Influenza Virus Polymerase Acidic (PA) Endonuclease Inhibitor

Subjects: Allergy

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Drug repurposing can quickly and effectively identify novel drug repurposing opportunities. The PA endonuclease catalytic site has recently become regarded as an attractive target for the screening of anti-influenza drugs. PA N-terminal (PAN) inhibitor can inhibit the entire PA endonuclease activity. In this study, we screened the effectivity of PAN inhibitors from the FDA database through in silico methods and in vitro experiments. PAN and mutant PAN-I38T were chosen as virtual screening targets for overcoming drug resistance. Gel-based PA endonuclease analysis determined that the drug lifitegrast can effectively inhibit PAN and PAN-I38T, when the IC50 is 32.82 ± 1.34 μM and 26.81 ± 1.2 μM, respectively. Molecular docking calculation showed that lifitegrast interacted with the residues around PA or PA-I38 T's active site, occupying the catalytic site pocket. Both PAN/PAN-I38T and lifitegrast can acquire good equilibrium in 100 ns molecular dynamic simulation. Because of these properties, lifitegrast, which can effectively inhibit PA endonuclease activity, was screened through in silico and in vitro research. This new research will be of significance in developing more effective and selective drugs for anti-influenza therapy.

lifitegrast virtual screening drug repurposing

1. Introduction

Influenza is an infectious disease that causes 300,000 to 500,000 human deaths globally per year [1][2]. The Influenza virus genome is composed of eight negative RNA segments encoded by multiple viral proteins, including PB2, PB1, PA, HA, NP, NA, M1, and NS1 [3][4]. RNA-dependent RNA polymerase (RdRp) is critical for virus RNA transcription and replication and comprises PA, PB1, and PB2 (**Figure 1**). The N-terminal of PA domain (PA_N) contains an endonuclease active pocket and plays a crucial role in influenza virus polymerase activity. Drugs that abolish PA_N endonuclease activity or disturb the assembly of RdRp can effectively inhibit the replication of the influenza virus [5]. The PA_N endonuclease domain is highly conserved among different influenza virus subtypes, indicating that PA_N is a promising broad-spectrum anti-influenza therapeutic target because of its ability to inhibit virus proliferation during the initial mRNA synthesis stage [6].

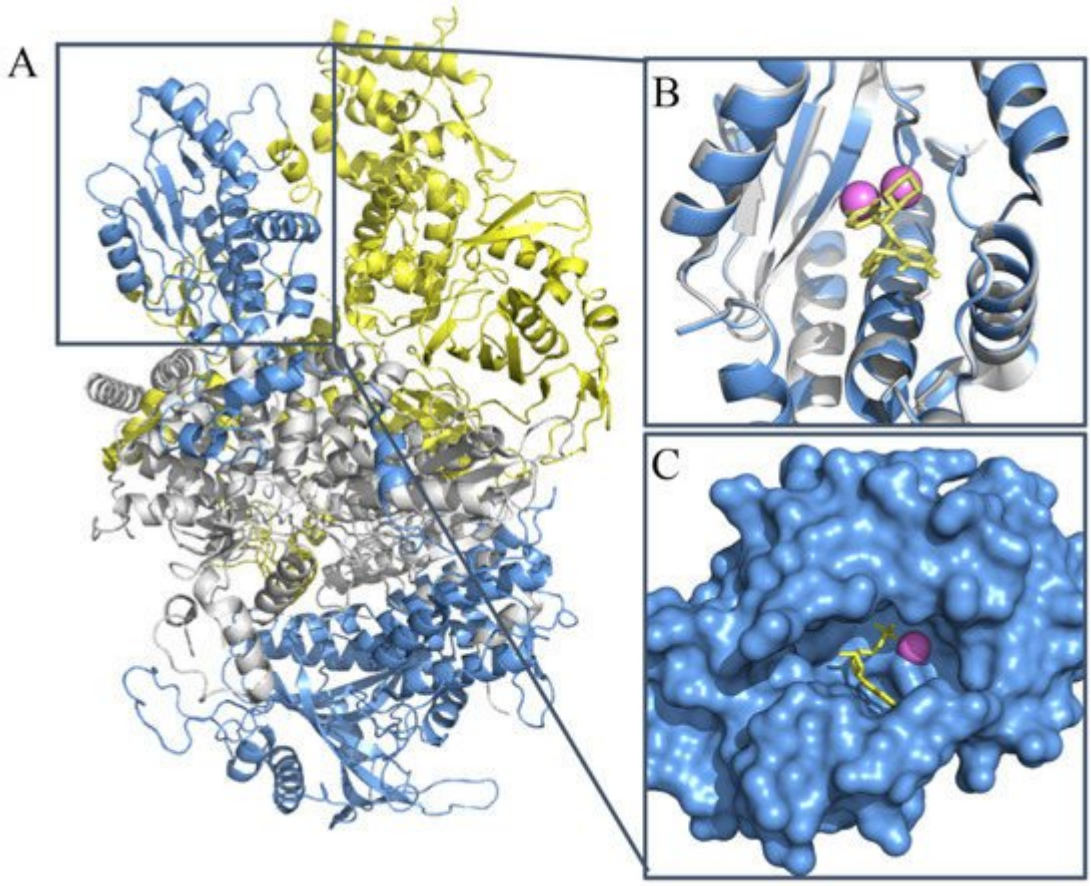


Figure 1. The structure of PA endonuclease. (**A**) The structure of RNA-dependent RNA polymerase (influenza A virus H5N1, PDB ID: 6QPF). The PA domain is shown in blue. The PB1 domain is shown in white. The PB2 domain is shown in yellow. (**B**) The cartoon structure of PA N-terminal (blue) (PDB ID: 6FS6) or PA-I38T N-terminal (white) (PDB ID: 6FS7) endonuclease domain complex with the inhibitor BXM. The BXM is shown in yellow. The Mn2+ is indicated with violet spheres. (**C**) The PA active site pocket complex with the substrate Amp. Amp is shown in yellow. The Mn2+ is indicated with violet spheres.

The three classes of FDA-approved anti-influenza drugs are: neuraminidase inhibitor (oseltamivir and zanamivir), M2-ion channel inhibitor (adamantanes), and PA_N endonuclease inhibitor (Baloxavir acid). Several classes of PA_N endonuclease inhibitors researched included: 4-substituted 2,4-dioxobutanoic acids and 3,4-substituted 2,6-diketopiperazines [7], flutamide and derivatives [8][9][10], N-hydroxamic acid-scaffold compounds [11], catechins [12], etc. Baloxavir acid, the inhibitor targeted on the PA_N endonuclease, was approved by the FDA as an inhibitor of influenza A and B after proving effective in clinical trials [13].

A mutation in the influenza viral genome led to the influenza's subsequent drug resistance. The hydrophobic interaction residues between PA_N endonuclease and Baloxavir acid were disturbed after the Ile 38 mutated to Thr [14]. The I38T substitution in the PA_N endonuclease domain is the primary mutant that leads to influenza's resistance to Baloxavir acid. This resistance reduces the effectiveness of Baloxavir acid as an anti-influenza drug [15]. Using the I38T mutant as the drug screening target may help to develop and refine the next-generation endonuclease inhibitors.

Drug repurposing research can effectively identify new drug repurposing opportunities, quickly expand the drug market, and reveal new commercially valuable uses for existing drugs [16]. Some examples of drugs that have been successfully repurposed include thalidomide [17], sildenafil [18], bupropion [19], and fluoxetine [20]. These drugs are currently used for applications beyond their initially approved therapeutic indications. The combination of in vitro and in silico methods will increasingly be used in the discovery of novel medicine [21].

This research screened the effectivity of PA_N endonuclease inhibitors from the FDA-approved database through in silico methods and in vitro experiments. PA_N inhibitors can inhibit the entire PA_N endonuclease activity. Since the purpose of this study is to identify drugs that overcome resistance, we screened inhibitors that target both PA_N and mutant PA_N -I38T. Experimental tests have verified that the drugs lifitegrast and saquinavir have an inhibitory effect on PA_N, while the drugs lifitegrast and conivaptan have an inhibitory effect on PA_N-I38T. In addition, molecular docking shows the interaction mechanism between lifitegrast and the active site of PA_N or PA_N-I38T. Research suggests that lifitegrast may be a potential anti-flu drug. Finally, the method employed in this work could be utilized as a fast and viable strategy for accelerating research in the treatment of influenza.

2. Analysis on Results

2.1. Virtual Screening and Compounds Selection

To develop the new PA_N endonuclease inhibitor structure, we performed the virtual screening protocol based on the PA_N and mutant PA_N-I38T structure. The residue Ile38 was not essential in either metal-ion binding or in catalytic activity. The I38 substitution does not block the endonuclease reaction but offers resistance to the inhibitor because the I38 side chain interacts with the Baloxavir acid. After the virtual screening, the compounds interacting with PA_N or PA_N-I38T were sorted by affinity score. The top-ranked compounds listed in Tables S1 and S2 were selected. Nine compounds closely interacted with PA_N and PA_N-I38T as hit compounds. Finally, six compounds, saquinavir, conivaptan, lifitegrast, rifaximin, dutasteride, and lurasidone, were used for further basic gel endonuclease determination. In comparison, three compounds, Teniposide [22], Simeprevir [23], and Nilotinib [24], were filtered according to levels of cytotoxicity.

2.2. The Inhibitory Effect of PA_N or PA_N-I38T with Compounds

We identified the five compounds that could inhibit the endonuclease activity of PA_N or PA_N-I38T, respectively. Next, we incubated different concentrations of compounds with PA_N or PA_N-I38T and then detected the cleavage of a substrate (**Figure 2** and **Figure 3**).

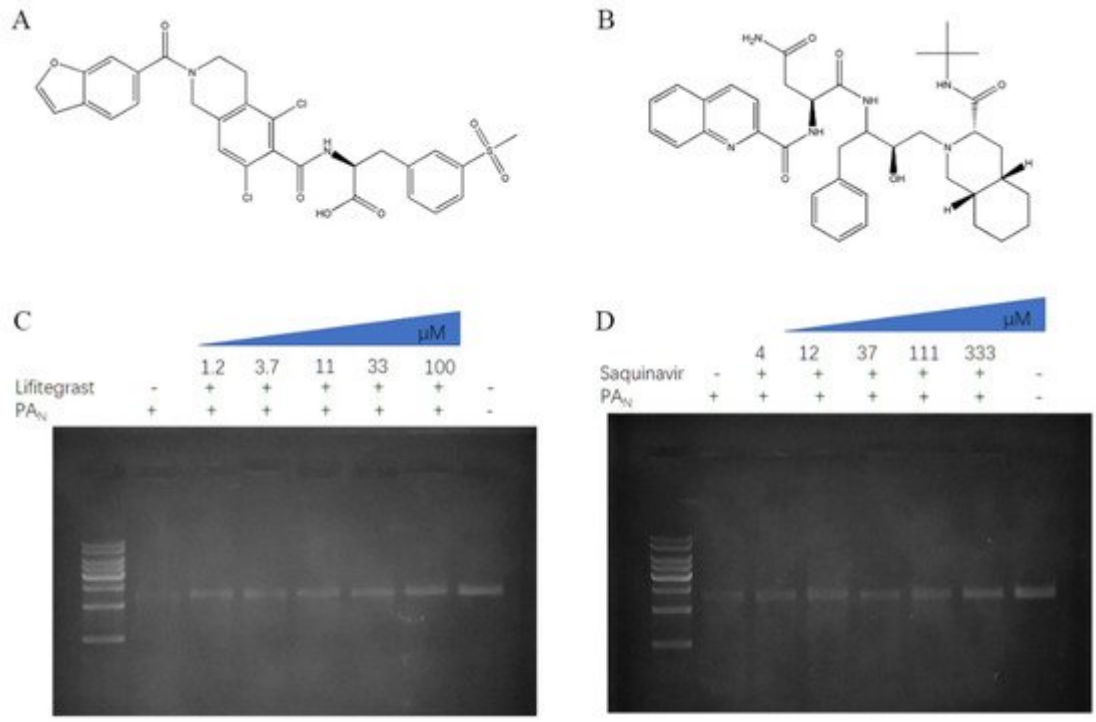


Figure 2. Compound inhibition of the endonuclease activity of PA_N. The chemical structure of lifitegrast (**A**) and saquinavir (**B**). For the inhibition assay, different concentrations of the compounds lifitegrast (**C**) and saquinavir (**D**) were incubated with the 1.5 μM PA_N and 100 ng ssDNA at 37 °C for 1 h. After the digestion, the products were resolved on agarose gel.

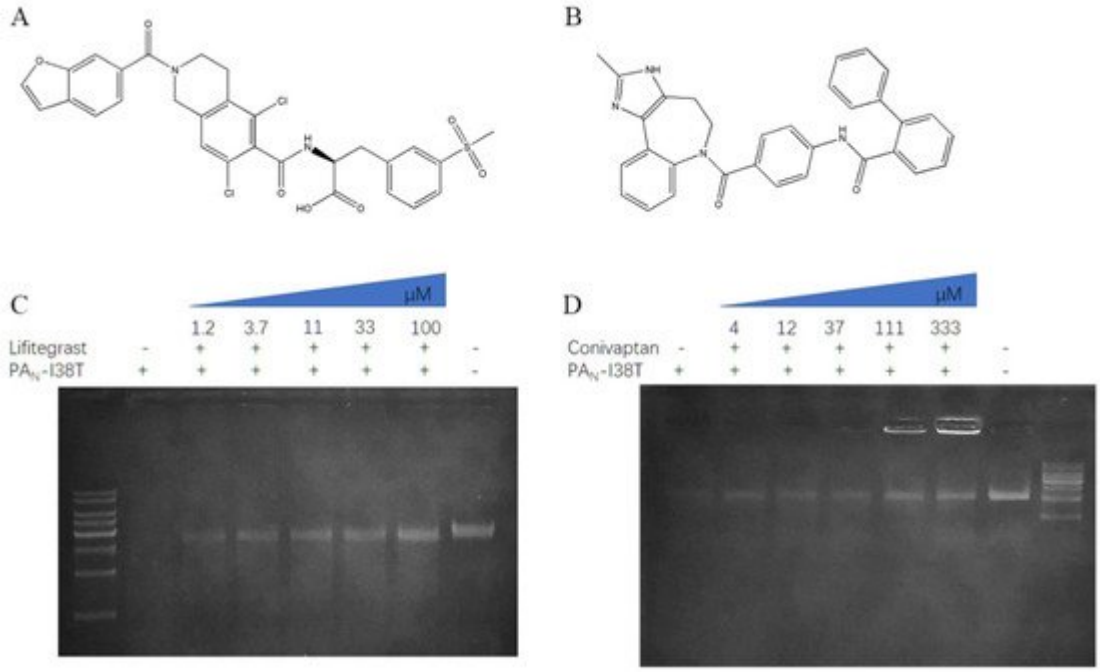


Figure 3. Inhibition of the endonuclease activity of PA_N-I38T. The chemical structure of lifitegrast (**A**) and conivaptan (**B**). For the inhibition assay, different concentrations of compounds lifitegrast (**C**) and conivaptan (**D**)

were incubated with the 1.5 μM PA_N-I38T and 100 ng M13mp18 at 37 °C for 1 h. After the digestion, the products were resolved on agarose gel.

The results showed that the compound saquinavir could effectively inhibit the cleavage of PA_N substrate (**Figure 2**D), but not PA_N-I38T substrate. Meanwhile, compound conivaptan can effectively inhibit the cleavage of PA_N-I38T substrate (**Figure 3**D), but not PA_N substrate. However, compounds conivaptan, dutasteride, rifaximin, and lurasidone showed no inhibition of the endonuclease activity of PA_N (Figure S1); compounds dutasteride, rifaximin, saquinavir, and lurasidone showed no inhibition of the endonuclease activity of PA_N-I38T (Figure S2). Finally, the compound lifitegrast was shown to effectively inhibit the substrate cleavage of both PA_N and PA_N-I38T at 32.82 ± 1.34 μM and 26.81 ± 1.2 μM, respectively (**Table 1**).

Table 1. IC_{50} values of compound inhibited PA_N and PA_N-I38T endonuclease.

Chemical Name	PA_N IC_{50} (μg/mL) [1]	PA_N-I38T IC_{50} (μg/mL)
lifitegrast	32.82 ± 1.34	26.81 ± 1.2
conivaptan	NI	227.7 ± 1.33
saquinavir	372.7 ± 1.38	NI

[1] Measurement IC_{50} values of compounds through five independent experiments and data were shown as mean ± SD.

2.3. Retained Stability of Conformations of PA_N/PA_N-I38T and Lifitegrast during MD Simulations

To gain further insight into the binding mode of the system after reaching equilibrium, 100 ns MD simulations were performed using an explicit solvent. The initial confirmation of lifitegrast was obtained from the optimal pose of molecular docking operations in virtual screening. The RMSD values are an essential parameter in assessing the stability of a protein–ligand complex. As shown in **Figure 4**A, the structure of both proteins and ligands acquired good equilibrium during 100.0 ns. Accordingly, we can draw the same conclusion from the radius of the gyration of protein analysis (**Figure 4**B).

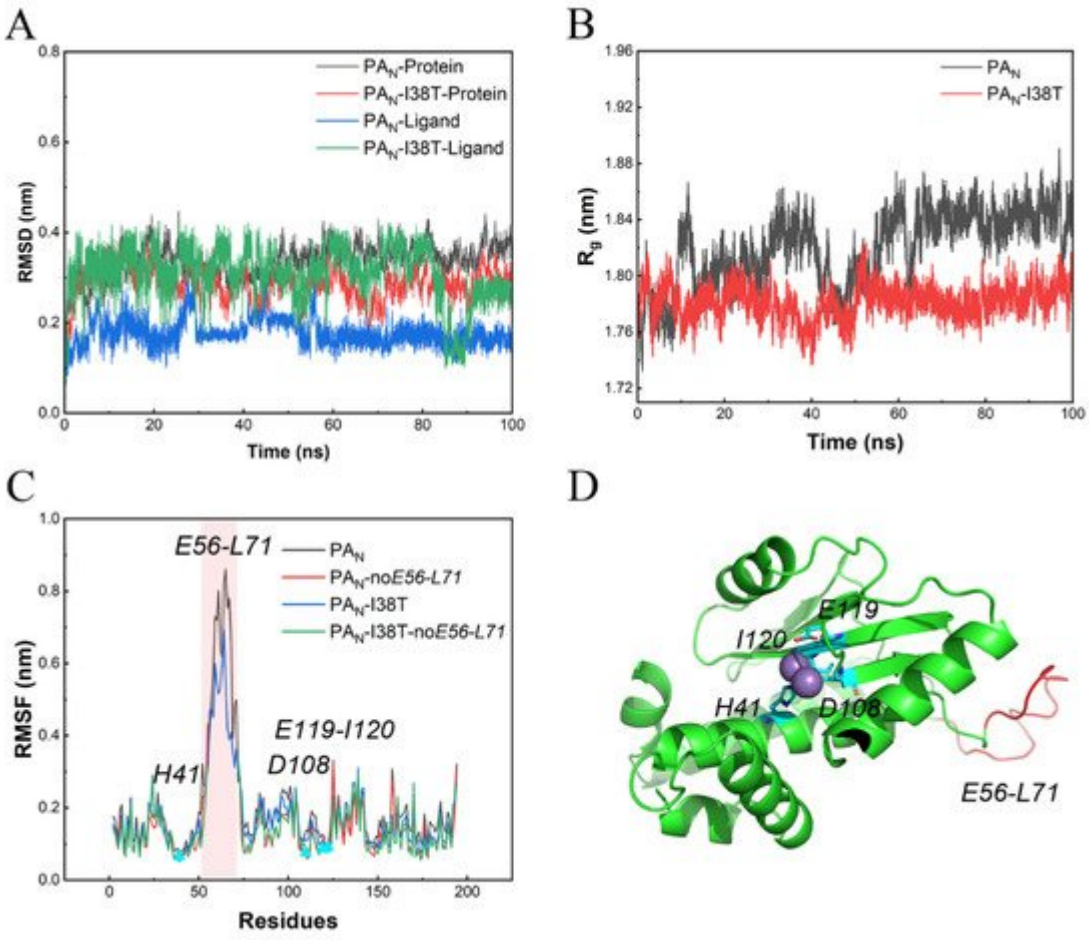


Figure 4. RMSD (**A**), Rg (**B**) and RMSF (**C**) propensities of PA_N and PA_N-I38T with ligand during molecular dynamic simulation. The highly flexible residue Asn55–Leu71 in the Loop region is colored red. The residue His41, Asp108, Glu119, and Ile120 associated with the active site is colored blue (**D**) and is stable.

Furthermore, to estimate the structural flexibility, the mean RMSF values were calculated. **Figure 4**C indicates that residues that interact with lifitegrast at the active pocket of protein remain stable. Although the residue Asn55-Leu71 in the Loop region is highly flexible, the overall protein conformation is stable (**Figure 4**D).

2.4. The Interaction Mechanism between PA_N/PA_N-I38T and Lifitegrast

Next, the protein–ligand complex structures in the 100 ns molecular simulation trajectories with similar conformations were divided into the same clusters. The representative frame of the largest cluster was extracted for analysis. **Figure 5**A,B show that lifitegrast binds to PA_N/PA_N-I38T at the structure active site and forms hydrogen bond interactions with lifitegrast (**Figure 5**C,D). **Table 2** gives the detailed non-bond parameters of lifitegrast and PA_N/PA_N-I38T. Comparing the number of hydrogen bonds between protein and lifitegrast, it can be concluded that lifitegrast has three hydrogen-bond interactions with residues Arg124 of PA_N, and four hydrogen-bond interactions with residues Trp88, Thr123, and Arg125 of PA_N-I38T.

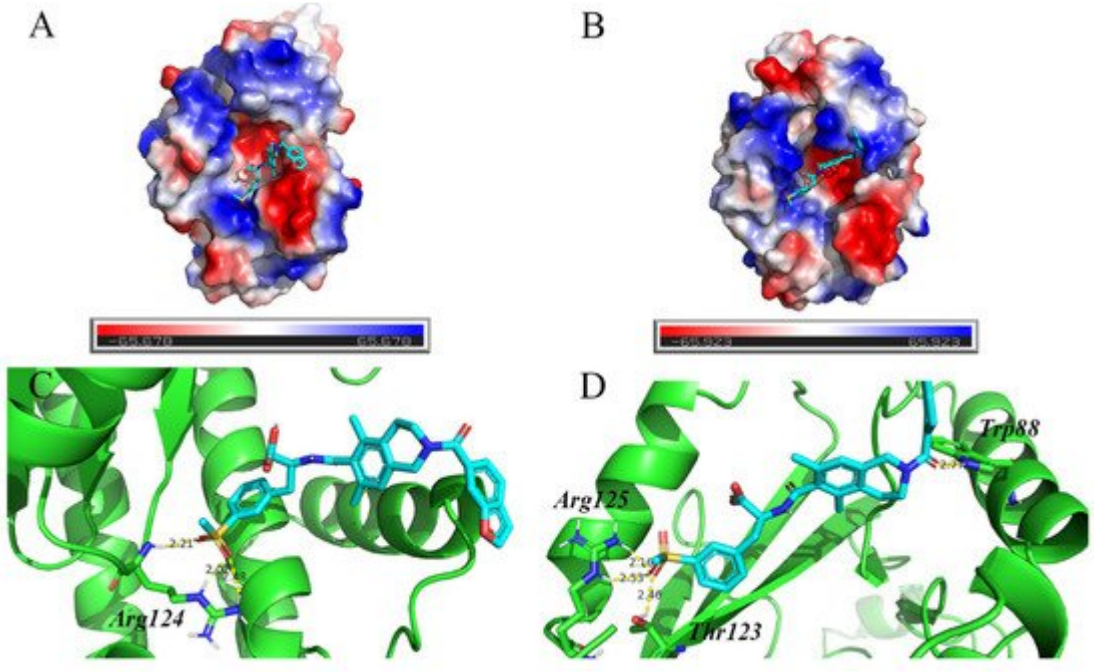


Figure 5. The interaction of lifitegrast within PA_N and PA_N-I38T active sites. The representative structure is extracted from the largest number of clusters in the system after molecular dynamics simulation. Electrostatic potential surface of PA_N (**A**) or PA_N-I38T (**B**) structure with lifitegrast in the active site pocket. Structure of PA_N (**C**) or PA_N-I38T (**D**) with lifitegrast. Manganese ions are indicated as gray spheres. Lifitegrast is shown using blue sticks. PA_N or PA_N-I38T are shown as green cartoons. Hydrogen bonds are shown as yellow dashed lines.

Table 2. Hydrogen bond parameters of lifitegrast and PA_N/PA_N-I38T.

PA_N			PA_N-I38T		
Donors Atom	**Receptor Atom**	**Distances (Å)** [1]	**Donors Atom**	**Receptor Atom**	**Distances (Å)**
Arg124:NH	lifitegrast:O4	2.21	Trp88:HE1	lifitegrast:O24	2.77
Arg124:HE	lifitegrast:O3	2.02	Thr123:HG1	lifitegrast:O3	2.46
Arg124:1HH2	lifitegrast:O3	2.48	Arg125:HE	lifitegrast:O3	2.16
			Arg125:1HH2	lifitegrast:O3	2.56

[1] The length of the hydrogen bonds.

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