness, developmental delays, and reduced survival rates—particularly those of Somali ancestry, Canadian Mennonite ancestry, and/or Amish ancestry from Juniata and Mifflin County, Pennsylvania.[8, 9] Although the mutation is typically caught by newborn screenings via blood testing or genetic testing,[6] if missed and/or left untreated, ADA-SCID becomes life-threatening before the age of two years.[7]

Regarding treatment, remarkable progress has been made in novel enzyme replacement therapies and allogeneic hematopoietic stem cell transplants (NSCT) with supplementation from enzyme replacement therapies.[10] Although HSCT survival has been historically superior, gene therapies have become the leading option with Cicalese et al. demonstrating a 100% survival rate (with HSCT treatment) in 18 ADA-SCID patients over a median follow-up time of 6.9 years.[11] However, 10-20% of patients have needed to either resume enzyme replacement therapy or receive subsequent HSCT or GT courses.[11] Further limitations are also presented with HSCT as matched siblings (86%) and family donors (81%) showcasing significantly better outcomes versus unrelated matched (66%) or haploidentical donors (43%).[12] All options and their potential impact, although life-saving and effective, are further restricted in terms of accessibility and cost, especially in low-resource settings. Moreover, all the treatments are, more importantly, constrained by technical limitations, ethical challenges, and knowledge gaps regarding long-term efficacy on immune reconstitution and survival outcomes, especially concerning gene therapy.[11] However, developments in computational modeling[13, 14] and ligand-based drug design[15] have opened new avenues for exploring potential therapeutics aimed at mitigating the effects of ADA deficiency. Among available ligands, 9-Deazainosine (9DI) has historically shown the most promise with its considerable strength as an inhibitor of ADA enzymatic activity.

In regards to FR0, it has only been previously investigated for its structural interactions with adenosine deaminase. Specifically, with its inclusion of a series of imidazole-4-carboxamides, FR0 has the potential to serve as a non-nucleoside inhibitor for adenosine deaminase, along with other favorable pharmacokinetic properties.[57] Tan et al. has also noted that FR0 helped maintain the stability and structure of adenosine deaminase, especially in its binding pocket site and the a12 helix.[57] Similar circumstances—no applicable research examining this area—apply to leucine mutations near the active site of ADA due to fear of potential loss of activity, an issue solved by the application of computational simulations. To tackle these gaps, this study investigates the impact of replacing ligand 9DI with alternative FR0 to evaluate the latter ligand's potential as a more favourable therapeutic candidate. This study will also examine the effects of mutating leucine into serine in the active site of ADA to investigate the potential of ultra-targeted amino acid-focused treatments as an alternative to

the typical gene-level ones. This study aims to inform and guide future strategies and research for therapeutics targeting ADA deficiency and other inflammatory disorders.[18]

METHODOLOGY

Utilizing models from the Protein Data Bank,[19] this study investigates the effect of different changes on the protein Adenosine Deaminase (PDB ID: 3KM8).[20] Following preprocessing to remove extraneous elements and separate the ligand from the molecule, the molecular model was redocked using GNINA, py3Dmol, and openbabel in Google Colab's Linux Command Line Interface[21] to verify box dimensions and parameters. Once several rounds of verification were complete, we utilized PyMOL to mutate leucine 193 to serine 193 in adenosine deaminase in the first case and changed the docking ligand from 9-Deazainosine to FR0 in the second case. Then, to observe the effects of the changes, we utilized GNINA[22-28] to dock the ligands into the protein and extracted affinity & RMSD calculations for analysis. PyMOL and py3Dmol were used to visualize the proteins and ligands throughout all steps of the process.

Protein Data Bank: Structure Models

The Protein Data Bank (PDB)[19] is a database for three-dimensional structural data of biological molecules physically obtained by scientists via techniques like X-ray crystallography, NMR spectroscopy, and cryo-electron microscopy. With thorough review from biocurators, the Protein Data Bank has become an increasingly trusted source for biological models.[29] In this case, for our base structure, we utilized an experimental crystal structure of *mus musculus* adenosine deaminase in its Assembly I complexed form with 9-Deazainosine obtained using X-Ray Diffraction at a resolution of 2.00 Å (PDB: 3KM8).[20] For our second experimental ligand, we selected N''-(4-(5-((1H-BENZIMIDAZOL-2-YLAMINO)METHYL)-2-THIENYL)-1,3-THIAZOL-2-YL)GUANIDINE, otherwise referred to as FR0[30] in PDB or FR117016, which is a ligand with unknown experimental binding effects on Adenosine Deaminase.

To ensure there were not any confounding effects, the molecules were then imported into PyMOL. PyMOL is a molecular visualization program that creates 3-dimensional models of biological macromolecules and smaller molecules and is widely utilized by structural biologists to analyze the structure and function of many biological molecules.[31] In PyMOL, the structures were cleaned by removing any excess molecules (water, hydrogen, ions, etc.), reflective copies, and other remnants of the crystallography. Then, in the case of adenosine deaminase, the ligand 9DI was separated from the overall protein. All ligands and molecules were finally coded to ensure maximum clarity when visualizing the effects.

GNINA: Re-Docking

The ligands and protein were transferred into a Google Colab Linux Command Line Interface built on Google Drive connectivity and storage. Additional components include GNINA[22-28] for integrated molecular docking support, Open Babel[32] for chemical file format conversions, and py3Dmol[33] for active visualization. To elaborate, py3Dmol is a python wrapper for the 3Dmol javascript library, which is used to create visualizations of molecular systems, while Open Babel is a chemical toolbox that enables the parsing of over 100 chemical file formats to extract inputs. On the

other hand, GNINA, a fork of smina[34] (a fork of AutoDock Vina),[35, 36] is a molecular docking program that involves applying convolutional neural networks (CNN) and deep learning for scoring and optimizing ligands. When the protein and ligand are clearly defined, GNINA demonstrates excellent docking performance and high accuracy at an efficient computational cost.[22] Considering that our protein and ligand are clearly defined and all extraneous elements were already removed from the model, GNINA is highly suitable for our application of protein docking via deep learning, simulations, and related tools; further details of our application are outlined as follows.

To ensure an accurate docking process, rigorous testing was used to find the optimal format: a Cartesian box surrounding the entire protein as chain A in position 4 (Figure 1) with a size of 20 Å by 20 Å by 20 Å, and center at (-25, -10, 5). This box was validated using Root Mean Square Deviation (RMSD). RMSD, measured in angstroms [Å], is utilized to quantify the distance between a re-docked ligand and its original docked position in relation to the protein.[37] The best RMSD of the redocking experiment was 0.358797 in position 1.

Table 1. The Root Mean Square Deviation (RMSD) of different poses during the redocking and docking of the ligand 9DI process and the ligand FR0 process with the best outcomes marked in yellow.

POSE [RMSD (Angstroms)]	REDOCKING (Leucine 193)	DOCKING (Serine 193)	DOCKING (Ligand FR0)
1	0.358797	6.07046	30.3305
2	2.17716	6.09601	29.032
3	3.67258	3.55575	26.3124
4	5.19042	0.403497	30.0749
5	5.23979	6.19387	27.4681
6	6.1055	5.96293	29.8459
7	2.6072	3.0841	31.9335
8	6.06772	5.79027	29.3549
9	7.12945	2.20489	26.7437

PyMOL & GNINA: Docking

Although, for analysis, we focused on the most accurate position (position 1), we did visualize the top nine poses in PyMOL before addressing our research questions. These top 9 poses were determined after iterative refinements to see what fit the model based; our final selection was that position 1 corresponded to frame 0, and so on and so forth till position 9 corresponded with frame 8. Subsequently, using PyMOL's reputed mutagenesis wizard tool,[38, 39] mutated the active site residue

L193 (leucine 193) to S193 (serine 193). Then, both files were again transferred to Google Colab and docked using a similar methodology. RMSD for position evaluations and affinity calculations for binding tendencies were drawn from the data for analysis. For the mutated residue, the best RMSD was 0.403497 in position 4.

For the docking process, in the case of FR0 & Adenosine deaminase, the original ligand (9-Deazainosine) was removed, and docking was performed using FR0. Then, using a similar methodology to the re-docking process, docking was completed using a Cartesian box of size 20 Å by 20 Å by 20 Å, and center at (-25, -10, 5). RMSD for position evaluations and affinity calculations for binding tendencies were drawn from the data for analysis. Visualizations were performed in PyMOL.

RESULTS

The results section will include a detailed analysis of the binding interactions and affinity changes observed due to the mutation of leucine 193 to serine 193 and the comparison of ligand 9DI with ligand FR0.

Residue Mutation of Leucine 193 to Serine 193

The mutation of leucine 193 to serine 193 represents a significant change in the protein's structure and, potentially, its function. Protein stability and ligand binding are closely tied to the energy levels. Replacing leucine, a hydrophobic amino acid, with serine, a polar amino acid, could destabilize the protein's structure, causing shifts in its energy profile. Similarly, amino acid location 193 was chosen for its role as an adenosine deaminase conserved residue [58] of unknown significance with potential mutations affecting the molecule's active site, where it resides. [59]

Table 2. The affinity level of different poses during the redocking and docking of the ligand 9DI process with the best outcomes marked in yellow.

POSE [affinity (Kcal/mol)]	REDOCKING (leucine 193)	DOCKING (serine 193)
1	-9.18	45.03
2	-4.51	53.00
3	10.59	38.00
4	4.40	36.94
5	11.43	51.89
6	1.14	39.18
7	-2.68	40.98
8	-6.36	41.97

9	7.44	40.47

The data presented in the table highlights a significant change in the energy levels following the mutation of residue leucine 193 to serine 193. Specifically, the mutation induced a transition from a low-energy state to a high-energy state. This elevated energy level suggests that the protein is unlikely to bind 9-Deazainosine, highlighting the mutation's potential implications. This occurrence, in turn, reinforces our hypothesis that the mutation will likely increase the distance from the binding site as a result of the noted reactivity potential of leucine and serine.

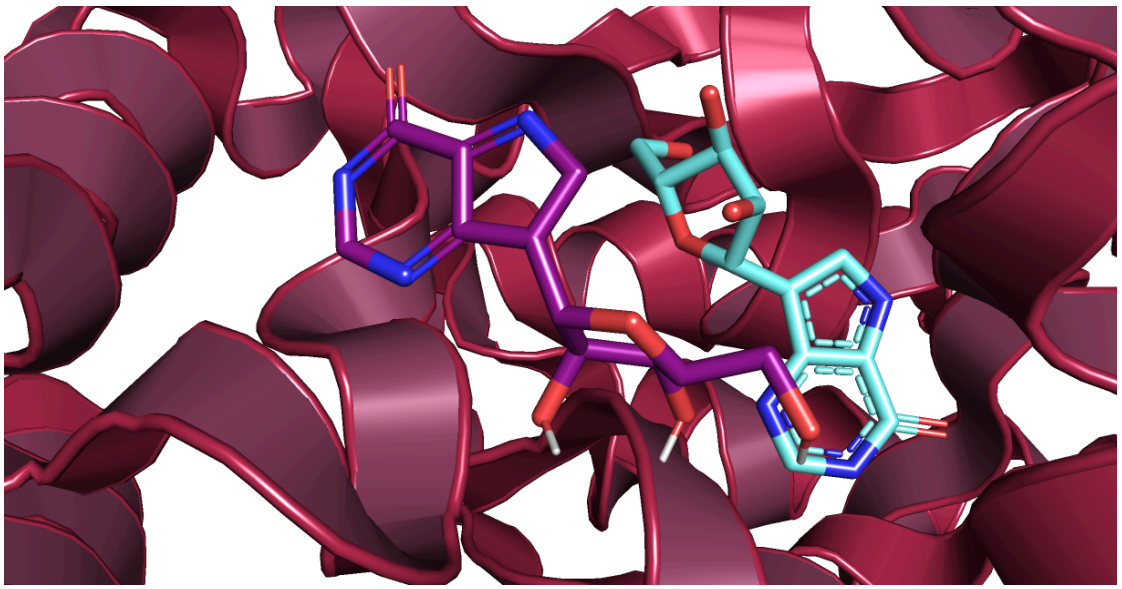

Figure 1. ADA [Blue] Bound to 9DI [Purple] with Serine 193 Replacing Leucine 193 in Pose 4 of 9.

Pose 4 has an affinity of 36.94 Kcal/mol (Docking, Serine 193), making it the best pose as it has the lowest affinity value of the nine tested poses, indicating the strongest and most stable binding.

Considering that binding affinities represent the free energy change upon ligand binding, lower values are more favorable as they suggest that less energy is required for the ligand to remain bound to the target, resulting in a more thermodynamically stable complex. In theory, the mutation leading to this altered energy state could prevent the inhibition of ADA by 9-Deazainosine. This could mean that the mutation could reverse the inhibition of ADA, allowing the enzyme to function properly again and alleviate symptoms of the deficiency in ADA. However, this therapeutic result can only be successful with the precondition that this mutation does not cause loss of the general enzymatic function of ADA. If this mutation negatively affects the active site of ADA or its structural integrity, it could negate any advantages of its restored activity. Additional research is needed to prove that normal enzymatic function is not altered when the mutation stops inhibition.

Replace Ligand 9DI with Ligand FR0

Replacing the ligand 9-Deazainosine (9DI) with the ligand FR0 introduces a significant shift in the energy dynamics of the protein-ligand interaction. Ligand binding plays a crucial role in stabilizing or destabilizing the protein structure, and different ligands can induce varying energy states depending on their binding affinity and interaction profile.[40]

Table 3. The affinity level of different poses during the redocking of the ligand 9DI and the docking of the ligand FR0 with the best outcome marked in yellow.

POSE [affinity (Kcal/mol)]	REDOCKING (Ligand 9DI)	DOCKING (Ligand FR0)
1	-9.18	-16.64
2	-4.51	-14.50
3	10.59	-17.99
4	4.40	-16.70
5	11.43	-13.18
6	1.14	-16.30
7	-2.68	-13.35
8	-6.36	-17.80
9	7.44	-13.12

Replacing the ligand 9-Deazainosine (9DI) with the ligand FR0 has resulted in a lower energy level, suggesting a higher likelihood of the ligand binding to the protein. This change in binding affinity may have serious implications for the protein's function. This finding is concerning since adenosine deaminase (ADA) deficiency is caused by dysfunction of the ADA enzyme. FR0 strengthens the inhibition of ADA by binding more effectively to the protein, which could worsen the condition by further impairing the enzyme's ability to break down adenosine. A deficiency in ADA results in the accumulation of adenosine, leading to severe immune dysfunction; hence, any enhancement in its inhibition is likely to worsen the disease.[41]

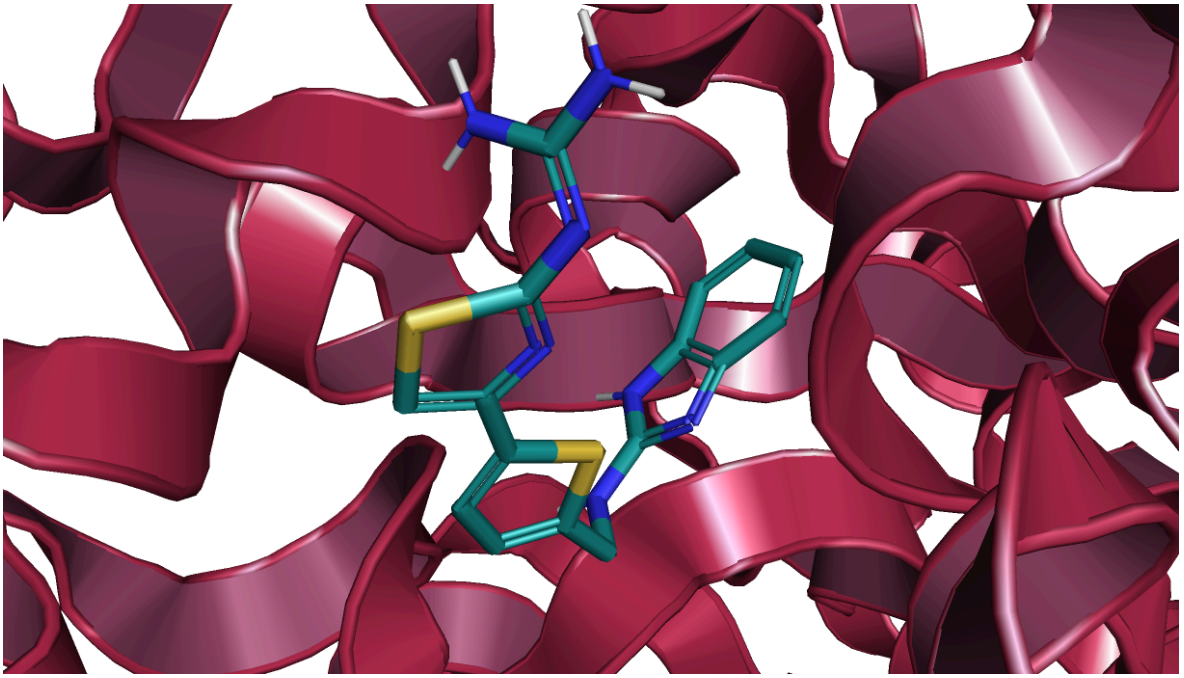

Figure 2. FR0 [Cyan] Docked Into Adenosine Deaminase [Red] in Pose 3 of 9.

Pose 3 has an affinity of -17.99 Kcal/mol (Docking, Ligand FR0). This makes it the best pose as it has the lowest affinity value of the nine tested poses, signifying the strongest and most stable binding.

However, it is important to point out that this would be the effect of FR0 if it does not interfere with the overall function of ADA. If the binding of FR0 changes the enzyme's active site or disrupts its overall structure, it could compound the detrimental effects of the inhibition, exacerbating the disease. Further research should be conducted to ensure that, while FR0 may increase binding affinity, it does not compromise ADA's normal enzymatic activity, which would lead to a more severe manifestation of ADA deficiency.

DISCUSSION

Adenosine deaminase is highly prone to result in adenosine deaminase deficiency if its amino acids are structurally modified, even on a singular site basis.[42] Particular modifications that result in ADA-deficient phenotypes include the substitution of Arg-101 to Trp-101 and/or Arg-211 to His-211 in ADA-deficient cell line GM2606,[43] along with substitution of arginine for leucine and lysine in deficient cell line GM2471.[44] In relation to leucine,[45] mutating it to arginine has been noted to cause a loss of ADA activity.[46] However, despite their significant combination, little research has been conducted on mutations near the active site of ADA nor mutating leucine to serine.

Nevertheless, in regards to other proteins, mutating leucine to serine increases polarity (non-polar to polar; removal of leucine's aliphatic side chain), raising the potential for protein destabilization, the modification of hydrophobic packing, and new folding dynamics for a given molecule.[47] The addition of a hydroxyl (-OH) group through serine, along with the replacement of leucine's large, branched side chain with serine's smaller structure, also increases opportunities for interactions (i.e. hydrogen bonding, etc.)[48, 49] and may alter static hindrance, potentially impairing

substrate binding or catalytic efficiency.[50, 51] Phosphorylation,[52] glycosylation,[53] and other post-translational modifications[54] may also occur with serine substitutions. These modifications in other proteins have also been linked with autosomal dominant strains of Alzheimer's disease[55] and mecillinam resistance in *Escherichia coli* via partial starvation of isoleucine and valine.[56] In our study, mutating leucine 193 to serine 193 in adenosine deaminase prevented the further binding of ligand 9-Deazainosine, an adverse reaction supporting our hypothesis. If this mutation does not otherwise impair the function of adenosine deaminase, this mutation poses the potential to override other loss-of-function mutations from binding with toxic ligands.

In regards to FR0, it has only been previously investigated for its structural interactions with adenosine deaminase. Specifically, with its inclusion of a series of imidazole-4-carboxamides, FR0 has the potential to serve as a non-nucleoside inhibitor for adenosine deaminase, along with other favorable pharmacokinetic properties.[57] Tan et al. has also noted that FR0 helped maintain the stability and structure of adenosine deaminase, especially in its binding pocket site and the a12 helix.[57] From

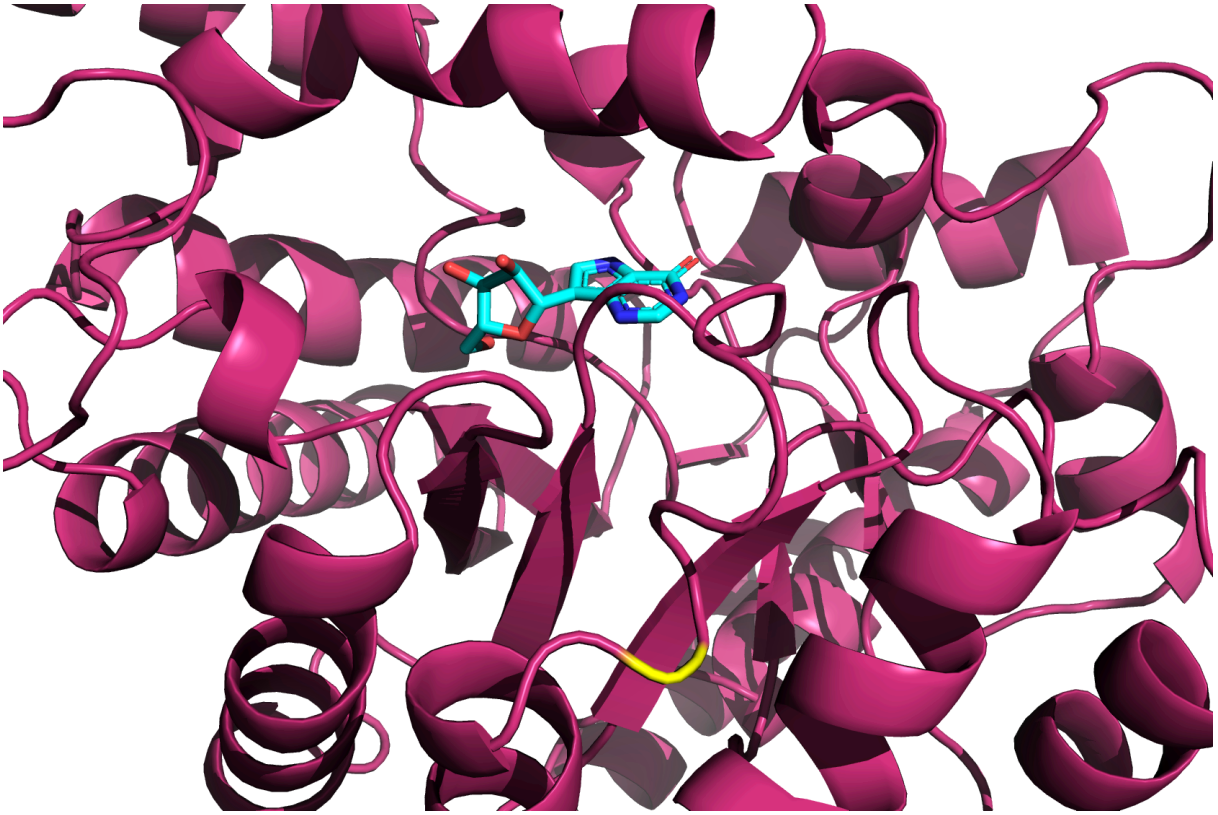

Figure 3. 9DI [Blue] Bound to Adenosine Deaminase [Red] with Site of Leucine 193 to Serine 193 Mutation Highlighted [Yellow].

our conclusions, it is likely that FR0's inhibitory effect derives from its prevention of adenosine deaminase's crucial flexing during its molecular binding process, thereby preventing binding at all. Interestingly though, as FR0 binds with adenosine deaminase in its open form, FR0's innate ease of electron transfer lends itself to a high potential to form additional interactions and bonds, particularly hydrogen-based ones.[57] Although previous research has focused primarily on such structural implications of FR0-adenosine deaminase bonding, our study instead examined what this may look like on an atomistic level. Notably, the relatively low-affinity level of the FR0-ADA binding means that the bonding is likely stable and has long-lasting equilibrium. Considering that ADA deficiency is

caused by failures in adenosine deaminase and the nature of FR0 as a potential inhibitor, this result would likely cause an aggravation of the disease as the loss-of-function that comes with ADA deficiency is exacerbated through greater ADA inhibition. This outcome, nevertheless, will only occur if the inhibitor, FR0, does not otherwise adversely affect the function of ADA.

CONCLUSION

Overall, our binding of 9-Deazainosine with modified adenosine deaminase (leucine 193 to serine 193) represents the first example to our knowledge of a mutation empowering the function of adenosine deaminase, versus debilitating it. With further research, this mutation represents the possibility of further modifying a malignant form of the enzyme to enable function versus replacing it with a wild-type form.

Conversely, our binding of typical adenosine deaminase to FR0 builds upon previous research, by taking a new perspective to it while reaffirming FR0's potential to serve as an inhibitor of ADA. Broadly, this confirmation has the potential to help create treatments for ADA-deficient severe combined immunodeficiency and other adenosine deaminase-related metabolic imbalances. Further research needs to examine these phenomena for adverse effects and other potential modifications to develop new, more effective, targeted treatments for malformed adenosine deaminase.

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