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Predictions in Clinical Efficiency of SARS-CoV-2 RNA-Dependent RNA Polymerase (RdRp) Inhibitors by Molecular Docking

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Abstract

This study utilizes the enzyme-substrate complex theory to predict the clinical efficacy of COVID-19 treatments at the biological systems level, using molecular docking stability indicators. Experimental data from the Protein Data Bank and molecular structures generated by AlphaFold 3 were used to create macromolecular complex templates. Six templates were developed, including the holo nsp7-nsp8-nsp12 (RNA-dependent RNA polymerase) complex with dsRNA primers (holo-RdRp-RNA). The study evaluated several ligands—Favipiravir-RTP, Remdesivir, Abacavir, Ribavirin, and Oseltamivir—as potential viral RNA polymerase inhibitors. Notably, the first four of these ligands have been clinically employed in the treatment of COVID-19, allowing for comparative analysis. Molecular docking simulations were performed using AutoDock 4, and statistical differences were assessed through t-tests and Mann-Whitney U tests. A review of the literature on COVID-19 treatment outcomes and inhibitors targeting RNA polymerase enzymes was conducted, and the inhibitors were ranked according to their clinical efficacy: Remdesivir > Favipiravir-RTP > Oseltamivir. Docking results obtained from the second and third templates aligned with clinical observations. Furthermore, Abacavir demonstrated a predicted efficacy comparable to Favipiravir-RTP, while Ribavirin exhibited a predicted efficacy similar to that of Remdesivir. This research, focused on inhibitors of SARS-CoV-2 RNA-dependent RNA polymerase, establishes a framework for screening AI-generated drug templates based on clinical outcomes. Additionally, it develops a drug screening platform based on molecular docking binding energy, enabling the evaluation of novel or repurposed drugs and potentially accelerating the drug development process.

Keywords

AlphaFold 3, RNA-Dependent RNA Polymerase, Anti-Viral Drugs, Molecular Docking

1. Introduction

The first reported case of COVID-19 can be traced back to December 2019, which reported a cluster of unexplained pneumonia cases. Initially, the illness was suspected to be viral pneumonia [1]. Further investigation revealed that the cause was a novel coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2]. The name SARS-CoV-2 was chosen due to its close genetic and evolutionary relationship with SARS-CoV, the virus responsible for the SARS outbreak in China during 2002 - 2003 [3] [4].

The SARS-CoV-2 genome consists of a large, non-segmented, positive-sense single-stranded RNA approximately 30 kb in length, which includes a 5'-cap structure and a 3'-poly-A tail [5] [6]. It encodes a total of 29 proteins: 25 putative non-structural and accessory proteins, along with four structural proteins [7]. The open reading frame 1ab (Orf1ab) at the 5' end encodes polyproteins PP1ab and PP1a, which are then cleaved into 16 non-structural proteins (NSP1 to NSP16). The 3' end of the genome encodes the four structural proteins—spike (S), envelope (E), membrane (M), and nucleocapsid (N)—as well as nine accessory proteins (Orf3a, Orf3b, Orf6, Orf7a, Orf7b, Orf8, Orf9b, Orf9c, and Orf10). Non-structural proteins (NSPs) are crucial for the replication of viral RNA and for evading the host's immune system, while accessory proteins assist in the infection, survival, and transmission of the virus within host cells [8]-[10]. Structural proteins play a key role in the assembly of the virus and form the mature viral particles. Newly synthesized NSPs, together with the structural protein N, constitute the multi-protein replicase-transcriptase complex (RTC), which is vital for the replication and transcription of the viral genome [11]. NSP12 functions as an RNA-dependent RNA polymerase and is a core component of the replication-transcription machinery [12]. The auxiliary factors NSP7 and NSP8 enhance NSP12's binding to the RNA template and its enzymatic activity [13]. The interaction of NSP7, NSP8, and NSP12 with the template-primer RNA forms the essential components of a functional holo-RdRp, which is a key element of the SARS-CoV-2 RTC [14]. Additionally, NSP14 has proofreading capabilities during RNA replication, detecting and removing mismatched nucleotides with its exoribonuclease (ExoN) activity, which may also play a role in viral RNA recombination [15].

The enzyme-substrate complex refers to the transient structure that forms during the catalytic process when an enzyme binds to its substrate. This intermediate complex is essential for facilitating the enzymatic reaction, representing a crucial step in the overall mechanism. Key features of the enzyme-substrate complex,

such as binding affinity and enzyme conformational changes, play a pivotal role in the reaction's efficiency and specificity, ultimately influencing the enzymatic process's outcome.

While molecular dynamics simulations can predict the binding affinity between target proteins and small molecules at the molecular level [16] [17], they are limited in their ability to forecast clinical outcomes. These simulations provide valuable insights into the early stages of drug discovery by identifying potential drug candidates through comparison of binding affinities, yet they do not sufficiently predict how these candidates will perform in clinical settings.

Artificial intelligence (AI) has emerged as a transformative tool in COVID-19 research and drug discovery. The advent of AlphaFold 3, an advanced AI model, has significantly enhanced the accuracy of predicting biomolecular interactions, such as those between proteins and ligands, or proteins and nucleic acids [18]. AI applications in drug discovery encompass various approaches, including de novo drug design, property prediction, and drug response analysis, utilizing methodologies like graph neural networks and reinforcement learning [19]. Specifically, AI has played a key role in identifying SARS-CoV-2 genomic sequences and variants of concern, as well as in drug development and repurposing efforts [20]. Noteworthy AI-assisted drug candidates include Atazanavir, Remdesivir, and PARP1 inhibitors. Moreover, AI has contributed to vaccine development through the use of bioinformatics, immunoinformatics, and machine learning. Despite these advancements, significant challenges remain, particularly in the areas of data collection, validation, and ethical considerations, as well as the need for robust clinical trials to validate AI-generated predictions [19] [20]. This study aims to integrate the enzyme-substrate complex theory with AI-generated biomolecular structures, using these as enzyme templates. By incorporating clinical drug testing data, we seek to develop a systematic approach for selecting suitable AI-generated molecular structure templates for subsequent drug development. This strategy will form the basis of a virtual screening and evaluation platform designed to identify novel inhibitors of SARS-CoV-2 RNA-dependent RNA polymerase (RdRp). Such an approach has the potential to expedite drug discovery efforts and improve the precision of therapeutic interventions against COVID-19.

2. Materials and Methods

2.1. Preparation of the Molecular Docking Template

The initial holo-RNA-dependent RNA polymerase complex with double-stranded RNA (holo-RdRp-RNA) was analyzed using cryo-electron microscopy (Cryo-EM) structural data (Protein Data Bank [PDB] ID: 7AAP). This structure represents the NSP7-NSP8-NSP12 complex of the SARS-CoV-2 RNA-dependent RNA polymerase, bound to a double-stranded RNA (dsRNA) template-primer and favipiravir ribonucleoside triphosphate (favipiravir-RTP). For the molecular docking studies, the ligands favipiravir-RTP and Mg^{2+}, which were located in the enzyme's active site, were removed from the complex, resulting in the macromolecular

structure of holo-RdRp-RNA prepared for docking analysis.

The second holo-RdRp-RNA was generated using the AlphaFold Service (AlphaFold 3). AlphaFold is a web-based service designed for predicting high-accuracy biomolecular structures, including proteins, DNA, RNA, ligands, ions, and chemical modifications, all within a single platform utilizing the latest AlphaFold 3 model [18]. AlphaFold employs an ensemble learning approach to improve prediction accuracy by generating multiple models under slightly varied conditions or parameters to account for potential structural variability and uncertainty. Each CIF file represents one of these predicted models, utilizing a "seed" for internal random number generation. By default, this seed is automatically sampled and resampled for job cloning, though running multiple models with different seeds can enhance accuracy. The seed can be any integer between 0 and 4,294,967,295, and when cloning a job with a set seed, the seed will revert to automatic selection by default (AlphaFold Service, [https://alphafoldserver.com/about]). The predicted template modeling (pTM) score and the interface predicted template modeling (ipTM) score, derived from the template modeling (TM) score, gauge the accuracy of the predicted structures [21] [22]. A pTM score above 0.5 suggests that the predicted fold of the complex may be similar to the actual structure. The ipTM score assesses the accuracy of the predicted relative positions of the subunits within the complex, with values over 0.8 indicating high confidence, while values below 0.6 suggest a likely failure. Scores between 0.6 and 0.8 are in a gray zone, indicating that predictions could be either correct or incorrect (AlphaFold Service, [https://alphafoldserver.com/about]).

The protein sequences for SARS-CoV-2 were obtained from the National Center for Biotechnology Information (NCBI) [https://www.ncbi.nlm.nih.gov/datasets/taxonomy/2697049/]. These sequences comprise NSP7, consisting of 83 amino acids; NSP8, containing 198 amino acids; and NSP12, the RNA-dependent RNA polymerase, which consists of 932 amino acids. Guanosine triphosphate (GTP) was utilized as the ligand. The RNA sequences employed in this study were 5'-UUUUUCAUAACUUAAUCUCACAUAGCACUG-3' and 5'-CAGUGCUAUGUGAGAUUAAGUUAU-3', which are identical to the RNA sequence of the first holo-RdRp-RNA complex. Molecular structures generated by AlphaFold resulted in five distinct models, which served as templates for the second through sixth holo-RdRp-RNA complexes in this research. In subsequent molecular docking simulations, GTP will be removed from these structures and replaced with five different small-molecule ligands (drugs) utilized in this study.

2.2. Preparation of Small Molecule Ligands

The molecular structures of the small-molecule ligands utilized in this study were sourced from the Protein Data Bank (PDB). The extraction process was conducted using PyMOL [https://www.pymol.org/]. To prepare the ligands for docking, hydrogen atoms were added, and molecular structures were converted to the mol2

format using Open Babel [https://openbabel.org/docs/Installation/install.html]. Favipiravir-RTP was modeled based on Cryo-EM data (PDB ID: 7AAP) with a resolution of 2.50 Å (PubChem CID: 492405). Oseltamivir was obtained from X-ray crystallographic data (PDB ID: 3CL0) with a resolution of 2.20 Å (PubChem CID: 65028). Remdesivir was derived from X-ray crystallographic data (PDB ID: 7BF6) with a resolution of 2.15 Å (PubChem CID: 121304016). Abacavir was sourced from X-ray crystallographic data (PDB ID: 3UPR) with a resolution of 2.00 Å (PubChem CID: 441300), and Ribavirin was collected from X-ray crystallographic data (PDB ID: 4PB1) with a resolution of 2.80 Å (PubChem CID: 37542).

2.3. Molecular Docking Simulations

Molecular docking simulations were performed using AutoDockTools [https://autodocksuite.scripps.edu/adt/] in conjunction with AutoDock 4 [https://autodock.scripps.edu/download-autodock4/]. Each simulation paired a single holo-RdRp-RNA template with five distinct ligands, resulting in five unique docking outcomes for each template. Six different templates were utilized, with grid boxes defined based on default grid center values in AutoDock 4, as follows:

Template 1: (x center: 108.090, y center: 103.698, z center: 105.881)

Template 2: (x center: 1.724, y center: 1.202, z center: 0.250)

Template 3: (x center: -0.019, y center: 1.982, z center: −1.386)

Template 4: (x center: 0.713, y center: 0.646, z center: 0.317)

Template 5: (x center: 0.688, y center: 1.834, z center: 0.786)

Template 6: (x center: −1.709, y center: −0.400, z center: 0.020)

All other conditions across the six templates were kept consistent, including a grid spacing of 0.375 Å and a grid size of 126 × 126 × 126 points. Ligand parameters were set to default values (randomized). For the genetic algorithm parameters, 30 genetic algorithm (GA) runs were performed per docking simulation, with all other settings retained as default. The output format was Lamarckian GA (4.2). For each ligand-template pair, 30 binding energy samples were generated. The average binding energies and their confidence intervals were calculated for comparative analysis, resulting in ten distinct comparison groups.

2.4. Statistical Methods

2.4.1. Shapiro-Wilk Test

Normality is a fundamental assumption in numerous statistical analyses, and the Shapiro-Wilk test, introduced by Samuel Shapiro and Martin Wilk in 1965 [23], is a widely used method to assess this assumption, especially in small sample sizes.

Sample Ordering: The initial step in the Shapiro-Wilk test is to arrange the sample data in ascending order. This ordered sequence serves as the basis for evaluating the data's conformity to normality.

Expected Values Calculation: The test then calculates theoretical expected values derived from the standard normal distribution. These expected values correspond to the quantiles of the normal distribution and serve as benchmarks against

which the observed sample data are compared.

Test Statistic Calculation: The test statistic, denoted as W, is computed to measure the extent of deviation from normality. The formula for W is as follows:

$$W = \frac{\left(\sum_{i=1}^{n} a_i x_{(i)}\right)^2}{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2}$$

where $x_{(i)}$ represents the ordered sample values, $\bar{x}$ is the sample mean, and a_i are constants derived from the expected values of the normal distribution.

In hypothesis testing utilizing the Shapiro-Wilk test, the null hypothesis (H_0) asserts that the sample data adheres to a normal distribution, whereas the alternative hypothesis (H_1) posits that the data do not conform to a normal distribution. The W statistic, calculated from the sample data, is compared against critical values to determine the corresponding p-value. If the W value is less than the critical value at a designated significance level, commonly set at $\alpha = 0.05$, the null hypothesis is rejected, suggesting that the sample may not be normally distributed. Conversely, if the W value exceeds the critical value, there is insufficient evidence to reject the null hypothesis, indicating that the sample may be normally distributed. In the present study, the sample size is 30, and the critical value for the Shapiro-Wilk test at a significance level of 0.05 ($\alpha = 0.05$) is established at 0.927. The Shapiro-Wilk test can be efficiently calculated using Microsoft Excel, providing researchers with a practical means to assess the normality of their data.

2.4.2. The Independent T-Test

The independent t-test is a statistical technique used to compare the means of two independent groups to determine whether a statistically significant difference exists between them. Key assumptions include: 1) Independence: The samples must be independent, meaning the selection of one sample does not influence the other. 2) Normality: The data in each group should be approximately normally distributed, especially for smaller sample sizes. 3) Homogeneity of Variance: The variances of the two groups should be roughly equal.

The t-statistic for the independent t-test is calculated using the following formula:

$$\mathrm{t} = \frac{\bar{X}_1 - \bar{X}_2}{S_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where:

$\bar{X}_1$ and $\bar{X}_2$ are the sample means of the two groups.

n_1 and n_2 are the sample sizes of the two groups.

s_p is the pooled standard deviation, calculated as:

$$S_p = \sqrt{\frac{\left(n_1 - 1\right)\cdot S_1^2 + \left(n_2 - 1\right)\cdot S_2^2}{n_1 + n_2 - 2}}$$

where:

s_1^2 and s_2^2 are the sample variances of the two groups.

Hypothesis testing involves the following: the null hypothesis (H_0) states that there is no difference between the means of the two groups ($\mu_1 = \mu_2$), while the alternative hypothesis (H_1) suggests a significant difference between the means of the two groups ($\mu_1 \neq \mu_2$). The t-statistic is compared to a critical value from the t-distribution table, based on a chosen significance level (commonly $\alpha = 0.05$). If the absolute value of the calculated t-statistic exceeds the critical value, the null hypothesis is rejected, indicating a significant difference between the group means. Conversely, if the t-statistic is less than the critical value, there is insufficient evidence to reject the null hypothesis, suggesting that there is no significant difference between the means. The independent t-test can be conveniently calculated using Microsoft Excel.

2.4.3. Mann-Whitney U Test

The Mann-Whitney U Test [24] is a non-parametric statistical method that does not require assumptions about the data distribution. This test is especially useful for comparing two independent samples and is applicable to ordinal or continuous data that may not follow a normal distribution, making it advantageous when working with small sample sizes.

Sample Preparation: The Mann-Whitney U test [https://www.socscistatistics.com/tests/mannwhitney/default2.aspx] requires two independent samples, denoted as Sample 1 and Sample 2. These samples can consist of any form of continuous or ordinal data.

Ranking: The data from both samples are combined and sorted in ascending order. Each value is assigned a rank. In cases of ties, the average rank is assigned to the tied values.

The U statistic is derived for two samples using the following formulas:

$$U_1 = n_1 \cdot n_2 + \frac{n_1 \cdot (n_1 + 1)}{2} - R_1$$

$$U_2 = n_1 \cdot n_2 + \frac{n_2 \cdot (n_2 + 1)}{2} - R_2$$

In these equations, R_1 and R_2 represent the rank sums of Sample 1 and Sample 2, respectively, while n_1 and n_2 denote the sizes of the two samples. The final U statistic is defined as the smaller value between U_1 and U_2.

The Z statistic is calculated using the formula:

$$\mathrm{Z} = \frac{U - \mu_U}{\sigma_U}$$

where μ_U represents the expected value of U, and σ_U signifies the standard deviation of U. The Z statistic is utilized for hypothesis testing.

$$\mu_U = \frac{n_1 \cdot n_2}{2}$$

$$\sigma_{U=}\sqrt{\frac{n_1 \cdot n_2 \cdot (n_1 + n_2 + 1)}{12}}$$

The null hypothesis (H_0) posits that the two samples are drawn from the same distribution, implying that their medians are equal. Conversely, the alternative hypothesis (H_1) asserts that the medians of the two samples differ. The U statistic is subsequently employed to calculate the p-value, which can be determined using statistical tables or software. Once μ_U and σ_U have been estimated, the Z statistic can be computed. Finally, the calculated p-value is compared to a predetermined significance level, typically set at 0.05. If the p-value is less than this significance threshold, the null hypothesis is rejected, indicating a statistically significant difference between the two groups.

3. Results

In this study, molecular docking simulations were carried out using six distinct templates of SARS-CoV-2 holo-RdRp-RNA macromolecular complexes. The first template was obtained from a structure resolved by electron cryomicroscopy (cryo-EM) and is available in the Protein Data Bank (PDB). The other five templates were derived from molecular structures predicted using AlphaFold 3 models, with the following parameters: Seed: 33157952, ipTM = 0.86, and pTM = 0.9. These generated models exhibit high confidence compared to the true structure. Each template, representing a macromolecular complex, was used to evaluate the binding stability and affinity of five different ligands under identical grid box conditions. The binding stability and affinity were assessed using binding energy as the metric, where lower values indicate higher stability and affinity. The differences in binding energies among the ligands were analyzed for each macromolecular complex template, with a total of ten ligand-template pairs evaluated.

The Shapiro-Wilk test was employed to evaluate whether the samples derived from the pairing of each template with its corresponding ligand followed a normal distribution. The critical value for the Shapiro-Wilk test at a significance level of 0.05 (α = 0.05) for a sample size of 30 is 0.927. This value serves as a benchmark for determining the appropriateness of subsequent statistical tests, as the assumption of normality is a prerequisite for conducting a t-test. In contrast, the non-parametric Mann-Whitney U test does not impose this requirement. Statistical comparisons were performed using both t-tests and the Mann-Whitney U test, with a two-tailed significance level set at 0.05 (α = 0.05). The inferential results were interpreted accordingly. Based on the statistical outcomes for the ten evaluated ligand-template pairs, relationships and classifications among the five ligands were inferred. Ligands belonging to the same category were indicated within parentheses, with connections represented by an "=" sign. Instances where the five ligands could not be distinctly classified and instead appeared across multiple categories were denoted by an "!".

For the first holo-RdRp-RNA template, as illustrated in **Figure 1**, the binding energies (mean ± 95% CI) for the five ligands were recorded as follows: Favipiravir-

RTP (−6.078 ± 0.253 kcal/mol), Oseltamivir (−5.028 ± 0.146 kcal/mol), Remdesivir (−6.496 ± 0.215 kcal/mol), Abacavir (−6.096 ± 0.316 kcal/mol), and Ribavirin (−6.162 ± 0.189 kcal/mol).

The W values calculated from the Shapiro-Wilk test for the respective ligands were: Favipiravir-RTP (0.984), Oseltamivir (0.986), Remdesivir (0.911), Abacavir (0.904), and Ribavirin (0.957). The results of the Shapiro-Wilk test indicated that the samples for Favipiravir-RTP, Oseltamivir, and Ribavirin conformed to a normal distribution. In contrast, the samples for Remdesivir and Abacavir did not exhibit a normal distribution.

The results of the statistical tests and inferences are summarized as follows (**Figure 1**):

- **t-test**: Oseltamivir > (Favipiravir-RTP = Abacavir = Ribavirin) > Remdesivir
- **Mann-Whitney U test**: Oseltamivir > (Favipiravir-RTP = Abacavir = Ribavirin)! > (Remdesivir = Ribavirin)!

For the second holo-RdRp-RNA template, as depicted in **Figure 2**, the binding energies (mean ± 95% CI) for the ligands were as follows: Favipiravir-RTP (−5.909 ± 0.304 kcal/mol), Oseltamivir (−5.463 ± 0.178 kcal/mol), Remdesivir (−6.569 ± 0.221 kcal/mol), Abacavir (−5.803 ± 0.245 kcal/mol), and Ribavirin (−6.415 ± 0.225 kcal/mol).

Comparisons between Models		t-test	Mann-Whitney U test
Model A [Template 1 : ligand]	Model B [Template 1 : ligand]	α: 0.05	α: 0.05
[Template 1 : Favipiravir-RTP]	[Template 1 : Oseltamivir]	*	*
[Template 1 : Favipiravir-RTP]	[Template 1 : Remdesivir]	*	*
[Template 1 : Favipiravir-RTP]	[Template 1 : Abacavir]	NS	NS
[Template 1 : Favipiravir-RTP]	[Template 1 : Ribavirin]	NS	NS
[Template 1 : Oseltamivir]	[Template 1 : Remdesivir]	*	*
[Template 1 : Oseltamivir]	[Template 1 : Abacavir]	*	*
[Template 1 : Oseltamivir]	[Template 1 : Ribavirin]	*	*
[Template 1 : Remdesivir]	[Template 1 : Abacavir]	*	*
[Template 1 : Remdesivir]	[Template 1 : Ribavirin]	*	NS
[Template 1 : Abacavir]	[Template 1 : Ribavirin]	NS	NS

* : significant at α: 0.05; NS: non-significant

Symbol: [Model]	Binding Energy (mean ± 95%CI) kcal/mol	W value for the Shapiro-Wilk test
A: [Template 1 : Favipiravir-RTP]	-6.078 ± 0.253	0.984◎
B: [Template 1 : Oseltamivir]	-5.028 ± 0.146	0.986◎
C: [Template 1 : Remdesivir]	-6.496 ± 0.215	0.911
D: [Template 1 : Abacavir]	-6.096 ± 0.316	0.904
E: [Template 1 : Ribavirin]	-6.162 ± 0.189	0.957◎

CI: confidence interval

◎: indicates that the sample exhibits a normal distribution.

Interpretation of Results	
t-test (α: 0.05)	Mann-Whitney U test (α: 0.05)
B > (A=D=E) > C	B > (A=D=E)! > (C=E)!

!: represents that the inference is vague and unclear.

Figure 1. Molecular docking interactions and variability analysis of Holo-RdRp-RNA from Template 1 (PDB ID: 7AAP) with Ligands.

Comparisons between Models		t-test	Mann-Whitney U test
Model A [Template 2 : ligand]	Model B [Template 2 : ligand]	α: 0.05	α: 0.05
[Template 2 : Favipiravir-RTP]	[Template 2 : Oseltamivir]	*	*
[Template 2 : Favipiravir-RTP]	[Template 2 : Remdesivir]	*	*
[Template 2 : Favipiravir-RTP]	[Template 2 : Abacavir]	NS	NS
[Template 2 : Favipiravir-RTP]	[Template 2 : Ribavirin]	*	*
[Template 2 : Oseltamivir]	[Template 2 : Remdesivir]	*	*
[Template 2 : Oseltamivir]	[Template 2 : Abacavir]	*	*
[Template 2 : Oseltamivir]	[Template 2 : Ribavirin]	*	*
[Template 2 : Remdesivir]	[Template 2 : Abacavir]	*	*
[Template 2 : Remdesivir]	[Template 2 : Ribavirin]	NS	NS
[Template 2 : Abacavir]	[Template 2 : Ribavirin]	*	*

* : significant at α: 0.05; NS: non-significant

Symbol: [Model]	Binding Energy (mean ± 95%CI) kcal/mol	W value for the Shapiro-Wilk test
A: [Template 2 : Favipiravir-RTP]	-5.909 ± 0.304	0.966◎
B: [Template 2 : Oseltamivir]	-5.463 ± 0.178	0.967◎
C: [Template 2 : Remdesivir]	-6.569 ± 0.221	0.965◎
D: [Template 2 : Abacavir]	-5.803 ± 0.245	0.797
E: [Template 2 : Ribavirin]	-6.415 ± 0.225	0.89

CI: confidence interval

◎: indicates that the sample exhibits a normal distribution.

Interpretation of Results	
t-test (α: 0.05)	Mann-Whitney U test (α: 0.05)
B > (A=D) > (C=E)	B > (A=D) > (C=E)

Figure 2. Molecular docking interactions and variability analysis of Holo-RdRp-RNA from Template 2, generated by AlphaFold 3 Model 1, with Ligands.

The *W* values obtained from the Shapiro-Wilk test for the respective ligands were: Favipiravir-RTP (0.966), Oseltamivir (0.967), Remdesivir (0.965), Abacavir (0.797), and Ribavirin (0.890). The results of the Shapiro-Wilk test indicated that the samples for Favipiravir-RTP, Oseltamivir, and Remdesivir conformed to a

normal distribution. In contrast, the samples for Abacavir and Ribavirin did not exhibit a normal distribution.

Statistical comparisons and inferences are detailed as follows (Figure 2):

- **t-test**: Oseltamivir > (Favipiravir-RTP = Abacavir) > (Remdesivir = Ribavirin)
- **Mann-Whitney U test**: Oseltamivir > (Favipiravir-RTP = Abacavir) > (Remdesivir = Ribavirin)

For the third holo-RdRp-RNA template, as shown in Figure 3, the binding energies (mean ± 95% CI) were as follows: Favipiravir-RTP (−5.983 ± 0.353 kcal/mol), Oseltamivir (−5.346 ± 0.187 kcal/mol), Remdesivir (−6.913 ± 0.273 kcal/mol), Abacavir (−6.174 ± 0.244 kcal/mol), and Ribavirin (−7.017 ± 0.214 kcal/mol).

Comparisons between Models		t-test	Mann-Whitney U test
Model A [Template 3 : ligand]	Model B [Template 3 : ligand]	α: 0.05	α: 0.05
[Template 3 : Favipiravir-RTP]	[Template 3 : Oseltamivir]	*	*
[Template 3 : Favipiravir-RTP]	[Template 3 : Remdesivir]	*	*
[Template 3 : Favipiravir-RTP]	[Template 3 : Abacavir]	NS	NS
[Template 3 : Favipiravir-RTP]	[Template 3 : Ribavirin]	*	*
[Template 3 : Oseltamivir]	[Template 3 : Remdesivir]	*	*
[Template 3 : Oseltamivir]	[Template 3 : Abacavir]	*	*
[Template 3 : Oseltamivir]	[Template 3 : Ribavirin]	*	*
[Template 3 : Remdesivir]	[Template 3 : Abacavir]	*	*
[Template 3 : Remdesivir]	[Template 3 : Ribavirin]	NS	NS
[Template 3 : Abacavir]	[Template 3 : Ribavirin]	*	*

* : significant at α: 0.05; NS: non-significant

Symbol: [Model]	Binding Energy (mean ± 95%CI) kcal/mol	W value for the Shapiro-Wilk test
A: [Template 3 : Favipiravir-RTP]	-5.983 ± 0.353	0.904
B: [Template 3 : Oseltamivir]	-5.346 ± 0.187	0.939◎
C: [Template 3 : Remdesivir]	-6.913 ± 0.273	0.903
D: [Template 3 : Abacavir]	-6.174 ± 0.244	0.852
E: [Template 3 : Ribavirin]	-7.017 ± 0.214	0.853

CI: confidence interval

◎: indicates that the sample exhibits a normal distribution.

Interpretation of Results	
t-test (α: 0.05)	Mann-Whitney U test (α: 0.05)
B > (A=D) > (C=E)	B > (A=D) > (C=E)

Figure 3. Molecular docking interactions and variability analysis of Holo-RdRp-RNA from Template 3, generated by AlphaFold 3 Model 2, with Ligands.

The *W* values calculated from the Shapiro-Wilk test for the respective ligands were: Favipiravir-RTP (0.904), Oseltamivir (0.939), Remdesivir (0.903), Abacavir (0.852), and Ribavirin (0.853). The results of the Shapiro-Wilk test indicated that only the sample for Oseltamivir conformed to a normal distribution, while the samples for Favipiravir-RTP, Remdesivir, Abacavir, and Ribavirin did not exhibit a normal distribution.

The results are summarized as follows (Figure 3):

- **t-test**: Oseltamivir > (Favipiravir-RTP = Abacavir) > (Remdesivir = Ribavirin)
- **Mann-Whitney U test**: Oseltamivir > (Favipiravir-RTP = Abacavir) > (Remdesivir = Ribavirin)

For the fourth holo-RdRp-RNA template, as illustrated in Figure 4, the binding energies (mean ± 95% CI) were as follows: Favipiravir-RTP (−6.56 ± 0.621 kcal/mol), Oseltamivir (−5.71 ± 0.185 kcal/mol), Remdesivir (−6.856 ± 0.275 kcal/mol), Abacavir (−6.37 ± 0.338 kcal/mol), and Ribavirin (−6.48 ± 0.281 kcal/mol).

The W values calculated from the Shapiro-Wilk test for the respective ligands were: Favipiravir-RTP (0.972), Oseltamivir (0.980), Remdesivir (0.870), Abacavir (0.930), and Ribavirin (0.908). The results of the Shapiro-Wilk test indicated that the samples for Favipiravir-RTP, Oseltamivir, and Abacavir conformed to a normal distribution. In contrast, the samples for Remdesivir and Ribavirin did not exhibit a normal distribution.

The statistical results are presented as follows (Figure 4):

- **t-test**: Oseltamivir > (Favipiravir-RTP = Remdesivir = Abacavir = Ribavirin)! > (Remdesivir = Ribavirin)!
- **Mann-Whitney U test**: Oseltamivir > (Favipiravir-RTP = Remdesivir = Abacavir = Ribavirin)! > (Remdesivir = Ribavirin)!

For the fifth holo-RdRp-RNA template, as shown in Figure 5, the binding energies (mean ± 95% CI) were recorded as follows: Favipiravir-RTP (−5.744 ± 0.351 kcal/mol), Oseltamivir (−5.263 ± 0.113 kcal/mol), Remdesivir (−6.607 ± 0.234 kcal/mol), Abacavir (−5.953 ± 0.178 kcal/mol), and Ribavirin (−6.205 ± 0.249 kcal/mol).

The W values calculated from the Shapiro-Wilk test for the respective ligands were: Favipiravir-RTP (0.981), Oseltamivir (0.963), Remdesivir (0.891), Abacavir (0.908), and Ribavirin (0.918). The results of the Shapiro-Wilk test indicated that the samples for Favipiravir-RTP and Oseltamivir conformed to a normal distribution, whereas the samples for Remdesivir, Abacavir, and Ribavirin did not exhibit a normal distribution.

The statistical results are detailed as follows (Figure 5):

- **t-test**: Oseltamivir > (Favipiravir-RTP = Abacavir)! > (Abacavir = Ribavirin)! > Remdesivir
- **Mann-Whitney U test**: Oseltamivir > (Favipiravir-RTP = Abacavir = Ribavirin)! > (Remdesivir = Ribavirin)!

For the sixth holo-RdRp-RNA template, as shown in Figure 6, the binding energies (mean ± 95% CI) were as follows: Favipiravir-RTP (−6.901 ± 0.344 kcal/mol), Oseltamivir (−5.31 ± 0.148 kcal/mol), Remdesivir (−6.567 ± 0.303 kcal/mol), Abacavir (−5.809 ± 0.264 kcal/mol), and Ribavirin (−6.486 ± 0.298 kcal/mol).

Comparisons between Models		t-test	Mann-Whitney U test
Model A [Template 4 : ligand]	Model B [Template 4 : ligand]	α: 0.05	α: 0.05
[Template 4 : Favipiravir-RTP]	[Template 4 : Oseltamivir]	*	*
[Template 4 : Favipiravir-RTP]	[Template 4 : Remdesivir]	NS	NS
[Template 4 : Favipiravir-RTP]	[Template 4 : Abacavir]	NS	NS
[Template 4 : Favipiravir-RTP]	[Template 4 : Ribavirin]	NS	NS
[Template 4 : Oseltamivir]	[Template 4 : Remdesivir]	*	*
[Template 4 : Oseltamivir]	[Template 4 : Abacavir]	*	*
[Template 4 : Oseltamivir]	[Template 4 : Ribavirin]	*	*
[Template 4 : Remdesivir]	[Template 4 : Abacavir]	*	*
[Template 4 : Remdesivir]	[Template 4 : Ribavirin]	NS	NS
[Template 4 : Abacavir]	[Template 4 : Ribavirin]	NS	NS

* : significant at α: 0.05; NS: non-significant

Symbol: [Model]	Binding Energy (mean ± 95%CI) kcal/mol	W value for the Shapiro-Wilk test
A: [Template 4 : Favipiravir-RTP]	-6.56 ± 0.621	0.972©
B: [Template 4 : Oseltamivir]	-5.71 ± 0.185	0.98©
C: [Template 4 : Remdesivir]	-6.856 ± 0.275	0.87
D: [Template 4 : Abacavir]	-6.37 ± 0.338	0.93©
E: [Template 4 : Ribavirin]	-6.48 ± 0.281	0.908

CI: confidence interval

©: indicates that the sample exhibits a normal distribution.

Interpretation of Results	
t-test (α: 0.05)	Mann-Whitney U test (α: 0.05)
B > (A=C=D=E)! > (C=E)!	B > (A=C=D=E)! > (C=E)!

!: represents that the inference is vague and unclear.

Figure 4. Molecular docking interactions and variability analysis of Holo-RdRp-RNA from Template 4, generated by AlphaFold 3 Model 3, with Ligands.

Comparisons between Models		t-test	Mann-Whitney U test
Model A [Template 5 : ligand]	Model B [Template 5 : ligand]	α: 0.05	α: 0.05
[Template 5 : Favipiravir-RTP]	[Template 5 : Oseltamivir]	*	*
[Template 5 : Favipiravir-RTP]	[Template 5 : Remdesivir]	*	*
[Template 5 : Favipiravir-RTP]	[Template 5 : Abacavir]	NS	NS
[Template 5 : Favipiravir-RTP]	[Template 5 : Ribavirin]	*	NS
[Template 5 : Oseltamivir]	[Template 5 : Remdesivir]	*	*
[Template 5 : Oseltamivir]	[Template 5 : Abacavir]	*	*
[Template 5 : Oseltamivir]	[Template 5 : Ribavirin]	*	*
[Template 5 : Remdesivir]	[Template 5 : Abacavir]	*	*
[Template 5 : Remdesivir]	[Template 5 : Ribavirin]	*	NS
[Template 5 : Abacavir]	[Template 5 : Ribavirin]	NS	NS

* : significant at α: 0.05; NS: non-significant

Symbol: [Model]	Binding Energy (mean ± 95%CI) kcal/mol	W value for the Shapiro-Wilk test
A: [Template 5 : Favipiravir-RTP]	-5.744 ± 0.351	0.981©
B: [Template 5 : Oseltamivir]	-5.263 ± 0.113	0.963©
C: [Template 5 : Remdesivir]	-6.607 ± 0.234	0.891
D: [Template 5 : Abacavir]	-5.953 ± 0.178	0.908
E: [Template 5 : Ribavirin]	-6.205 ± 0.249	0.918

CI: confidence interval

©: indicates that the sample exhibits a normal distribution.

Interpretation of Results	
t-test (α: 0.05)	Mann-Whitney U test (α: 0.05)
B > (A=D)! > (D=E)! > C	B > (A=D=E)! > (C=E)!

!: represents that the inference is vague and unclear.

Figure 5. Molecular docking interactions and variability analysis of Holo-RdRp-RNA from Template 5, generated by AlphaFold 3 Model 4, with Ligands.

Comparisons between Models		t-test	Mann-Whitney U test
Model A [Template 6 : ligand]	Model B [Template 5 : ligand]	α: 0.05	α: 0.05
[Template 6 : Favipiravir-RTP]	[Template 6 : Oseltamivir]	*	*
[Template 6 : Favipiravir-RTP]	[Template 6 : Remdesivir]	NS	NS
[Template 6 : Favipiravir-RTP]	[Template 6 : Abacavir]	*	*
[Template 6 : Favipiravir-RTP]	[Template 6 : Ribavirin]	NS	NS
[Template 6 : Oseltamivir]	[Template 6 : Remdesivir]	*	*
[Template 6 : Oseltamivir]	[Template 6 : Abacavir]	*	*
[Template 6 : Oseltamivir]	[Template 6 : Ribavirin]	*	*
[Template 6 : Remdesivir]	[Template 6 : Abacavir]	*	*
[Template 6 : Remdesivir]	[Template 6 : Ribavirin]	NS	NS
[Template 6 : Abacavir]	[Template 6 : Ribavirin]	*	*

* : significant at α: 0.05; NS: non-significant

Symbol: [Model]	Binding Energy (mean ± 95%CI) kcal/mol	W value for the Shapiro-Wilk test
A: [Template 6 : Favipiravir-RTP]	-6.901 ± 0.344	0.972◎
B: [Template 6 : Oseltamivir]	-5.31 ± 0.148	0.9
C: [Template 6 : Remdesivir]	-6.567 ± 0.303	0.863
D: [Template 6 : Abacavir]	-5.809 ± 0.264	0.787
E: [Template 6 : Ribavirin]	-6.486 ± 0.298	0.849

CI: confidence interval

◎: indicates that the sample exhibits a normal distribution.

Interpretation of Results	
t-test (α: 0.05)	Mann-Whitney U test (α: 0.05)
B > D > (A=C=E)	B > D > (A=C=E)

Figure 6. Molecular docking interactions and variability analysis of Holo-RdRp-RNA from Template 6, generated by AlphaFold 3 Model 5, with Ligands.

The W values calculated from the Shapiro-Wilk test for the respective ligands were: Favipiravir-RTP (0.972), Oseltamivir (0.900), Remdesivir (0.863), Abacavir (0.787), and Ribavirin (0.849). The results of the Shapiro-Wilk test indicated that only the sample for Favipiravir-RTP conformed to a normal distribution, while the samples for Oseltamivir, Remdesivir, Abacavir, and Ribavirin did not exhibit a normal distribution.

The results are summarized as follows (**Figure 6**):

- **t-test**: Oseltamivir > Abacavir > (Favipiravir-RTP = Remdesivir = Ribavirin)
- **Mann-Whitney U test**: Oseltamivir > Abacavir > (Favipiravir-RTP = Remdesivir = Ribavirin)

Although the Shapiro-Wilk test indicated that the sample distributions for all six holo-RdRp-RNA template-ligand pairs were not suitable for subsequent independent t-test analysis, notable differences were still observed in the statistical comparisons for the second, third, fourth, and sixth holo-RdRp-RNA template-ligand pairs. The classification results for the ligands were consistent with the outcomes of the non-parametric Mann-Whitney U test, excluding instances of ambiguous category differentiation. A comparative statistical analysis of the binding affinities for all six holo-RdRp-RNA template-ligand pairs revealed consistent results for the second, third, and sixth templates.

4. Discussion

Remdesivir is a nucleoside analogue initially developed to treat Ebola virus disease [25] and has since demonstrated efficacy against SARS-CoV, MERS-CoV, and SARS-CoV-2 in vitro and in animal studies [26]-[28]. Its therapeutic action is attributed to its inhibition of viral RNA-dependent RNA polymerase [28]. A systematic review of nine randomized controlled trials (RCTs) involving a total of 11,218 participants with SARS-CoV-2 infection, with a mean age of 53.6 years, assessed the impact of remdesivir. Of these participants, 5982 were administered remdesivir. Most participants required low-flow oxygen at baseline, and the trials were mainly conducted in high- and upper-middle-income countries. The review indicates that remdesivir likely has a minimal effect on reducing all-cause or in-hospital mortality among individuals with moderate to severe COVID-19. While one study reported a reduction in hospitalization rates for patients with mild to moderate COVID-19, the overall clinical benefits of remdesivir for both hospitalized

and non-hospitalized patients remain unclear. The applicability of these findings might be limited by the fact that participants were predominantly from largely unvaccinated populations and were exposed to early variants of SARS-CoV-2 [29].

Favipiravir represents a new class of RNA-dependent RNA polymerase inhibitors and is effective against a wide range of RNA viruses. Inside cells, Favipiravir is converted to its active form through phosphorylation. This active form is then recognized by viral RNA polymerase, inhibiting its activity [30]. However, unlike Remdesivir, Favipiravir shows limited effectiveness in halting RNA replication when natural nucleotides are present [31]. The binding interactions of Favipiravir with RdRp and its low incorporation efficiency as Favipiravir-RTP are detailed in the cryo-EM structure of the Favipiravir-bound SARS-CoV-2 RdRp-RNA complex [32]. A review of 25 randomized trials, involving 5750 adults from various countries including Bahrain, Brazil, China, India, Iran, Kuwait, Malaysia, Mexico, Russia, Saudi Arabia, Thailand, the UK, and the USA, predominantly focused on hospitalized patients with mild to moderate COVID-19 (89%). Of these trials, 22 compared Favipiravir to placebo or standard care, two compared it to lopinavir/ritonavir, and one compared it to umifenovir. The review concluded that the evidence supporting the efficacy of Favipiravir for COVID-19 is of low to very low certainty. Although treatment with Favipiravir may be associated with an increase in adverse events, it does not seem to lead to a higher rate of serious adverse events [33].

Oseltamivir, a neuraminidase inhibitor used for treating influenza A and B [34], was assessed as a potential antiviral treatment for SARS-CoV-2. However, its efficacy in treating COVID-19 has not been conclusively demonstrated [35]. The virological, laboratory, and radiological response rates did not show significant benefit from oseltamivir, although improvements were observed in electrocardiographic safety parameters in the oseltamivir group. Despite these findings, additional research is necessary to provide strong evidence regarding oseltamivir's effectiveness for COVID-19 treatment [35]. Furthermore, there is no evidence that oseltamivir inhibits RdRp through interaction. In this study, oseltamivir was designated as the control group.

According to clinical drug testing results for RdRp during the COVID-19 pandemic, treatments are categorized based on their therapeutic efficacy as follows:

1) Effective: Remdesivir

2) No Significant Effect: Favipiravir-RTP

3) Non-RdRp Target Control Group: Oseltamivir

In alignment with the experimental outcomes from the six groups in this study, the results from the second and third sets produced by AlphaFold 3 satisfied the criteria for therapeutic efficacy. Consequently, these findings were utilized to predict and assess the clinical effectiveness of nucleoside analogue reverse transcriptase inhibitors, specifically Abacavir and Ribavirin, in the treatment of COVID-19.

Abacavir, a nucleoside analogue reverse transcriptase inhibitor, is clinically used

as an anti-HIV medication [36] [37]. Currently, there are no clinical reports on its antiviral efficacy against SARS-CoV-2. This study assessed Abacavir's binding affinity to RNA-dependent RNA polymerase (RdRp) and found that its binding values are statistically similar to those of Favipiravir with RdRp. This suggests that Abacavir is unlikely to have significant therapeutic effects against COVID-19.

In addition, the study evaluated Ribavirin's binding affinity to RdRp and found its binding stability to be comparable to that of Remdesivir, a known anti-COVID-19 viral RNA polymerase inhibitor. Ribavirin has shown efficacy in inhibiting viral RNA-dependent RNA polymerase and demonstrated in vitro effectiveness against SARS replication [38]. However, clinical research on Ribavirin's effectiveness for severe COVID-19 has yielded mixed results. Two studies reported no significant benefits regarding clinical outcomes, mortality rates, or viral clearance in hospitalized adults with severe COVID-19 [39] [40]. Conversely, an open-label trial of Ribavirin aerosol showed some improvement in clinical status and ventilator discontinuation in certain patients [41], while another observational study reported a 100% 28-day survival rate and reduced viral positivity in critically ill patients [42]. Ribavirin was generally well-tolerated in these studies, with no significant adverse effects reported [39] [40] [42]. Nonetheless, due to the mixed clinical outcomes and the known liver toxicity associated with Ribavirin [38] [43] further research, especially randomized controlled trials, is needed to definitively determine its efficacy in treating severe COVID-19.

In the face of pathogenic threats, the development of potential therapeutics can be significantly hindered when structural data of target molecules, essential for drug design, are lacking in protein structure databases. Molecular docking and molecular dynamics simulations, both critical for drug development, rely heavily on accurate molecular structures. In this context, AlphaFold 3, an advanced AI system, can predict and generate multiple versions of target protein structures, providing valuable virtual models for subsequent drug design efforts. However, determining which of these AI-generated models closely resembles a biologically active structure, comparable to those found in the real world, is a key objective for future research. Identifying such structures is crucial for ensuring they serve as reliable references in drug development workflows. AlphaFold typically generates five different models for each target protein, offering a range of predictions. To evaluate which model most closely approximates the true structure, the following criteria can be considered: AlphaFold assigns a pLDDT (Predicted Local Distance Difference Test) score [18] to each residue in the predicted structure, representing the confidence level of the prediction on a scale from 0 to 100. A higher score indicates greater reliability of the predicted structure. Regions with pLDDT scores above 90 are considered highly accurate, while scores between 70 and 90 indicate moderate confidence, 50 to 70 suggest lower reliability, and below 50 indicate inaccurate predictions. By assessing the overall pLDDT scores across the five models, the model with the highest scores is generally more trustworthy. AlphaFold also generates a PAE (Predicted Aligned Error) matrix [18], visualizing the predicted

errors between pairs of residues. This helps assess the accuracy of the model across different regions and evaluate the relative positioning of structural domains. Models with minimal errors in the PAE matrix are more likely to reflect the true structure. After selecting a model with high pLDDT scores and low PAE, molecular dynamics simulations [16] [17] can further test the stability of the predicted structure. Stable models are more likely to be biologically relevant. Additionally, if experimental data such as X-ray crystallography or cryo-electron microscopy are available, the AlphaFold models can be compared against these experimental results to validate their accuracy. For proteins with multiple domains, AlphaFold's predictive accuracy may vary across different regions. It is important to assess whether the predicted domains are biologically plausible and whether their relative positions make sense in the context of the protein's function. Careful analysis of these domains can help identify the most accurate and biologically meaningful models. By combining pLDDT and PAE analyses, molecular dynamics simulations, and potential experimental validation, it is possible to identify which AI-generated protein structure is most likely to resemble the real-world, biologically active conformation. These models can then be used with greater confidence in the drug discovery and development process.

This study investigates molecular interactions by leveraging estimated binding energies of specific template molecules and ligands as crucial indicators. The primary hypothesis posits that binding and catalytic reactions adhere to the "enzyme-substrate complex" theory, which encompasses multiple binding states. To validate this hypothesis, comprehensive sampling is essential to implement statistical methods that correlate the clinical therapeutic efficacy of specific ligands. The findings suggest that the second and third holo-RdRp-RNA templates, derived from AlphaFold 3 models, may function as promising molecular structure templates for drug screening. However, the study does not provide a detailed elucidation of the mechanisms of action in clinical treatments. Additionally, the potential interactions of ligands with other molecules, along with their effects and reactions within biological systems, introduce variables that the proposed drug screening platform is unable to account for. These confounding factors may affect biological system functionality and subsequently influence clinical outcomes. To enhance the effectiveness of this drug screening platform, it is advisable to prioritize candidate drugs based on the absence of significant side effects, such as repurposed drugs or candidates that have already passed metabolic and toxicological evaluations. This approach aims to minimize potential confounding variables, thereby improving the accuracy of predictions regarding clinical outcomes.

5. Conclusion

Recent advancements in artificial intelligence (AI) have facilitated the generation of numerous virtual molecular structures, each with distinct characteristics. However, amidst this extensive array of virtual models, the identification of those that closely resemble authentic biological structures and demonstrate biological activity

presents a considerable challenge. This study focuses on SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) inhibitors, utilizing clinical treatment outcomes for COVID-19 as a reference point. Our objective is to employ clinical statistical data regarding drug efficacy as a classification basis, correlating this information with the molecular docking interactions of various virtual molecular structures. By selecting versions that are consistent with both clinical and molecular docking classifications, we create a robust framework for subsequent drug design and screening initiatives. This methodology not only enhances the evaluation process for novel or repurposed drugs with clinical potential but also accelerates the overall drug development pipeline.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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