Evaluating Associations with the Mechanism of the K65R Mutation in HIV-1 Reverse Transcriptase Using Computational Molecular Docking

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ABSTRACT. Nucleoside reverse transcriptase inhibitors (NRTIs) are common antiretroviral HIV medications, targeting the function of reverse transcriptase (RT), a protein designed to transcribe viral RNA into DNA. An emerging problem is the development of resistance against such medications. For example, the K65R mutation in RT has been shown to decrease the binding affinity of RT to the NRTI tenofovir, while conserving affinity to tenofovir's nucleotide analog, adenosine. This has been associated with the decreased distance between the guanidinium planes of residues 65 (R65) and 72 (R72) and, alternatively, with the increased distance between tenofovir and DNA already bound to RT. These theories have never been tested. This study aims to evaluate these theories through computational methods. Mutations were generated within the β3-β4 loop of K65R-mutant reverse transcriptase (accessed from the Protein Data Bank). For each mutation introduced, data was recorded on the distance between the R65 and R72 guanidinium planes, as well as the distance between RT-bound ligand and RT-bound DNA. In addition, the binding affinity of ligands tenofovir and adenosine to RT was calculated through the docking software Autodock Vina. After 95 mutations, linear correlations indicated that the binding affinity of tenofovir to R65/R72 increased with the decreased distance between the guanidinium planes of R65/R72, contrary to previous literature. In addition, a significant, increasing relationship was seen between binding affinity and the increased distance between either tenofovir and adenosine and RT-bound DNA, challenging the previously postulated association of this distance with the mechanism of the K65R mutation.

KEYWORDS. K65R mutation, antiretroviral resistance, reverse transcriptase, HIV-1, molecular docking

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INTRODUCTION

As society transitions from the 2-year-long COVID-19 pandemic back to "normalcy," the spread of human immunodeficiency virus (HIV) is overlooked, constituting a pandemic that has spanned 4 decades. Since the beginning of the HIV pandemic in the 1980s, the virus has infected 84.2 million people, killing over 40 million; today, almost 39 million live with HIV, and in 2021 alone, nearly 650,000 people died of HIV-related illness (UNAIDS 2022). As quantified by a December report from the Centers for Disease Control and Prevention (CDC), 38,000 new HIV cases develop annually in the United States, and in response, the United States government launched an initiative in February 2019 titled "Ending the HIV Epidemic: A Plan for America" (Harris et al. 2019). This plan dictates that, by 2030, at least 95% of HIV patients need to know their diagnosis. At least 50% of those at high risk for HIV need to be under a prescription for pre-exposure prophylaxis (PrEP), and at least 95% of those with HIV need to be virally suppressed (Harris et al. 2019). "Viral suppression" refers to the phenomenon in which infected individuals eliminate almost all the HIV virus within their bodies. This prevents the development of acquired immunodeficiency syndrome (AIDS) even while an undetectable amount of the virus persists, allowing patients with HIV to live normal, healthy lives.

The mechanism through which viral suppression is achieved involves the use of antiretroviral medications dedicated to lowering the HIV viral load. An HIV virion has a complex life cycle in a host cell, from entry and uncoating to the production

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of mature virions that leave their host cells and infect other cells within an individual (Engelman and Cherepanov 2012). A critical step within the life cycle of an HIV virion is the production of DNA from genomic RNA, which takes place through the process of reverse transcription. Reverse transcription produces HIV virion-coding DNA, which can then be integrated within the host cell's genetic makeup, turning an innocuous human cell into a virus-producing machine (Engelman and Cherepanov 2012). Many HIV medications used today specifically target reverse transcription, interrupting and blocking the function of a viral enzyme by the name of reverse transcriptase (RT). This enzyme functions by working off a tRNA primer bound to the complementary primer binding site (PBS) within single-stranded viral RNA, using free nucleotides to polymerize and form negative-stranded DNA complementary to viral RNA. This negative-stranded DNA is used as a template to form complementary plus-strand DNA, creating double-stranded viral DNA from single-stranded genomic RNA (Sarafianos et al. 2009). RT conducts this arduous task through two subunits: the p51 subunit and the p66 subunit. The p51 subunit exists to maintain the structure of reverse transcriptase, while p66 acts with a wider range of functionality. The p66 is further divided into the RNase H, connection, thumb, palm, and fingers subunits (Sarafianos et al. 2009, Jacobo-Molina et al. 1993). The fingers subunit has been especially highlighted to bind to free nucleotides (at a space within the subunit known as the N site) and position them to be added to the DNA strand already bound to RT at the polymerase active site (P site), located spatially between residues 110, 185, and 186 (Jacobo-Molina et al. 1993). Without the fingers subunit of RT and its involvement in DNA polymerization, HIV virions are rendered unviable in human systems.

RT-targeting antiretrovirals are divided into two classes: nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs are nucleotide analogs, resembling real deoxynucleotides in chemical structure and binding behavior with RT and its N site; however, with the lack of a 3' OH group required to continue the DNA strand, NRTIs block the future polymerization of RT-bound DNA at the P site. Meanwhile, NNRTIs function as noncompetitive inhibitors, binding away from RT's active site yet altering the structure of its N site indefinitely, preventing RT from binding to any nucleotides/nucleotide analogs whatsoever (Sluis-Cremer and Tachedjian 2008).

As people use antiretrovirals, HIV virions are slowly evolving and developing resistance to antiretroviral medications (Tarasova et al. 2018). The mechanisms of resistance to NRTIs remain ambiguous. One mutation that perfectly exemplifies this is the K65R mutation, a substitution of the 65th amino acid in RT (within the fingers subunit) from lysine (K) to arginine (R), which causes resistance to the antiretrovirals tenofovir and didanosine (Tarasova et al. 2018) (Figure 1). There are two theories in recent literature hypothesizing the potential mechanism of the K65R mutation. One theory focuses on the interaction between tenofovir and residues 65 and 72 (R65 and R72) within reverse transcriptase, which form bonds with every chemical component of tenofovir (Das et al. 2009). One study proposes that as the distance between the guanidinium planes of residues 65 and 72 decreases, the binding affinity with the NRTI tenofovir significantly decreases, while the binding affinity with tenofovir's nucleotide counterpart, adenosine monophosphate (AMP), is relatively unchanged (Das et al. 2009). If RT-interactions with tenofovir are inhibited when compared to interactions with adenosine triphosphate, RT-AMP interactions will be favored in comparison to RT-tenofovir interactions. However, the issue over the mechanism of the K65R mutation becomes significantly more nebulous when considering another hypothesis regarding the K65R mutation. A study conducted by Fourati et al. used computational molecular docking to look at structures of tenofovir bound to RT models with the K65R mutation, as well as more rare K65 mutations like K65E and K65N. It was found that an increase in the distance between the terminal phosphate group of tenofovir and the 3' OH end of RT-bound DNA led to a decrease in the binding affinity of tenofovir, especially when the distance exceeded 6 Angstroms (Å) (Fourati et al. 2013). Therefore, this study postulated that this distance may also be associated with the mechanism of the K65R mutation. As these hypotheses have not been thoroughly evaluated, the scientific community remains undecided over how the K65R mutation

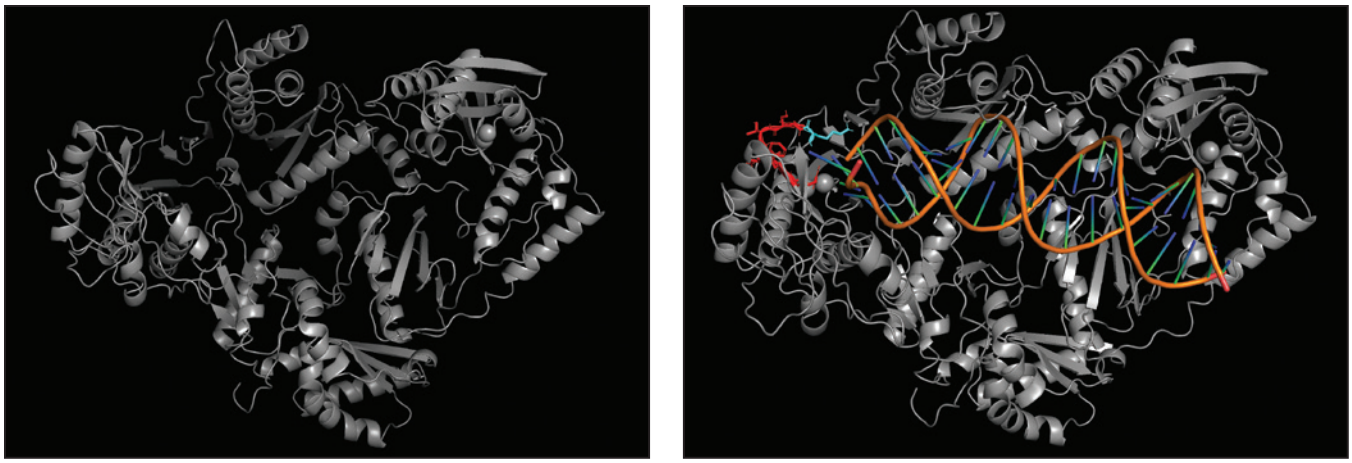

Figure 1. A comparison is shown between RT without bound DNA (left) and RT with bound DNA (right). The 65th residue of RT (highlighted in cyan, boxed in white) is within the β3-β4 loop of RT (highlighted in red, boxed in blue). Located at the end of a DNA strand, these residues are critical in the incorporation of incoming nucleotides into DNA.

functions in creating resistance to NRTIs, as well as how current theories may/may not accurately point to the mutation's true mechanism.

The mechanism of the K65R mutation can potentially be studied through molecular docking, a computational, in silico technique of binding small molecules to protein macromolecules. Such molecular docking can give important information about the interactions between ligands and various protein structures, including mutant protein structures, providing an opportunity to investigate whether the distance between the guanidinium planes of residues 65/72 and/or the distance between a ligand and RT-bound DNA influence the binding of RT to tenofovir and antiretroviral resistance (Tarasova et al. 2018).

This directly points to the research question of this study regarding the mechanism of the K65R mutation in HIV-1 reverse transcriptase (RT). To what extent can associations with the distance between the guanidinium planes of residues 65 and 72 and the distance between nucleotide analogs and RT-bound DNA be supported through computational molecular docking? Answering this question will facilitate the investigation of the K65R mutation's true mechanism. Such research can lead to the development of new antiretrovirals that can combat the K65R mutation, potentially saving the lives of millions in the future as this mutation spreads around the world. In addition, answering this research question can form the framework for future studies on other mutations within reverse transcriptase, providing a way for antiretroviral resistance to be combated as antiretroviral use increases with increased HIV infection.

METHODS AND MATERIALS

In this study, the RT model that was used was the 1RTD model found in the Protein Data Bank (PDB), a database of protein models that can be downloaded and computationally manipulated through programs like Schrödinger's PyMol. The 1RTD model was specifically selected because of the reliable x-ray crystallography techniques used to construct the model, as well as the fact that the RT protein was in complex with a section of DNA. This DNA-bound complex was needed to examine associations with the distance between RT-bound DNA and the ligand interacting with RT (Huang et al. 1998, Fourati et al. 2013).

The 1RTD model was manipulated in such a way as to introduce various mutations within the protein structure. The 65th residue within RT is critical in its chemical interactions with RT-bound ligands (Das et al. 2009), so all trials with the 1RTD model were done with the 65th residue replaced with arginine and optimized using PyMol, in accordance with the K65R mutation. This mutation is part of a 9-residue loop (known as the β3-β4 loop) within the reverse transcriptase protein, between the 64th and 72nd residues, inclusive (Kew et al. 1998). For each individual position within this loop, PyMol was used to substitute the wild-type amino acid with each of the 19 other naturally occurring amino acids and optimize the protein structure for these mutations. This was done through employing an MMFF94 force field that optimized the bond lengths and angles between the residues along the β3-β4 loop, taking into account electrostatic and hydrogen-bonding effects without changing the R65 rotamer. This created 19 different protein

structures for each of 9 positions, leading to a total of 171 protein structures, each containing the K65R mutation and another, random mutation within the β3-β4 loop, with the R65 rotamer constant in all structures. The reason for creating all these mutations was to generate various, viable protein structures with slightly different characteristics, leading to different values for selected structural variables (e.g., the distance between the guanidinium planes of residues 65 and 72) that could then be correlated to how the RT protein interacts with various ligands.

This study tests whether the distance between the guanidinium sidechains of residues 65 and 72 and the distance between a ligand and RT-bound DNA have a real effect on the binding of RT to ligands like the antiretroviral tenofovir. Therefore, these were the 2 independent, structural variables for which data was collected for each mutant-RT structure. Using PyMol, the distance between the guanidinium planes of residues 65 and 72 for each mutant-RT structure was measured by finding the distance between the NH1 atoms of both residues (Das et al. 2009). The positions of the NH1 and NH2 atoms relative to each other in residues 65 and 72 stayed constant across mutant-RT structures, making NH1 a valid measuring point (Das et al. 2009). The distance between RT-bound DNA and interacting ligand was found by measuring the distance between the terminal phosphate atoms of both molecules (Fourati et al. 2013). These measurements could then be used to test the impact of the structural variables being studied on RT binding across various random mutations, determining whether it's likely that these variables are associated with the mechanism of the K65R mutation.

After these measurements were collected, data had to be collected on RT binding itself. For this study, Autodock Vina, a state-of-the-art program commonly used for docking studies, was used to conduct all docking trials (Eberhardt et al. 2021, Trott and Olson 2010). Each structure of reverse transcriptase (generated through the mutagenesis done with PyMol) was tested as a receptor for the ligands of tenofovir and adenosine. The computational models of these ligands were downloaded from the PubChem® database, converted to PDB format through the NIH Online SMILES Translator, and converted into PDBQT files through standard protocol described in Autodock Tools documentation (Forli et al. 2016). Regarding the preparation of the mutant-RT structures themselves for molecular docking, these structures were stored in PDBQT files using standard docking preparation procedures, involving the addition of carbon-bound hydrogens and Kollmann charges (Forli et al. 2016). The reasoning behind using tenofovir and adenosine as the ligands for RT is that tenofovir is a well-established NRTI that is rendered ineffective by the K65R mutation, while adenosine is its corresponding analog. This leads to an accurate measure of how the K65R mutation affects the function of various antiretrovirals (Tarasova et al. 2018, Das et al. 2009). In other words, by comparing the binding of RT with tenofovir to the binding of RT with tenofovir's analog for each mutant RT structure, information can be gathered on how the structural variables being studied impact RT-tenofovir interactions relative to RT-adenosine interactions (Das et al. 2009).

Molecular docking with tenofovir and adenosine was carried out in two ways. Firstly, to examine the binding of these ligands to residues 65 and 72, the computational models of tenofovir *diphosphate* and adenosine *triphosphate* were docked with each mutant RT structure. This is specifically because both tenofovir and adenosine originally contain triphosphate "tails" when binding to the N site of RT at residues 65 and 72; therefore, these tails needed to be accounted for in studying the interactions between tenofovir/adenosine and residues 65 and 72 in mutant RT structures (Jacobo-Molina et al. 1993, Das et al. 2009). In addition, the grid box for Autodock Vina, defining the space in which tenofovir diphosphate and adenosine triphosphate were docked, was created with the center x-y-z parameters (53.304, 25.212, 32.683) and the size x-y-z parameters (16, 11, 16).

To dock tenofovir and adenosine to RT-bound DNA, the computational models of tenofovir monophosphate (labeled simply as tenofovir) and adenosine monophosphate were used. This is because, after binding to the N site of RT, both tenofovir diphosphate and adenosine triphosphate lose two phosphates when interacting with RT-bound DNA (Fourati et al. 2013). As for the grid box for Autodock Vina, it was defined by the center x-y-z parameters (49.291, 21.271, 36.927) and the size x-y-z parameters (19, 19, 18).

In addition, Autodock Vina ran with an exhaustiveness of 8. This allowed for multiple binding conformations to be given as output. After running a docking command, Autodock Vina returned data on the binding affinity between reverse transcriptase and the selected ligand for each of these conformations (in units of kcal/mol). For each docking result, the conformation selected aligned with the accepted structure of tenofovir/adenosine in complex with K65R-mutant RT. Thus keeping the pose of RT and the selected ligand constant with the corresponding binding affinity recorded (Das et al. 2009). This binding affinity was the dependent variable for this study and was collected for each trial binding a mutant-RT binding structure to tenofovir/adenosine in interaction with residues 65 and 72 or RT-bound DNA. Certain mutations weren't included in the final data, only because no resulting conformations aligned with the accepted tenofovir/adenosine-RT complex, resulting in a final dataset with 95 mutant RT structures (included in the Appendix). To draw viable conclusions from recorded trials, each of the two structural variables in this study was correlated to the binding affinity between RT and a given ligand. For this correlation, the computer program RegressIt was used to generate regression models between structural data and binding affinity. These models also show how the impact of binding affinity differs between RT-tenofovir interactions and RT-adenosine interactions, leading to viable conclusions on which structural variables impact binding with reverse transcriptase and evaluating current theories on the mechanism of the K65R mutation.

RESULTS

In the graph correlating the binding affinity between RT and tenofovir diphosphate and the distance between residues 65 and 72, while binding affinity seems to increase (become more negative) as distance increases, the correlation is deemed statistically insignificant with a p-value of 0.666, well above the threshold of 0.05 (Fig. 2).

The outlying points observed in the graph originate from data collected by mutating D67 within the mutant-1RTD structure. When removing those points, a positive, statistically significant trend is observed, with a slope of 0.4527 suggesting that binding affinity decreases (becomes more positive) by 0.4527 kcal/mol for every 1 Å increase in the distance between R65/R72 guanidinium planes (Fig. 3).

In the graph correlating the binding affinity between RT and adenosine monophosphate and the distance between the guanidinium planes of

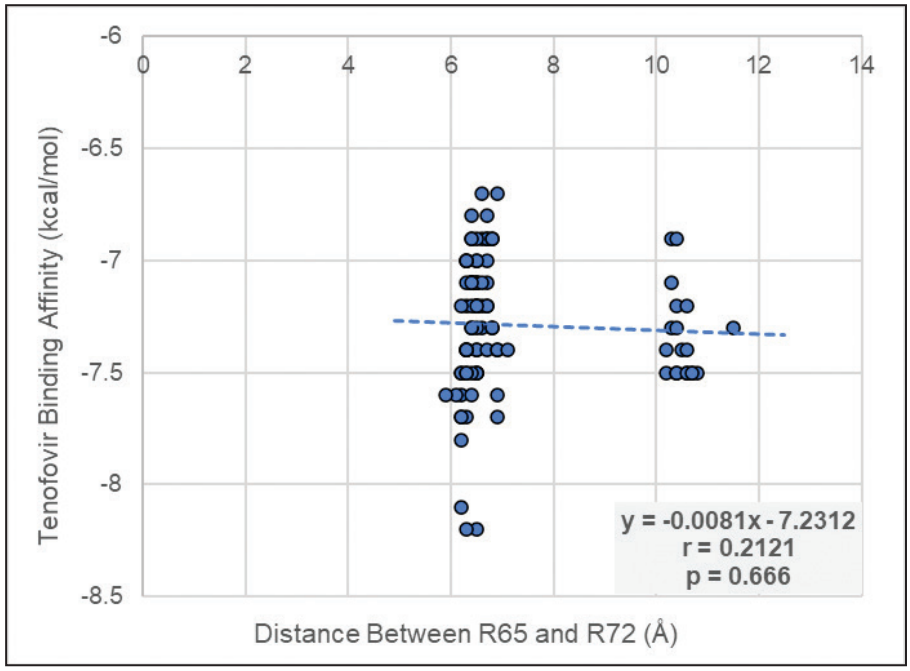

Figure 2. Distance Between Guanidinium Planes of R65 and R72 (Å) and Binding Affinity with Tenofovir (kcal/mol). The unaltered, linear correlation of RT-tenofovir binding affinity and the distance between the guanidinium planes of residues 65 and 72 reveals a statistically insignificant correlation, with a p-value of 0.666.

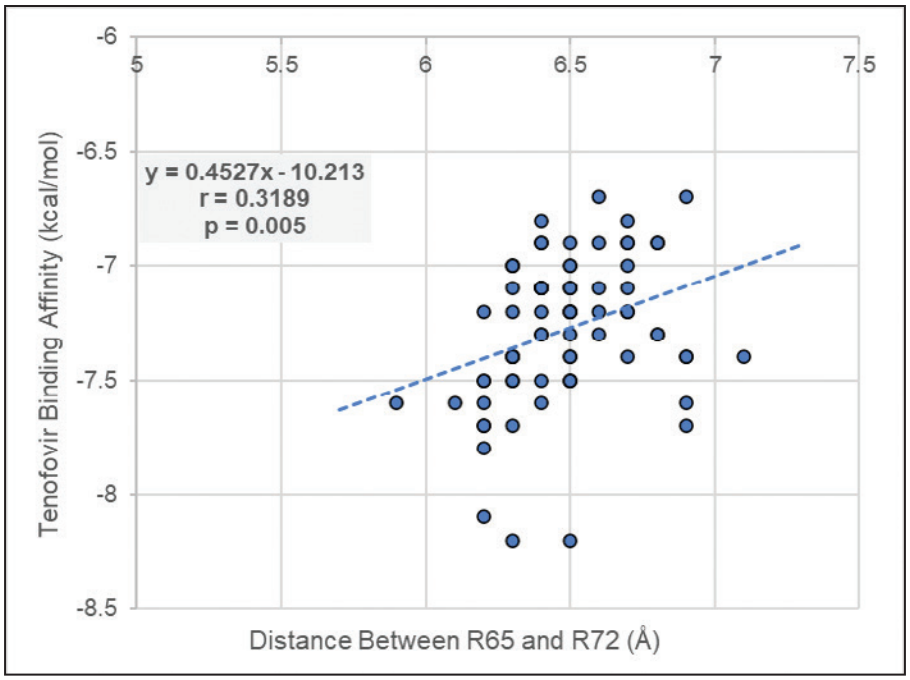

Figure 3. Distance Between Guanidinium Planes of R65 and R72 (Å) and Binding Affinity with Tenofovir (kcal/mol)—Without D67 Points. Without outlying data from mutating D67, a statistically significant correlation is observed, suggesting that that binding affinity between tenofovir and RT decreases as the R65/R72 guanidinium plane distance increases.

residues 65 and 72, binding affinity decreases as distance increases in a regression with a statistically significant p-value less than 0.001 (Fig. 4). In fact, a trend line with a slope of 0.0835 suggests that binding affinity decreases by 0.0835 kcal/mol for every 1 Å increase in the distance between the guanidinium planes of residues 65 and 72. That being said, this correlation, however significant, is weak with a moderately low r-value of 0.5122, suggesting that there is some variability of data away from the trend line. Furthermore, when removing the outlying data originating from D67 mutations, a statistically insignificant correlation is observed (Fig. 5).

Meanwhile, when correlating the binding affinity between RT and tenofovir and the distance between tenofovir and the free 3' OH group on RT-bound DNA, the resulting trend (statistically significant with a p-value of 0.05) line has a slope of –0.0753, meaning that the binding affinity increases by 0.0753 kcal/mol for a 1 Å increase in the distance between the free 3' OH group on RT-bound DNA and tenofovir (Fig. 6). However, it is imperative to acknowledge that this correlation may be very weak, given a relatively high, albeit statistically significant, p-value as well as an r-value of 0.2017, suggesting that much of the data is far from the trend line itself. A similar correlation is observed when removing previously outlying data from mutating D67 (Fig. 7).

Finally, in the correlation between RT-adenosine binding affinity and the distance between adenosine monophosphate and RT-bound DNA, the statistically significant regression is represented with a slope of –0.0468, meaning that the binding affinity increases by 0.0468 kcal/mol for a 1 Angstrom increase in the distance between RT-bound DNA and adenosine monophosphate (Fig. 8). However, it is imperative to acknowledge that this correlation may also be weak, despite a p-value of 0.013, due to an r-value of 0.2534, suggesting that there is a large quantity of separation between a portion of the data and the regression line itself. Once again, a similar correlation is observed when removing previously outlying data from mutating D67 (Fig. 9).

DISCUSSION

Implications

Past literature has clearly hypothesized that, as the distance between the guanidinium planes of residues 65 and 72 within RT decreased, binding affinity with tenofovir would also decrease (Das et al. 2009). However, that specific hypothesis has been rejected because the correlation with the distance between the guanidinium planes

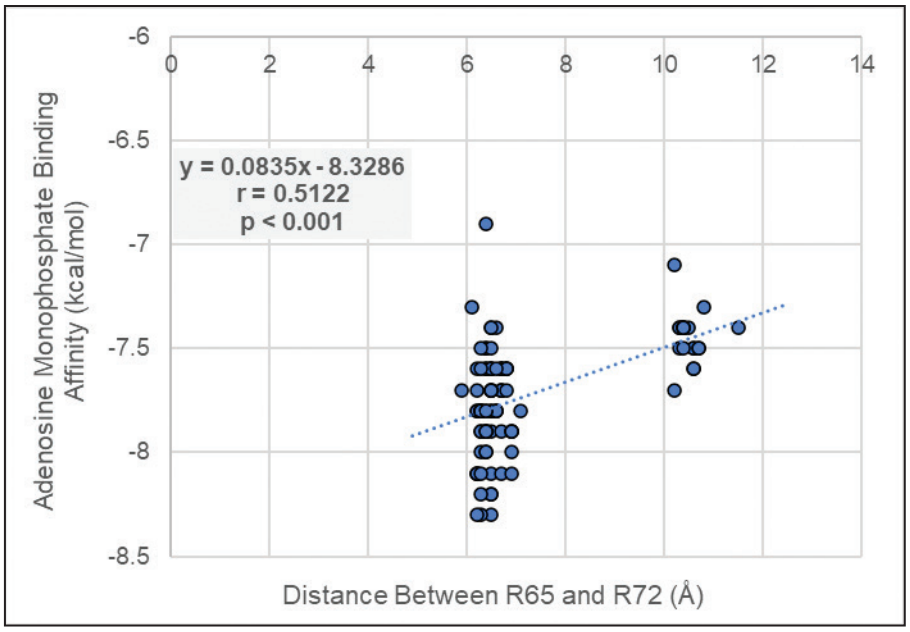

Figure 4. Distance Between Guanidinium Planes of R65 and R72 (Å) and Binding Affinity with Adenosine Monophosphate (kcal/mol). The unaltered correlation of RT-adenosine binding affinity with the distance between the guanidinium planes of residues 65 and 72 reveals a statistically significant relationship, with a linear slope of 0.0835 and an r-value of 0.5122.

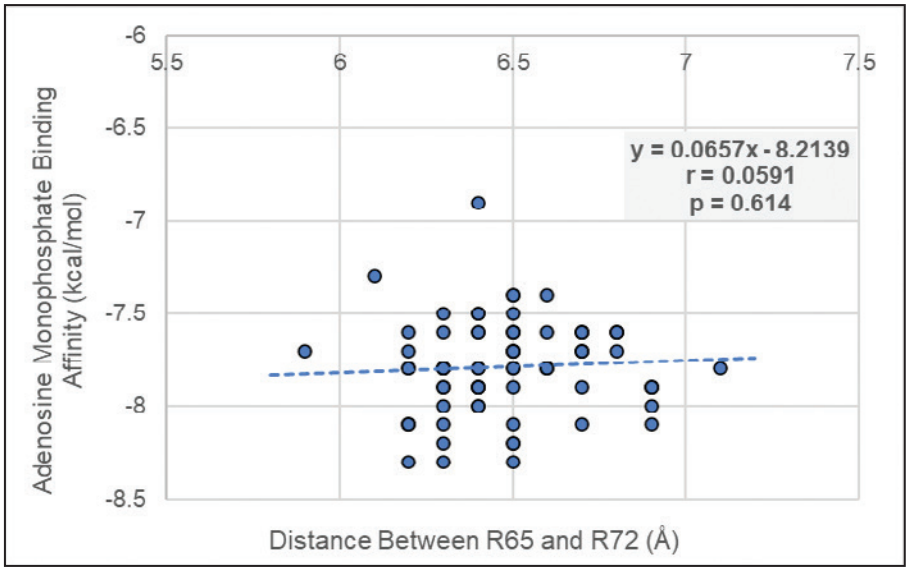

Figure 5. Distance Between Guanidinium Planes of R65 and R72 (Å) and Binding Affinity with Adenosine Monophosphate (kcal/mol)—Without D67 Points. Without outlying points from mutating D67, the correlation between RT-adenosine binding affinity and the distance between the guanidinium planes of residues 65 and 72 is statistically insignificant, with a p-value of 0.614.

and tenofovir binding affinity, when adjusted for outliers, suggests that the binding affinity between RT and tenofovir increases (becomes more negative) as the distance between the R65/R72 guanidinium planes decreases (Figure 3). However, given a low r-value of 0.3189 in figure 3, this association must be studied further with more conclusive data. The element of the aforementioned hypothesis that is *supported* by the data of this study is that there is not a significant change in the binding affinity between RT and adenosine triphosphate that is attributable to the change in the distance between R65's and R72's guanidinium planes. This is especially true considering a p-value of 0.614 when adjusting a correlation studying these variables, as seen in Figure 5.

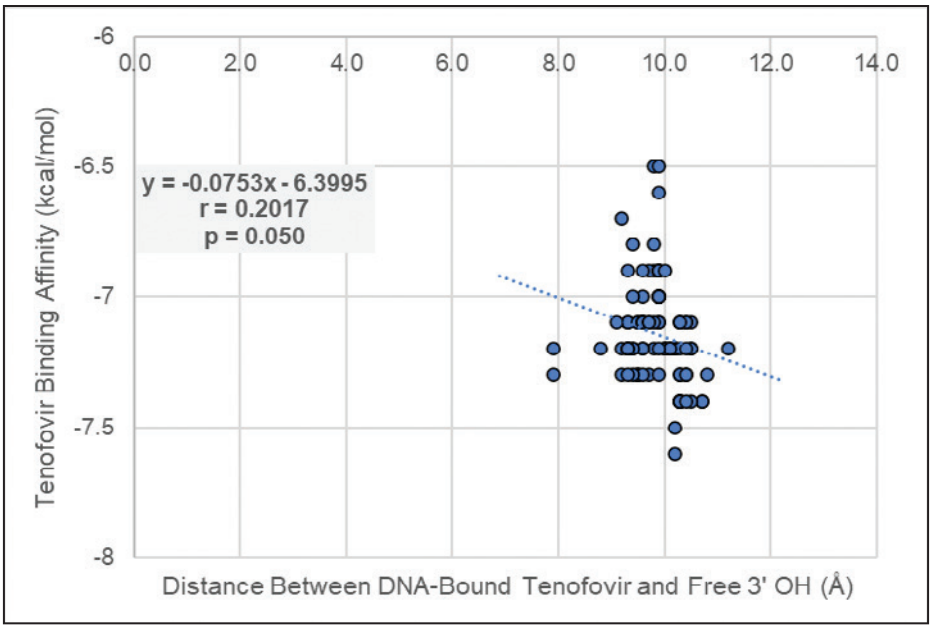

Figure 6. Distance Between DNA-Bound Tenofovir and Free 3′ OH (Å) and Binding Affinity with Tenofovir (kcal/mol). The correlation of RT-tenofovir binding affinity with the distance between tenofovir and RT-bound DNA proves to be statistically significant, with a slope of –0.0753 and a relatively weak r-value of 0.2017.

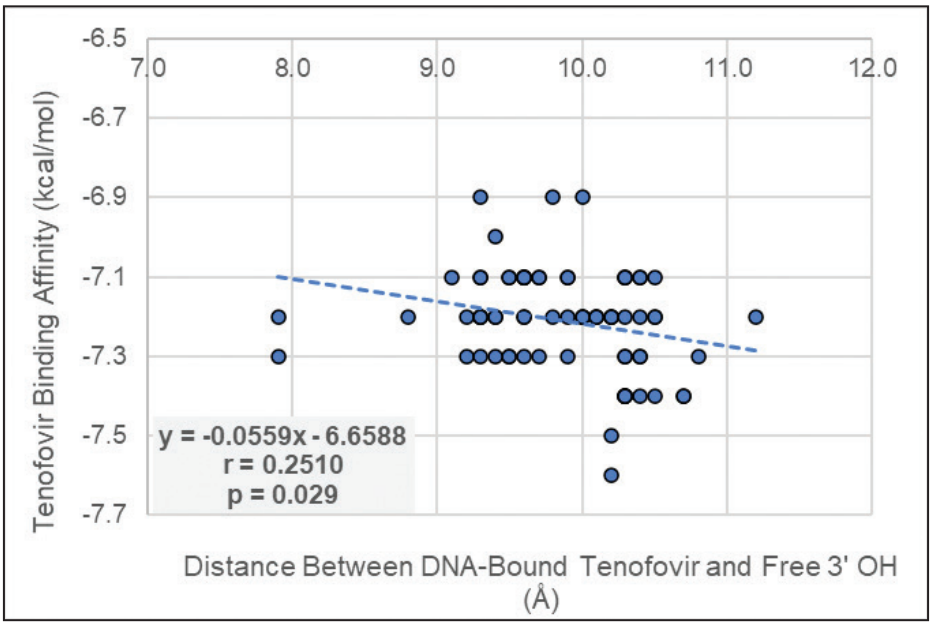

Figure 7. Distance Between DNA-Bound Tenofovir and Free 3′ OH (Å) and Binding Affinity with Tenofovir (kcal/mol)—Without D67 Points. Removing data from mutating D67 reveals a similar, statistically significant correlation, with a slope of –0.0559 suggesting that binding affinity increases (becomes more negative) by 0.0559 kcal/mol for every 1 Å increase in the distance between RT-bound DNA and tenofovir.

Results show that this increased distance *increases* binding affinity not only with tenofovir but also with adenosine, despite the theory that increased distance between tenofovir and interacting RT-bound DNA decreases binding affinity. In fact, within the correlation between distance from RT-bound DNA and tenofovir binding affinity, an adjusted trend line with a slope of –0.0559 indicates that binding affinity becomes more negative by 0.0559 kcal/mol for a 1 Angstrom increase in the distance from RT-bound DNA (Figure 7). Similarly, within the correlation between the distance from RT-bound DNA and adenosine binding affinity, an adjusted trend line with a slope of –0.0488 suggests that binding affinity becomes more negative by 0.0488 kcal/mol for a 1 Å increase in the distance from RT-bound DNA

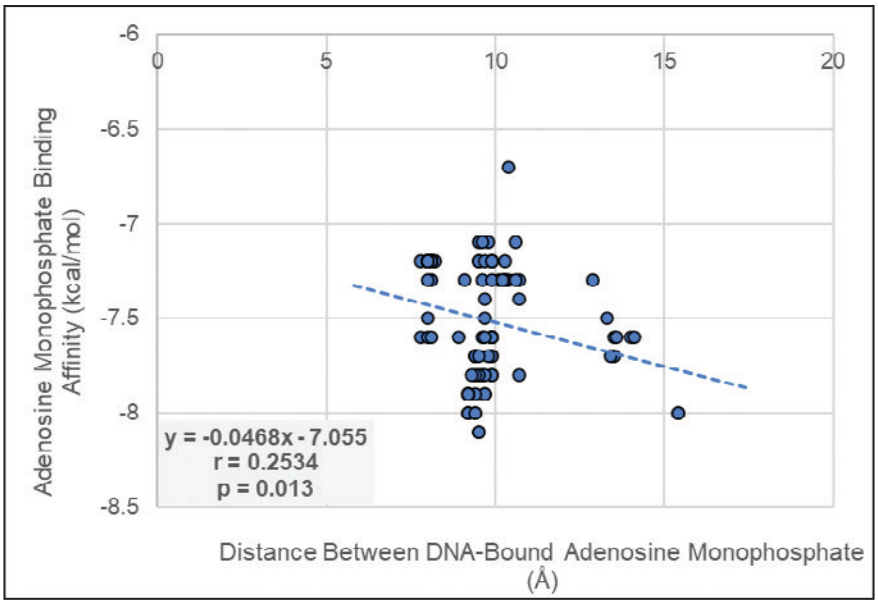

Figure 8. Distance Between DNA-Bound Adenosine Monophosphate and Free 3′ OH and Binding Affinity with AMP (kcal/mol). The correlation of RT-adenosine binding affinity with the distance between adenosine and RT-bound DNA proves to be statistically significant with a slope of −0.0468 and a relatively weak r-value of 0.2534.

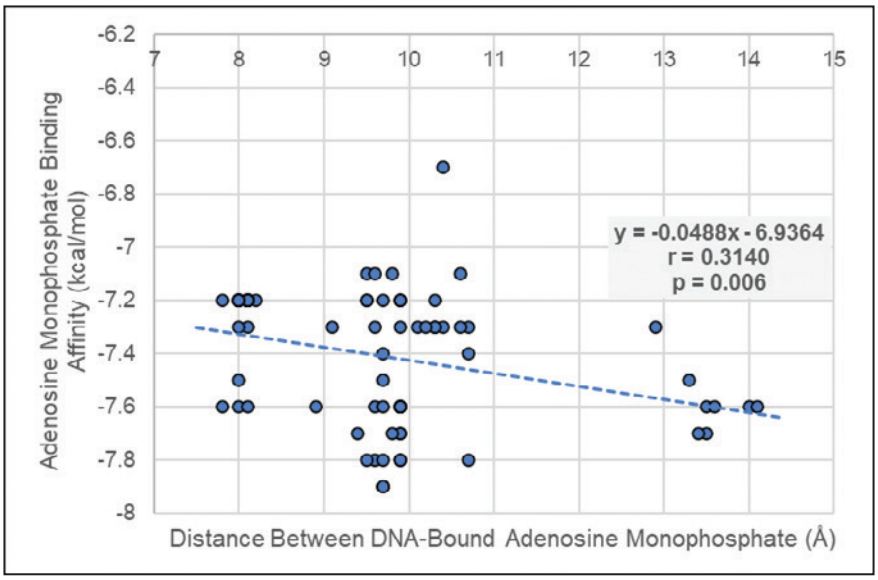

Figure 9. Distance Between DNA-Bound Adenosine Monophosphate and Free 3′ OH and Binding Affinity with AMP (kcal/mol)—Without D67 Points. Removing data from mutating D67 reveals a similar, statistically significant correlation, with a slope of −0.0488 suggesting that binding affinity increases (becomes more negative) by 0.0448 kcal/mol for every 1 Å increase in the distance between RT-bound DNA and adenosine monophosphate.

(Figure 9). The r-values of these correlations are extremely low (0.2017 and 0.2534, respectively), but it can still be concluded that binding affinity between RT and either adenosine or tenofovir does not become more positive as the distance from RT-bound DNA increases. This refutes the conjecture that implicates this mechanism for the K65R mutation (Fourati et al. 2013).

Limitations

These findings cannot go without saying that this study has its own limitations. For one, the mutagenesis of various residues within the β3-β4 loop may have caused additional changes within RT, besides the changes in the distance between the guanidinium planes of residues 65 and 72 and the distance between tenofovir/adenosine and

RT-bound DNA. For example, studies show that changes in thermal stability, which often follow single amino acid mutations, are often critical predictors of protein function outside various structural variables (Hoie et al. 2022). Such confounding effects are simply unaccounted for in this study's data, acting as a limiting factor in regard to the viability of this data and its conclusions. In addition, the weak correlations in the data described could be attributable to interactions between tenofovir/adenosine and side chains outside those being studied. For example, the side chains of residues 112–114 are close enough in proximity to the β3-β4 loop such that they could influence interactions between tenofovir/adenosine and residues 65/72 (Figure 10).

Lastly, it is unclear whether the same conclusions drawn from the computational methods described in this study would be drawn if this study's methods were replicated using in vitro laboratory techniques. Recent studies suggest that local docking using Autodock Vina, as performed in this study, has an average scoring accuracy of about 80% (Gaillard 2018). In addition, one study compared 800 protein-ligand complexes available in the Protein Data Bank to the conformational results for these complexes on Autodock Vina, finding that it has an accuracy of 81% in predicting binding conformations (Nguyen et al. 2020). Therefore, even as Vina stands out as the most powerful docking software to date, there is still room for error when compared to in vitro techniques, the effect of which cannot be accurately determined within this study. It remains ambiguous as to how that difference may affect the conclusions drawn in this study.

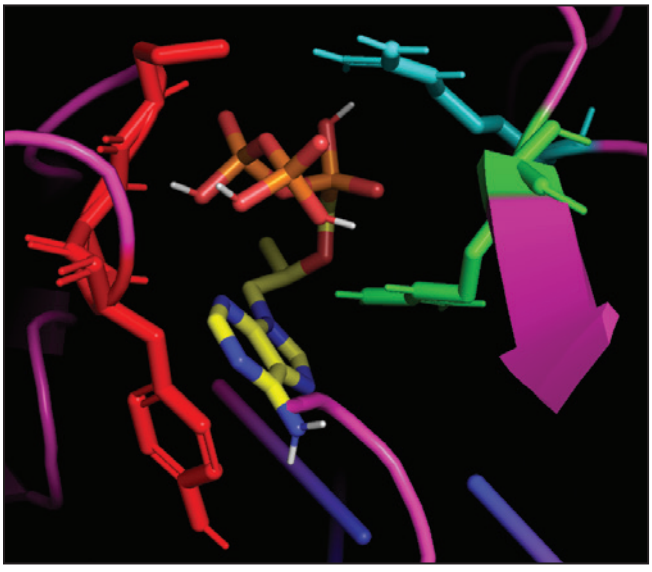

Figure 10. The tenofovir molecule shown in the center can interact with residues 65 and 72 of reverse transcriptase, highlighted in blue and green, respectively (also boxed on the right, residue 65 on the top and residue 72 on the bottom). However, tenofovir diphosphate can also interact with the sidechains of residues 112–114 (highlighted in red, boxed on the left), which are near residues 65 and 72.

Future Experimentation

Despite its limitations, this study is crucial in guiding future research of the K65R mutation. Of course, the methodology of this study should be repeated, with the collection of additional structural variables, data on thermal stability, and even data from RT structures with mutations outside the β3-β4 loop. This will help to more conclusively determine the effects of these certain structural factors on RT binding affinity with tenofovir and adenosine (Høie et al. 2022). In addition, future experiments could specifically center on interactions between tenofovir/adenosine and specific amino acids. For example, it's highly plausible that, within the K65R mutation, a specific characteristic within the R group of arginine itself causes decreased binding with tenofovir. So, future experiments may need to introduce other substitution mutations at the 65th residue to find common characteristics between amino acid residues that cause decreased binding affinity with tenofovir. Finally, even though the entire methodology of this study is simply irreproducible using in vitro techniques given cost and time constraints, binding affinities and conformations should be confirmed in the laboratory for certain mutations across the volume of data in this study. This would provide greater credence to not only the data in this study but also the conclusions drawn from it. Only with such future experimentation can additional insight into the K65R mutation be gained, offering the chance for the understanding of the true mechanism of the K65R mutation and the development of antiretrovirals combating the resistance conferred by the mutation.

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