

# Design of Small Molecules with HIV Fusion Inhibitory Property Based on Gp41 Interaction Assay

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## Abstract

**Background:** Gp41 of HIV (Human Immunodeficiency Virus) is a protein that mediates fusion between viral and cellular membranes. The agent, T-20, which has been approved for HIV inhibition, can restrain Gp41 function in the fusion process; nevertheless, it has disadvantages like instability, high cost of production and injection form to be delivered twice a day.

**Methods:** Several molecules like NB-2 and NB-64 have been discovered that can inhibit HIV infection. These molecules were used as template compounds to design and develop more effective small molecules functioning as HIV-1 fusion inhibitors targeting Gp41. The process included *in silico* docking protocols using HEX and ArgusLab applications. A multisource database was created, after choosing the best molecules; they were tested *in vitro* for inhibitory activity by HIV-1 single-cycle model, transfected in HEK cells (293T).

**Results:** Computational analysis and experimental data were combined to explore molecular properties and the most potent ones were found, with the best suitable criteria for interaction with Gp41. Several examples (DAA-6, DAA-9 and DAA-12) could inhibit infection *in vitro* as effective as NB-2, NB-64.

**Conclusion:** Since disadvantages of available fusion inhibitor (T-20), it seems necessary to find similar molecules to be approved and have small size providing suitable bioactivity profile. The molecules explored in this study can be good candidates for further investigations to be used as oral HIV fusion inhibitors in the future.

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**Keywords:** Antagonists and inhibitors, Drug design, Flow cytometry, HIV fusion inhibitors

## Introduction

Human immunodeficiency virus type 1 (HIV-1) penetrates cells by membrane fusion<sup>1,2</sup>. This process requires the trimeric viral envelope glycoprotein gp160, which is lysed by a single proteolytic reaction, and then the stable associated Gp120, Gp41 is formed. These

coupled proteins are located on the virion surface<sup>3,4</sup>.

Binding of the Gp120 to CD4 and a co-receptor (CCR5 or CXCR4) causes conformational changes in Gp41<sup>1,3,5,6</sup>. Therefore, CHR (C-terminal Heptad Repeat) and NHR (N-ter-

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minal Heptad Repeat) join together and consequently a 6-Helix Bundle (6HB) is formed<sup>7</sup>. Finally, the two membranes move towards each other and merge<sup>3,8,9</sup>. Internal core is a helical coiled coil formed by N-terminal regions of the three polypeptide chains<sup>8</sup>, and it is surrounded by an external layer consisting of C-terminal region of those chains. There are interactions between NHR and CHR<sup>10</sup> which involve the connection among residues (Leu565, Leu566, Leu568, Thr569, Val570, Trp571, Gly572, Ile573, Lys574, Leu576, and Gln577) in inner core and the side chains of Trp628, Trp631, and Ile635 in outer layer<sup>8,10,11</sup>.

Each step in HIV envelope fusion procedure can be a target to design a drug. Also, a part of the hydrophobic pocket, which is located near the Trp-60 and Lys-63 positions on the inner core, would be a suitable site for an inhibitory drug<sup>7,8,12</sup>. Any molecule which can block the process of six-helix bundle formation by targeting the Gp41 NHR would be a good lead as a HIV fusion inhibitor<sup>6</sup>. To date, one Gp41 inhibitor has been approved by FDA (Food and Drug Administration) for the treatment of HIV infection and others are in the advanced stage of clinical trials<sup>1,6,7,13</sup>. Peptides derived from the NHR and CHR of Gp41 regions has confirmed potent inhibitory activity on the HIV fusion. T-20 is one of the entry inhibitors which are approved by FDA.

Recently, scientists are working on another HIV peptide fusion inhibitor that it is under Phase II clinical trials, and is named Sifuvirtide (SFT)<sup>14</sup>. However, for peptides such as T-20, several limitations are expected, for instance, lack of oral usage<sup>7</sup> and high cost of production<sup>1,6,15</sup>. NB-64 and NB-2<sup>7</sup> are two compounds introduced in 2003, as the small molecule instead of peptide inhibitors, with more advantageous properties like stability, low cost of production and oral usage. These compounds have close molecular weights and may inhibit HIV-1 through blocking the fusion process or suppressing HIV-1 replication just when they are added to the cells at the first hour post infection.

NB-2 and NB-64 are "drug-like" molecules and significantly inhibit HIV-1 mediated syncytium formation and the six-helix bundle formation between NHR and CHR. Both NB-2 and NB-64 are N-substituted pyrrole derivatives with molecular weight of 231 and 222 Da, respectively. And ClogP (a calculated measure of partition of a drug in water and octanol phase) of 4.28 and 3.15, respectively which is in an optimal range for a drug or drug-like compound<sup>7</sup>.

In this study, we tried to find alternative structures through *in silico* and *in vitro* methods with fusion inhibition activity.

## Materials and Methods

### *In silico* operations and dock projects

After exploring the lead compounds based on literature study, similarity search was started based on NB-64 and NB-2 to make a database for each lead compound. A multisource database was created according to similarity search via different network sources such as <http://zinc.docking.org> (500 molecules), <http://www.chemspider.com> (about 20 molecules), and <http://pubchem.ncbi.nlm.nih.gov> (about 20 molecules). Marvin Sketch (5.0.0 2008) was used to draw the structures; after 3-D structure energy minimizing, they were added to our database. Through this database the similarity between small molecules was calculated with Tanimoto coefficient using ChemOffice package, 2008 (Chem Finder for Office, 9.0) to select the compounds with more than 70% similarity according to lead compounds.

For this purpose, we used substructure similarity search. The new database was saved in both sdf. and mol. formats to be used as a database for docking through different applications such as ArgusLab (4.0.1, 2004) and Hex (4.5, 2005). Hex application was used to find the correlation between shape only and shape/electrostatic docking. Because there is a linear correlation ( $R^2=0.90$ ), we then decided to continue docking process with ArgusLab which can use molecular library as sdf format. Also, ArgusLab uses gridbox dock for a targeted

section in the molecule, yet Hex will consider all parts of the molecule.

Crystallographic structures of Gp41 (PDB ID: 1F23 and 1AIK) were taken from PDB (Protein Data Bank) [http://www.rcsb.org] and used as a target in docking process<sup>2</sup>. The hydrophobic packet of Gp41 has been chosen as a grid box for docking<sup>16</sup>. This region includes strategic residues which involve an ionic bridge between NHR and CHR. Because of mismatches in these sequences and *in vitro* protein sequence, we made mutations (I35V, E85K, D87E, N91D, S95H, L96I, H98Y, E103Q) followed by energy minimization on the molecular structure file (using SPDB viewer 3.7, 2001). After docking process the results were considered according to normalized values based on none hydrogen atoms to increase accuracy.

Finally, the best ligands, according to their interaction energy, were obtained for *in vitro* testing<sup>17</sup>. We used NB-64 as a control in the docking process and *in vitro* testing and then the results were compared with each other.

### Compounds

The tested compounds with designated codes (DAA series, DAR series and DKHB series) were prepared in Department of Medicinal Chemistry, Pharmaceutical branch of the Azad University and were kindly provided to us<sup>18</sup>. The structures were confirmed by spectroscopic methods and the data are available upon request.

### Inhibition of HIV-1 single-cycle infection

Human embryonic kidney, HEK cells and MT-2 were obtained from National Cell Bank of Iran. To measure the inhibition effect, HEK 293T cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (Fetal Bovine Serum), 100 U/ml of penicillin, and 100 µg/ml of streptomycin.

To produce HIV pseudo type GFP (Green fluorescent protein) expressing reporter virus, HEK cells were separately co-transfected with 1.6 µg of pLOX-EWgfp (Addgene, www.addgene.org), 1.8 µg of pE7-HX Env (kindly provided by J. Sodroski, DFCI) and 450 ng of

psPAX Gag/Pol expression vector<sup>19</sup> using the PolyFect reagent (Qiagen).

Briefly,  $5 \times 10^5$  cells/well were seeded in a 6-well plate, and after overnight incubation a transfection mixture containing DNA plasmids (total amount of 2 µg) and PolyFect reagent (20 µl) were added to each well. Supernatants of the transfected cells were harvested at 24, 48 and 72 hrs and then followed by centrifugation for 15 min at 10,000 g. Final centrifugation was performed for 120 min at 60,000 g. The virions pellet were shaken gently overnight in 1/20 volume of RPMI 1640 at 4°C, p24 ELISA assay kit (Cell Biolabs) was used to quantified the virions and stored at -70°C<sup>20</sup>.

### Cytotoxicity assay

MT-2 cells were infected by recombinant HIV-1 virions expressing normal Env protein, but carrying GFP coded genome and this system was used to distinguish the rate of infection; as a result, the virus suppression can be detected. psPAX2 plasmid encodes gag and gag-pol polyproteins for the HIV-1, and in addition, it expresses the viral accessory proteins while lacks the packaging signal (ψ)<sup>20</sup>.

MT-2 cells were counted and seeded ( $10^4$  cells per well) into 96-well culture plates (Falcon; BD Biosciences) after 4 hr incubation at 37°C with 5% CO<sub>2</sub>, cells were treated with various concentration of the compounds in triplicates. Also 0.1% DMSO treated cells were used as a reference. The plates were incubated at 37°C with 5% CO<sub>2</sub> for 3 days, after that 50 µl XTT sodium 39-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid (Cell Proliferation Kit II, Roche, Germany) reagent was added to each well and incubated for another 4 hr for color development. The color was measured at 450 nm by using micro plate reader (ELISA READER)<sup>21</sup>. Percentage of viability was calculated in each concentration according to untreated cells and cells treated with 0.1% DMSO as reference. IC<sub>50</sub> was calculated via GraphPad prism® (version 5.04, 1992-2010).

### Flow cytometry and statistical analysis

MT-2, target cell lines were cultured in RPMI-1640 and 15% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. All inhibitors were dissolved in 1/10 DMSO and diluted 100-fold in the final assay. The final concentration of DMSO in each well was 0.1%. Inhibitors should be added in viruses and incubation at 37°C. After 72 hr, the infection rate was measured via flow cytometry using PAS machine (Partec, Germany) the results were analyzed using FloMax software (Partec, Germany).

### Results

Small molecules with Gp41 inhibitory effects have been reported before<sup>2,6,8,22</sup>. In this study we attempted to look for more molecules through database mining of sources like ZINC [http://zinc.docking.org] and Pubchem [http://pubchem.ncbi.nlm.nih.gov]. Over 500 molecules were selected and their interaction energy checked via computational methods. Then the promising molecules were tested *in vitro*. ArgusLab and HEX were used as *in silico* application tools for molecular docking. More than 15 molecules in four different concentrations were tested experimentally, but only three compounds could reduce the fusion based infection. However, we were able to find two other molecules which stimulated HIV infection and its results are to be considered in future.

Also, several molecular structures used for the *in vitro* test are demonstrated in table 1. In figure 1 we indicate docking results of the best compounds compared to NB-2 and NB-64.

To confirm the findings obtained from docking procedure, the *in vitro* assay was carried out for more than 15 molecules. However, five compounds showed an acceptable p-value indicating the significant difference with the control groups. Three molecules were found as inhibitor and two compounds induced the infection process (Table 2).

We used DMSO (dimethyl sulfoxide) as the solvent. Also, BMS-378806<sup>21,23</sup> and Nelfin-

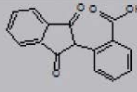
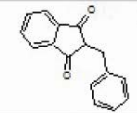
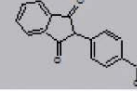
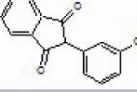
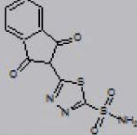
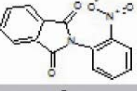
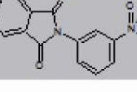
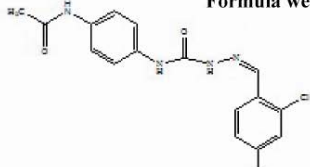
Compounds	Structures and molecular weights
DAA-4	 Formula weight=2662432
DAA-5	 Formula weight=23626623
DAA-6	 Formula weight=2662452
DAA-9	 Formula weight=25663376
DAA-12	 Formula weight=30932036
DAR-1	 Formula weight=26822432
DAR-2	 Formula weight=26822432
DHB-74	 Formula weight=365.21

Table 1. The structures and molecular weights of several compounds which used for *in vitro* test

avir were used as control inhibitors and NB-64<sup>7</sup> as a Gp41 fusion inhibitor. DMSO plus MT-2 (human, lymphocyte) cells and virus were used to normalize the solvent effect. Also, virus with cells was used to figure out the other effects. The assay was repeated for all the wells in triplicates. After 72 hr, the infection rate was measured via flow cytometry and the results were analyzed. The t-test was performed to calculate the significant differences among the special experimental groups. By this system, we could determine the infection rate. Hence, the inhibitory effect can be calculated via the formula below.

$$\text{Inhibitory effect} = 1 - \frac{\text{mean of sample infection}}{\text{mean of control infection}}$$

To determine cell viability, XTT assay was performed<sup>24</sup>. The assay is based on formation



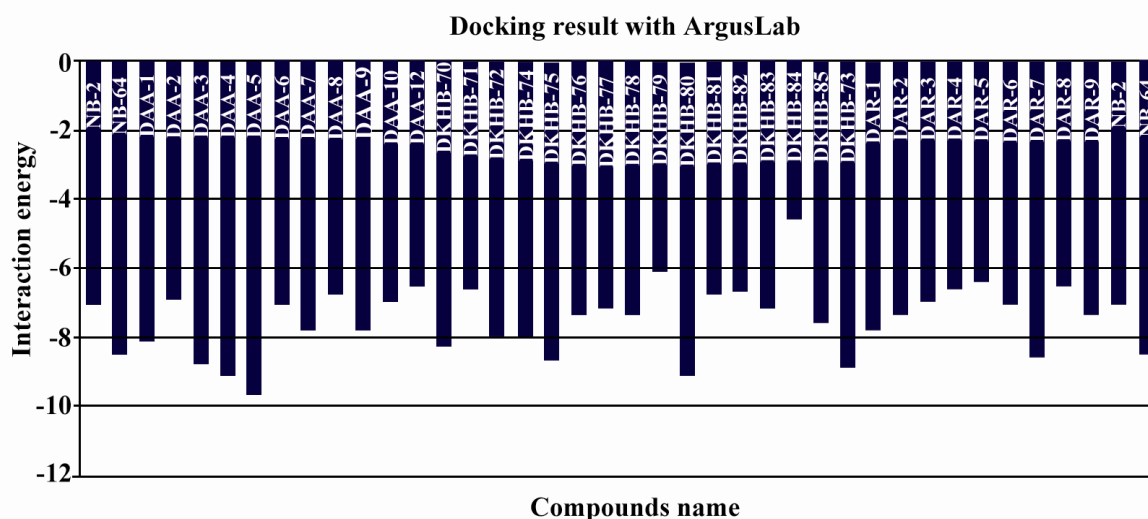


Figure 1. The interaction energy between NHR Gp41 and the tested compounds as measured by ArgusLab applications

Table 2. *In vitro* result of testing compounds

	1000 $\mu$ m			100 $\mu$ m			10 $\mu$ m			1 $\mu$ m		
	Average of infection (%)	p-value	Inhibition (%)	Average of infection (%)	p-value	Inhibition (%)	Average of infection (%)	p-value	Inhibition (%)	Average of infection (%)	p-value	Inhibition (%)
DAA4	10.09	0.43205	3.90	10.39	7.5006E	-26.38	9.42	0.03761	-14.58	9.71	0.0052	-18.12
DAA5	11.34	0.70266	-8.07	9.98	0.00016	-21.41	10.395	0.01178	-25.45	9.80	0.0298	-19.31
DAA6	13.26	0.03129	-25.33	6.48	0.000935	2117	6.3175	8.27E-05	23.14	6.37	0.0009	22.41
DAA9	8.19	0.02335	21.95	6.33	5.37E	2293	6.32	4.40E-05	2311	7.06	0.0300	34.08
DAA12	9.36	0.35488	10.80	6.45	1.2205E	2150	6.7675	0.000904	1757	6.93	0.0087	15.50
DAR1	--	--	--	--	--	--	--	--	--	8.07	0.2151	-9.54
DAR2	--	--	--	6.48	0.154259	1185	8.7225	0.04859	-1851	8.41	0.1013	-14.35
DKHB-50	--	--	--	7.07	0.629155	3.590	7.935	0.374156	-7.81	7.74	0.4935	-5.23
DKHB77	--	--	--	7.31	0.933355	0.51	7.965	0.323508	-8.22	8.41	0.1042	-14.33
DKHB74	--	--	--	7.57	0.674355	285	7.605	0.711123	-3.32	8.69	0.0751	-18.13
PIR	--	--	--	Dead	Dead		8.47	0.039175	-15.08	8.6	0.1772	-15.84
MIR	--	--	--	7.61	0.682399	-3.39	8.39	0.274113	-13.99	8.60	0.0538	-15.93
BMS-806							0.475	0.035355	93.54			
NB-64	--	--	--	6.16	0.000511	25	--	--	--	--	--	--
DMSO	10.5	--	--	8.22	--	--	--	--	--	--	--	--

This test was done in triplicate and the average was normalized by the control group. Several compounds with inhibitory or stimulatory effect had good p-value. All concentration cannot be tested due to their solubility. BMS-806 used as a control test, NB-64 as a lead compound, and DMSO as a solvent control. Our new compounds are effective as lead compound

of orange colored formazan from yellow tetrazolium XTT salt by viable cells. After reading the absorbance by ELISA reader at 450 nm, p-value was calculated for various concentrations of each compound. No significant effect was shown; therefore, these compounds did not have toxic effect on uninfected cells (data not shown).

The value for IC<sub>50</sub> was calculated by GraphPad<sup>®</sup>. Due to solubility issue limita-

tions in preparing higher concentrations of some compounds such as DAA-6, results did not present a good regression value for these compounds; therefore, those results were not shown. Yet, the applied concentrations at the infection assay were too low to have a cytotoxic effect, and no effect was reported in XTT test.

To find a correlation between bioinformatics data and *in vitro* results, we checked

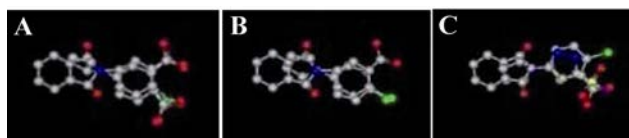


Figure 2. NB64 RMSE superimposed over with; A) DAA-6; B) DAA-9 and C) DAA-12 RMSE values are 1.7158, 2.0479 and 1.6425, respectively, which indicate that they are similar in their 3-D structures

RMSE (Root-Mean-Square Error) that is the square root of the variance of the residues for each molecule compared to NB-64 (Figure 2). These assessments show the minimum RMSE for molecules whose *in vitro* results are nearest to NB-64 (starting template compound). Two of our compounds, DAA-6 and DAA-12, have the lowest RMSE compared to others.

### Discussion

NB-64 and NB-2<sup>7</sup> are two compounds introduced in 2003 as the small molecule instead of peptide inhibitors. They have advantageous properties like stability, low cost of production and oral usage. These compounds have similar molecular weights and may inhibit HIV-1 through blocking the fusion process or suppressing HIV-1 replication just when they are added to the cells at the first hour of post infection.

Flow cytometry was used to detect GFP in MT-2 cells. The GFP ratio in this method identifies the infection rate; accordingly, the inhibitory effects of our compounds can be measured (Table 2).

RMSE comparison among the compounds demonstrated the correlation between small-molecule 3-D similarity and their affinity to bind to our target protein. However, this method may not be sufficient solely to predict their protein binding patterns<sup>25</sup>. In our study, we found the relationship between small molecules RMSE and their protein binding affinity; although, one of our (DAA-9) compounds did not comply with this general pattern. As mentioned above, the RMSE is not sufficient to predict protein binding affinity, but it is cost benefit to screen large molecule databases and as need, can perform their *in vitro* testing.

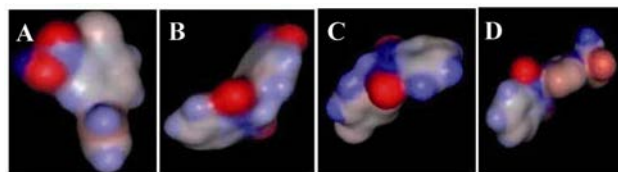


Figure 3. The electrostatic surface of compounds; A) NB-64; B) DAA-6; C) DAA-9 and D) DAA-12. These compounds are similar in their electric surface

The interaction between proteins (especially enzymes) and small compounds at a molecular level is a multi-factorial event, as it is influenced by entropic effects and different molecular forces between protein groups and the ligand. In recent years, the concept of protein-small molecule binding has also been supported by computational docking studies. In our study, we checked the charged surface to explore the electrostatic effect. The general pattern of charge distribution and locations on the surface of the most active compounds as obtained from the superposed status were also similar in the space fill model to that of NB-64, as indicated by the electrostatic centers of the molecules (Figure 3).

In order to get the meticulous docking results, we had to make some point mutations (I35V, E85K, D87E, N91D, S95H, L96I, H98Y, and E103Q) in the available PDB protein sequence to make it more similar to the protein used in the bioassay system of ours; also we deleted one chain of Gp41 to have critical position of hydrophobic pocket. In our docking procedure, we changed the environmental conditions such as waterless docking or global and local docking, but there is no meaningful difference in the results (data not shown).

Compounds that are similar to NB-2 and NB-64 possess functional groups that can mimic the chemical structure and functional groups that would place a similar interaction with the target protein. For example, the carboxyl group of the NB compounds is present in the selected compounds. Other functionally biosimilar groups with resembling activity are chlorine or sulfoxide moieties. In addition, the presence of two ring systems seems to be an-

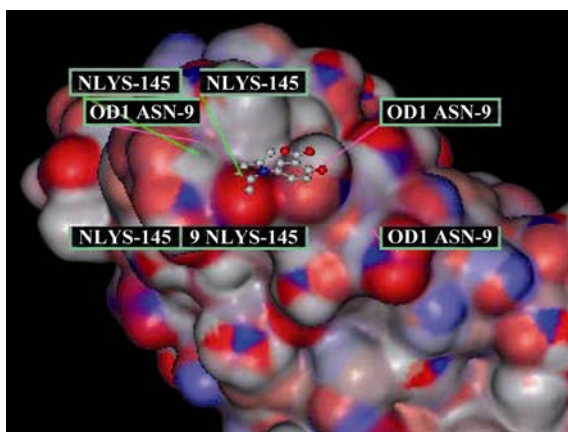


Figure 4. This picture shows the docking between receptor (PDB ID: 1F23) and NB-2 by Hex application. Several involving amino-acids were marked by Hex

other necessity among the compounds to play a similar role to NB compounds.

According to their interaction pose and the type of interaction points between the ligand and protein, it seems that the interaction is based on electrostatic forces (in case of existing carboxyl group in the ligand, Figure 4) and partial dipoles (in case of sulfoxide) and permanent dipoles (in case of chlorine) from one side and the partial positive charges on the protein, from the other side.

The recombinant virus is produced by HEK 293T (human embryonic kidney cells). These particles in our bioassay system do not have essential proteins for the next cycle replication. This would be an important biosafety advantage to use in the aforementioned system. These particles are carried only GFP as a reporter and Env protein on the surface in order to be fused with the host cell membrane<sup>20</sup>.

Our *in vitro* result indicates that the enhancement effect is in higher concentration (1000  $\mu$ M), and it is related to DMSO effect. This could be due to that we have to use 1% DMSO as a solvent and it increases transcription of viral RNA<sup>26</sup>. The compounds DAA-6, DAA-9 and DAA-12 can inhibit the infection rate in 1, 10 and 100  $\mu$ M concentrations and this suppression is close to the NB-64 effect, which was used as a starting point template compound (Table 2).

In flow cytometry data, it was noted that the infected cells have a larger size than the control (cells without added virus). This may be related to any change in the cell cycle proteins or cytokinesis.

In addition, we found molecules with enhancing effect on the infection that needs more experiments to understand the mechanism of infection enhancement and cell size enlarging. At last, we are trying to prove these compounds and similar small molecules as effective inhibitors and find the exact mechanism of these compounds by recombinant production of Gp41 and then via experiments like Circular Dichroism (CD) and ELISA assays.

## Conclusion

Due to disadvantages of enfuvirtide (fuz-eon), scientists have tried to discover small molecules which inhibit fusion process. We selected NB-64 and NB-2 as starting point template compounds in our study, since they have a simple structure. We found small molecules which can inhibit HIV fusion as NB-64 does. In this respect, computational methodology, which is a cost effective way to screen large databases, was used. As few molecules with enhancement effect on HIV infection process were found, more experiments are needed to explore their mechanism(s).

On the other hand, this study will open the way of screening the natural sources and finding molecules with potential HIV suppressive or enhancing property. Consequently, certain diets including or devoiding of these molecules, can be designed and recommended to HIV positive people to possibly provide them with a higher chance of survival.

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