RESULTS AND DISCUSSION

CHAPTER I: Virtual Screening of K103N and Y181C HIV-1 Reverse Transcriptase Mutants Based on Nevirapine and Some NNRTIs

In order to find novel inhibitors insensitive to the K103N and Y181C mutations of HIV-1 Reverse Transcriptase by using virtual screening method, the procedures used to screen with an 'in-house' database of a set of 500k commercially available drug-like compounds are pharmacophore database searching, molecular docking and topological classification, respectively, as shown Figure 15. The compounds in database were filtered by using pharmacophore searching with the UNITY4.4 program. The 3D pharmacophore models were constructed based on the known important interactions between the amino acid in the binding pocket and NNRTIs. After filtering the database, the selected compounds were applied to docking with the selected docking method. The hits from docking were selected and classified by scaffold diversity using ClassPharmer program. Finally, the selected compounds from the classification were tested for HIV-1 RT inhibition.

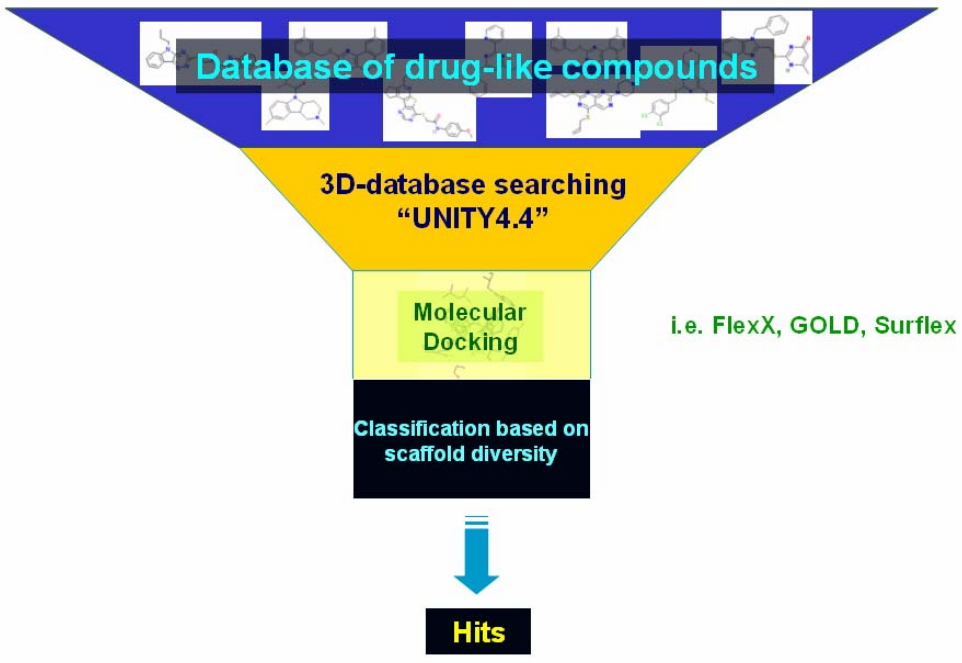


Figure 15 Virtual screening procedure.

1. Validation of the docking method

Three docking methods, FlexX, GOLD and Surflex, have been used to dock nevirapine into K103N and Y181C HIV-1 RT and PNU-142721 into K103N HIV-1 RT. The rmsd of the docked pose from the X-ray pose of nevirapine and PNU-142721 by using three docking methods are shown in Table 8. The rmsd values of nevirapine by using FlexX are 5.07 Å and 13.38 Å for K103N and Y181C mutations, respectively. They showed that the FlexX method failed to reproduce the X-ray bound conformation of nevirapine for both mutations. Surflex successfully reproduced the X-ray bound conformation for the Y181C mutation with a 0.98 Å rmsd but failed to reproduce the X-ray pose for the K103N mutation. The rmsd of the docked pose from the X-ray pose of PNU-142721 using three docking methods are 4.91 Å, 0.94 Å and 2.00 Å, respectively. The result showed that FlexX and Surflex failed to reproduce the x-ray bound conformation of PNU-142721. Last, the GOLD method revealed a good ability to reproduce the X-ray bound conformation with rmsd less than 1.0 Å for both protein mutants. From Table 8, GOLD was the most accurate method to reproduce the X-ray bound conformation of nevirapine to both mutants and PNU-142721 to its complex. Therefore, the GOLD method was selected for further docking steps.

Table 8 Rmsd (Å) of the docked pose from the X-ray pose of nevirapine and PNU-142721 by using FlexX, GOLD and Surflex methods.

Method	rmsd (Å)		
	nevirapine		PNU-142721
	K103N (1fkp)	Y181C (1jlb)	K103N (1ikx)
FlexX	5.07	13.38	4.91
GOLD	0.41	0.42	0.94
Surflex	4.91	0.98	2.00

2. Docking of other NNRTIs

The NNRTIs, nevirapine, efavirenz and PNU-142721, have been docked to the nevirapine's K103N binding pocket (pdb code 1fkp) using GOLD. The GoldScore of docked nevirapine, efavirenz and PNU-142721 were 57.16, 58.88 and 56.47, respectively. The conformations of docked NNRTIs are shown in Figure 16a. The docked nevirapine conformation was used to define model 1 of 3D database searching for K103N inhibitors. The conformations of docked nevirapine, efavirenz and PNU-142721 were used to define model 2 of 3D database searching for the same case. For the K103N binding pocket of PNU-142721 (pdb code 1ikx), PNU-142721 was docked into its binding pocket using GOLD. The docked conformation was shown in Figure 16b. The GoldScore of docked PNU-142721 is 59.99. It was used to define model 3 of 3D database searching for K103N inhibitors.

For the Y181C mutant, the NNRTIs, nevirapine, efavirenz and 8-Cl TIBO, have been docked to the Y181C binding pocket by using GOLD. The conformations of docked NNRTIs were shown in Figure 16c. The GoldScore of docked nevirapine, efavirenz and 8-Cl TIBO were 54.23, 56.05 and 56.81, respectively. To define the pharmacophore models for 3D database searching, the conformations of docked nevirapine was used to define model 1 and the conformations of docked nevirapine and efavirenz were used to define model 2.

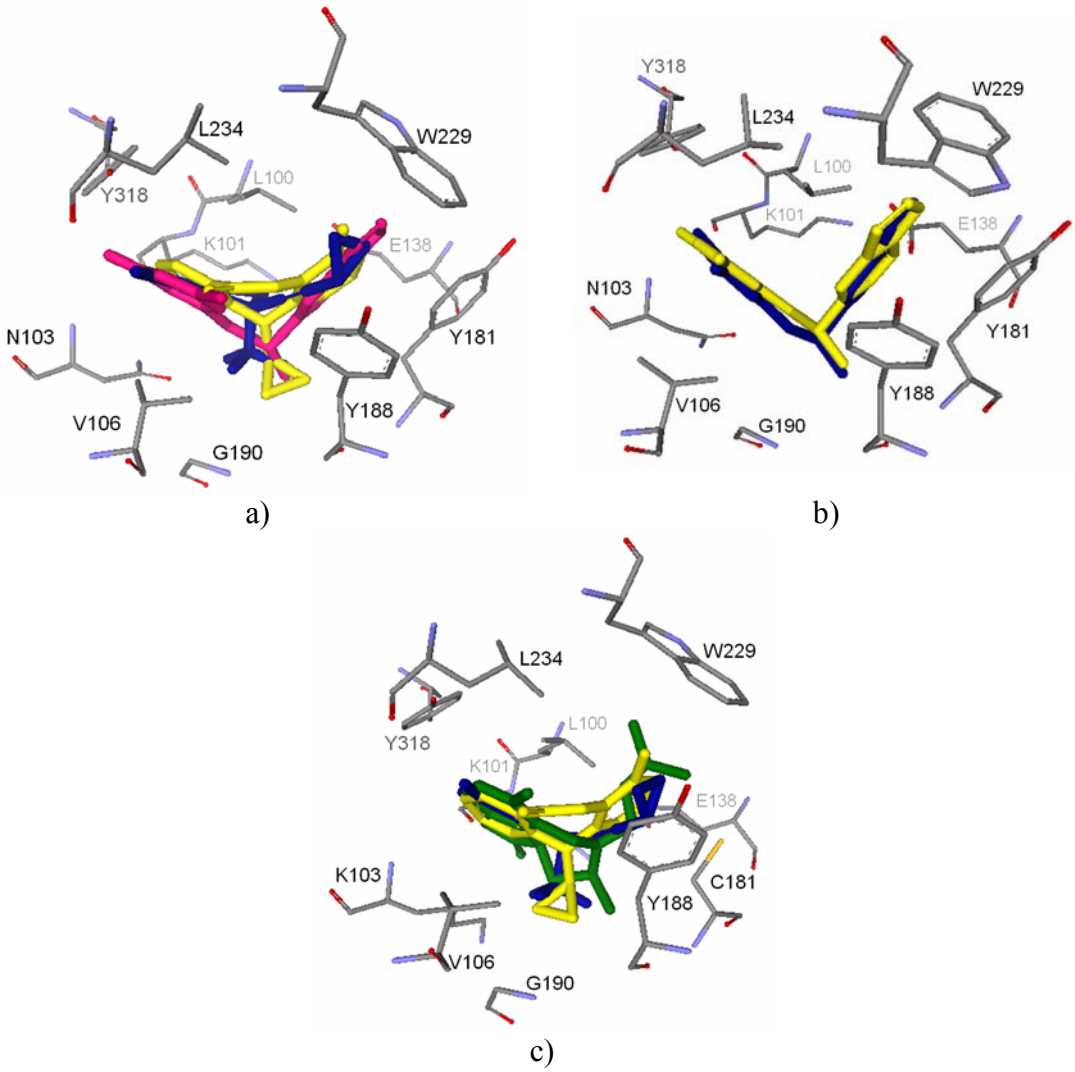


Figure 16 Conformations of docked NNRTIs;

a). Docked conformations of nevirapine (yellow), efavirenz (blue) and PNU-142721 (pink) in the K103N binding pocket (PDB code 1fkp).

b). Docked conformations of docked PNU-142721 (blue) compared with the orientation of X-ray pose (yellow) in the K103N binding pocket (PDB code 1ikx).

c). Docked conformations of nevirapine (yellow), efavirenz (blue) and 8-Cl TIBO (green) in the Y181C binding pocket (PDB code 1jlb).

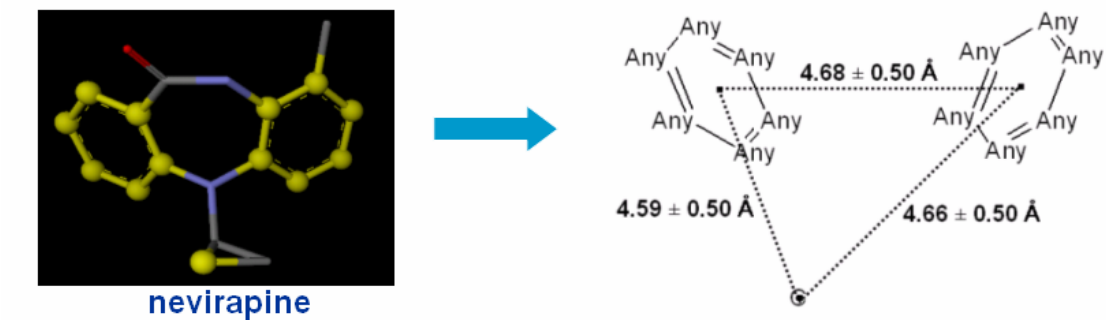
3. Pharmacophore database searching

The conformations of docked NNRTIs were used to construct pharmacophore models for 3D database searching. Three pharmacophore models used in 3D database searching for the K103N protein mutant have been defined. Model 1 and 2 were used for the K103N protein mutant (PDB code 1fkp). Model 3 was used for the K103N protein mutant (PDB code 1ikx). The first model was derived from the docked conformation of nevirapine and the second one was derived from docked conformation of nevirapine, efavirenz and PNU-142721 in K103N binding pocket (PDB code 1fkp). The features of the first model consist of two centroids of 6-membered aromatic ring and one sp^3 carbon atom. Two centroids of 6-membered aromatic ring represented the stacking interactions with the aromatic amino acids Y181, Y188 and Y318. The sp^3 carbon atom represented the van der waals interaction with G190. The features of the second model consist of a centroid of 6-membered aromatic ring from ring B of nevirapine, two nitrogen atoms from efavirenz and PNU-142721. The nitrogen atom of efavirenz represented the H-bond interactions with the K101 backbone atom and the N103 side- chain atom. The nitrogen atom of PNU-142721 represented the H-bond interactions with the N103 backbone atom. For the third model, the conformation of docked PNU-142721 in K103N binding pocket (PDB code 1ikx) and the position of the N103 backbone nitrogen atom were used to construct the pharmacophore model. The model consists of a centroid of 6-membered aromatic ring from the pyridine ring, any atom at the chlorine position of PNU-142721, carbon atom from the methyl group of PNU-142721 and a ligand acceptor (LA) atom that H-bonds to the N103 receptor donor (RD) backbone nitrogen atom. The 6-membered aromatic ring represented the stacking interactions with the aromatic amino acids Y181 and Y188. The any atom at the chlorine position of PNU-142721 represented the van der waals interaction with V106 and L234. The carbon atom represented the van der waals interaction with V179, Y181, Y188 and G190.

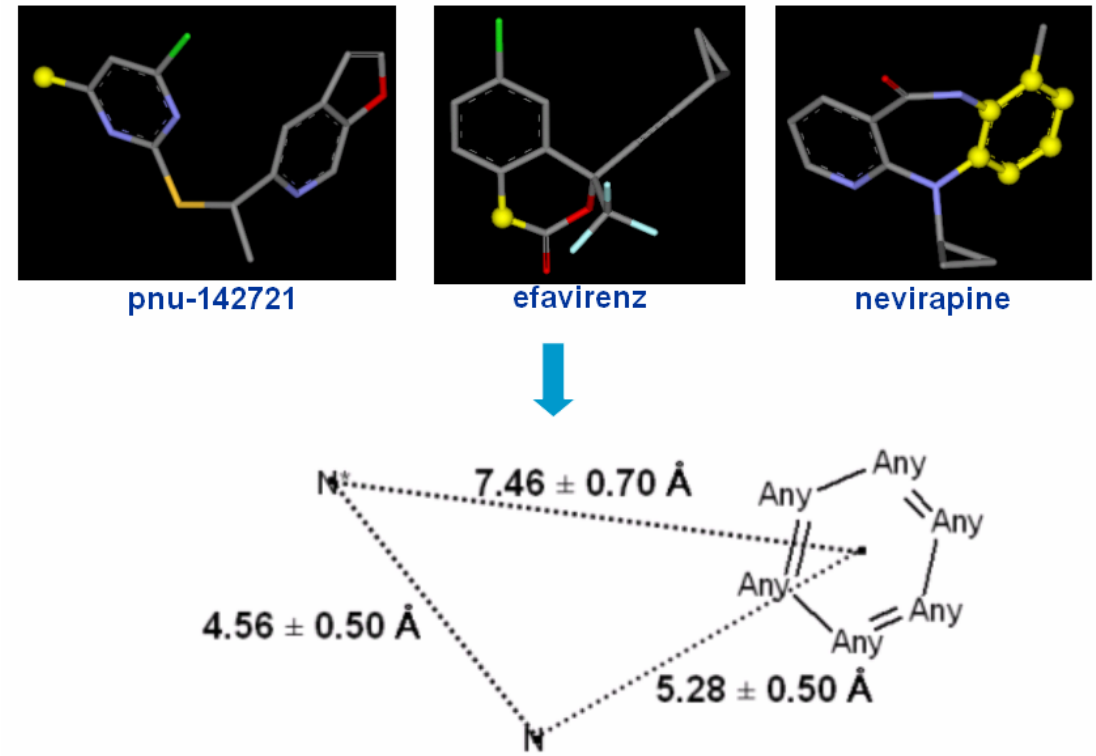
For the Y181C protein mutant (PDB code 1jlb), two pharmacophore models have been defined. The first model derived from the docked conformation of nevirapine consists of a centroid of 6-membered aromatic ring from ring A, the

methyl group and a CH_2 or CH_3 group. The 6-membered aromatic ring from ring A represented the stacking interactions with the aromatic amino acids Y318. The methyl group represented the van der waals interaction with W229 and L234. The CH_2 or CH_3 group represented the van der waals interaction with G190. The second model was derived from docked conformations of nevirapine and efavirenz. It consists of a centroid of 6-membered aromatic ring from ring A of nevirapine, a CH_2 or CH_3 group from the carbon atom in methyl position of nevirapine and the nitrogen atom from efavirenz. The features from nevirapine represented the same interactions described in the first model and the nitrogen atom from efavirenz represented the H-bond interaction with K101. The tolerances of the distance constraints were set to ± 10% of each interatomic distance. The features and constraints of all models were shown in Figure 17.

All pharmacophore models were used as constraints for searching the "Bioinfo" database for both protein mutant inhibitors. 10,047 compounds and 10,343 compounds were found to matching pharmacophores from the K103N mutation pharmacophore model 1 and model 2, respectively. After combining the compounds and removing the duplicated compounds from two models, there are 20,200 compounds. From the K103N pharmacophore model 3, there are 3,733 compounds matching pharmacophore. Searching with the same database for Y181C inhibitors, 13,005 compounds and 7,620 compounds were found as matching pharmacophores from model 1 and 2, respectively. After combining the compounds and removing the duplicated compounds from two models, there are 19,761 compounds. These compounds were selected for further molecular docking with GOLD as previously described.

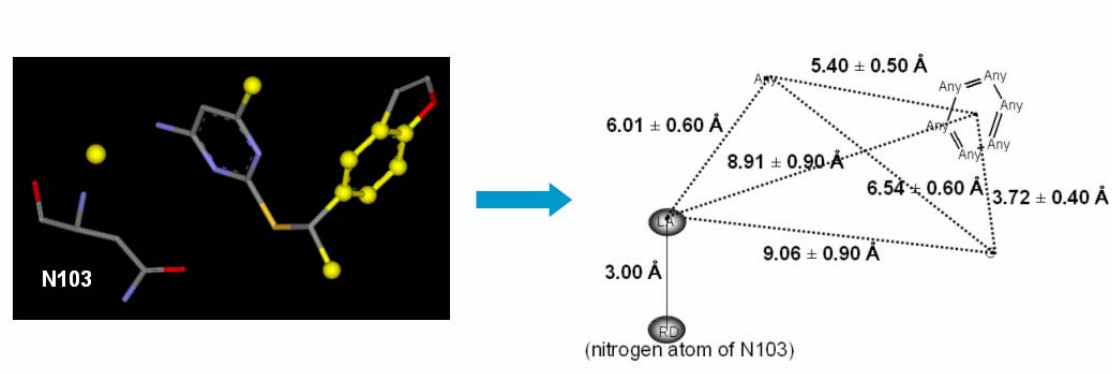


a) Model 1of K103N protein mutant.



b) Model 2 of K103N protein mutant.

Figure 17 Features and constraints of all pharmacophore models.



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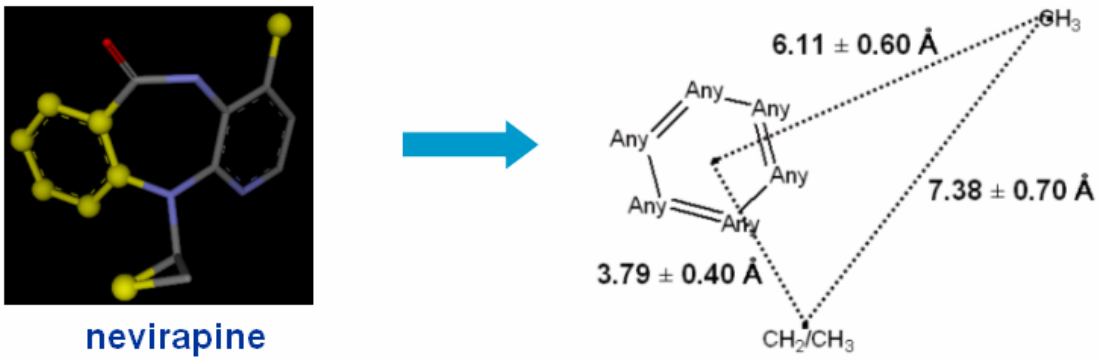
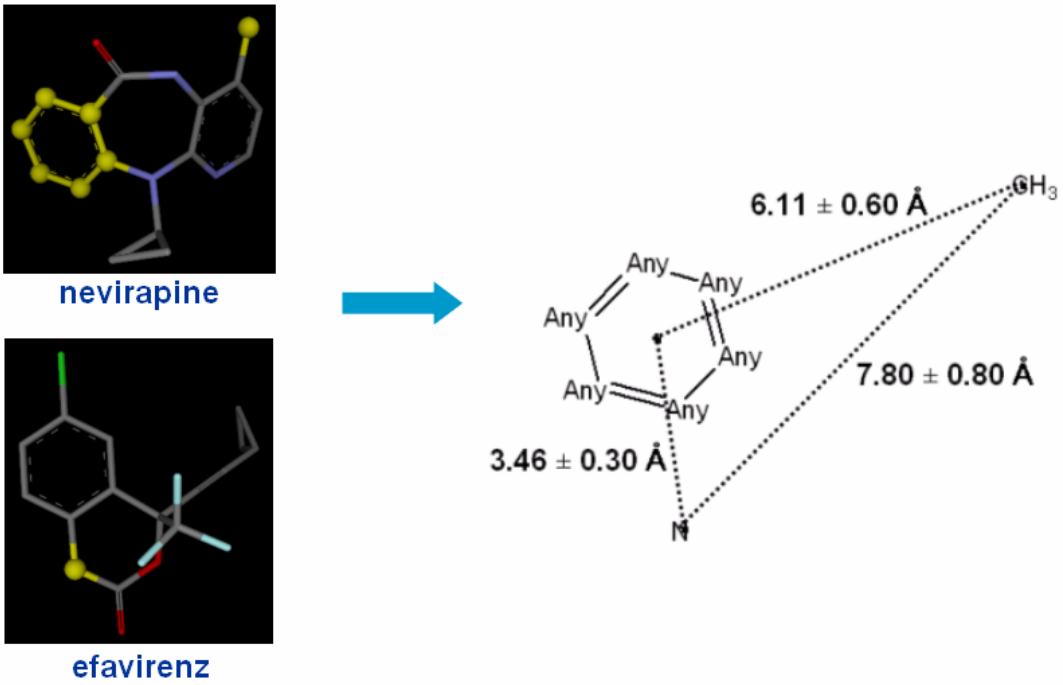


d) Model 1 of Y181C protein mutant.



e) Model 2 of Y181C protein mutant.

Figure 17 (cont'd)

4. Database docking

The previously generated pharmacophore hitlists of 20,200 compounds, 3,733 compounds and 19,761 compounds were docked to the K103N protein mutants (PDB code 1fkp and 1ikx) and Y181C protein mutant (PDB code 1jlb), respectively. Two hitlists (GoldScore higher than 45 and GoldScore higher than 50) were saved for each protein mutant, respectively (Table 9).

Table 9 Numbers of hits with GoldScore higher than 45 and 50 for K103N and Y181C protein mutants.

	K103N (PDB code 1fkp)	K103N (PDB code 1ikx)	Y181C (PDB code 1jlb)
no. of hits (GoldScore ≥ 45)	4,052	599	1,768
no. of hits (GoldScore ≥ 50)	1,996	255	748

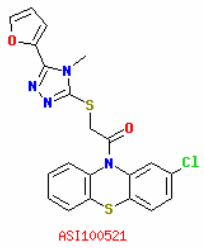
5. Classification of the hits

5.1 K103N protein mutant inhibitors of PDB code 1fkp

In the first procedure, the compounds having a GoldScore higher than 45 were classified into 192 classes and 4 singletons. After calculating the probability distribution, there are 47 classes having PrbDst score higher than 0.2, out of which 38 representative compounds were finally selected. For the second procedure, the compounds having a GoldScore higher than 50 were classified into 133 classes and 2 singletons. After removing the classes with less than 4 compounds and importing the compounds having a GoldScore lower than 50, the percent of compounds having the GoldScore higher than 50 in the class (%Val) was calculated. 67 classes with a percentage higher than 20% were prioritized, out of which 58 compounds were finally selected. The 24 common compounds from both were selected for biological evaluation. The structures of selected compounds from procedure1, procedure2 and common compounds to both procedures were shown in Table 10.

Table 10 Number and structure of selected compounds from procedure1, procedure2 and common compounds to both procedures for K103N protein mutant.

	No. of selected compounds
- Procedure 1	38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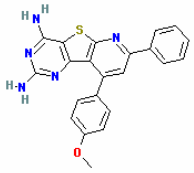
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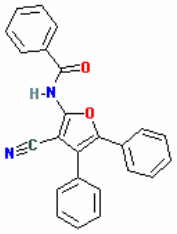
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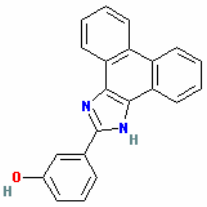
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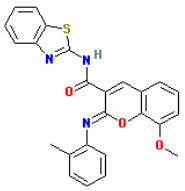
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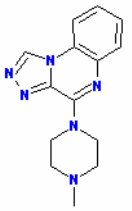
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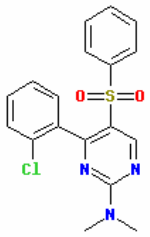
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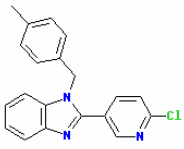
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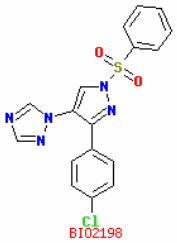
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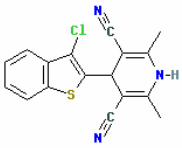
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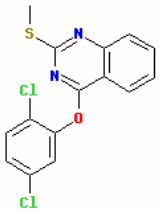
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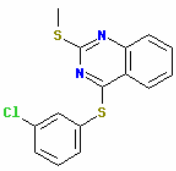
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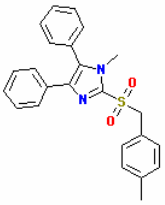
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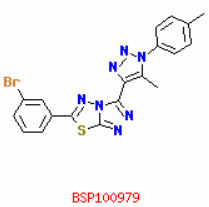
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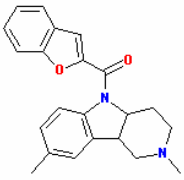
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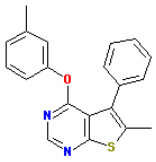
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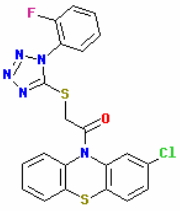
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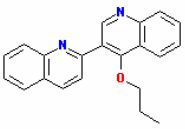
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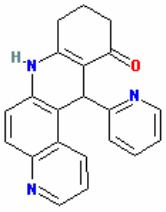
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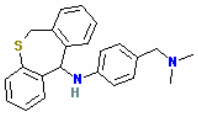
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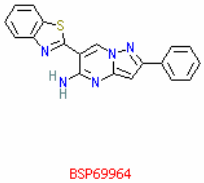
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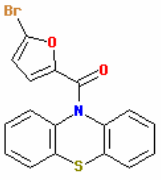
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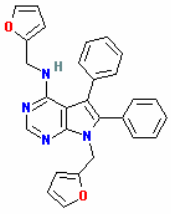
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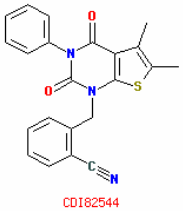
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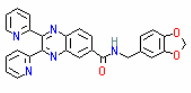
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Table 10 (cont' d)

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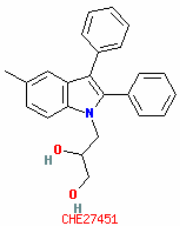
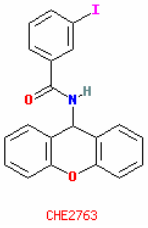
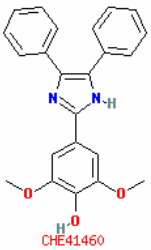
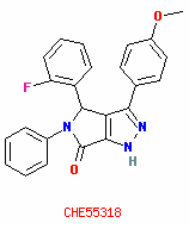
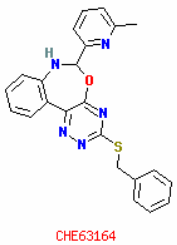
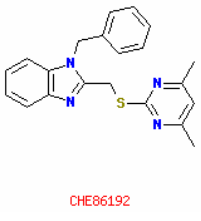
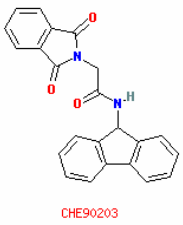
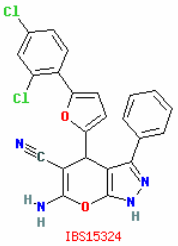
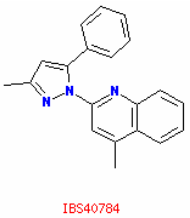
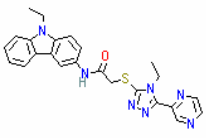
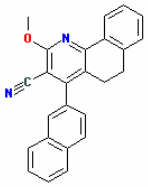
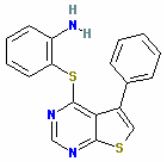
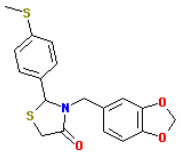
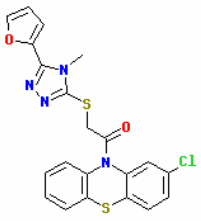
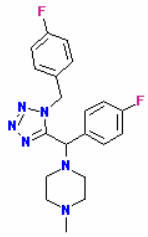
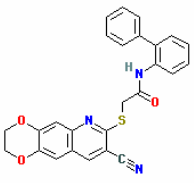
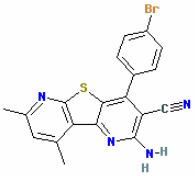
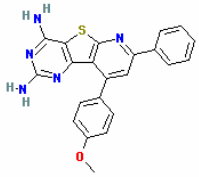
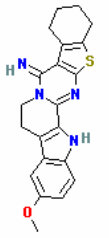
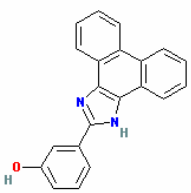
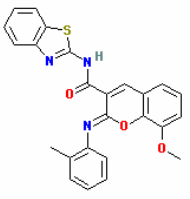
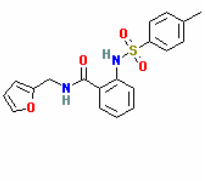
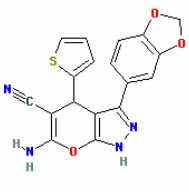
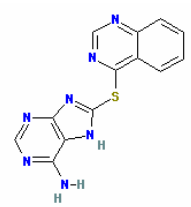
 CHE27451  CHE2763  CHE41460  CHE55318  CHE63164  CHE86192  CHE90203  IBS15324  IBS40784  IBS84013  MAY4062  MAY9  TRI29535	
- Procedure 2	58
 ASI100521  ASI118272  ASI122735  ASI15047  ASI15057  ASI17161  ASI29516  ASI42496  ASI52163  ASI52277	

Table 10 (cont' d)

No. of selected compounds
- Procedure 2 (continued)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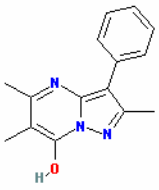
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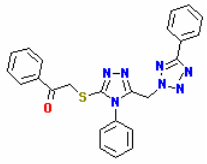
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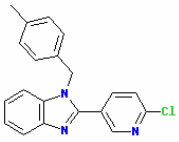
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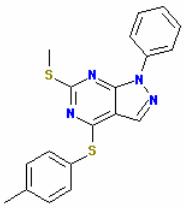
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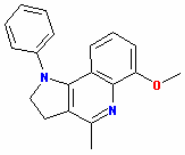
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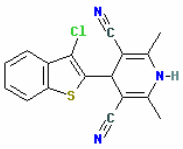
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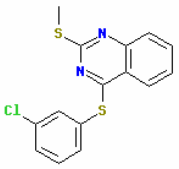
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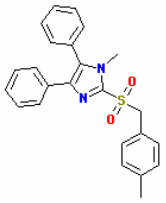
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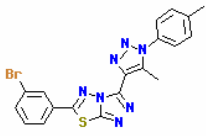
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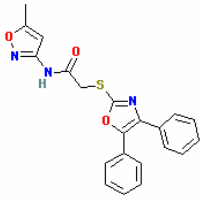
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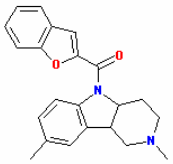
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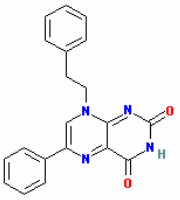
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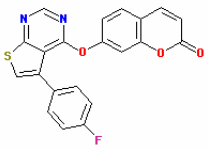
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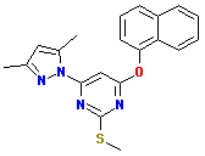
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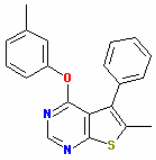
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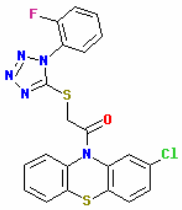
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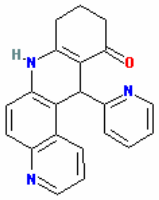
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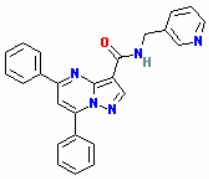
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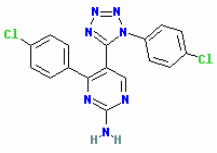
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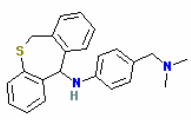
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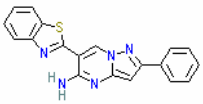
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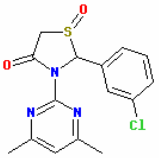
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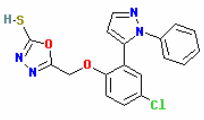
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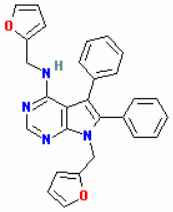
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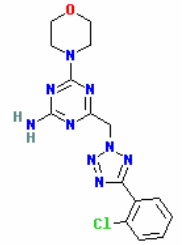
Table 10 (cont' d)

	No. of selected compounds
- Procedure 2 (continued)	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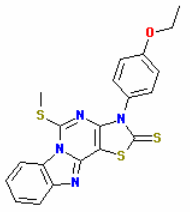
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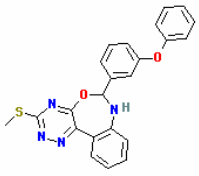
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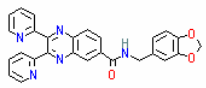
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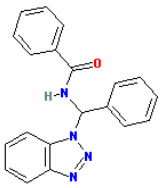
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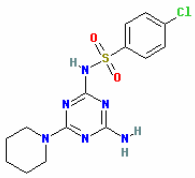
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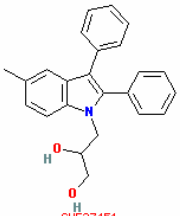
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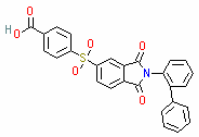
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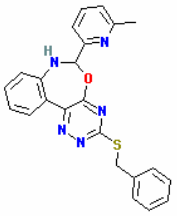
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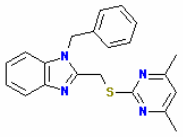
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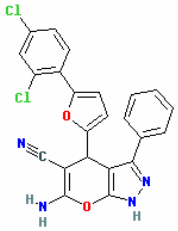
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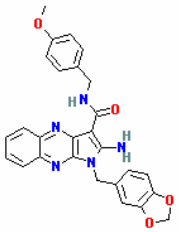
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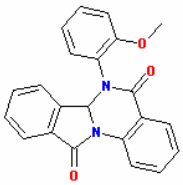
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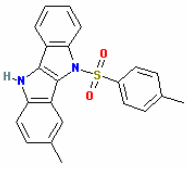
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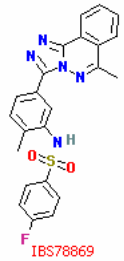
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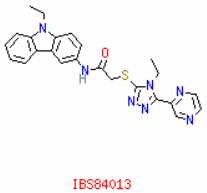
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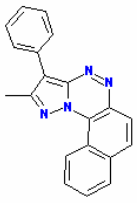
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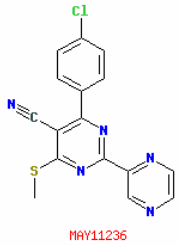
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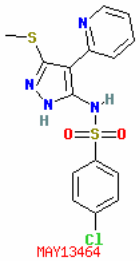
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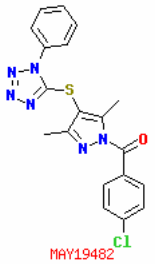
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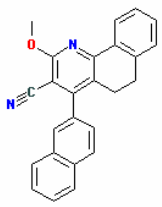
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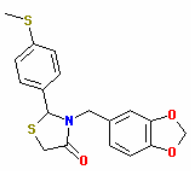
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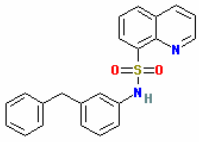
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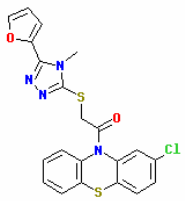
Table 10 (cont' d)

	No. of selected compounds
- Common compounds to procedures 1 and 2	24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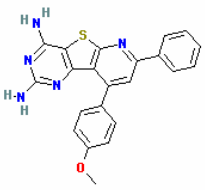
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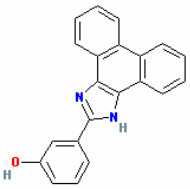
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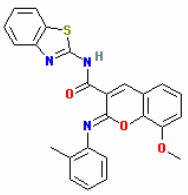
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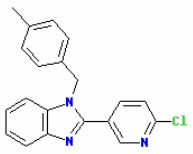
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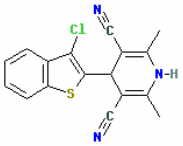
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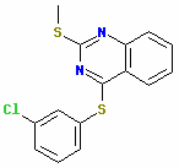
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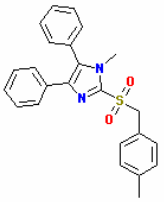
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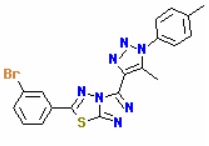
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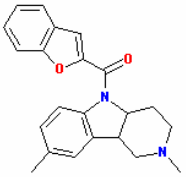
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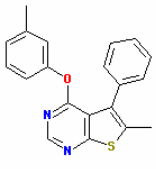
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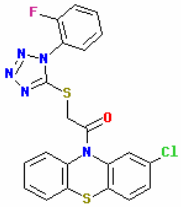
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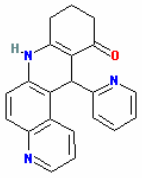
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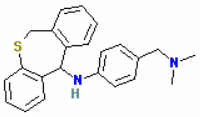
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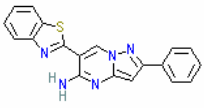
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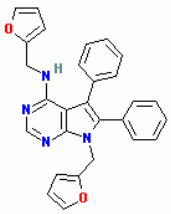
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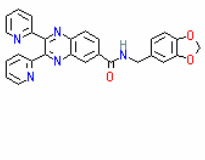
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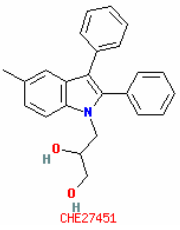
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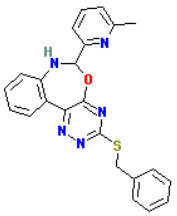
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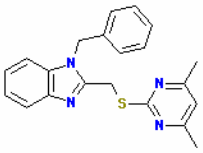
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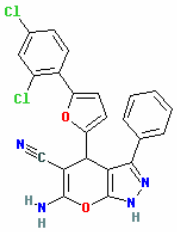
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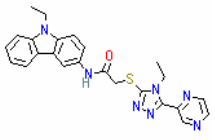
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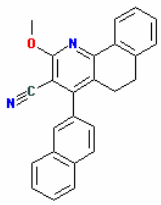
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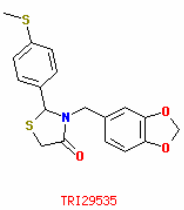

TRI29535

5.2 K103N protein mutant inhibitors of PDB code 1ikx

In the first procedure, the 599 compounds having a GoldScore higher than 45 were classified into 81 classes and 9 singletons. After calculating the probability distribution, there are 12 classes having the probability distribution higher than 0.2, out of which 11 representative compounds were selected. In the second procedure, the 255 compounds having a GoldScore higher than 50 were classified into 53 classes and 3 singletons. After removing the classes with less than 4 compounds and importing back the compounds having a GoldScore lower than 50, the percent of compounds having a GoldScore higher than 50 in each class (%Val) was calculated. There are 18 classes with a %Val higher than 20, out of which 15 compounds were selected. From two procedures of classification, 9 compounds were finally selected for biological evaluation. The structures of selected compounds from procedure1, procedure2 and common compounds to both procedures were shown in Table 11.

Table 11 Number and structure of selected compounds from procedure1, procedure2 and common compounds to both procedures.

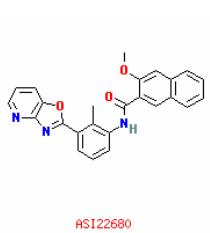
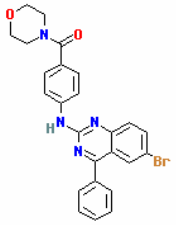
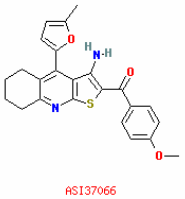
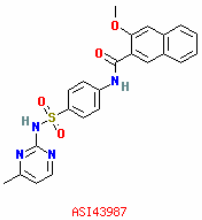
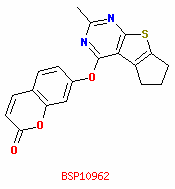
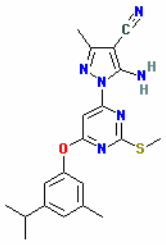
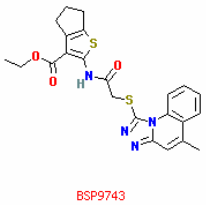
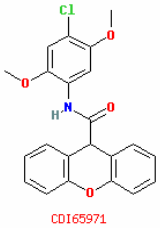
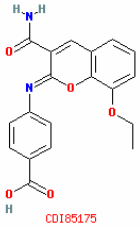
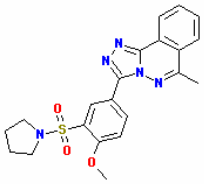
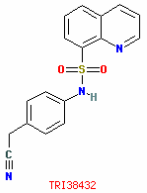
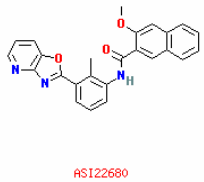
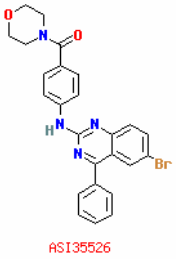
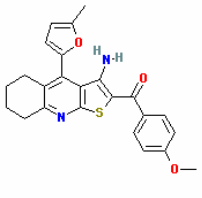
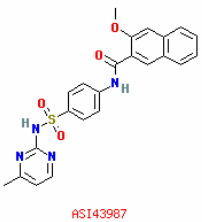
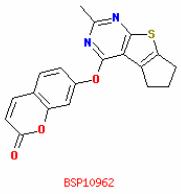
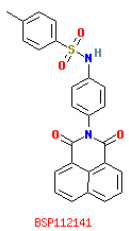
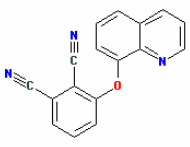
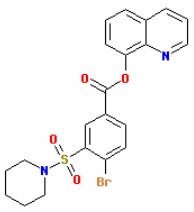
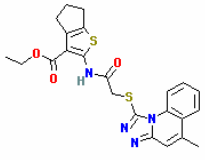
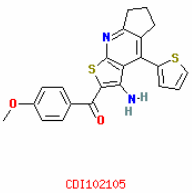
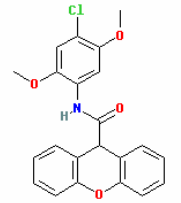
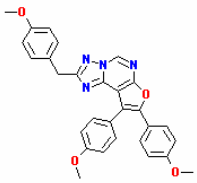
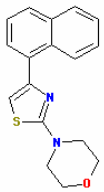
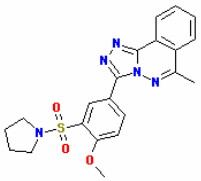
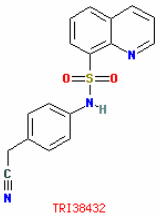
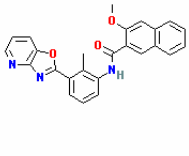
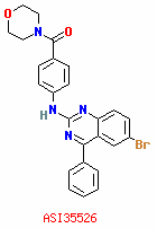
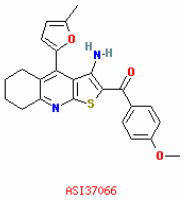
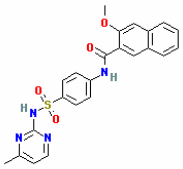
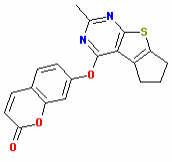
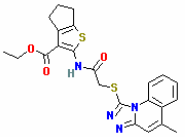
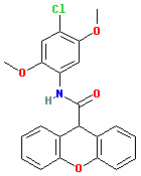
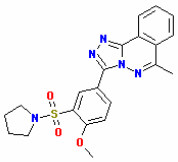
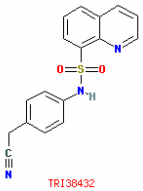
	No. of selected compounds
- Procedure 1	11
 ASI22680  ASI35526  ASI37066  ASI43987  BSP10962  BSP111232  BSP9743  CDI65971  CDI85175  IBS85975  TRI38432	
- Procedure 2	15
 ASI22680  ASI35526  ASI37066  ASI43987  BSP10962  BSP112141  BSP73788  BSP74249  BSP9743  CDI102105	

Table 11 (cont' d)

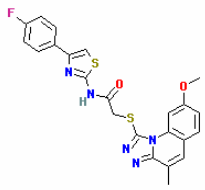
	No. of selected compounds
- Procedure 2 (cont' d)	
 CDI65971  CHE107378  CHE49771  IBS85975  TRI38432	
- Common compounds to procedures 1 and 2	9
 ASI22680  ASI35526  ASI37066  ASI43987  BSP10962  BSP9743  CDI65971  IBS85975  TRI38432	

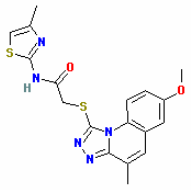
5.3 Y181C protein mutant inhibitors of PDB code 1jlb

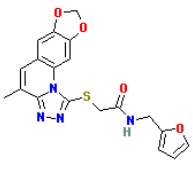
The same post-processing procedures have been performed to GOLD hits. In the first procedure, the compounds having a GoldScore higher than 45 were classified into 162 classes and 5 singletons. The probability distribution of each class was calculated and 37 classes having a PrbDst score higher than 0.2 were selected, out of which 25 representative compounds were finally chosen. For the second procedure, 748 compounds having a GoldScore higher than 50 were classified into 104 classes and 6 singletons. The classes with less than 4 compounds were removed and the compounds having GoldScore lower than 50 were imported back and classified into the earlier classified classes. After calculating the percent of compounds having a GoldScore higher than 50 in each class, 31 classes with a percentage higher than 20% were studied, out of which 23 compounds were selected. The 17 common compounds to both procedures were finally selected for biological evaluation. The structures of selected compounds from procedure1, procedure2 and common compounds to both procedures were shown in Table 12.

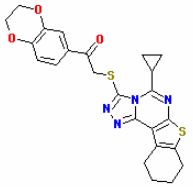
Table 12 Number of selected compounds from procedure1, procedure2 and common compounds to both procedures for Y181C protein mutant.

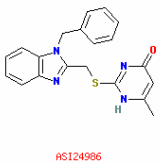
	No. of selected compounds
- Procedure 1	25

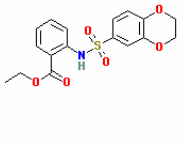


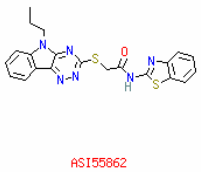


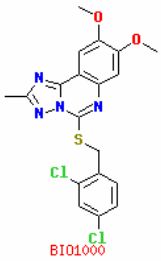


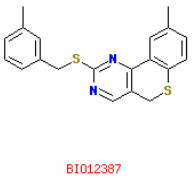


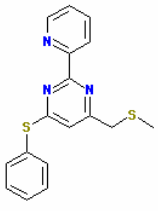


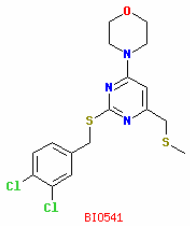


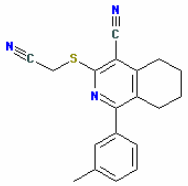


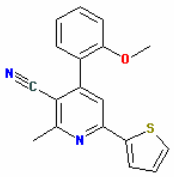


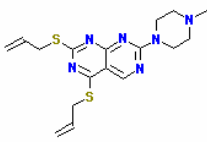


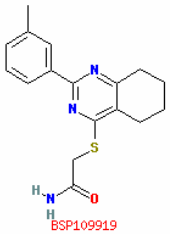


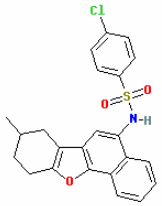


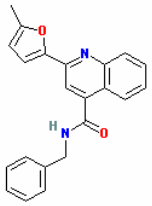


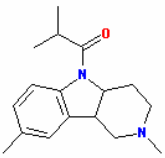


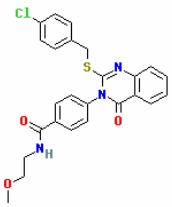


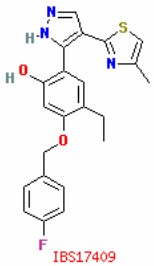


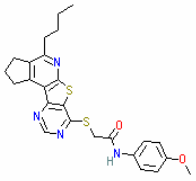


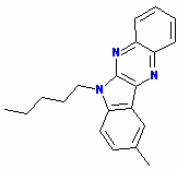


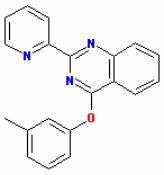


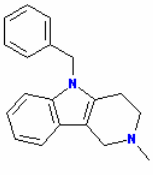


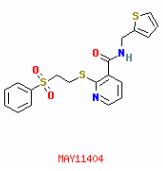












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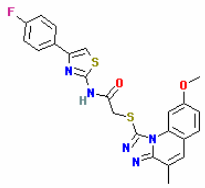
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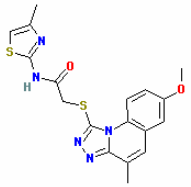
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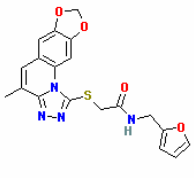
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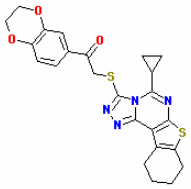
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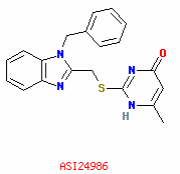
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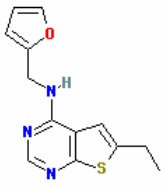
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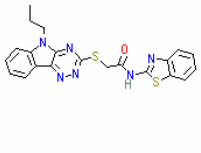
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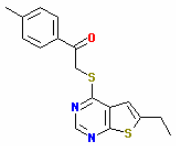
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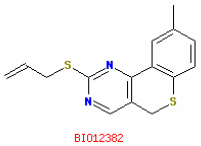
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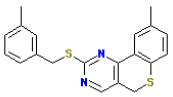
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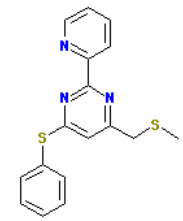
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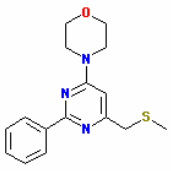
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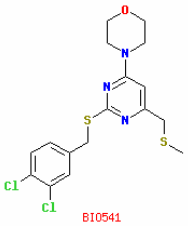
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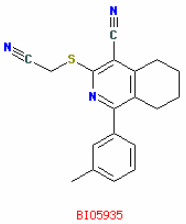
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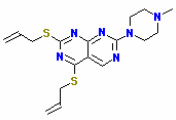
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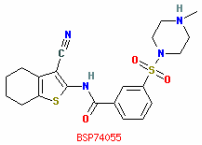
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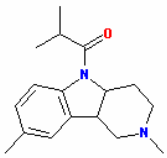
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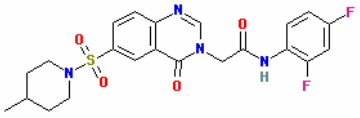
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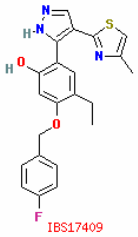
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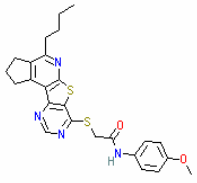
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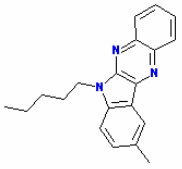
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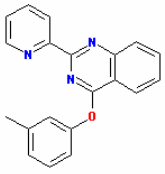
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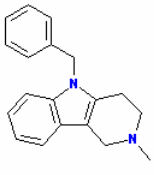
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Table 12 (cont' d)

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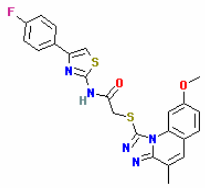
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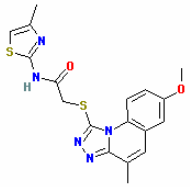
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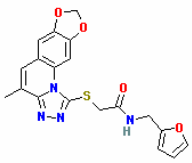
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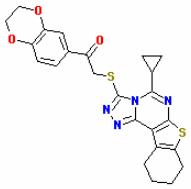
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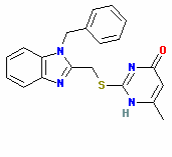
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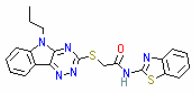
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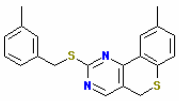
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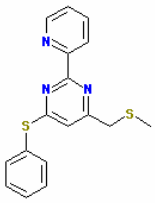
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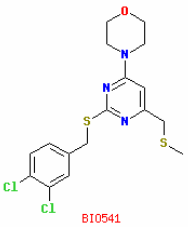
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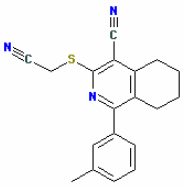
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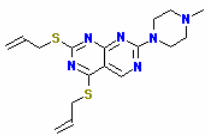
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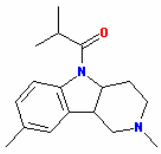
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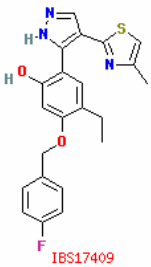
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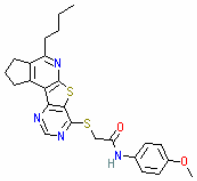
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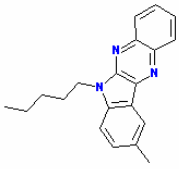
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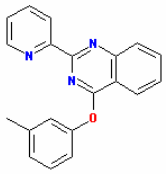
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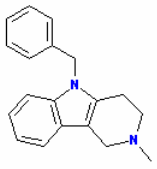
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6. Test for HIV-1 inhibition

From the classification of the hits, 50 selected common compounds (24, 9, and 17 compounds from Table 10, 11, and 12, respectively) are selected for purchase. Eighteen of them are unavailable from the suppliers, therefore, only 32 compounds are purchased from the suppliers and tested for HIV-1 RT inhibition ultrasensitive reverse transcriptase assay with wild-type, K103N, and Y181C HIV-1 RT at Pr615-Virus, Department of Microbiology, Faculty of Science, Mahidol University. Nevirapine is also tested the inhibition of wild-type, K103N and Y181C HIV-1 RT. The IC_{50} of nevirapine are 104.84 μM, 2,459 μM, and 5,254 μM for wild-type, K103N and Y181C HIV-1 RT, respectively. The %inhibition of each selected compounds are shown in Table 13. %Fold is calculated by %inhibition of wild-type divided by %inhibition of K103N or Y181C. There are three compounds having %inhibition higher than 50, i.e. compounds no. 18, 21 and 25. Compound no. 18, BIO5935, has 75.85% of wild-type inhibition with an IC_{50} = 7.84 mM. Compound no. 21, BSP12957, has 58.66% of K103N inhibition with an IC_{50} = 15.64 mM for K103N HIV-1 RT. Compound no. 25, CHE63164, has 51.16% wild-type inhibition with an IC_{50} = 6.15 mM and 55.90 % of K103N inhibition with an IC_{50} = 5.63 mM.

Table 13 Testing results of wild-type, K103N and Y181C HIV-1 inhibition of the selected compounds by using enzyme assay.

No.	Name	Code	Ref.[a]	Conc. (mM)	% inhibition (%fold)		
					Wild-Type	K103N	Y181C
1	ASI100521	ASN4210712	K103N screen1	12.53	19.89	23.98 (1.20)	-10.67 (-)
2	ASI29516	BAS0914944	"	16.11	13.03	16.41 (1.26)	-38.13 (-)
3	ASI42496	BAS1532395	"	8.83	-12.28	17.16 (-)	-46.49 (-)
4	ASI22680	BAS0706686	K103N screen2	5.76	22.06	16.03 (0.73)	-34.43 (-)
5	ASI35526	BAS1130832	"	8.38	26.22	8.08 (0.31)	-32.62 (-)
6	ASI121185	ASN5443828	Y181C	0.77	10.61	-31.99 (-)	-1.56 (-)
7	ASI106801	ASN4482181	"	9.01	20.98	27.34 (1.30)	-9.68 (-)
8	ASI103467	ASN4371565	"	8.55	16.15	18.28 (1.13)	-1.56 (-)
9	ASI106879	ASN4482652	"	4.03	23.47	12.94 (0.56)	-23.36 (-)
10	ASI55862	BAS2729623	"	4.6	25.20	29.92 (1.19)	-0.65 (-)
11	BIO7687	5K-511S	K103N screen1	10.68	1.63	-17.88 (-)	-21.15 (-)
12	BIO12186	9J-334S	"	14.98	24.17	5.12 (0.21)	-48.49 (-)
13	BIO695	10N-667S	"	5.96	23.47	-12.67 (-)	-24.48 (-)
14	BIO4483	2M-704	"	15.04	16.53	10.54 (0.64)	-40.02 (-)
15	BIO541	10L-603S	Y181C	16.09	26.89	-10.15 (-)	-31.42 (-)
16	BIO4447	2L-570S	"	50 mg (Liquid)	-1.56	-47.79 (-)	-38.75 (-)
17	BIO12387	9N-324S	"	15.41	-21.31	-47.12 (-)	-104.72 (-)
18	BIO5935	3T-0325	"	11.9	75.85	-0.31 (-)	19.15 (0.25)
19	BSP8968	AE-848/41541184	K103N screen1	10.3	6.44	17.04 (2.64)	18.79 (2.92)
20	BSP10962	AF-399/15031032	K103N screen2	2.86	18.42	18.90 (1.03)	28.77 (1.56)
21	BSP12957	AF-399/40703852	K103N screen1	18.35	43.95	58.66 (1.33)	29.09 (0.66)
22	CHE27451	5667084	"	6.43	-8.26	-4.01 (-)	16.17 (16.17)
23	CHE86192	6725551	"	2.77	14.21	-3.54 (-)	20.24 (1.42)
24	CHE103108	7028396	"	2.82	30.16	15.91 (0.53)	9.47 (0.31)
25	CHE63164	6376229	"	6.29	51.16	31.70 (0.62)	55.90 (1.09)
26	IBS40039	STOCK2S-88593	Y181C	0.607	0	2.37 (2.37)	38.37 (38.37)
27	IBS85975	STOCK4S-23237	K103N screen2	5.94	9.6	24.90 (2.59)	37.24 (3.88)
28	IBS67296	STOCK3S-79310	Y181C	13.8	15.7	16.29 (1.04)	19.15 (1.22)
29	IBS58085	STOCK3S-47234	"	9.82	9.6	13.20 (1.38)	28.77 (3.00)
30	IBS17409	STOCK1S-84182	"	23.2	15.7	5.84 (0.37)	15.79 (1.01)
31	IBS15324	STOCK1S-73707	K103N screen1	13.1	20.30	43.77 (2.16)	43.44 (2.14)
32	IBS90270	STOCK1N-00932	Y181C	24.1	32.9	18.53 (0.56)	4.86 (0.15)

a. References:

K103N screen 1 represented the selected compounds from Table 10.

K103N screen 2 represented the selected compounds from Table 11.

Y181C represented the selected compounds from Table 12.