RESULTS AND DISCUSSION

1. Interaction energy of efavirenz with individual residues of HIV-1 RT binding pocket

The interaction energies between efavirenz and the individual residues (Xi) of HIV-1 RT binding pocket for wild type, Y181C and K103N/Y181C enzymes were calculated at B3LYP/6-31G(d,p) and MP2/6-31G(d,p) levels of theory and are shown in Table 1. As the MP2 method includes the dispersion interactions it is expected to give more accurate interaction energies than the B3LYP method. The results show that MP2 calculations give lower interaction energies compared to B3LYP results at the same basis set, and the relative interaction energies ($^{a}\Delta E$ and $^{b}\Delta E$ in Table 1) for both B3LYP/6-31G(d,p) and MP2/6-31G(d,p) methods are consistent. In wild type RT, the main contributions to the interaction with efavirenz come from L100, K101, K103, Y181, Y188 and W229 which produce attractive interactions greater than 3 kcal/mol, calculated at MP2/6-31G(d,p) level. Considered in greater detail (Figure 8), the cyclopropylethylnyl of efavirenz is flexible to bind to HIV-1 RT and interacts with aromatic side chains of Y181, Y188 and W229 via H-π interaction. The main part of the efavirenz molecule is a benzoxazin-2-one ring which interacts mainly with L100, K101 and K103. Moreover, the backbone carbonyl oxygen (C=O) and backbone amino hydrogen (-NH) of K101 can exhibit moderate hydrogen bonding with the benzoxine-2-one (-NH and –C=O) of efavirenz causing the strongest interaction: -14.81 kcal/mol at the MP2/6-31G(d,p) method. Comparing the wild-type and Y181C enzymes at the MP2/6-31G(d,p) method, there is no significant difference between the interactions except for V179 which interacts with trifluoromethyl group of efavirenz. Figure 9 shows the residues color-coded according to attractive and repulsive interactions. It can be seen that there are more repulsive interactions between efavirenz and residues of the binding pocket for the double mutant (K103N/Y181C) enzyme (Figure 9c) than in the wild-type (Figure 9a) and Y181C (Figure 9b) HIV-1 RTs. Also, the attractive interactions between efavirenz and K101 and K103 in the K103N/Y181C enzyme were reduced to 5.52 and 3.62 kcal/mol, respectively ($^{b}\Delta E$ in Table 1, MP2/6-31G(d,p) method), compared to the wild type RT. These results indicate that the mutated residues (K103N and Y181C) not only reduce binding stability of efavirenz,

but also induce destabilization in the cavity leading other residues to loose contact or have reduced interactions with the inhibitor.

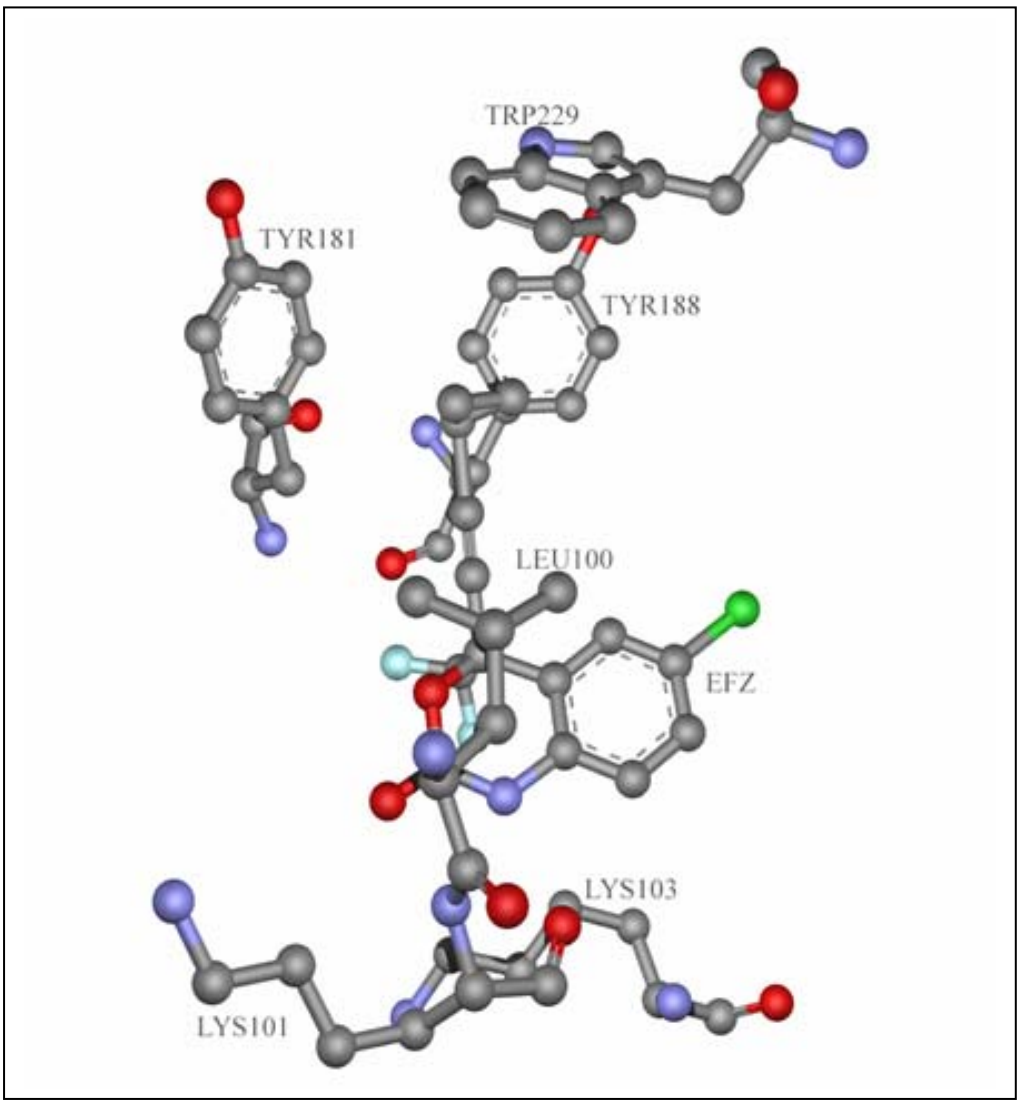

Figure 8 Orientation of efavirenz and residues with largest interactions in the binding pocket of wild-type HIV-1 RT.

Table 1 Calculated interaction of efavirenz with individual residues (X_i) from B3LYP/6-31G(d,p) and MP2/6-31G(d,p) methods.

Residue(X_i)	Interaction energy (kcal/mol)									
	B3LYP/6-31G(d,p)					MP2/6-31G(d,p)				
	Wild-type	Y181C	K103N/Y181C	ΔE^a	ΔE^b	Wild-type	Y181C	K103N/Y181C	ΔE^a	ΔE^b
Pro95	-0.33	-0.13	-0.35	-0.20	0.02	-1.09	-0.91	-1.09	-0.18	0.00
Leu100	-0.56	0.58	0.16	-1.13	-0.71	-7.66	-7.68	-6.11	0.02	-1.54
Lys101	-12.49	-12.68	-8.08	0.19	-4.41	-14.81	-15.00	-9.29	0.19	-5.52
Lys102	1.01	1.05	0.47	-0.04	0.54	0.17	0.11	-0.37	0.06	0.54
Lys103(Asn)	-1.08	-0.65	(0.42)	-0.43	-1.50	-5.15	-3.87	(-1.53)	-1.28	-3.62
Lys104	0.11	0.11	-0.16	-0.01	0.27	0.04	0.05	-0.29	-0.01	0.33
Ser105	0.06	0.14	0.35	-0.08	-0.28	-0.06	0.01	0.22	-0.07	-0.28
Val106	1.08	0.24	0.71	0.84	0.37	-2.59	-3.60	-3.76	1.01	1.17
Val179	1.61	-1.57	-1.80	3.18	3.41	0.06	-2.84	-3.60	2.90	3.67
Ile180	-0.17	-0.09	-0.17	-0.08	0.00	-0.50	-0.32	-0.40	-0.18	-0.11
Tyr181(Cys)	-0.25	(-1.39)	(-1.17)	1.13	0.92	-3.87	-3.56	-3.32	-0.31	-0.55
Tyr188	-1.14	-0.26	-0.52	-0.88	-0.62	-6.21	-6.67	-5.54	0.45	-0.68
Val189	-0.49	-0.62	-0.52	0.13	0.02	-1.09	-1.31	-1.17	0.22	0.08
Gly190	-1.03	-1.15	-0.83	0.12	-0.19	-1.75	-1.97	-1.45	0.22	-0.30
Phe227	0.07	-0.12	0.28	0.19	-0.21	-1.04	-1.24	-1.59	0.20	0.55
Leu228	-0.06	-0.16	0.02	0.10	-0.08	-0.08	-0.18	-0.02	0.10	-0.06
Trp229	-0.86	-1.07	1.44	0.21	-2.30	-3.24	-4.13	-4.07	0.90	0.83
Leu234	0.15	1.28	2.95	-1.13	-2.80	-1.96	-1.53	-0.31	-0.43	-1.66
His235	-1.58	-2.21	-0.78	0.63	-0.79	-2.87	-3.43	-2.32	0.56	-0.56
Pro236	-0.55	0.23	0.50	-0.78	-1.06	-2.81	-1.81	-1.45	-1.01	-1.36
Tyr318	-0.37	-0.54	-0.83	0.17	0.46	-2.87	-3.51	-3.50	0.65	0.64
Glu138(b)	1.83	0.61	0.12	1.22	1.72	1.26	-0.22	-0.09	1.48	1.35

[a] $\Delta E = E_{\text{wild- type}} - E_{\text{Y181C mutant}}$

[b] $\Delta E = E_{\text{wild- type}} - E_{\text{K103N/Y181C mutant}}$

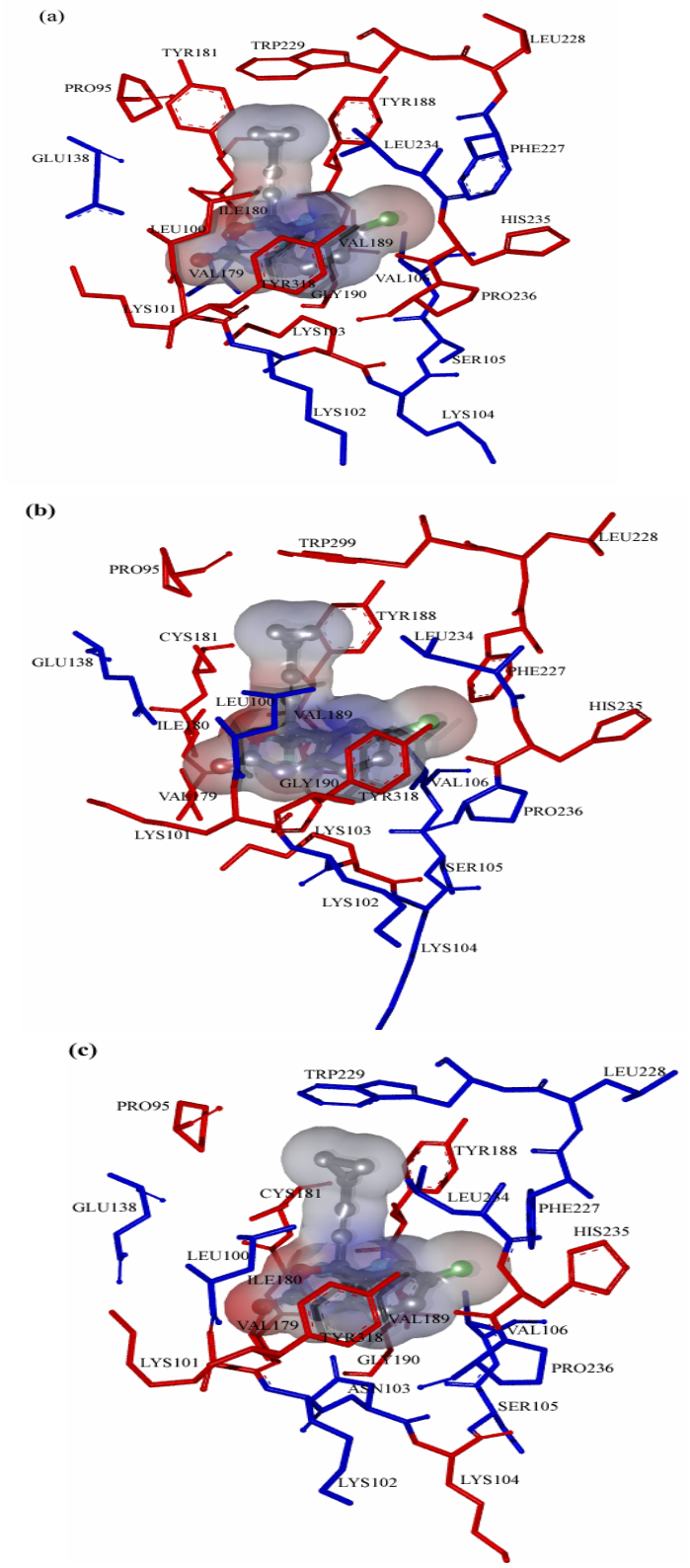

Figure 9 Residues which have attractive interactions (red) and repulsive interactions (blue) with efavirenz are shown for (a) wild-type, (b) Y181C and (c) K103N/Y181C enzymes.

2. Binding energy calculations

2.1 Comparing binding energy calculations between the wild type and Y181C enzymes

From Table 1, it can be seen that the K101 residue has the strongest interaction with efavirenz. The interaction energies between efavirenz and each residue of both the wild-type and Y181C enzymes are similar excepted for V179. Moreover, the mutated Y181C has less contact between efavirenz and C181. This helps confirm that it was correct to include K101, V179 and Y181 (C181) in the inner layer of ONIOM 2 calculations (Figure 6). Moreover, efavirenz only interacting with Y181 via hydrogen atoms of its cyclopropylethylnyl group with the aromatic ring of the residue was considered as part of the interacting core for ONIOM3 calculations. For better accuracy, the MP2 method was used in ONIOM3 calculations to include these dispersion interactions. With all the ONIOM methods used, it was found that the binding energy differences (Table2) between wild type and Y181C enzymes are small, with 1.03 kcal/mol from MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3 calculations being the greatest difference. This is in good agreement with the experimentally observed 2.5-fold binding loss. Only the interaction energy in region (A+B) [ΔE (High, A+B)] of Y181C enzyme is less than that in the wild- type, with a greatest difference of 1.65 kcal/mol (-10.21 kcal/mol for wild type and -8.56 kcal/mol for Y181C enzymes) using B3LYP/6-31G(d,p):PM3 method (Table2).

With the ONIOM3 method (Table2), binding energy of efavirenz is determined from three terms; (i) the interaction energy in region A [ΔE (High, A)], (ii) interaction between the regions A and B [ΔΔE (Mid, AB-A)], and (iii) the interaction energy between the regions A+B and C [ΔΔE (low, ABC-AB)]. The main contributions come from both the interaction energy between regions A and B (-11.02 kcal/mol for wild type and -10.79 kcal/mol for Y181C enzyme), and the interaction energy between regions A+B and C (-8.68 kcal/mol for wild type and -9.56 kcal/mol for Y181C enzyme). In contrast, the calculated interaction energy in region A, between the cyclopropylethylnyl group and Y181 (Figure 10a), is weakly attractive for wild

type (–0.82 kcal/mol) and, between the cyclopropylethylnyl group and C181 (Figure 10b), is weakly repulsive for Y181C HIV-1 RT (0.86 kcal/mol). Thus the Y181C enzyme has approximately 1.6 kcal/mol less attractive interaction in region A, indicating that change from an aromatic side chain of Y181 to a non-aromatic side chain of C181 leads to a reduction of contact between efavirenz and this residue. However, the main contribution to binding energy from K101 is not much changed, which corresponds well with the little change in hydrogen bonding distance between benzoxine-2-one (-NH and –C=O) of efavirenz and the carbonyl (-C=O) and amino (-NH) groups of K101 (Table 3). This also agrees with the X-ray structure data (Table 3).

Table 2 Binding energy and components of the binding energies with BSSE corrections for the wild-type and Y181C mutant HIV-1 RT complexed with efavirenz, by various ONIOM2 and ONIOM3 methods.

Methods	Calculated energies (kcal/mol)			
	ΔE	ΔE (High, A)	ΔΔE (Mid, AB-A)	ΔΔE (Low, ABC-AB)
HF/6-31G(d,p):PM3				
Wild-type	-17.46	-9.55		-7.91
Y181C mutant	-17.44	-8.56	-	-8.88
B3LYP/6-31G(d,p):PM3				
Wild-type	-18.69	-10.21	-	-8.48
Y181C mutant	-18.24	-8.56	-	-9.68
B3LYP/6-31G(d,p):HF/6-31G(d,p):PM3				
Wild-type	-18.67	0.25	-10.12	-8.82
Y181C mutant	-17.75	1.44	-10.00	-9.19
MP2/6-31G(d,p):HF/6-31G(d,p):PM3				
Wild-type	-19.36	-0.85	-10.23	-8.28
Y181C mutant	-18.45	0.81	-10.04	-9.22
MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3				
Wild-type	-20.52	-0.82	-11.02	-8.68
Y181C mutant	-19.49	0.86	-10.79	-9.56
Experiment binding loss			2.5 fold	

Table 3 Calculated distances of the hydrogen bonds (Å) between benzoxine-2-one (-NH and –C=O) of efavirenz and the backbone carbonyl oxygen (-C=O) and backbone amino hydrogen (-NH) of K101, based on X-ray structure, ONIOM2 and ONIOM3 methods for wild-type and Y181C enzymes.*

Methods	Wild-type		Y181C mutant	
	$-C=O_K\cdots H-N_B-$	$-N-H_K\cdots O=C_B-$	$-C=O_K\cdots H-N_B-$	$-N-H_K\cdots O=C_B-$
B3LYP/6-31G(d,p):PM3	2.84	3.07	2.79	3.16
MP2/6-31G(d,p):B3LYP/631G(d,p):PM3	2.83	3.06	2.79	3.16
X-ray	2.75	3.17	2.67	3.30

*Hydrogen bond distances of ($-C=O_K\cdots H-N_B-$) and ($-N-H_K\cdots O=C_B-$) are in Å

The calculated deformation energies of the binding pocket and efavirenz for the wild type and Y181C enzymes are shown in Table 4. It can be seen that, all three ONIOM3 calculations give more accurate results than the ONIOM2 calculations. The deformation energies in the Y181C enzyme are more than that of the wild type. This corresponds with experimental data that the binding of efavirenz to the Y181C enzyme is less strong. The obtained results indicated that binding of efavirenz to its binding pocket of Y181C enzyme is slightly more hindered than wild- type to adapt its structure to bind within the binding pocket, and the pocket of Y181C enzyme needs slightly more energy that than the wild type to rearrangement of the residue side chain in the pocket. This can be concluded that efavirenz shows similar high inhibitory affinities for both wild type and the single mutant (Y181C) enzymes.

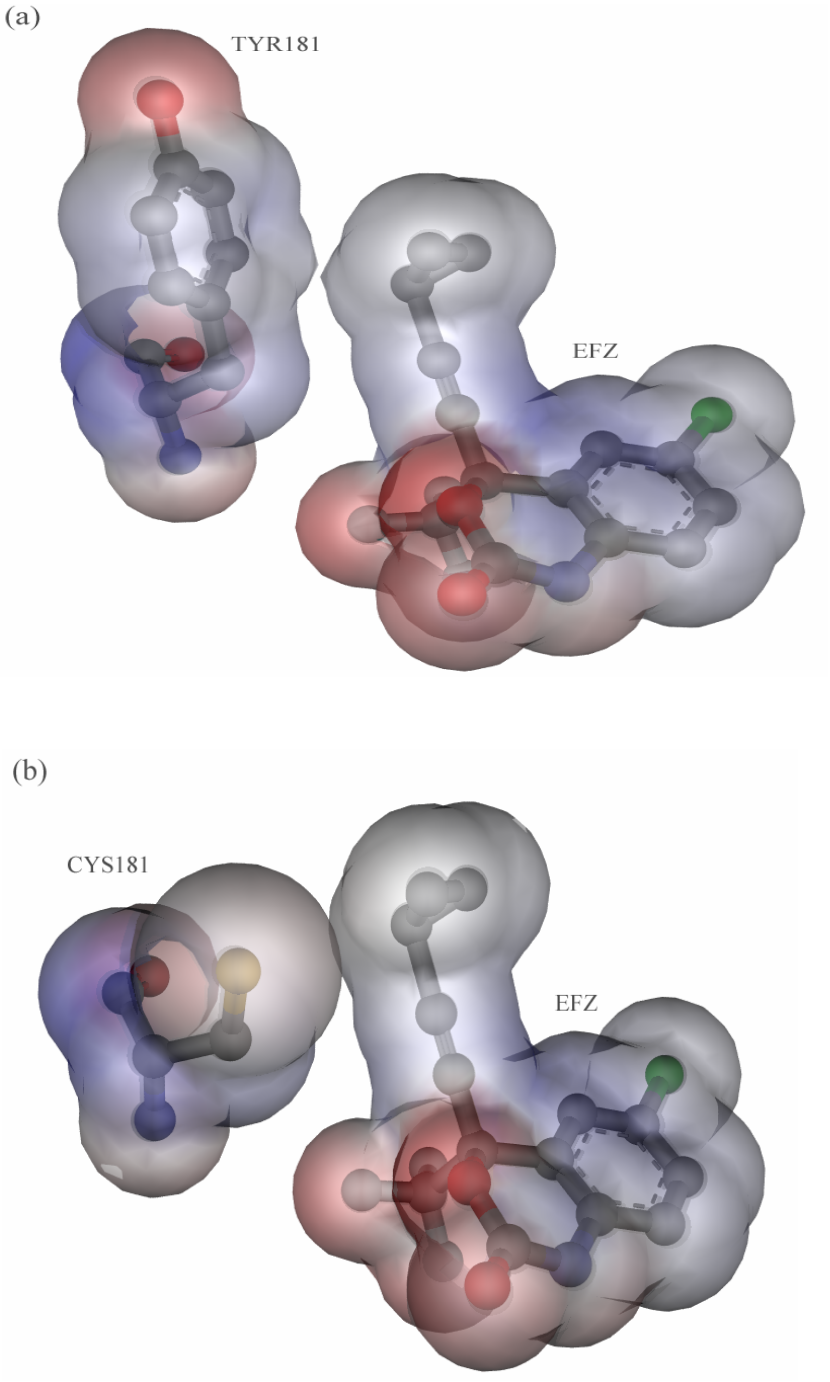

Figure 10 Electrostatic potential is shown on the VDW(van der Waals) accessible surface with red for negative and blue for positive values for (a) efavirenz interacting with Y181 and (b) efavirenz interacting with C181.

Table 4 Interaction and deformation energy components of the binding energies of HIV-1 RT/efavirenz complex for both wild-type and Y181C mutant enzymes, calculated by different ONIOM models.

Method	Binding energy components (kcal/mol)			
	INT	DEF_{pk}	DEF_{EFZ}	DEF
HF/6-31G(d,p):PM3				
Wild-type	-19.48	0.71	1.31	2.02
Y181C mutant	-20.23	1.11	1.68	2.79
ΔE^a	0.75	-0.40	-0.37	-0.77
B3LYP/6-31G(d,p):PM3				
Wild-type	-20.97	0.68	1.60	2.28
Y181C mutant	-21.59	1.30	2.04	3.34
ΔE^a	0.62	-0.62	-0.44	-1.06
B3LYP/6-31G(d,p):HF6-31G(d,p):PM3				
Wild-type	-21.00	0.80	1.52	2.32
Y181C mutant	-20.51	1.08	1.68	2.76
ΔE^a	-0.49	-0.28	-0.16	-0.44
MP2/6-31G(d,p):HF/6-31G(d,p):PM3				
Wild-type	-21.47	0.88	1.23	2.11
Y181C mutant	-21.13	1.07	1.62	2.69
ΔE^a	-0.34	-0.19	-0.39	-0.58
MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3				
Wild-type	-22.96	0.85	1.59	2.44
Y181C mutant	-22.59	1.18	1.92	3.10
ΔE^a	-0.37	-0.33	-0.33	-0.66

$BE = INT + DEF, \quad DEF = DEF_{Pk} + DEF_{EFZ}$

$DEF = DEF_{Pk} + DEF_{EFZ}$

[a] $\Delta E = E_{wild\text{-}type} - E_{Y181C\ mutant}$

2.2 Comparing binding energy calculations between the wild type and K103N/Y181C enzymes

From Table 1, the double mutations K103N and Y181C lead to the loss of contact between efavirenz and K101, N103 and Y181, with the interaction energies between efavirenz and these residues reduced by 5.52, 3.62 and 0.55 kcal/mol, respectively, as compared with the wild-type. Therefore, in ONIOM2 calculations, the three residues K103 (N103), Y181 (C181) and K101 were considered as part of the interacting core (Figure 7), region A+B) and were treated by the HF/6-31G(d,p) and B3LYP/6-31G(d,p) methods. Table 5 shows the binding energies with basis set superposition error (BSSE) for the wild type and K103N/Y181C complexes using two- and three-layer ONIOM calculations. It can be seen that the relative binding energy [a]ΔE from all the ONIOM methods are consistent, and the difference in binding energy between the wild type and K103N/Y181C enzymes is far more significant (7.91 kcal/mol with the MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3 method). The interaction energy in region A+B and the interaction energy between regions AB and C of the K103N/Y181C enzyme are less than the wild type by 5.14 and 3.66 kcal/mol, respectively, using B3LYP/6-31G(d,p):PM3 calculations (Table 5). This indicates that the mutations K103N and Y181C induce the loss of contact of residues in region (A+B) and region C with efavirenz. From MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3 calculations, the significant difference in binding energy comes from the interaction energy between regions A and B (-9.21 kcal/mol for wild type and -3.78 kcal/mol for K103N/Y181C enzyme). This indicates that the double mutation causes a large reduction in attractive interactions between efavirenz and residues in region B (K101 and N103). This corresponds with increased hydrogen bond distances between –C=O of benzoxine-2-one of efavirenz and backbone amino hydrogen (-NH) of K101 (Table 6). Moreover, N103 creates repulsive interactions with efavirenz (Figure 11b) when compared with the interaction between K103 and efavirenz (Figure 11a). The hydrogen bond distances between the benzoxine-2-one and K101 from B3LYP/6-31G(d,p):PM3 and MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3 calculations correspond well with the X-ray structure data (Table 6). The mutation Y181C causes a loss of contact between efavirenz and C181 leading to a weakly repulsive interaction in region A

(0.20 kcal/mol at MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3 calculations). The calculated binding energy of the K103N/Y181C complex, from all the ONIOM calculations, is smaller than the wild type by approximately 8 kcal/mol, which agrees well with the experimentally observed 9-fold reduction in binding.

Table 5 Binding energies and components of the binding energies with BSSE corrections for the wild-type and K103N/Y181C HIV-1 RT complexed with efavirenz, by various ONIOM2 and ONIOM3 methods.

	Calculate energies (kcal/mol)			
Methods	ΔE	ΔE (High, A)	ΔΔE (Mid, AB-A)	ΔΔE (Low, ABC-AB)
HF/6-31G(d,p):PM3				
Wild-type	-14.22	-7.67	-	-6.55
K103N/Y181C mutant	-6.01	-3.19	-	-2.82
B3LYP/6-31G(d,p):PM3				
Wild-type	-15.62	-8.38	-	-7.24
K103N/Y181C mutant	-6.82	-3.24	-	-3.58
B3LYP/6-31G(d,p): HF/6-31G(d,p):PM3				
Wild-type	-15.01	0.57	-8.56	-7.02
K103N/Y181C mutant	-7.03	1.07	-3.54	-4.56
MP2/6-31G(d,p): HF/6-31G(d,p):PM3				
Wild-type	-15.60	-0.67	-8.61	-6.32
K103N/Y181C mutant	-8.09	0.14	-3.50	-4.73
MP2/6-31G(d,p): B3LYP/6-31G(d,p):PM3				
Wild-type	-16.91	-0.66	-9.21	-7.04
K103N/Y181C mutant	-9.00	0.20	-3.78	-5.42
Experiment binding loss			9 fold	

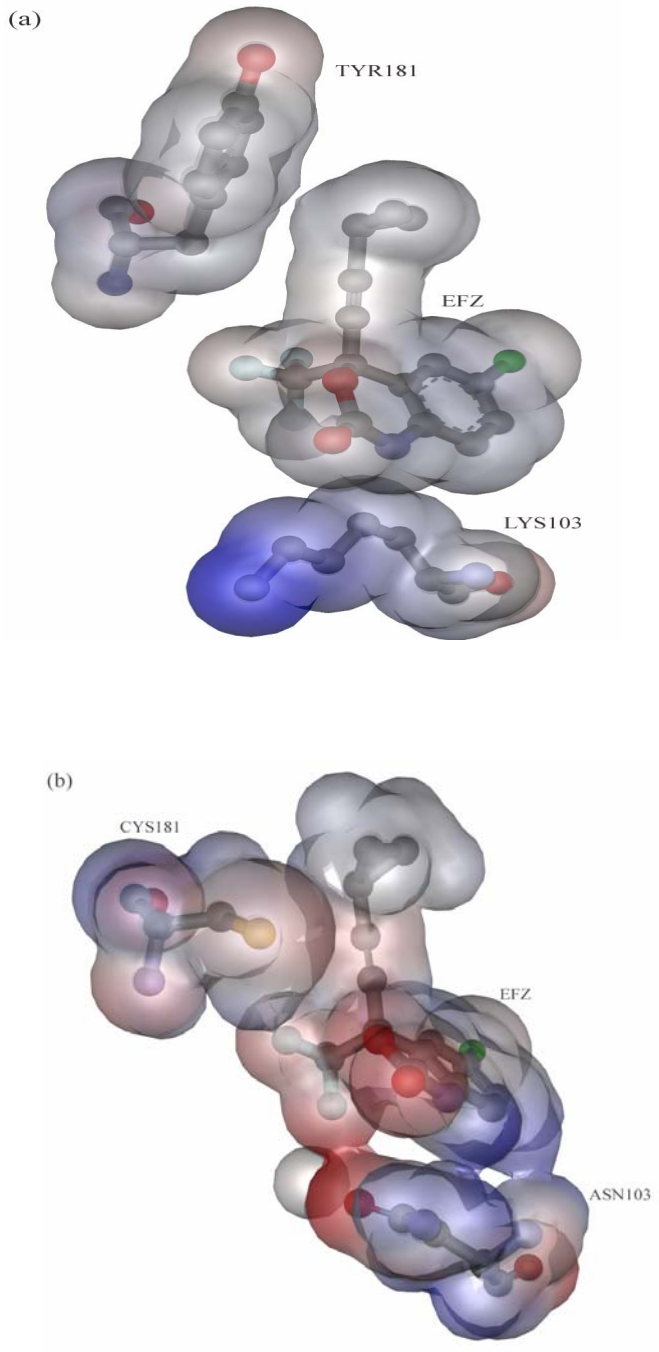

Figure 11 Electrostatic potential is shown on the VDW accessible surface with red for negative and blue for positive values for (a) efavirenz interacting with Y181 and K103, and (b) efavirenz interacting with C181 and N103.

Table 7 shows interaction energies and deformation energy of efavirenz bound to HIV-1 RT for both wild-type and K103N/Y181C enzymes. The differences in interaction energies show that they are significant, especially the $^{a}\Delta E$ of INT, compared to the smaller differences in the deformation energies of the double mutant complex where they are only slightly more than in the wild-type. This shows that the binding of efavirenz is more difficult in the pocket of the K103N/Y181C enzyme compared with the wild-type, as more energy is needed to adapt its structure and to rearrange the residue side chain around the pocket. From the MP2/6-31G(d,p): B3LYP/6-31G(d,p):PM3 method, the calculated binding energy and interaction energy are reduced by 7.91 and 6.35 kcal/mol, respectively, in the K103N/Y181C enzyme compared to the wild-type. These are a significant difference. Moreover, the deformation energy of efavirenz and the pocket of the K103N/Y181C enzyme is slightly greater than that of the wild type. These results indicate that the mutations in the K103N/Y181C enzyme eliminate favorable contacts of the aromatic ring of the Y181 and hydrocarbon side chain of K103 with efavirenz. This leads to a reduction in the stabilization energy of the complex and induces destabilization in the cavity by reducing contact between K101 and efavirenz. This is consistent with the observation that efavirenz shows higher inhibitory affinities with the wild type compared to the double mutation K103N/Y181C enzyme.

Table 6 Calculated distances of the hydrogen bonds (Å) between benzoxine-2-one (-NH and –C=O) of efavirenz and the backbone carbonyl oxygen (-C=O) and backbone amino hydrogen (-NH) of K101, based on X-ray structure, ONIOM2 and ONIOM3 methods for the wild-type and K103N/Y181C HIV-1 RT.

Methods	Wild type		K103N/Y181C mutant	
	$-C=O_K\text{----}H-N_B-$	$-N-H_K\text{----}O=C_B-$	$-C=O_K\text{----}H-N_B-$	$-N-H_K\text{----}O=C_B-$
B3LYP/6-31G(d,p):PM3	2.79	3.03	2.85	3.47
MP2/6-31G(d,p): B3LYP/6-31G(d,p):PM3	2.79	3.03	2.84	3.46
X-ray	2.75	3.17	3.01	3.61

*Hydrogen bond distances of ($-C=O_K\text{----}H-N_B-$) and ($-N-H_K\text{----}O=C_B-$) are in Å

Table 7 Interaction and deformation energy components of the binding energies of HIV-1 RT/efavirenz complex for both wild-type and K103N/Y181C enzymes, calculated by different ONIOM models.

Methods	Binding energy components (kcal/mol)			
	INT	DEF_{pk}	DEF_{efz}	DEF
HF6-31G(d,p):PM3				
Wild-type	-17.47	1.70	1.55	3.25
K103N/Y181C mutant	-9.02	1.93	1.08	3.01
ΔE^a	-8.45	-0.23	0.47	0.24
B3LYP/6-31G(d,p):PM3				
Wild-type	-18.83	1.55	1.67	3.22
K103N/Y181C mutant	-10.70	2.56	1.32	3.89
ΔE^a	-8.13	-1.01	0.34	-0.67
B3LYP/6-31G(d,p):HF/6-31G(d,p):PM3				
Wild-type	-18.42	1.86	1.55	3.41
K103N/Y181C mutant	-11.05	2.20	1.82	4.02
ΔE^a	-7.37	-0.34	-0.27	-0.61
MP2/6-31G(d,p):HF/6-31G(d,p):PM3				
Wild-type	-18.58	1.69	1.29	2.98
K103N/Y181C mutant	-12.03	2.09	1.85	3.94
ΔE^a	-6.55	-0.40	-0.56	-0.96
MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3				
Wild-type	-19.98	1.56	1.51	3.07
K103N/Y181C mutant	-13.63	2.59	2.05	4.64
ΔE^a	-6.35	-1.03	-0.54	-1.57

[a] $\Delta E = E_{\text{wild-type}} - E_{\text{K103N/Y181C mutant}}$

CONCLUSION

The multi-layered integration or ONIOM method has been applied to determine the binding energies of efavirenz to Y181C and K103N/Y181C enzymes as compared with the wild type RT. The calculated binding energy for efavirenz/Y181C HIV-1 RT complex is -19.49 kcal/mol by using MP2/6-31G(d,p):B3LYP/6-31G(d,p):PM3 method which is similar to the efavirenz/wild type complex. Moreover, it was found that interaction energies calculated at MP2/6-31G(d,p) level between efavirenz and each individual residues of the binding pocket for both wild type and Y181C HIV-1 RT are not significantly different except for Val179. The interaction between K101 and efavirenz for both wild type and Y181C enzymes was found to be the major component, typically -15.0 kcal/mol. These results are consistent with the observation that efavirenz shows high inhibitory affinities against for both wild type and Y181C enzymes, and that efavirenz has a higher inhibitory affinity for the Y181C enzyme than first generation drugs such as nevirapine.

With the K103N/Y181C enzyme, the two mutations K103N and Y181C eliminate favorable contacts of the aromatic ring of the Y181 and hydrocarbon side chain of K103 with efavirenz leading to reduce the stabilization energy of the complex. It was observed that this then leads to more repulsive interactions between efavirenz with residues of the binding pocket of the K103N/Y181C enzyme compared to the wild type and Y181C enzymes. The binding energy for efavirenz/K103N/Y181C HIV-1 RT complex was calculated to be 8 kcal/mol less than that of the wild type complex. Also, hydrogen bonding between efavirenz and K101 was disturbed, and N103 in the binding pocket of K103N/Y181C enzyme creates a repulsive interaction with efavirenz. Overall, deformation energies of the K103N/Y181C complex were slightly greater than in the wild-type complex. This is consistent with the observation that efavirenz shows lower inhibitory affinities against K103N/Y181C HIV-1 RT than wild type and Y181C HIV-1 RT.

Thus it can be concluded that the use of ONIOM methods, especially ONIOM3, is accurate and efficient for modeling interactions between inhibitor

molecules and HIV-1 RT. Also understanding the interactions involved in binding within the pocket, and the structural changes that occur, can be useful for the design of higher potency inhibitors specific to double mutant enzyme target.