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ORIGINAL ARTICLE **OPEN ACCESS**

Molecular Recognition and Structural elucidation studies of tenofovir/β-Cyclodextrin complex using ^{1}H-NMR, qualitative ROESY analysis and molecular modeling studies

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ABSTRACT
Structure determination of TFR/β-CD complex was determined using experimental as well as computational studies. Experimental studies used are ^{1}H-NMR and ROESY while theoretical methods include molecular mechanics (MM) and molecular dynamics (MD). The initial characterization of inclusion complex formed was done using ^{1}H-NMR. The down field shift in ^{1}H-NMR signal of cavity protons (H-3' and H-5') of β-CD confirmed the inclusion of TFR into CD cavity. Also, large chemical shift in H-3' as compared to H-5' showed that guest has entered from wider end of cavity. Further characterization was done using ROESY spectrum analysis, which also confirms the inclusion complex formation. Based on experimental observation, MM studies were performed, in different orientations and modes, from both the ends of cavity, to find inclusion depth and mode of entry. The conformation with minimum steric energy and having large negative binding energy was considered as final structure. Based on MM studies, the MD simulations were performed to get the final structure. The negative value of binding energies show that process of inclusion is spontaneous.
***Keywords**: Cyclodextrin, TFR, NMR, COSY, ROESY, MM, MD*

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INTRODUCTION

Biological systems like lipids, nucleic acids (RNA, DNA) etc., and other intracellular biomolecules act as fundamental units in the organization of a cell. The various aspects of life are controlled by the interaction of these biological systems with each other and extracellular particles. A lot of biological processes that are enabled by systematic functioning of these bio-systems, occur inside the cell body, in a well-defined manner, to maintain the life of an organism. This basic functioning of these biological systems can be well understood by basics of supramolecular chemistry [1].In supramolecular chemistry we study multimolecular systems that are formed as a result of non-covalent interactions. This chemistry has demonstrated some important concepts likemolecular self-assembly, molecular and chiral recognition, etc. Of all these concepts, molecular as well as chiral recognition are of great importance, because they are the basis of supramolecular chemistry. Molecular recognition is a type of specific interaction between two or more interacting molecules having complementarity in geometries, functional groups and electronic structures. Chiral recognition is a type of chemical interaction by which a receptor molecule preferably binds to particular substrate enantiomer. To understand the molecular and chiral recognition phenomenon in these biological processes, the detailed study of non-covalent interactions is required. But the biological systems being large in size are not suitable for practical study. Therefore, to understand these biological phenomena, model molecules of suitable sizes are required. Since, the chemists have considered cyclodextrin (CD) inclusion complexes as good models to explore these non-covalent interactions [2]. CDs resemble biological systems because biological systems are aqueous and CDs are water soluble. Therefore, structural exploration of CD inclusion complexes can be found useful in understanding molecular and chiral recognition phenomena[3].

The structural exploration of CD inclusion complexes is under investigation worldwide using different experimental as well as computational techniques. The experimental techniques include UV, FTIR, NMR, etc. and computational methods consists of both classical as well as quantum mechanical methods. Of all the experimental methods ^{1}H-NMR spectroscopy was the ist technique to provide direct evidence on inclusion of guest into CD cavity. The high field shift in the ^{1}H-NMR signals of CD cavity protons, and

downfield shift in protons of aromatic guests, was the first evidence to confirm the inclusion of guest molecule into CD cavity [4].The stoichiometry and association constant in CD inclusion complexes were obtained from ^{1}H-NMR titration[5].Later on, little advancements in ^{1}H-NMR spectroscopy i.e. rotatory frame overhauser effect spectroscopy (ROESY)was used to identify the part of the guest included into CD cavity, orientation, entry mode and depth of guest in the CD cavity [6]. Computational studies like PM3, AM3, MD Molecular docking, etc. were performed in light of ROESY peaks to obtain 3D model of inclusion complex. Based on the energy of 3D models obtained from computational studies, the minimum energy structure was proposed as the final structure. But the atom accuracy of these proposed structures was probably never tested. Based on the work of Butts and coworkers, Mashhood Ali and team developed a method to analyze the structures of CD inclusion complexes. In this method they convert the interproton distances into calculated ROESY intensities, which were further compared with experimental ROESY intensities. The model for which calculated and experimental ROESY intensities better matched each other was proposed as the final structure[2]. Using this method of quantitative ROESY analysis Mashhood Ali and his team has achieved highly atom accurate structures of CD inclusion complexes [7].

Later on, they reach the conclusion that maximum times calculated and experimental ROESY intensities match each other for minimum energy structures and hence, are proposed as final structures of CD inclusion complexes. To, further test atom accuracy of the structures obtained by their method, the compared their method to a significant method DFT. The results showed that their method produces structures with high atom accuracy. Their method showed significance over other methods that it is less expensive and less time consuming. Only disadvantage of their method is it need to be performed several times, because final structure depends on the initial coordinates taken.

In this article we have studied complexation of tenofovir (TFR) with β-CD. Initial characterization was done using 1H-NMR and then further complexation was confirmed from analysis of ROESY NMR. In light of ROESY interaction peaks, computational studies (molecular mechanics (MM), molecular dynamics (MD)) were done. Based on Mashhood Ali and his coworker's conclusion the minimum energy structure was proposed as final structure. In our previous article we have determined structure of inclusion complex using MM and molecular docking studies [8].

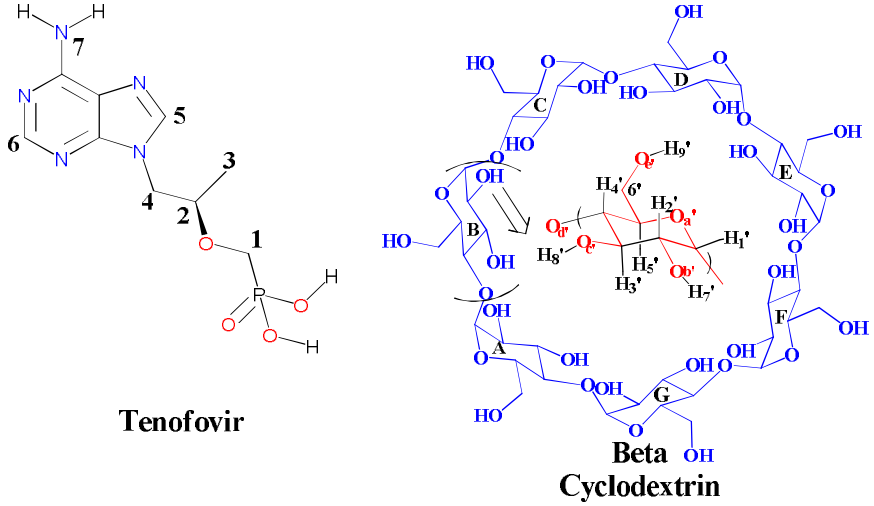


Fig.1. Chemical structure of Tenofovirand β-CD.

MATERIAL AND METHODS

Experimental

1H-NMR (fig. 2), 2D-ROESY (fig. 5) and 2D-COSY (fig. 3) spectra for TFR/β-CD mixture (1:1 ratio) were recorded on 500 MHz instrument at room temperature in D_2O. 1:1 pulse field gradient was utilized to record COSY spectrum. The COSY spectrum was recorded with relaxation delay and repetition time of 1.5s and 1.609s respectively. The mixing time of 500ms was utilized for recording ROESY spectrum under spin lock conditions. The value of relaxation time and repetition time were kept same s in case of COSY spectrum. Computational studies (MMand MD) studies were done utilizing Allinger`s force field (MM2) at room temperature in CS Chem3D Proin the vapor phase [9]. TFR was drawn in CS ChemDraw Pro 2D and converted in C3D format by CS Chem3D Pro. This was further minimized using Aligners force field (MM2) and was used for MM and MD studies. Published neutron diffraction coordinates of hydrated β-CD were used, after removal of water coordinates [10].

RESULTS AND DISCUSSION

^{1}H-NMR spectrum

The proton NMR spectra were recorded for pure TFR, pure CD and TFR/β-CD complex (1:1 ratio). Comparison of 1H-NMR spectrum of pure CD with complexed CD spectrum showed the chemical shift in cavity protons (H-3′ and H-5′) of CD. It can be seen that there is large highfield shift in CD cavity protons (H-3′ and H-5′). This highfield shift in CD cavity proton is due to shielding which in turn occur because of presence of the π- electron-rich group such as aromatic compounds. This shielding of CD cavity protons is inferred due to the anisotropic effect of the aromatic ring entering the cavity. The chemical shift changes of H-3′ and H-5′ of CD cavity towards highfield suggested TFR has entered cavity. Figure 2 shows the expansion of the ^{1}H NMR showing the up-field shift of H-3′ and H-5′ protons of β-CD in TFR/β-CD(1:1) complex, compared to pure β-CD. Therefore, it can be concluded that guest molecule (TFR) has formed inclusion complex with β-CD. Further, it is clear from spectrum (figure 2) H-3' has shifted much highfieldas compared to H-5' confirming the wide side mode of entry. The exact orientation, and depth of inclusion of TFR into CD cavity cannot be predicted. It was considered from previous studies that the ratio of TFR and CD in complex formation is 1:1 as the cavity of CD is of size that it can accommodate only one aromatic ring.

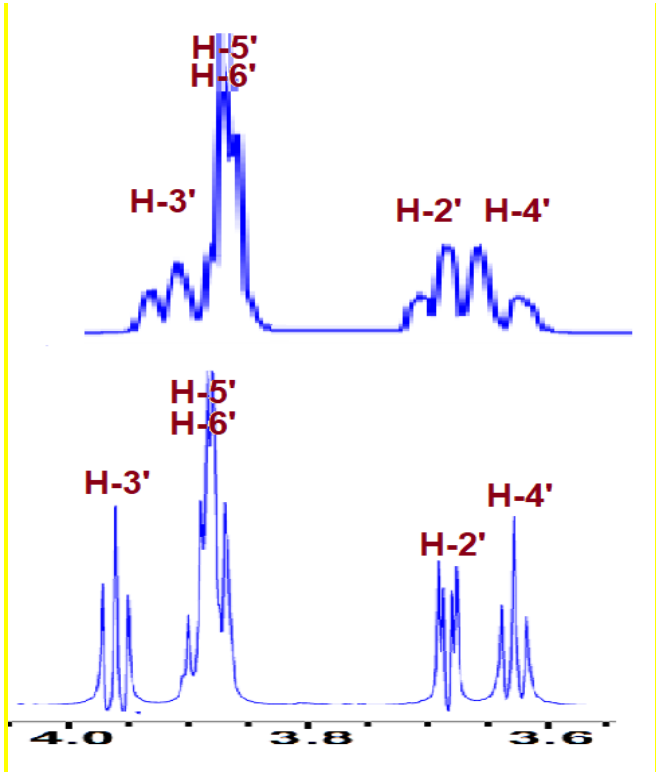


Fig.2. NMR of pure CD as well as TFR-β-CD mixture(1:1 ratio).

^{1}H-^{1}H COSY spectrum

Correlation spectroscopy (COSY) is a type of 2D NMR spectroscopy which is used to assign NMR peaks in spectra. The technique uses correlation between diagonal and cross peaks to assign NMR signals.2D COSY spectrum of TFR/β-CD mixture (1:1 ratio) was recorded on a 500 MHz instrument inD_2O at room temperature. In COSY spectrum (figure 3) the proton assigned by red color belong to guest molecule (TFR) and protons shown in blue color with superscript belongs to CD. The assignment of protons in pure CD and TFR was easy but, difficult in case of mixture. The assignment of peaks is shown in figure 3. The correlation between diagonal peaks and cross peaks is also shown in figure 3. We are mainly interested in aromatic protons of guest molecule as it is considered that generally aromatic part enters CD cavity. The guest molecule TFR contains only two aromatic protons i.e., H-5 and H-6. The peaks for both these protons appear to merged with each other at about 8.5 ppm.

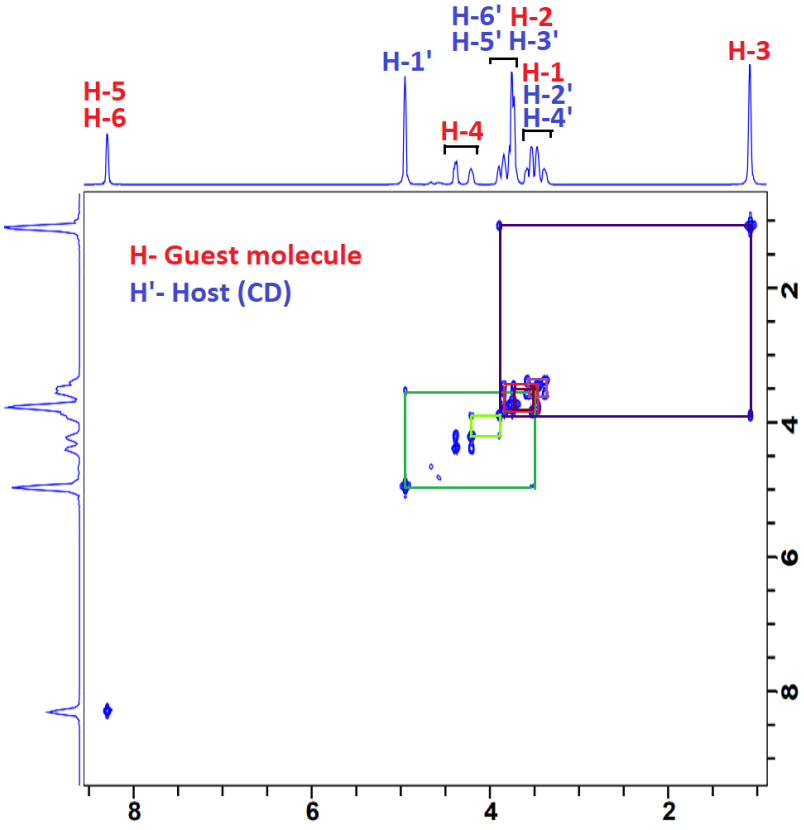


Fig. 3.COSY spectrum of TFR-β-CD mixture (1:1 ratio).

^{1}H-^{1}H ROESY spectra

The β-CD is composed of seven D-glucopyranose units joined by α-1, 4-linkage with each other in cyclic manner. Each glucose unit in β-CD has of six different types of protons directly attached to its carbons. Of these six types of hydrogens H-3' and H-5' are present inside theβ-CD cavity. These H-3' protons form a band on wider side of cavity and H-5' protons form the band on narrow side of cavity. The other protons i.e., H-1', H-2' and H-4' lie on exterior surface of CD cavity. The H-6' protons are present on the narrow rim of the cavity. Thenarrower rim of CD cavity is covered with primary -OH groups and wider rim is covered with secondary –OH groups, making its exterior hydrophilic. However, the interior of the cavity is less hydrophilic than exterior surface, hence making it suitable to accommodate hydrophobic guests into it. Fig. 4 shows the position of β-CD protons in relation with the wide and narrow ends of the cavity.

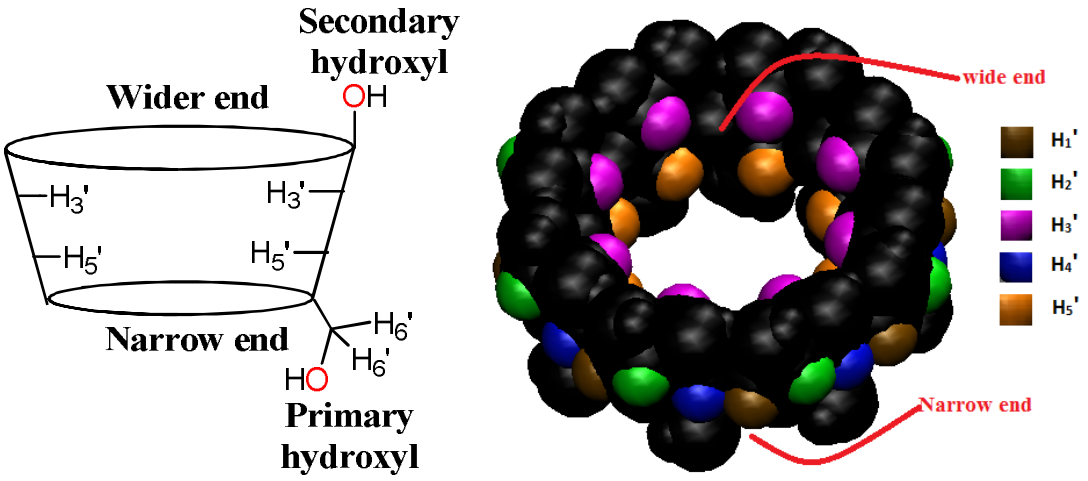


Fig. 4.2D and 3Dstructure showing hollow cavity of CDs.

In 2D-ROESY spectrum cross peaks arises between neighboring nuclei, that are close to each other in space. Detailed analysis of these cross peaks, between the aromatic protons of guest and CD cavity

protons, provides valuable information on the depth of inclusion, mode of entry and part of the guest encapsulated into the cavity. A qualitative analysis of a ROESY contact observed between a guest proton and any one of the cavity protons, provides a little information about their closeness. A minor change in the position of the guest can lead to change in all interproton distance between guest and CD cavity. ROESY spectrum of TFR/β-CD mixture (fig.5.) shows a peak of interaction (encircled in figure) between aromatic protons (H-5 and H-6) of guest and CD cavity protons (H-3'and H-5') on both side of diagonal. These cross peaks do not make clear its mode of entry, but it is clear that both the protons of guest molecule (TFR) interact with cavity proton. The cavity size of β-CD is of size so that it can accommodate only one aromatic ring inside the cavity. Therefore, it was considered from our previous studies that host is to guest ratio is 1:1. Further, computational studies were done to confirm mode of entry, orientation and depth of inclusion.

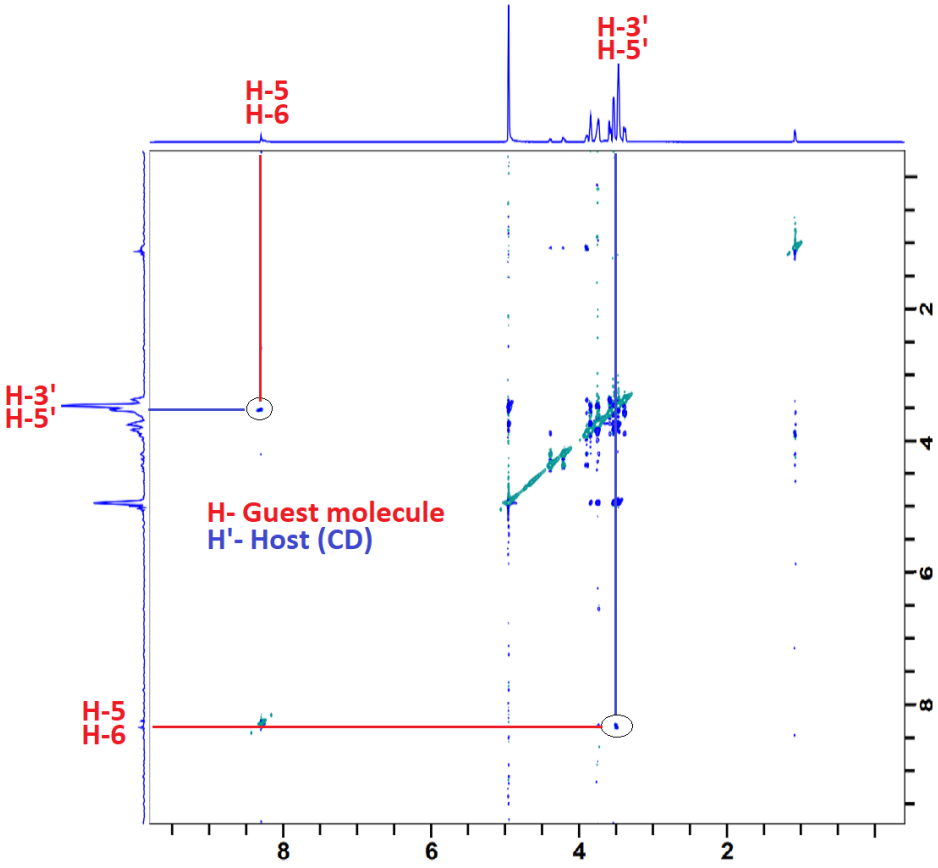


Fig.5.ROESYspectrum of TFR-β-CD mixture (1:1 ratio).

Computational studies

For detailed explanation of complex formation betweenTFR and β-CD, molecular mechanics (MM) and molecular dynamics (MD) simulations were carried out in CS Chem3D Pro(Cambridge soft corp.), using Allinger`s force field (MM2) in the vaporphase [9] at room temperature. The structure of TFR/β-CD complex was drawn by minimizing its geometry to root mean square gradient of 0.1 kcal/mol. During the simulations host molecule(β-CD) were kept static and guest molecule (TFR) was allowed to move because CDs adopt more symmetrical geometry in complexes of solid as well as solution state.

Molecular mechanics (MM)

In the MM study of TFR/β-CD complexation, the guest molecule (TFR) was placed manually inside the CD cavity, and then energy minimization was done. The energy minimization process was done at different depths and different modes of entry. The different depths and different modes of entry for placing the TFR in CD cavity are shown in figure 6 and 7 respectively. The MM studies were done at three different depths from both the narrow side (NS) and wide side (WS). The three different depths are denoted as Narrow Surface (NA), Narrow Centre (NB) and Narrow Bottom (NC) on narrow side. Similarly, these modes on wide side are named as Wide Surface (WA), Wide Centre (WB) and Wide Bottom (WC). In mode 1 entry of paranitrogen of aromatic ring into cavity was studied and in other two modes entry of aromatic ring was done by bonds alternate to paranitrogen. The binding energy (B.E.) for the inclusion complex formed was calculated using formula

$E_{\text{binding energy}} = E_{\text{(complex)}} - E_{\text{(free CD + freeCTZ)}}$ .....(1)

Higher the negative value of the binding energy more stable is the complex and proves the spontaneity of inclusion process [11].

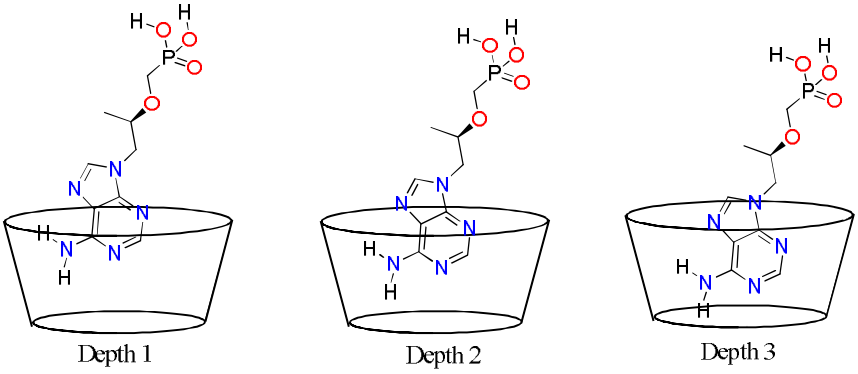


Fig.6. Different depths at which molecular mechanics studies were done.

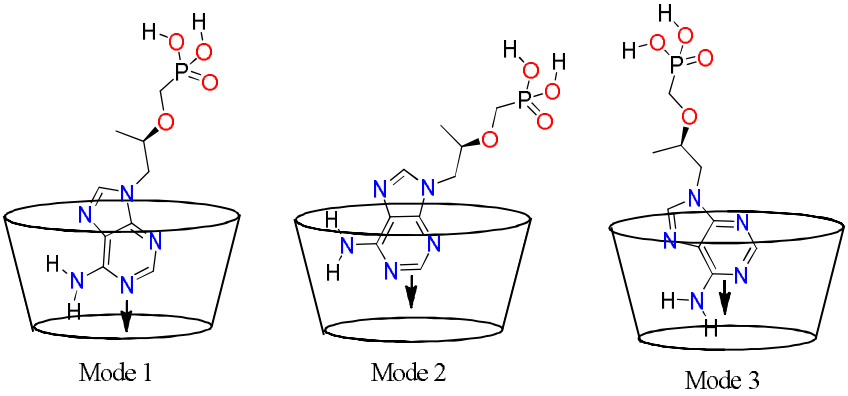


Fig.7. Different modes of study used in molecular mechanics.

As analyzed from 1-HNMR spectra the entry of TFR was seen from wider end and ROESY spectrum was not able to reveal the mode of entry. So, to confirm mode of entry MM studies was done from both wider as well as narrow end. The minimum energy structure was obtained from mode 2 with the WC inclusion depth i.e., W2C was found to stable of all. Further narrow side entry was found to be unfavorable as compared to wide side entry based on steric energy calculations. The steric energy of molecule was found to be 88.212 kcal/mol (B.E = -23.961kcal/mol). The top and side view for this minimum energy conformation is shown in fig.8. The Binding energy values for all the studied modes and depths are given in Table 1.

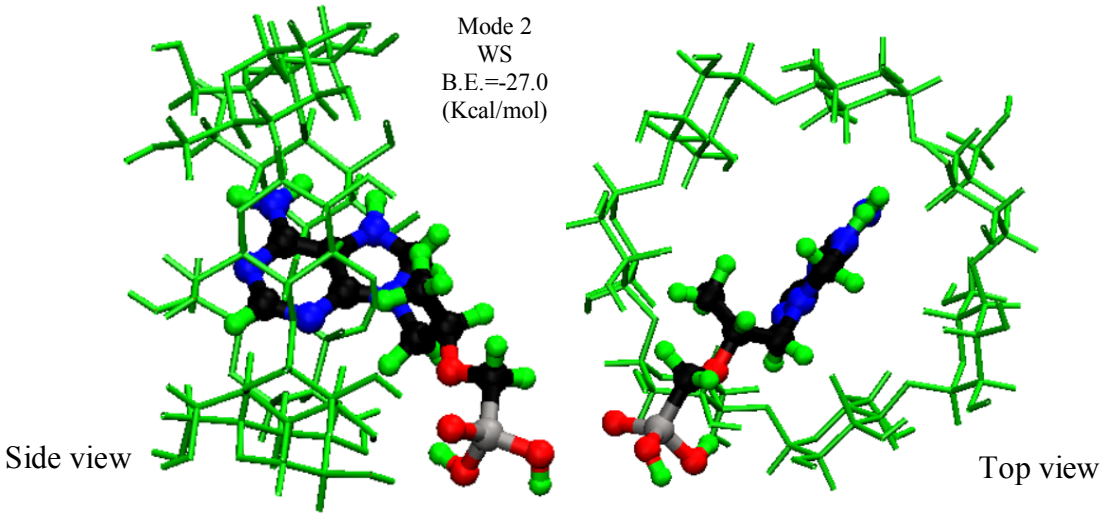


Fig.8. Lowest energy structure of TFR-β-CD from MM studies.

Table 1. Binding energies (Kcal/mol) obtained from MM studies for complex of β-CD with TFR in different inclusion depths and different modes studied.

(a) TFR/β-CD						
Modes	WS			NS		
	WA	WB	WC	NA	NB	NC
Mode 1	-9.435	-13.257	-14.980	-10.170	-11.252	-15.478
Mode 2	-14.424	-19.139	**-23.961***	-11.312	-15.001	-15.645
Mode 3	-9.690	-11.874	--9.132	-9.698	-12.715	-9.132

* Minimum energy conformation

Molecular dynamics (MD)

The MD provides global minima however MM provides local minima. MD study explores all the possibilities for guest molecule orientation in CD cavity. To provide the atomic level representations of molecular systems CD inclusion are studied on large scale using MD simulations. The results obtained from 1H-NMR spectrum and MM studies were further extended by simulating inclusion process. The MD simulation was done to 10000 equilibrium into as a result trajectory with 1000 frames was obtained, with a step interval of 1.0 fs and frame interval of 10fs for each run. The geometries of these structures were minimized to root mean square gradient of 0.1 $kcalmol^{-1}Å^{-1}$. During the simulation process the guest molecule (TFR) was allowed to move and the host molecule (β-CD) were kept static to prevent symmetry of the CD cavity. The MD simulation studies were carried in Chem3D Pro using Aligners force field [9] in vapor phase [12]. The frame with minimum energy was taken as final structure. To further confirm the mode of entry the MD studies were performed from wide as well as narrow side of CD cavity. During MD simulation of TFR/β-CD complexation process, several MD simulations were done with different possibilities of guest orientation exploring large area in front of CD cavity. Based on steric energy calculation wide side entry was found to be more favorable. The energy time plot for both wide side and narrow side entry is shown in figure 9 and 10 respectively.

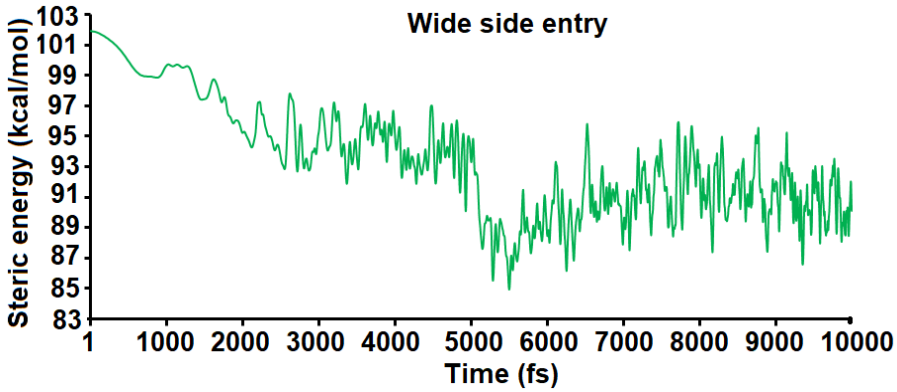


Fig. 9. Energy-time plot of MD run for complexation between β-CD and TFR from wide side entry.

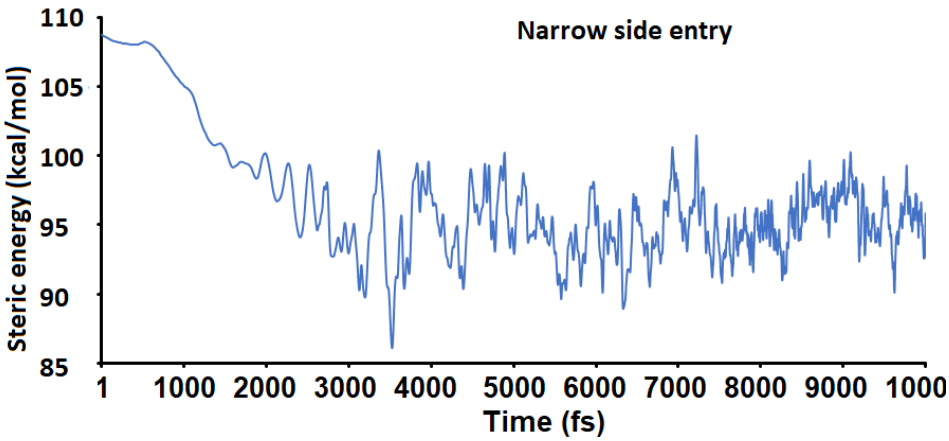
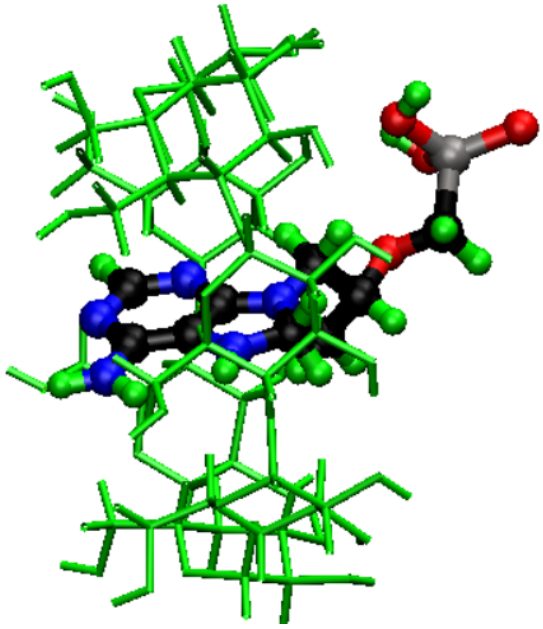


Fig. 10. Energy-time plot of MD run for complexation between β-CD and TFR from narrow side entry.



Side view

Frame 549
B.E. = -34.6
Kcal/mol

Top view

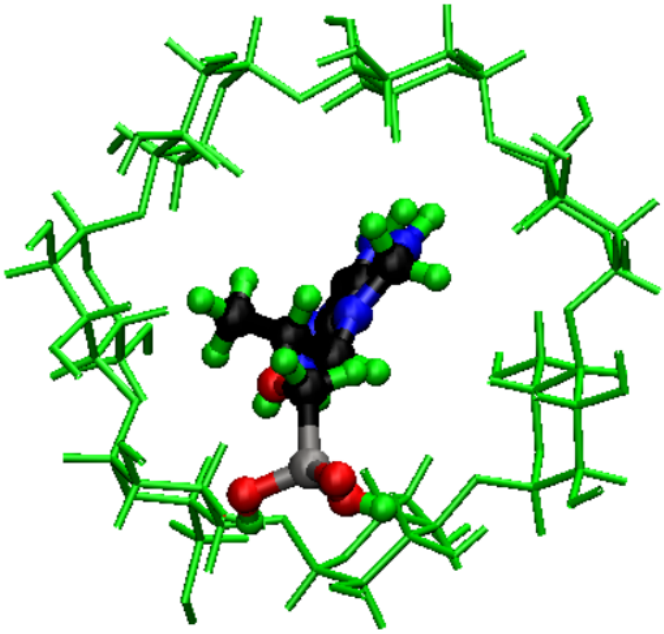


Fig. 11. Side and top view of least energy frame of MD simulation between TFR and β-CD.

In the TFR/β-CD simulation studies fused aromatic ring was placed near the cavity such that para nitrogen, H-6 and NH_2groupwere present in line oh H-3' cavity protons (fig.12,frame 1). The aromatic fused ring of TFR was placed perpendicular to the diameter of CD cavity. As the command for the MD run was commenced ring of TFR molecule showed the movement towards the bottom of the cavity till 1440fs and started rotating round at same position up to the 1740fs. The ring then started moving back to the surface of the cavity till 2790fs. At the same time other part of TFR have also shown large movement. The aromatic part of TFR than showed the motion on surface diameter till 4130fs. Afterward the ring again showed movement towards the bottom of cavity where para nitrogen, -NH2 and H-6 of TFR were seen

out of narrow end at 5460fs. The ring continued the motion and showed minimum energy frame (549) at 5490fs. After this, the aromatic part of TFR showed movement to come out narrow end and again return back into center of cavity. During whole simulation process aliphatic part of TFR also showed a large movement. The minimum energy frame (frame 549) with steric energy 84.939 kcal/mol(B.E. =-27.234 kcal/mol) was considered as final structure (fig.11) of this complex formed. Few MD snapshots are shown in fig.12.

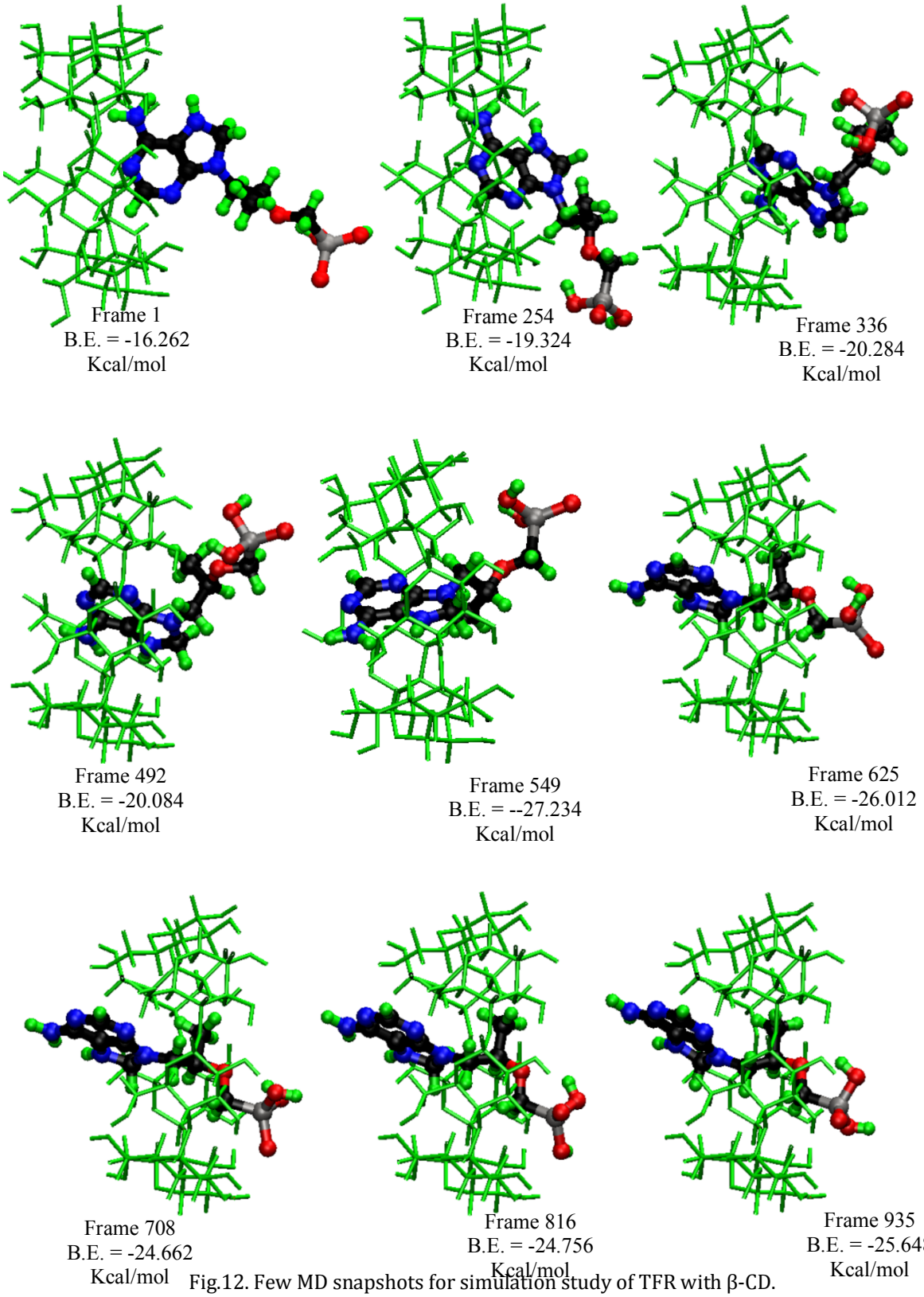


Fig.12. Few MD snapshots for simulation study of TFR with β-CD.

CONCLUSION

^{1}H-NMR chemical shift in CD cavity protons reported that complexation has occurred between TFR and β-CD. ^{1}H-NMR analysis showed that the guest molecule (TFR) has entered CD cavity from wide side. The complexation of TFR with β-CD was further confirmed from ROESY analysis. The MM studies provided with mode of entry, inclusion depth based on steric energy calculations. The minimum energy conformation was proposed as final structure from MM studies. Further, TFR was simulated into CD cavity which showed clear path for the movement of guest molecule. The mode of entry was found to be wider side entry of guest molecule. The minimum energy structure from MD simulation was proposed as final structure. The Binding energy were calculated for both the structures obtained from MM and MD studies. The negative value of binding energy showed that the process of inclusion is spontaneous. These findings may raise the possibility of industrial applications developing novel therapeutic molecules targeting such complexes.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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