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Virtual Screening: Small Molecular Inhibitors of Human Norovirus RNA-dependent RNA-polymerase

Lufei Liu

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, China.

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***Corresponding author:** Lufei Liu, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, China.

Abstract

Noroviruses, belonging to the family of *Caliciviridae*, are non-enveloped RNA viruses with single strands that cause illnesses such as acute gastroenteritis. Every year, it can bring about 685 million acute gastroenteritis cases, which leads to a serious situation for medical care. About one-third of patients are children under 5 years old. Even more dangerous, especially in developing countries, five thousand kids die every year. Currently, there is no specific medicine to treat human norovirus yet. Vaccine treatments and potential drugs are still in clinical trials and are not available yet. To prevent the outbreak of human norovirus, inhibiting the replication of viral genomes can be a suitable treatment method. Since RNA-dependent RNA-polymerase (RdRp) plays an important role during crucial steps of viral replication and is highly conserved during the amplification of RNA viruses, this protein can be a prospective target for selecting future anti-viral drugs. In this context, eleven small molecular compounds (drugs) are selected during the process of blind docking using Auto Dock Vina and tested in vitro experiments as potential human norovirus inhibitors. The enzyme activity is tested in experiments in vitro, indicating the inhibitory activities of these small molecular compounds.

Keywords

RNA-dependent RNA-polymerase inhibitor, human noroviruses, molecular docking

1. Introduction

Noroviruses, belonging to the family of *Caliciviridae*, are non-enveloped RNA viruses with single strands that cause illnesses such as acute gastroenteritis. They are named after a remarking case happened in the school of Norwalk in 1968, and then are categorized into 10 genogroups (from GI to GX) and 48 genotypes. Every year, human norovirus can cause about 685 million acute gastroenteritis cases, which leads to a serious situation for medical care. About one-third of patients are children under 5 years old. Even more dangerous, especially in developing countries, five thousand kids die every year [1, 2]. None of the medicine is approved to treat people with norovirus illness. Traditionally, people drink plenty of liquids like water to prevent dehydration resulting from vomiting and diarrhea [3, 4, 5]. Antibody treatment for norovirus by injecting vaccines is still in progress. Researchers are now developing new vaccines using particles such as P particles, virus-like particles (VLPs), and recombinant adenoviruses in the laboratory. Recently, a bivalent VLP-based injectable vaccines and oral vaccine are still being tested in clinical trials [6]. Moreover, there are also potential treatments related to small molecular inhibitors. For example,

naphthalene di-sulfonate (NAF2) and pyridoxal-50-phosphate-6-(20-naphthylazo-60-nitro-40,80-disulfonate) tetrasodium salt (PPNDS) are tested as potential small molecular inhibitors for human norovirus by inhibiting RNA-dependent RNA-polymerase protein essential for genome replication.

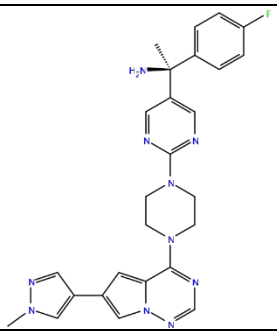
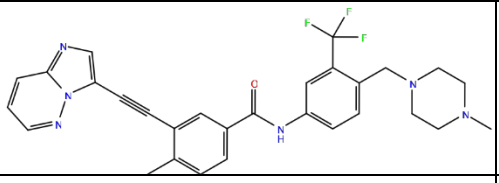
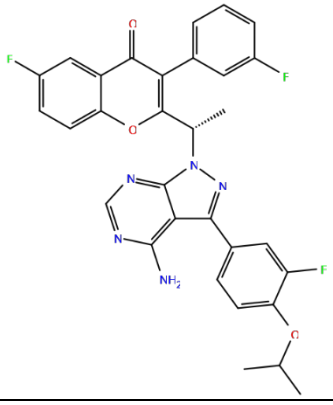
RNA-dependent RNA-polymerase (RdRp) is a multifunctional enzyme that is irreplaceable for genome replication and transcription. Although there are some differences in RdRps sequences within viruses, their basic structural features of them are highly conserved. By inhibiting its function, the replication and amplification of the RNA genome will be affected and therefore halt the spread of RNA viruses such as human norovirus. Because this protein is highly conserved during the replication and mutation of viruses, it can be a suitable research object for developing potential drugs to treat RNA viruses. Our finding is based on inhibiting human norovirus RdRp protein to find potential small molecular drugs which can involve in the future treatment of norovirus. Using blind docking drug-sized compounds to human RdRp protein with accession codes 4LQ9 preserved on the PDB website, eleven potential inhibitors were selected among 2092 compounds.

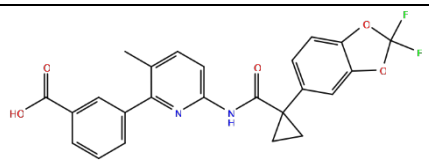
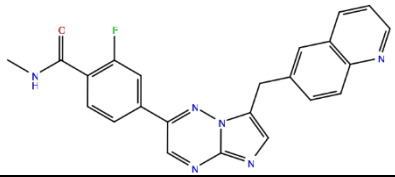
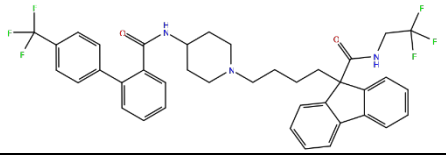
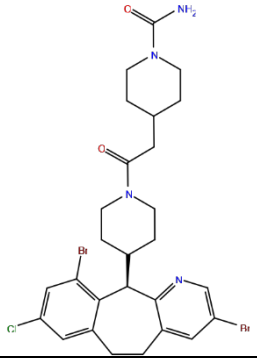
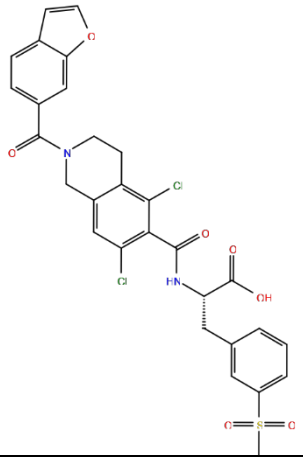
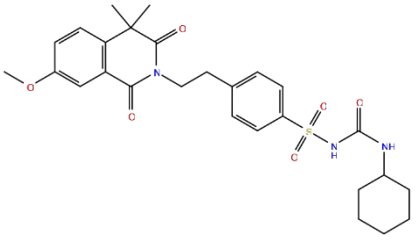
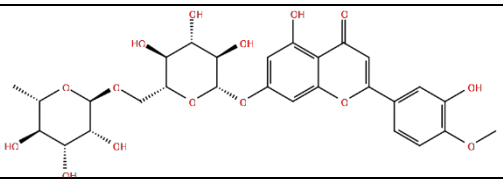
2. Materials and Methods

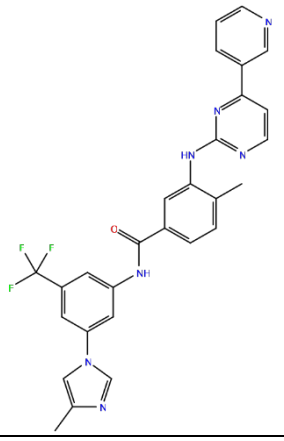
2.1 Chemicals

During the inhibition assay of 4LQ9-RdRp, compounds in the Approved Drug Library, (*Targetmol*, n.d.) named Avapritinib, Ponatinib, TGR1202, Lumacaftor, Capmatinib, Lomitapide, Lonafarnib, Lifitegrast, Gliquidone, Diosmin, and Nilotinib (monohydrochloride monohydrate), numbered 1,5,6,9,11,19,20,35,36,37,39 accordingly, were purchased and tested. All of these compounds were proven to be old drugs for various treatments.

Table 1. Names and structures of small molecular compounds chosen from the Approved Drug Library from Targetmol (https://www.targetmol.com). Figures of molecular structures were created using Maestro 13.0 (https://www.schrodinger.com/products/maestro). Schrodinger Software ModulesCopyright (c) Schrodinger, LLC. All rights reserved

Number	Name	Formula	Structure	Previous Treatment
1	Avapritinib	C26H27FN10		Cancer
5	Ponatinib	C29H27F3N6O		Cancer
6	TGR1202	C31H24F3N5O3		Cancer

9	Lumacaftor	C24H18F2N2O5		Chromosomal disease
11	Capmatinib	C23H17FN6O		Cancer
19	Lomitapide	C39H37F6N3O2		Metabolism
20	Lonafarnib	C27H31Br2ClN4O2		Infection
35	Lifitegrast	C29H24Cl2N2O7S		Inflammation
36	Gliquidone	C27H33N3O6S		Metabolism
37	Diosmin	C28H32O15		Cardiovascular system

39	Nilotinib	C28H22F3N7O.HCl.H2O		Cancer

2.2 Molecular docking by utilizing Auto Dock Vina

Software such as Auto Dock Vina, Open Babel, PyMOL, and Maestro was used in the process of virtual screening. The first step started with downloading the three-dimensional structure for hNV-RdRp 4LQ9 in PDB and SDF files of small molecular compounds from the bank. Second, used PyMOL to delete redundant water molecules and metal atoms, as well as the original inhibitors in complexed with 4LQ9 structure. PDB file of hNV-RdRp was converted into a pdbqt file. Then, sdf files of small molecular compounds were converted into pdbqt files by using Open Babel. Auto Dock Vina [7] was utilized to do blind docking and directed docking on Site A and Site B [8].

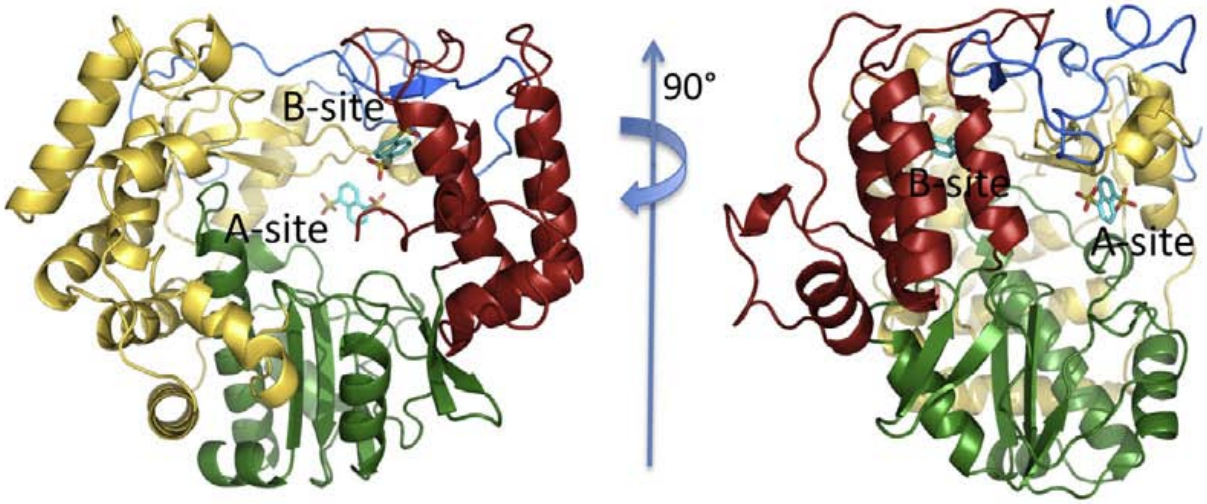


Figure 1. ***Crystal structure of hNV-RdRp bound to two NAF2 molecules in the A-site and B-site.***

To obtain a rapid virtual screening process, a Batch file (.bat in Windows) was utilized in the process of molecular docking. The last step was to collect data and select compounds with the lowest binding free energy with 4LQ9 hNV- RdRp. Free Maestro could help to visualize the specific binding point of small molecular compounds. It should be noticed that during this research, some metallic atoms would cause an error in Auto Dock Vina. Among the 2092 small molecular compounds, 7 compounds were reported as errors and not considered in the research.

2.3 Expression and purification of the Human Norovirus RNA-dependent RNA-polymerase (hNV-RdRp)

Inserted the DNA of human norovirus-RdRp between two restriction enzyme cutting sites BamH I and Not I sites of PET-21A (+) to obtain the recombinant plasmid named hNV-RdRp (human norovirus- RdRp). During the expression of a recombinant plasmid, the upstream link of hNV- RdRp DNA is "ATGGCTAGCATGACTGGTGGA CAGCAAATGGGTCGCGGATCC"; the downstream link is "GCGGCCGCACTCGAGCACCACCACCACCA CCACTGA". In this way, a fusion gene expressing hNV-RdRp with His 6 label was formed. The recombinant

plasmid hNV-RdRp was introduced into Escherichia coli BL21(DE3) to obtain recombinant bacteria. Then, the recombinant bacteria were inoculated in LB liquid medium containing 100μg/ml ampicillin and incubated at 37℃ at 200rpm until the 0D 600nm value =0.6. IPTG was added with a concentration of 0.5mM in the system. The recombinant bacteria were incubated at 16℃ at 200rpm for 18 hours when the previous steps were finished. Lysozyme and DNaseI were added into the system (0.2mg/ mL lysozyme and 0.02mg/ mL DNasel in the system), followed by ultrasonic crushing and centrifugation at 12000rpm at 4℃ for 10min. The collected supernatant was passed through the filter membrane of 0.45 nm. HNV-RdRp was filtered with nickel columns and desalting columns to purify the obtained hNV-RdRp from ions and other redundant elements (Cen et al., 2020). Purified hNV-RdRp was tested by bicinchoninic acid assay (BCA assay) to gain the concentration of protein. First, the Working Reagent is prepared in several steps, including mixing Reagent A and Reagent B at a ratio of 50:1 and adding it to the clear liquid in the color yellow-green. Then the Standard Reagent is prepared later. After that, lateral concentrations of 0, 0.125, 0.25, 0.5,1, 2, 4 mg/ml are diluted from 5 mg/ml BSA Standard. Each test tube is added by 200μl Working Reagent, 20 μl Test or Standard reagent, and incubated at 37℃ for 30 min in total. Next, use a spectrophotometer to measure the absorbance of samples at 562 nm and minus the reading of absorbance average in order to get the accurate absorbance. Finally, plot absorbance reading for each BSA standard versuscorresponding concentration on the graph to draw a standard curve. Utilize this curve to analyze and conclude the concentration of protein for each unknown sample [9].

2.4 Inhibitory activity of the small molecular compounds related to 4LQ9 hNV-RdRp

First, researchers should measure the polymerase activity by figuring out how poly(C) helps form RNA with double strands, and PicoGreen [10], the dye which can show fluorescent part in the sample, is used to visualize the result. In a black 96-well plate, the RdRp testing assay was performed, protecting the reaction mixture from being damaged by light. RNA recombining assays were tested in vitro: RNA poly(C) with single strand serves for the template for researchers, whereas GTP is consume as the substrate for the mixture with 25 μL. Being dissolved in the reaction buffer containing 2.5 mM MnCl2, 10 mM PBS with a standard pH of 7.5, and 5 mM DTT, 0.5 μL of 90 μM hNV-RdRp was incubated with the compound DMSO preserved for 10 min at 30◦C or 1 μL of 1 mM compound. Noticeably, PPNDS is the positive control for inhibiting human norovirus RNA-dependent RNA-polymerase. Thereafter, add 1 μL poly(C) with the concentration about 1 mg/mL and 1 μL GTP with 625 μM to the mixture and incubate at 30◦C for 30 min. Finally, there are several components researchers need to add in: 40 ng/mL prepared poly(C), 1.8 μM NV-RdRp, 40 μM reaction compound mixture, 25 μM GTP, 4% DMSO, 2.5 mM MnCl2, 10 mM PBS with standard pH of 7.5, and 5 mM DTT reagent. Researchers may end the reaction process with 10 mM EDTA. Next, visualization is accomplished by adding 175 μL of 200-fold diluted fluorescent dye PicoGreen to each well for five minutes at 25◦C. Last but not least, perform the PicoGreen dying and figure out the number of dsRNA formed at standard wavelengths, usually with excitation of 480 nm and emission of 520 nm, using a microplate reader.

Blank control was conducted using DMSO to replace compound solutions, and Total activities were measured. The average value was recorded as meanTA. Negative control was conducted with inactivated compound solutions (100℃, 2min) and the average result was recorded as meanNSA. Then the relative activity of hNV-RdRp was calculated by equation: Relative Activity=100·(sample-meanNSA)/ (meanTA- meanNSA).

3. Results

3.1 Virtual screening by using Auto Dock Vina

In the process of molecular docking, blind docking, docking based on Site A, and docking based on Site B [8] were conducted. Because nearly all the AutoDock scores (kcal/mol) of Site A and Site B for the tested compound NAF2 were higher than -7.0 (the score of NAF2 originally tested in Tarantino's essay was about -7.0 to -4.0 at both Site A and Site B), the docking result for Site A and Site B were not counted while selecting potential inhibitors.

The docking results of the eleven selected compounds are shown in the table below.

The visualized docking results of compound 1 and compound 9 are shown in Figure 3 and Figure 4.

3.2 Inhibitory activity of eleven small molecular compounds on hNV-RdRp

The result of experiments in vitro is recorded. By analyzing the relative hNV-RdRp activity after being treated with eleven small molecular compounds, the inhibitory activity of each compound can be figured out.

The relative activity of hNV-RdRp when each compound was added is shown below.

Table 2. Vina Molecular Blind Docking Result (Score and Interactions)

Tested Small Molecular Compound	Auto Dock Score (kcal/mol)	H-Bonding numbers	H-Bonding (amino acid)	Others
1*	-10.0/-10.0	Site a: 2 bonds Site b: 4 bonds	Site a: L183 G301 Site b: S306 V302 D247 D343	/
5	-10.2	2 bonds	T117 S300	/
6	-9.5	4 bonds	L169 R392 D242	/
9*	-10.0/ -9.6	Site c: 4 bonds Site d: 6 bonds	Site c: R393 D344 Site d: E168 K180 R182 L183	/
11	-9.9	5 bonds	L391 R393 N505	/
19	-10.6	3 bonds	R392 Q414	/
20	10.0	4 bonds	D247	two bonds are formed among the compound itself
35	-9.9	7 bonds	R182 W185 Y243 R245 D247	/
36	-10.1	5 bonds	R413 Q414 T418 R419 Q435	/
37	-10.1	12 bonds	L169 R182 R392 R393	/
39	-10.3	3 bonds	Q414 N505	/

"*" means that there is more than one suitable docking result. The number of hydrogen bonds will vary slightly.

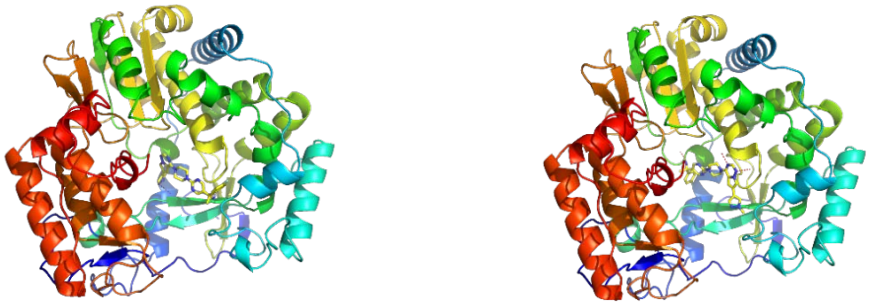


Figure 2. (A) is the structure of hNV-RdRp bound to compound 1 in Site a; (B) is the structure of hNV-RdRp bound to compound 1 in Site b. Figures were created using PyMol (http://www.pymol.org). The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

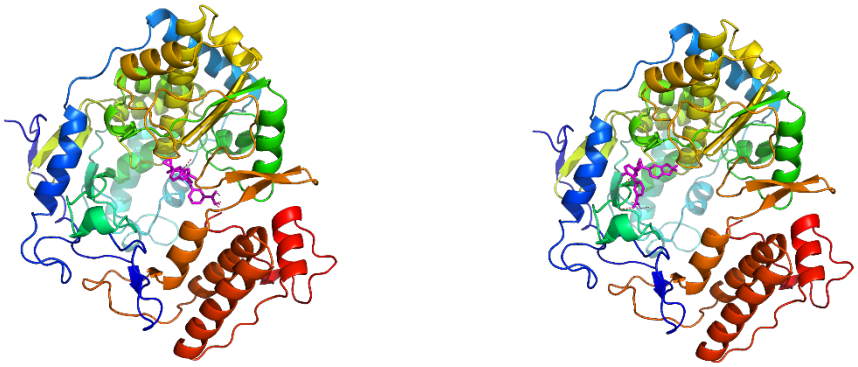


Figure 3. (C) is the structure of hNV-RdRp bound to compound 9 in Site c; (D) is the structure of hNV-RdRp bound to compound 9 in Site d. Figures were created using PyMol (http://www.pymol.org). The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

Table 3. The relative hNV- RdRp Activity was treated with eleven compounds

Compounds Experiment	1	5	6	9	11	19	20	35	36	37	39
1	88.49%	126.13%	76.50%	61.79%	72.64%	81.80%	54.82%	71.66%	62.37%	77.75%	80.62%
2	44.77%	85.23%	49.56%	54.86%	50.90%	44.91%	59.31%	52.06%	64.87%	50.96%	68.02%
3	50.52%	70.82%	58.38%	53.62%	56.67%	62.33%	48.75%	51.00%	58.09%	51.13%	57.39%
Average	61.26%	94.06%	61.48%	56.76%	60.07%	63.01%	54.29%	58.24%	61.78%	59.95%	68.68%

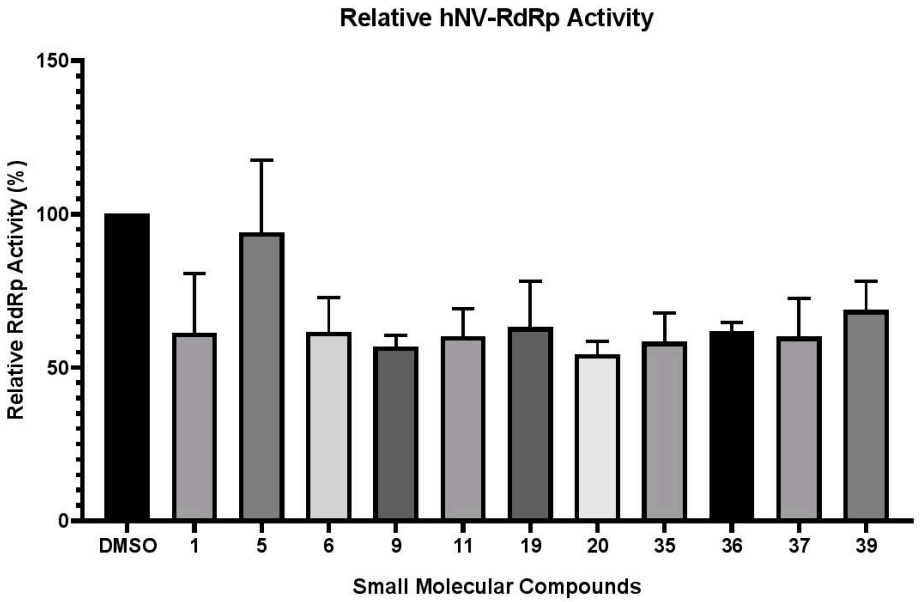


Figure 4. The Relative hNV-RdRp Activity with eleven small molecular compounds added. DMSO is the control group with 100% activity.

It is well-indicated that hNV-RdRp treated with compound 20 (Lonafarnib) has the lowest relative activity of 54.29% on average; in contrast, the polymerase with compound 5 has the highest activity of 94.06% on average, and one experiment among the assay even measured a relative activity of 126.13%. The other compounds have all decreased the relative activity of hNV-RdRp in the assay.

4. Discussion

4.1 Potential Value for the Experiment

The current studies related to human noroviruses are diverse. Scientists are still trying to develop a suitable solution for the treatment, and some focus on hNV-RdRp, which is highly conserved. There are two vital regions for RdRp to start the human norovirus RNA synthesis, which include the sequences near the antisense genomic stem region and the stem-loop structure in the 31 nt end, according to [11], so a loss of specific region in hNV-RdRp will disable the amplification for human norovirus. Using RdRp as the target protein provides researchers with a broader aspect to develop potential drugs dealing with viruses that have a high mutation rate. Inhibitors for the viral RdRp are likely to inhibit the growth and replication of other viruses in the family of *Caliciviridae* because, during the replication of human norovirus, RdRp helps with the synthesis of RNA that encodes theORF2 and 3' which are similar virus genomes for all the viruses in the same family with human noroviruses [1]. This suggests that hNV-RdRp has a high research value.

The structure and function of hNV-RdRp are getting clearer, yet a lot of potential targets are still unraveled. By employing the process of virtual screening, the binding site and interactions between small molecular compounds and proteins are well-visualized, which can help researchers to explore the potential RdRp binding targets. Also, screening by computer and models can save financial costs and time costs for the experiment.

In this article, all the screened small molecular compounds are drugs approved by FDA. By re-studying the novel

usage of old drugs, the time cost for conducting toxicity experiments and setting up clinical trials is saved since these drugs are well-studied in previous research.

4.2 Binding Sites Similar to Sites Discovered in Previous Researches Related to NAF2 Compound and Novel Binding Sites

It is suggested from Tarantino's experiment in vitro that NAF2 compounds have a relatively low inhibitory activity, yet Site A and Site B of NAF2 can hopefully be potential targets for future studies (2014). According to the docking results of Auto Dock Vina, most AutoDock scores of both sites are higher than -7.0 kcal/mol, suggesting that NAF2 is not likely to bind with hNV-RdRp because of the high binding free energy, which verified the previous studies. However, during the process of blind docking, we found some docking sites similar to what Tarantino's experiment described.

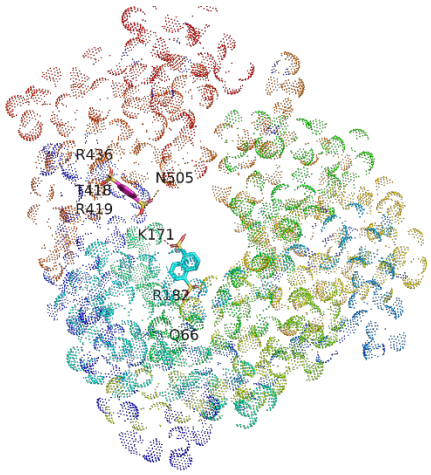


Figure 5. The docking result of NAF2 in Site A and Site B is shown (cyan for Site A and purple for Site B). Figures were created using PyMol (http://www.pymol.org). The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

It is suggested in PyMOL that there are 4 hydrogen bonds formed in Site A and 5 hydrogen bonds formed in Site B respectively when docking 4LQ9 with NAF2. The related amino acids are Q66, R182, and K171 at Site A and R436, T418, R419, and N505 at Site B. Notably, the binding site of 9 (Site d) is similar to the binding site in Site A in Tarantino's research; the binding sites of 11, 39, and 36 are similar as Site B in the same way. Thus, it is proved that Site A and Site B can be potential binding sites for drugs. However, the AutoDock score during molecular docking did not suggest this result, which needs to be studied further.

Moreover, in the docking result, it is suggested that compounds 1, 5, and 35 have a similar binding site (Site a) with center (-22.368, -29.233, -15.263) for x, y, z coordinates and size for 16.5, 19.5, and 15.0 at x, y, z. Figure X indicated the binding site of compound 1 (a) as green, 5 as yellow, and 35 as pink.

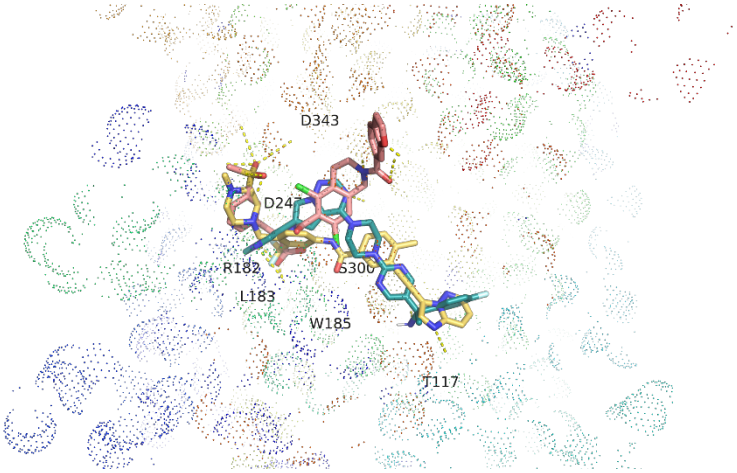


Figure 6. The binding site for 1(a), 5, and 35.

4.3 Effectiveness for Inhibition

Comparing the relative activity of hNV-RdRp when treated with eleven selected small molecular compounds, it is clearly shown that except for compound 5, compounds 1,6,9,11,19,20,35,36,37,39 inhibit the activity of hNV-RdRp, and compound 20 (Lonafarnib) has the greatest inhibitory effect on the polymerase.

4.4 Future Study for hNV-RdRp Inhibition and Prospects

The research related to human norovirus will be continued by carrying out other experiments in vitro that provide different aspects of analysis. To estimate how effective the compound is for inhibiting the replication of human norovirus, semi-maximum effect concentrations (EC50) of small molecular compounds will be calculated by GraphPad5.0 software [12]. By conducting hNV-RdRp inhibition assays in vitro experiment, IC50 values for each small molecular inhibitor will also be measured using the equation: Activity= minimum activity +(maximum activity-minimum activity)/ (1+ (inhibitor concentration/ IC50)×hill coefficient) [13]. Notably, the compound Lonafarnib will be tested and analyzed individually. Also, the combining capacity between ligands and the target protein will be tested in vitro using the method called biolayer interferometry assay (BLI). In this way, KD (dissociation constant) = Koff/ Kon can be calculated to indicate the binding ability between the ligand and the polymerase.

Further research will involve studies on the effect of selected small molecular inhibitors on other viruses in the family of *Caliciviridae*, such as Sapovirus. The inhibitory activities for viruses in other families will also be tested [14, 15]. Also, the reason for the high free binding energy at Site A and Site B should be further studied. The size of the docking pocket will be adjusted and compounds in the bank will be tested again. Furthermore, the new binding site found in this research will be tested to screen more small molecular compounds.

5. Conclusion

According to the research above, it can be concluded that among the eleven compounds tested, compound Lonafarnib has the greatest potential for treating human norovirus. Also, Site A and Site B described in Tarantino's essay are potential binding sites for drugs binding with hNV-RdRp, based on the molecular docking result of approved drugs from the database. Moreover, Site a suggested in this research is probably a new binding site for the treatment, which needs further study.

Acknowledgment

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