WHERE ARE WE IN HIV RESEARCH? A NOVEL, COMPUTER-BASED STRATEGY PREDICTING RESISTANCE TO HIV-1 NNRTIs

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INTRODUCTION

The introduction of Highly Active Antiretroviral Therapy (HAART) has had a dramatically positive effect on the natural history of HIV-1 disease in the developed world (1,2). However, incomplete suppression leading to drug resistance has often impaired the HAART efficacy. For example, resistance towards non-nucleoside HIV-1 reverse transcriptase inhibitors (NNRTIs) develops rapidly in the clinical setting. Accordingly, the optimal use of NNRTIs will require both the appreciation of the potential for the development of drug resistance, and the recognition that this problem can be avoided. Thus, an approach to the issue of rapid emergence of NNRTI resistance could be the attempt to provide sufficient levels of potent inhibitors in order to inhibit not only wild type HIV-1, but also preexisting resistant viral variants found at low levels as a result of de novo mutations during ongoing virus replication, or at high levels as a consequence of NNRTI treatment failure.

Non-nucleoside HIV-1 reverse transcriptase inhibitors (NNRTIs) are a structurally diverse group of compounds which can be highly effective inhibitors of the enzymatic activity of HIV-1 reverse transcriptase in vitro and of HIV-1 viral replication in cell culture and in infected people. To qualify as an NNRTI, a compound should interact specifically with an allosteric binding site, which is physically separated from the catalytic domain or the substrate binding site, of the HIV-1 RT, but not HIV-2 (or any other retrovirus) at a concentration that is significantly lower than the concentration required to affect normal cell viability (3,4). HIV-1 RT is a heterodimer composed of a p66 subunit carrying both DNA polymerase and RNase H domain, and a proteolytically processed p51 subunit comprising only the DNA polymerase domain. Both subunits are encoded in the same region of the viral genome, and a single mutation in the RT coding region will result in a heterodimer carrying the same amino acid substitution in both subunits. The three-dimensional structure of each subunit in the heterodimer is different, and the amino acid substitution in each subunit cannot be considered structurally or functionally equivalent. Pioneeristic structural studies, followed by a plethora of other experimental evidences, have demonstrated that all NNRTIs, independently of their structure, bind in a hydrophobic pocket, located in the p66 subunit, approximately 10 Å from the polymerase binding site (5-7) (see Figure 1).

Resistance to NNRTIs develops rapidly in the clinical setting. All three of the NNRTIs formally licensed for clinical use (Nevirapine, Delavirdine, and Efavirenz) select HIV-1 mutations in two different regions of RT: codons 98-108 and codons 179-190 (8). A single point mutation, such as K103N can induce resistance to almost all known NNRTIs (8). Further, resistance to the NNRTI class of drugs appears to be quite stable, with little impact on viral fitness (9). Resistance mutations function primarily by altering the binding of NNRTIs to RT. Several mechanisms have been proposed, that can change the susceptibility of mutant forms of HIV-1 RT to NNRTIs, including loss of favorable contacts between enzyme and inhibitor, and slower inhibitor binding due to changes as the entrance to the NNRTI binding pocket.

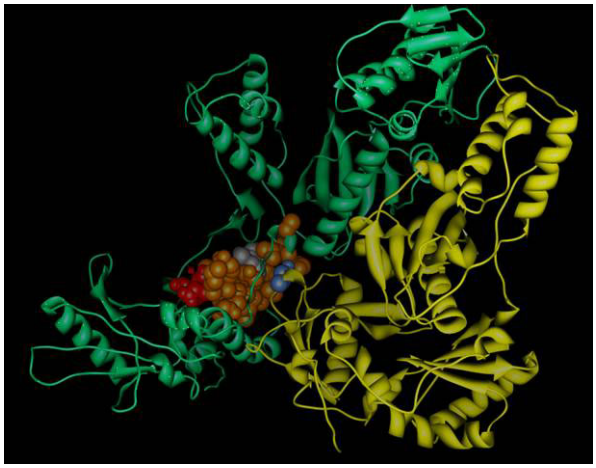

Figure 1 – HIV-1 RT hydrophobic binding pocket of NNRTIs.

According to the foregoing discussion, it is clear that the optimal use of NNRTIs in clinical practice will require an appreciation of the potential for the development of resistance, but also the recognition that this potential resistance problem can be avoided. Thus, an approach to the issue of rapid emergence of NNRTI resistance could be to attempt to provide sufficient levels of potent inhibitors to inhibit or 'cover' not only wild type HIV-1, but also preexisting resistant viral variants, found at low levels as a result of de novo mutations during ongoing virus replication, or at potentially higher levels as a consequence of prior NNRTI treatment failure.

Under this perspective, in this work we developed a computational procedure for the evaluation of the free energy contribution of each residue in the HIV-1 RT in binding to several, different NNRTIs which are known to fail in the presence of given mutations. The purpose of these computations is twofold: 1) to obtain indication for the design of resistance-evading drugs and, 2) to calculate the values of an empirical parameter, *GV*, which combines free energy calculations and sequence analysis to suggest possible drug resistance mutations on the RT. In practical terms, this parameter is defined as the product of a given residue contribution to the total binding free energy and the variability of that residue. This quantity could, in principle, be used in assisted resistance-evading drug design for HIV-1 RT, as well as for any other proteic viral target.

COMPUTATIONAL PROCEDURE

In the late '90s, Peter Kollman and colleagues (10) proposed a new, computational method to predict free energies of complexed biopolymers, termed Molecular Mechanics/Poisson-Boltzmann Surface Area (MM/PBSA). In short, this method makes use of a molecular dynamics (MD) simulation of the ligand-protein complex in explicit solvent to generate a thermally averaged ensemble of conformations of the molecules that are involved in the complex formation. Based on this set of structures, a free energy of binding is calculated using molecular mechanical and continuum solvent energies as well as including estimates of the nonpolar solvation free energy and solute entropy. According to this method, the total free energy of binding for a given complex, ΔG_{bind}, is calculated as:

$$\Delta G_{bind} = \Delta E_{MM} + \Delta G^{C}_{sol} - \Delta G^{L}_{sol} - \Delta G^{P}_{sol} - T\Delta S \quad [1]$$

where ΔE_{MM} is the interaction energy between the ligand and the protein, ΔG^{C}_{sol}, ΔG^{L}_{sol} and ΔG^{P}_{sol} are the solvation free energy for the complex, the ligand and the protein, respectively, and $-T\Delta S$ is the conformational entropy contribution to the binding. ΔE_{MM} can be obtained from the molecular mechanics (MM) interaction energies as:

$$\Delta E_{MM} = \Delta E^{ele}_{MM} + \Delta E^{vdW}_{MM} \quad [2]$$

where ΔE^{ele}_{MM} and ΔE^{vdW}_{MM} are the electrostatic and van der Waals contributions to the interaction energy between the ligand and the receptor.

The total solvation energy, ΔG_{sol}, is divided in two parts: the electrostatic contribution, ΔG^{ele}_{sol}, and the non-polar term, ΔG^{np}_{sol}:

$$\Delta G_{sol} = \Delta G^{ele}_{sol} + \Delta G^{np}_{sol} \quad [3]$$

The polar component of ΔG_{sol}, ΔG^{ele}_{sol}, is computed in continuum solvent, usually using a finite-difference Poisson-Boltzmann (PB) model; whereas the non-polar solvation term, ΔG^{np}_{sol}, can be derived from the solvent-accessible surface area (SA). The last term in eq. (1), $T\Delta S$, is solute entropy and is usually estimated by a combination of classical statistical formulas and normal-mode analysis.

In order to proceed with the calculations of the *GV* parameter, a search of the SWISS-PROT database was carried out, using PSI-BLAST (11). Multiple sequence alignment was carried out on 78 HIV-1 RT sequences with the MODELLER program (12). In the sequences considered, the human HIV-1, the feline immunodeficiency virus (FIV), and the simian immunodeficiency virus (SIV) reverse transcriptases were included. In the process for identification of those residues critical for binding, we introduced an empirical parameter *GV*, defines as the product of the contribution to free energy of binding of a given amino acid (ΔG_{AA}) and the variability of that amino acid ($V_{i,AA}$). According to the MM/PBSA method, the value of ΔG_{AA} can be calculated with the following equation:

$$\Delta G_{AA} = \Delta E^{vdW,AA}_{MM} + \Delta E^{ele,AA}_{MM} + \Delta G^{AA}_{sol} \quad [4]$$

in which the first two terms of the right-hand side represent the van der Waals and electrostatic interaction energy between the amino acid and the whole ligand, respectively. The last term in the above equation is the contribution of solvation penalty for the given amino acid, and is estimated from the following relationship:

$$\Delta G^{AA}_{sol} = \Delta G_{sol} + \Delta G^{0}_{sol} \quad [5]$$

where the two terms on the left-hand side are the solvation energies calculated from the ΔG_{sol} equation, considering normal partial charges and zero charges on the specific residue, respectively.

As the variability of a given amino acid $V_{i,AA}$, this can be calculated as:

$$V_{i,AA} = \sum_{j} \left(1 - \frac{P_{ij}}{P_{ii}} \right) \times W_j \qquad [6]$$

in which W_j is the weight of the j-th sequence, and is calculated for each sequence in the alignment on the basis of sequence identity. The sum of all sequences in the alignment is then normalized to 1. This weighting procedure should prevent over-presenting very similar sequences in the PSI-BLAST query results. The mixed term P_{ij} in the above equation gives an indication on how likely the residue a_j in the j-th sequence can be mutated into the amino acid a_i in the i-th sequence, and is obtained according to the following expression:

$$P_{ij} = 2^{(2 \times M_{ij})} \qquad [7]$$

where M_{ij} is the element of the BLOSUM62 matrix for a_i and a_j, and is assigned a penalty score of - 4 for gaps.

RESULTS AND DISCUSSION

The development of drug resistant mutants of the HIV-1 RT results in a much lower inhibitor binding efficacy but in almost unaltered reverse transcriptase own function. Intuitively, therefore, the catalytically or structurally topical residues, as for instance the catalytic aspartic triad 110, 185 and 186, are intolerant to any variation. The results of our analysis on the variability of each position in the allosteric, NNRTI binding site of the HIV-1 RT (codons 95-110, 176-190, 227-238 and 318) are graphically reported in Figure 2. In this Figure, single mutations on any open circle cause resistance to at least one drug, according to the Stanford HIV database, maintained by Robert Shafer at http://hivdb.stanford.edu.

This Figure immediately reveals that practically no NNRTI resistant mutations have ever been observed for positions characterized by values lower than 0.25 (P95, L100, L109, D110, Y183, M184, D185, D186, G231, L234). In absolute harmony with a recent and elegant genetic approach, based on oligonucleotide-directed saturation mutagenesis techniques (13) these conserved residues result to play a key catalytic (e.g., D110, D185 and D 186), or functional role (e.g., P95, Y183 and M184) in wild type RT activity. One notable exception is position 100, as the L100I mutation has been reported to give resistance to 9-Cl-TIBO (14). This case will be discussed later in details. Under drug pressure, then, these residues apparently mutate very little if at all; thus, single drug resistant mutations occur at those positions which are crucial for drug binding but, at the same time, are tolerant of mutations for viral activity.

The case of TIBOs

The simulation protocol adopted in this work leads to reasonably converged structures, and does not cause a significant drift of both the protein and the ligand from the original complex structure. Figure 3 shows a snapshot of the 8-Cl TIBO/RT complex taken from the

corresponding equilibrated MD trajectory.

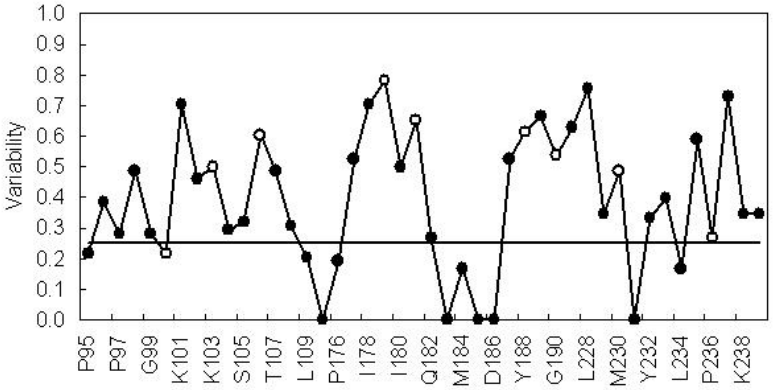

Figure 2 - Variability of each position in the allosteric, NNRTI binding site of the HIV-1 RT (codons 95-110, 176-190, 227-238 and 318). Single mutations on any open circle cause resistance to at least one drug, according to the Stanford HIV database, maintained by Robert Shafer at http://hivdb.stanford.edu.

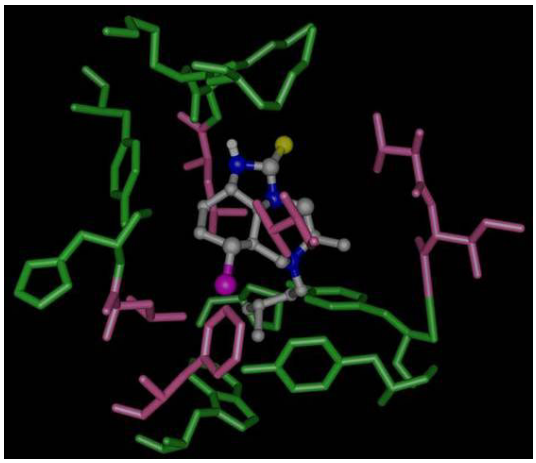

Figure 3 – Snapshot of the 8-Cl TIBO/RT complex taken from the corresponding equilibrated MD trajectory.

In all analyzed MD snapshots, the inhibitor overcomes the R-to-S energetic barrier several times in both directions, whereas the diazepine ring system remains in the TC/BS conformation, the TS configuration never being observed. Table 1 reports the corresponding results of free energy calculations. The estimated values of ΔG_{bind} (kcal/mol) are in excellent agreement with both the corresponding experimental evidences [15,16], and other values reported from other simulations [17-19].

In details, comparing the van der Waals/nonpolar with the electrostatic contributions, we can verify that the association between the TIBO ligands and the RT is mainly driven by more favorable nonpolar interactions in the complex than in solution. However, as indicated by the energy components of 9-Cl TIBO, this driving force can be considerably weakened when the nonpolar groups do not find an adequate bonding pattern in the protein compared to water.

The free energy penalty for electrostatics is indeed lower for 8-Cl TIBO than for the other derivative (23.40 kcal/mol and 25.54 kcal/mol for 8-Cl and 9-Cl TIBO, respectively, see Table 1), and this, together with its large van der Waals contribution, renders 8-Cl TIBO a stronger inhibitor.

Table 1 – Free energy of binding between TIBOs and HIV-1RT. All energies are in kcal/mol.

Cpd	ΔE_{MM}^{vdW}	ΔE_{MM}^{ele}	ΔE_{MM}	ΔG_{sol}^{ele}	ΔG_{sol}^{np}	$\Delta G_{MM/PBSA}$	$T\Delta S$	ΔG_{bind} (calc.)	ΔG_{bind} (exp.)[a]
8-Cl TIBO	-46.84	-27.40	-74.24	50.80	-4.34	-27.78	16.10	-11.68	-11.87
9-Cl TIBO	-47.22	-25.12	-72.34	50.66	-4.48	-26.16	15.82	-10.34	-10.60

Now, the main purpose of defining the *GV* parameter is to fuse free energy and evolutionary information into a single value. In order to fix a lower limit for regarding a mutation as drug resistant, we decided to consider only those RT mutations which, according to the literature, cause a relative resistance fold increase (RRFI) ≥ 10. Accordingly, a threshold of -0.35 (= -1.4×0.25) was selected for the *GV* value in identifying resistance, which corresponds to 1.4 kcal/mol (10-fold change in inhibition constant K_i) and variability > 0.25 (most positions included). Table 2 reports the prediction of drug resistant mutations on the basis of the calculated *GV* values for the two TIBO derivatives.

Interestingly, in the case of 8-Cl TIBO all three reported mutations (L100I, V106A and Y181C) are correctly predicted. At the same time, the *GV* value finds K103 resistant for this compound. Following our analysis, K103 is characterized by a variability of 50% (see Figure 2), mostly being non-conservative mutations such as N, T and S. Residue K103 is involved in the formation of the NNRTI binding pocket, being located at the outer rim of the NNRTI-BP and in the vicinity of its entrance. Thus, this residue can be classified as external. External side chains are not in contact with other critical residues or inhibitor, and are expected to be insensitive to substitution; therefore, they are all characterized by a high variability. This is in accordance with Wrobel et al. (13), who classified as external those RT positions at which all mutations have wild-type characteristics for both RT activity and protein stability, as measured by Western blot analysis. Likely, the calculated favorable electrostatic interactions (-1.60 kcal/mol) between the positively charged K103 and the nitrogen atoms of the imidazole ring of 8-Cl TIBO, coupled with the high residue variability account for the experimentally unobserved resistance. A further source of error can be traced to the fact that, as said, K103 is on the protein surface, and therefore at the boundary of the interior and exterior region when solving the BP equation; thus, more error may be introduced in the relevant PB calculations.

For 9-Cl TIBO, towards which a more consistent number of resistant mutants have been reported, the accordance with predicted and observed resistance is striking: indeed, if we consider the criterion according to which the mutant is considered to be resistant (i.e., RRFI ≥ 10), we have an agreement of 100% between experiment and prediction. If we take into consideration also those literature reported mutations for which 1< RRFI < 10, we see that an excellent agreement is maintained (90%).

Table 2 - Prediction of HIV-1 RT resistant mutations towards 8-Cl and 9-Cl TIBO on the basis of the free energy/variability parameter (*GV*) calculated values. *If the relative resistance fold increase is ≥ 10 for a single mutation, that residue is considered to be resistant. #Experimental evidence is reported in parenthesis. Italics: reported mutations with a relative resistance fold increase ≤ 10. †Prediction calculated considering only resistant mutations as defined above.

Residue #	Reported mutations*	*GV*	Resistance#	Prediction accuracy†
8-Cl TIBO				75%
A98	-	+2.9	N	
L100	L100I	-0.68	Y (Y)	
K101	-	-0.10	N	
K103	-	-0.41	Y (N)	
V106	V106A	-0.87	Y (Y)	
V179	-	-0.32	N	
Y181	Y181C	-1.3	Y (Y)	
Y188	-	-0.20	N	
G190	-	+1.1	N	
9-Cl TIBO				100%
A98	A98G	0.0	*N (N)*	
L100	L100I	-1.0	Y (Y)	
K101	K101E	+0.24	*N (N)*	
K103	K103N	-3.9	Y (Y)	
V106	V106A	-0.80	Y (Y)	
V179	V179D, V179E	-0.40	*Y (N)*	
Y181	Y181I, Y181V, Y181C, Y181S	-2.4	Y (Y)	
Y188	Y188C, Y188L, Y188H	-1.3	Y (Y)	
G190	G190E	-1.9	Y (Y)	

Interestingly, the GV value predicts a sensitivity towards resistant mutations for residue V179, for which at least two different mutations have been reported - V179D and V179E - characterized by RRFI values of 6.1 and 8.6, respectively (20). In both cases, the replacement of a highly variable (0.79), neutral residue with a negatively charged one (D or E) in the β-strand 9 must overcome both the strongly favorable van der Waals and electrostatic components of ΔG_{AA}: $\Delta E_{MM}^{vdW,V179}$ = -1.22 kcal/mol and $\Delta E_{MM}^{ele,V179}$ = -0.31 kcal/mol, respectively. The high degree of solvent exposure of this hydrophobic residue (92%), however, can be considered as an argument in favor of D and E replacement, as both hydrophilic amino acids could better interact with water solvent. Finally, we also predict resistant mutations at positions L228 and L234 (data not shown). Residue L234 is one of the most well conserved residues across the species considered in our analysis, with a variability of 0.17. Thus, the *GV* value of – 0.34 originates principally from the interactions existing between the CB of the residue side chain and the 9-chlorine atom (see above). In the case of L228, the high residue variability (0.76) is the leading factor to resistant mutation prediction.

Entirely analogous results have been obtained for HBY097, efavirenz, MCK442, delavirdine, HEPT and nevirapine, with prediction accuracy level never lower than 75%.

CONCLUSIONS

The developed computational method allowed to correctly predict all the resistance mutations found in vitro and/or in vivo for a substantial number of NNRTIs. Moreover, it was also able to highlight aminoacid residues classified as sensitive to resistance. The method proposed is highly predictive in all cases considered. More importantly, for those compounds for which the X-ray structure of the relevant complex with HIV-1 RT is not available, an "*ab initio*" procedure is devised, starting from the docking of the drug within the enzyme to the prediction of the relevant site susceptible to mutation. This method can be employed for the *a priori* prediction of the insurgence of resistance of newly designed drugs, or to aptly modify existing drugs towards a more efficacious binding even in the presence of resistant HIV-1 RT mutants.

We are currently testing this procedure with NNRTIs for which no crystallographic data are available, and the first results performed on some DABOs inhibitors are highly encouraging, being of the same quality as those described above.

REFERENCES

1. Palella, F.J.; Delaney, K.M.; Moorman, A.C.; Loveless, M.O.; Fuhrer, J.; Satten, G.A.; Ashman, D.J.; Holmberg, S.D. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *N. Engl. J. Med*. 1998, **338**, 853-860.
2. Palella, F.J.; Chmiel, J.S.; Moorman, A.C.; Holmberg, S.D. Outpatient Study Investigators. Durability and predictors of success of highly active antiretroviral therapy for ambulatory HIV-infected patients. *AIDS* 2002, **16**, 1617-1626.
3. De Clercq, E. The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. *Antiviral Res*. 1998, **38**, 153-179.
4. De Clercq, E. Non-nucleoside reverse transcriptase inhibitors (NNRTIs): past, present, and future. *Chem. Biodiv*. 2004, **1**, 44-64.
5. Kohlstaedt, L.A.; Wang, J.; Friedman, J.M.; Rice, P.A.; Steitz, T.A. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 1992, **256**, 1783-1790.
6. Ren , J.; Esnouf, R.; Garman, E.; Somers, D.; Ross, C.; Kirby, I.; Keeling, J.; Darby, G.; Jones, Y.; Stuart, D.; Stammers, D. High resolution structures of HIV-1 RT from four RT-inhibitor complexes. *Nature Struct. Biol*. 1995, **2**, 293-302.
7. Ding, J.; Das, K.; Tantillo, C.; Zhang, W.; Clark, A.D.J.; Pauwels, R.; Moereels, H.; Koymans, L.; Janssen, P.A.J.; Smith, R.H.J.; Kroeger Koepke R.; Michejda, C.J.; Hughes, S.H.; Arnold, E. Structure of HIV-1 reverse transcriptase in a complex with the nonnucleoside inhibitor a-APA R 95845 at 2.8 Å resolution. *Structure* 1995, **3**, 365-379.
8. Deeks, S.G. Non-nucleoside reverse transcriptase inhibitor resistance. *J. AIDS* 2001, **26**, S25-S33.
9. Havlir, D.V.; Eastman, S.; Gamst, A.; Richman, D.D. Nevirapine-resistent human immunodeficiency virus: kinetics of replication and estimated prevalence in untreated patients. *J. Virol*. 1996, **70**, 7894-7899.

10. Srinivasan, J.; Cheatham, T.E. III; Cieplak, P.; Kollman, P.A. Continuum solvent studies of the stability of DNA, RNA, and phosphoramidate-DNA helices. *J. Am. Chem. Soc*. 1998, **120**, 9401-9.
11. Altschul, S.F.; Madden, T.L.; Schaffer, A.A., Zhang, J.H.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein data base search programs. *Nucleic Acid Res*. 1997, **25**, 3389-3402.
12. Šalj, A.; Blundell, T.L. Comparative protein modeling by satisfaction of spatial restraints. *J. Mol. Biol*. 1993, **234**, 779-815.
13. Wrobel, J.A.; Chao, S.-F.; Conrad, M.J.; Mekker, J.D.; Swanstrom, R.; Pielak, G.J.; Hutchison, C.A. III. A genetic approach for identifying critical residues in the finger and palm subdomains of HIV-1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* 1998, **95**, 638-645.
14. Vandamme A.M.; Debyser Z.; Pauwels R.; DeVreese K.; Goubau P.; Youle M.; Gazzard B.; Stoffels P.A.;Cauwenbergh C.F.; Anne J.; Andries K.; Janssen P.A.J.; Desmyter J.; De Clercq E. Characterization of HIV-1 strains isolated from patients treated with TIBO R82193. *AIDS Res. Human Retro*. 1994, **10**, 39-46.
15. Pauwels R.; Andries K.; Debyser Z.; Kukla M.J.; Schols D.; Breslin H.J.; Woestenborghs R.; Desmyter J.; Janssen M.A.;De Clercq, E. New tetrahydroimidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and - thione derivatives are potent inhibitors of human immunodeficiency virus type 1 replication and are synergistic with 2',3'- dideoxynucleoside analogs. *Antimicrob. Agents Chemother*. 1994, **38**, 2863-2870.
16. Ho, W.; Kukla, M.J.; Breslin, H.J.; Ludovici, D.W.; Grous, P.P.; Diamone, C.J.; Miranda, M.; Rodgers, J.D.; Ho, C.Y.; De Clercq, E., Pauwels, R.; Andries, K.; Janssen, M.A.C.; Janssen, P.A.J.. Synthesis and anti-HIV-1 activity of 4,5,6,7-tetrahydro-5-methylimidazo-[4,5,1-jk]-[1,4]benzodiazepin-2(1H)-one (TIBO) derivatives. 4. *J. Med. Chem*. 1995, **38**, 794-802.
17. Eriksson, M.A.L.; Pitera, J.; Kollman, P.A. Prediction of the binding free energy of new TIBO-like HIV-1 reverse transcriptase inhibitors using a combination of PROFEC, PB/SA., CMC/MD, and free energy calculations. *J. Med. Chem*. 1999, **42**, 868-881.
18. Smith R.H. Jr.; Jorgensen W.L.; Tirado-Rives J.; Lamb M.L.; Janssen P.A.; Michejda C.J.; Kroeger Smith M.B. Prediction of binding affinities for TIBO inhibitors of HIV-1 reverse transcriptase using Monte Carlo simulations in a linear response method. *J. Med. Chem*. 1998, **41**, 5272-5286.
19. Wang J.; Morin P.; Wang W.; Kollman P.A. Use of MM-PBSA in reproducing the binding free energies to HIV-1 RT of TIBO derivatives and predicting the binding mode to HIV-1 RT of efavirenz by docking and MM-PBSA. *J. Am. Chem. Soc*. 2001, **123**, 5221-5230.
20. Byrnes, V.W.; Sardana, V.V.; Schleif, W.A.; Condra, J.H.; Waterbury, J.A.; Wolfgang, J.A.; Long, W.J.; Schneider, C.L.; Schlabach, A.J.; Wolanski, B.S.; Graham, D.J.; Gotlib, L.; Rhodes, A.; Titus, D.L.; Roth, E.; Blahy, O.M.; Quintero, J.C.; Staszewski, S.; Emini, E. Comprehensive mutant enzyme and viral variant assessment of human immunodeficiency virus type 1 reverse transcriptase resistance to nonnucleoside inhibitors. *Antimicrob. Agents Chemother*. 1993, **37**, 1576-1579.

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