MATERIALS AND METHODS

Models

1. Methods of calculations

In this study, the starting geometry of nevirapine was obtained from X-ray crystallographic data at 2.2 Å resolution of the enzyme-inhibitor complex structure (1vrt). (Berman *et al*., 2000) First, full geometrical optimization was performed, based on semiempirical (AM1) method, *ab initio* at the HF/3-21G, HF/6-31G and HF/6-31G** level and DFT at the B3LYP/6-31G** level. Then the obtained structures were compared to the X-ray diffraction data of nevirapine in the complex with RT. The compared structure parameters obtained by each method are presented in Table 1. It indicates that all considered methods provide sufficiently good results for the torsion angles. As expected, the standard deviations are smaller for results obtained from *ab initio* and density functional theory calculations than for that obtained by semiempirical method. It is generally known that the HF/3-21G, HF/6-31G and HF/6-31G** levels lead to a good geometry close to the results of the accurate B3LYP/6-31G** level of theory. The dihedral angles which determine the position of the cyclopropyl ring (C15-N11-C17-C19, α) are similar for all calculations and differ slightly from X-ray investigation data. Superimposition of the geometry optimization at B3LYP/6-31G** level on the crystal structure of nevirapine in the complex shows good agreement (root mean squares deviation of 0.08). It can be seen that at B3LYP/6-31G** level shows the smallest standard deviation (7.7). All optimized structures in this study were done at B3LYP/6-31G** level of theory.

Table 1 Comparison of the selected torsion angles of the fully optimized geometries of nevirapine, obtained by different methods and compared to experimental X-ray crystallographic data

		Starting geometry obtained from X-ray crystallographic data				
		AM1	HF/			B3LYP/
	X-ray[a]		3-21G	6-31G	6-31G**	6-31G**
Torsion angle (deg)						
O20-C6-C14-C7	30.0	34.8	21.1	24.6	24.1	21.2
C12-N11-C17-C19	68.7	78.4	69.6	69.1	73.3	71.2
C12-N11-C17-C18	136.8	147.5	137.7	138.1	142.0	139.9
C14-C15-N11-C17	168.3	154.1	154.8	153.1	157.0	159.9
C13-C12-N11-C17	200.1	206.6	208.3	209.3	206.9	205.4
C15-N11-C17-C19	**208.5**	**211.3**	**220.6**	**220.1**	**216.4**	**217.4**
C15-N11-C17-C18	276.6	280.5	288.7	289.7	285.1	286.1
C6-N5-C13-C14	144.4	144.6	135.8	137.6	131.1	134.8
N5-C6-C14-C7	212.9	217.5	202.2	205.4	204.1	201.1
C15-N11-C12-N1	238.8	250.0	237.6	238.1	243.3	238.0
C12-N11-C15-N10	126.7	111.8	127.4	126.0	122.2	127.9
SD[b]		9.3	9.0	8.7	8.3	7.7

[a] Data obtained from resolution of 2.2 Å (Ren et al., 1995)

[b] Standard deviation (SD) $=[\sum(x_{Cal.}-x_{Expt.})^2/n-1]^{1/2}$

2. NMR calculations

The NMR calculations typically benefit from an accurate geometry and large basis set such as at the B3LYP/6-311++G(2d,p) level of theory. Moreover, for the large basis sets, errors for ^{1}H chemical shifts could be consistently less than 1 ppm and sometimes less than 0.10 ppm (Cheeseman *et al.*, 1996). Therefore, in this study the chemical shifts were calculated by single point calculations at the B3LYP/6-311++*G*** level of theory.

The ^{1}H, ^{13}C and ^{15}N chemical shielding constants, $\sigma(^{1}H_i)$, $\sigma\ (^{13}C_i)$ and $\sigma\ (^{15}N_i)$ in gas, DMSO, chloroform, dichloromethane, ethanol and water were calculated by the GIAO method at B3LYP/6-311++G** level of theory on the structure optimized at B3LYP/6-31G** level. The calculations in solvents utilize the self-consistent reaction field (SCRF) method: integral equation formalism polarized continuum model (IEF-PCM). The ^{1}H and ^{13}C chemical shifts, $\delta(^{1}H_i)$ and $\delta(^{13}C_i)$, are referred to the usual standard tetramethylsilane (TMS) and the ^{15}N chemical shifts, $\delta(^{15}N_i)$ are referred to nitromethane through the relation

$$\delta(^{1}H_i) = \sigma(^{1}H)_{TMS} - \sigma(^{1}H_i)_{NEVIRAPINE} \quad (1)$$

$$\delta(^{13}C_i) = \sigma\ (^{13}C)_{TMS} - \sigma\ (^{13}C_i)_{NEVIRAPINE} \quad (2)$$

$$\delta(^{15}N_i) = \sigma\ (^{15}N)_{NITROMETHANE} - \sigma\ (^{15}N_i)_{NEVIRAPINE} \quad (3)$$

To give accurate chemical shifts, the isotropic shielding constant of hydrogen and carbon in TMS, $\sigma(^{1}H)_{TMS}$ and $\sigma(^{13}C)_{TMS}$, and isotropic shielding constant of nitrogen in nitromethane, $\sigma\ (^{15}N)_{NITROMETHANE}$, were calculated at the same computational level of the nevirapine compound. In addition, solvation energies of nevirapine were calculated on the optimized geometries. For the IEF-PCM model, the solvent is assumed to be a continuous medium with a dielectric constant ε that surrounds a cavity, adjusted to fit the shape of the solute molecule to afford more

accurate solvation energies. The dielectric constant for DMSO (ε = 46.7), chloroform (ε = 4.9), dichloromethane (ε = 8.93), ethanol (ε = 24.55), methanol (ε = 32.63) and water (ε = 78.39) were used in the calculations.

These calculations were done using GAUSSIAN98 (Frisch *et al*., 1998) on a 2.53 GHz Pentium IV PC running on Redhat Linux 9.

3. Conformational analysis

Because the alpha angle of cyclopropyl ring (C15-N11-C17-C19, α) plays an important role to determine the structure of nevirapine, where only a limited flexibility of the ring system exists (Hannongbua *et al*., 2001). To study the structural energy minimum, corresponding to the conformation of nevirapine as obtained from X-ray investigation of the association complex will give more information and it is useful in structure activity relationship studies and the design of new potent NNRTI compounds. In this study the conformational analysis of nevirapine was determined with a stepsize of 10° of rotational bond between the nitrogen of the tricyclic system and the carbon atom of the cyclopropyl group (C15-N11-C17-C19, α) by keeping the α angle constant based on semiempirical (AM1) method, *ab initio* at HF/3-21G, HF/6-31G and HF/6-31G**, levels and DFT at B3LYP/6-31G** level. All possible alpha conformations were fully optimized at B3LYP/6-31G** level of theory. The calculated ^{1}H ^{13}C and ^{15}N-NMR chemical shifts were compared to the experimental data.

Due to the main improvement of polarizable continuum model (PCM) in GAUSSIAN03 program respect to the previous versions, (i) definition of the solute cavity, and solvation charges (ii) use of weights instead of charges in the definition of the PCM operator (iii) iterative procedure to compute the solvation charges for very large solutes (vi) exact formulation of free energy gradients (v) completely new implementation (Cossi *et al*., 2002), the NMR chemical shifts calculations should be improved and agree well with experimental results.

These calculations were done using GAUSSIAN03 program (Frisch *et al*., 2004) on a 2.53 GHz Pentium IV PC running on Redhat Linux 9. The GAUSSIAN 03 program calculations results were compared with experimental results.

To compare to the previous study of Dokalik (Dokalik *et al*., 1999), thirty nitrogen-containing compounds were built by using HyperChem 6.0 program and ^{15}N chemical shifts were calculated at B3LYP/6-311++G**//B3LYP/6-31G** level. Then correlations between experimentally determined chemical shifts and GIAO-calculated isotropic shielding constants in DMSO and chloroform, $\delta_{expt} = a + b\sigma_{calc}$ were made.

4. Solvent optimization effects

Due to the improvement of polarizable continuum model (PCM) in GAUSSIAN03 program, it can be used to optimize nevirapine in solvent model which can not be done by GAUSSIAN98 version. To study the effect of solvent optimization, nevirapine were optimized in DMSO and chloroform IEF-PCM solvation models at B3LYP/6-31G** level and compared to the calculations in gas phase. Then NMR calculations were performed in gas phase and IEF-PCM solvation models to the solvent optimized structures.

5. Molecular Dynamics simulations (MD)

To study effect of short-range interaction, molecular dynamics simulations (MD) were used to generate clusters of nevirapine and solvent molecules in order to complete short-range and long-range interaction effect to the model of calculations. MD simulations were performed on AMBER 7 program (Case *et al*., 2002) with AMBER 1999 force field.

5.1 Force field set up for nevirapine

Starting geometry of nevirapine was obtained from the complex HIV-1 RT/nevirapine crystallographic data (1vrt), and then hydrogen atoms were added.

After that the hydrogen atoms were optimized at B3LYP/6-31G** level using GAUSSIAN03 program. Single point calculation at HF/6-31G* level was performed to calculate force and Merz-Kollman Shigh charge method (MK) (Singh *et al*., 1984) was used for calculated charge of nevirapine. Antechamber module in AMBER7 was used to generate nevirapine topology form the output of single point calculation. Finally, force field for nevirapine molecule was generated by parmchk subprogram.

5.2 Molecular Dynamics simulations of nevirapine in DMSO

DMSO molecule was generated by using HyperChem7 program and was optimized at B3LYP/6-31G** level. Single point calculation was performed at HF/6-31G* level to calculate charge. With DMSO force-field parameters of Fox and Kollman (Fox *et al*., 1998), the parameters of DMSO were modified to amber. A box of DMSO was created and then equilibrated. A new DMSO box library was created by using the equilibrated box structure. The nevirapine was solvated in the DMSO model with 15 Å cubic box size from the surface of the nevirapine to the faces of the box. There are 182 solvent DMSO molecules which were generated in the model. Cutoff distance for the Lennard-Jones interactions is 10 Å. The integration time step was 2 fs and SHAKE (Case *et al*., 2002) was applied to constrain bonds involving hydrogen atoms.

After initial minimization for DMSO solvent molecules and whole system of nevirapine and solvent, equilibration was performed by using NVT for 20 ps. The temperature was raised from 0 to 300 K. Then NPT ensemble was performed for 30 ps at 300 K and 1 atm. Data acquisition was carried out for 2 ns at 300 K using periodic boundary conditions in the NPT ensemble. Coordinates trajectories were save every 1 ps. A sample set of nevirapine-DMSO structures was generated from snapshots of system taken every 100 ps during the last 1 ns of the production period.

5.3 Molecular Dynamics simulations of nevirapine in chloroform

The nevirapine was immersed in chloroform using CHCl3 model. The maximum distance of cubic box from the surface of the nevirapine to the faces of the box was 10 Å. There are 141 solvent chloroform molecules which were generated in the model. Cutoff distance for the Lennard-Jones interactions is 10 Å. The integration time step was 2 fs and SHAKE was applied to constrain bonds involving hydrogen atoms.

After initial minimization for chloroform solvent molecules and whole system of nevirapine and solvent, equilibration was performed by using NVT and NPT ensembles. The temperature was raised from 0 to 300 K for 20 ps. Then nevirapine molecule was maintained with constraining force at 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 kcal/mol respectively, at 300 K and 1 atm for 30 ps each. Data acquisition was carried out for 2 ns at 300 K using periodic boundary conditions in the NPT ensemble. Coordinates trajectories were saved every 1 ps. A sample set of nevirapine-CHCl3 structures was generated from snapshots of system taken every 200 ps during the last 1 ns of the production period.

6. Molecular Dynamics simulations-Quantum Mechanics approach (MD-QM approach)

The nevirapine-DMSO structures generated from MD simulations were considered on the basis of cutoff distance (r_{cut}). The radial distribution functions (RDFs) would be used to define the size of the clusters used in NMR calculations regarding the structure of the solvent in the nearest neighborhood of nevirapine. All the sets of structures were optimized at B3LYP/6-31G** level and then single point calculations were performed to obtain ^{1}H-NMR chemical shifts at B3LYP/6-311++G** level with and without IEF-PCM solvation model.

These cropped samples also provided the discrete nevirapine-DMSO and nevirapine-$CHCl_3$ molecules for Our-own-2-layered integrated molecular orbital

(ONIOM2) calculations that examined the effects of the hydrogen bonding. With ONIOM2 applied at the B3LYP/6-31G**:PM3 level, the nevirapine molecule was treated as the high-level layer, and the DMSO and $CHCl_3$ molecules as the low-level layer, and the system energy minimised to give the geometry for NMR simulation. The two layers could be defined this way as there is no covalent bonding between the solute and solvent molecules.

For ONIOM2, the total energy of the system is given by:

$$E_{ONIOM2} = E_{High,\ Model} + E_{Low,\ Real} - E_{Low,\ Model} \quad (4)$$

where $E_{High,\ Model}$ and $E_{Low,\ Model}$ are the total energies calculated for only nevirapine at high and low levels respectively, and $E_{Low,\ Real}$ is the total energy for the nevirapine-solvent system at low level. When E_{ONIOM2} was minimised, the given geometry of the system was taken and the ^{1}H-NMR shielding tensors were calculated at the B3LYP/6-311++G**:HF/STO-3G level. The isotopic shielding tenser for each hydrogen atom in nevirapine is given by an expression analogous to the ONIOM2 energy, *viz*.:

$$\sigma(^1H_i)_{ONIOM2} = \sigma(^1H_i)_{High,\ Model} + \sigma(^1H_i)_{Low,\ Real} - \sigma(^1H_i)_{Low,\ Model} \quad (5)$$

With TMS as the standard reference, the ^{1}H-NMR chemical shifts are given by:

$$\delta(^1H_i) = \sigma(^1H)_{TMS} - \sigma(^1H_i)_{ONIOM2} \quad (6)$$

with the isotropic shielding constant of the hydrogen atoms in TMS, $\sigma(^1H)_{TMS}$, calculated at the same computational level as nevirapine.

These calculated ^{1}H-NMR shifts of nevirapine where then compared to experimentally measure shifts to show the accuracy of the model used, and to show that it is an acceptable and efficient improvement over previous models. All quantum calculations were done by using GAUSSIAN03 program.

7. Infrared (IR) frequency calculations

The determination of vibrational frequencies by computational method is important to help in many areas of chemistry such as identification of experimentally observed reactive intermediates and derivation of themochemical and kinetic information through statistical thermodynamics. To identify of experimentally observed of nevirapine, calculated IR frequencies were performed at B3LYP/6-31G*//B3LYP/6-31G** level. Random and Scott presented the scaling factors for the theoretical harmonic vibrational frequencies by comparison with the corresponding experimental fundamentals utilizing a total of 1066 individual vibrations. (Radom *et al*., 1996)

Furthermore, calculated IR frequencis at B3LYP/6-31G*//B3LYP/6-31G** level were used to study and observe the nevirapine, discrete nevirapine-DMSO model and discrete nevirapine-$CHCl_3$ model structures.

Experiment

1. NMR experiment

Nevirapine was extracted from Viramune® and recrystallized from CH_2Cl_2. ^{1}H-NMR spectra were recorded in deuterated dimethyl-*d6* sulfoxide (DMSO-*d6*) and $CDCl_3$ with a VarianINOVA 400 MHz spectrometer operating at 300 K. The typical spectral conditions used were a spectral width of 6387.7 Hz and 16-64 scans per spectrum. Digital resolution was 0.195 Hz per point. Deuterium from the solvent was used as the lock signal and TMS as the internal standard. Nevirapine concentration was 10-20 mg ml^{-1}.

^{1}H-NMR (DMSO-*d6*), δ (ppm): 0.33 (m, 2H), 0.86 (m, 2H), 2.32 (s, 3H), 3.61 (m, 1H), 7.04 (d, *J* = 12 Hz, 1H), 7.17 (dd, *J* = 8,11 Hz, 1H), 8.00 (dd, *J* = 3,14 Hz, 1H) 8.06 (d, *J* = 12 Hz, 1H), 8.50 (dd, *J* = 4,8 Hz, 1H), 9.86 (broad s, 1H).

^{1}H-NMR ($CDCl_3$), δ (ppm): 0.50 (m, 2H), 1.00 (m, 2H), 2.41 (s, 3H), 3.77 (m, 1H), 6.94 (d, *J* = 13 Hz, 1H), 7.07 (dd, *J* = 7,12 Hz, 1H), 8.11 (dd, *J* = 5,14 Hz, 1H) 8.16 (d, *J* = 13 Hz, 1H), 8.54 (dd, *J* = 5,7 Hz, 1H), 8.69 (br., s, 1H)

^{13}C proton decoupled spectra were recorded with the same spectrometer, also in DMSO-*d6* and $CDCl_3$. The spectral conditions used were a spectral width of 25157.2 Hz. and 2560-25600 scans per spectrum. The concentration was 20-30 mg mL^{-1} and the digital resolution was 0.7677 Hz per point.

^{13}C-NMR (DMSO-*d6*), δ (ppm): 8.52 (CH_2); 8.75 (CH_2); 17.57 (CH_3); 29.29 (CH); 119.35 (CH); 120.91 (CH); 122.27 (C); 124.92 (C); 140.01 (C); 140.73 (CH); 143.59 (CH); 151.33 (CH); 154.20 (C); 159.99 (C); 167.02 (C).

^{13}C-NMR ($CDCl_3$), δ (ppm): 8.82 (CH_2); 9.11 (CH_2); 17.80 (CH_3); 29.61 (CH); 118.97 (CH); 120.22 (CH); 122.08 (C); 124.90 (C); 139.47 (C); 140.36 (CH); 144.31 (CH); 152.10 (CH); 153.95 (C); 160.55 (C); 168.85 (C).

^{15}N NMR spectra were recorded in DMSO-*d6* and $CDCl_3$ with the same temperature and spectrometer by using gHMBC pulse sequence.

^{15}N-NMR (DMSO-*d6*), δ (ppm): -89.6, -91.6, -252.3, -269.4.

^{15}N-NMR ($CDCl_3$), δ (ppm): -86.3, -91.1, -248.6, -264.4.

2. Infrared (IR) experiment

Nevirapine crystals extracted from Viramune® were used. DRIFT fourier-transform infrared spectra (FT-IR) were recorded at room temperature using Bruker FT-IR IFS66S/S equipped with KBr beam splitter and a liquid-nitrogen-cooled mercury cadmium telluride (MCT) detector. The spectra were measured in 400-4000

cm^{-1} region at 2 cm^{-1} resolution and the number of scans was 32. IR (cm^{-1}): 1355, 1383 (CN), 1415 (CH), 1586, 1600 (C=C), 1649 (C=O), 3063 (CH), 3500 (NH).

Nevirapine crystals were dissolved in dry DMSO and $CHCl_3$ solvents and FT-IR spectra were recorded at room temperature in 400-4000 cm^{-1} region using Perkin-Elmer 2000FT-IR.