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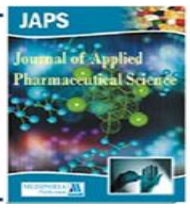

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Taxifolin acts as type I inhibitor for VEGFR-2 kinase: Stability evaluation by molecular dynamic simulation

Sharad Verma, Amit Singh and Abha Mishra

Sharad Verma, Abha Mishra
School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi-221005, India.

Amit Singh
Department of Pharmacology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India.

For Correspondence
Dr. ABHA MISHRA
Assistant Professor,
School of Biochemical Engineering,
Institute of Technology,
Banaras Hindu University,
Varanasi-221005, India
Phone : +915422307070

ABSTRACT

The VEGFR-2 kinase specific intracellular signalling cascades leading to proliferation, migration, survival of endothelial cells and increased permeability of vessels which contributes to angiogenesis. ATP is essentially required by VEGFR-2 to perform phosphorylation of specific proteins and to maintain cascade downstream. Taxifolin (plant polyphenol) inhibit the VEGFR-2 kinase by binding at ATP-binding pocket revealed by molecular docking study. Further, stability of VEGFR-2 kinase-taxifolin complex is validated by molecular dynamic simulation. RMSD analysis for 3800 ps confirmed the stability of complex. Furthermore, thermodynamic stability was evidenced by stable total energy, potential energy, and, temperature and pressure profile. After MD simulation taxifolin was found to stably interact with pocket residues Cys 917 and Lys 1053 along with water molecules. These results suggest that therapeutic inhibition of VEGFR-2 by taxifolin as a type I inhibitor may be a promising ways to retard signaling cascade of specific proteins which play crucial role in cancer proliferation and also in development of second generation type II inhibitors.

Keywords: VEGFR-2, taxifolin, molecular dynamic simulation, type I inhibitor, DFG-out

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a potent growth promotor that is highly specific for vascular endothelial cells (Dvorak *et al.*, 1995). VEGF is a strong angiogenic agent that increases vessel permeability and enhances endothelial cell growth, migration, proliferation and differentiation (Ferrara *et al.*, 2003). Angiogenesis plays crucial role in the pathogenesis of a variety of disorders such as cancer, proliferative retinopathies, and rheumatoid arthritis (Folkman, 1990; Klagsbrun *et al.*, 1991; Folkman *et al.*, 1992). VEGFs and their tyrosine kinase receptors (VEGFRs) have been recognized as attractive targets for the inhibition of angiogenesis (Shawver *et al.*, 1997; Traxler, 2003). The growth promoting and angiogenic effects of VEGF are mainly mediated by two receptor tyrosine kinases (RTKs): VEGFR-1 kinase and VEGFR-2 kinase (kinase insert domain receptor (KDR) kinase) (De Vries *et al.*, 1992; Shalaby *et al.*, 1995). Expression of VEGFRs varies in specific endothelial cell layers. The VEGFR-2 is located on almost all endothelial cells; however, the VEGFR-1 and -3 are alternatively located on endothelial cells in distinct vascular layers (Hicklin *et al.*, 2005). The inhibition of VEGF signaling not only blocks angiogenesis in tumors but can also change or destroy tumor vessels (Yang *et al.*, 2003).

Therefore, VEGFR-2 is an attractive target for biological cancer therapies (Lee *et al.*, 2010). It is possible to interfere with VEGF signailing from the extracellular as well as from the intracellular site. In the extracellular region antibodies, soluble receptors and VEGF antagonists can avoid binding of the VEGF to the ligand binding site of the receptor (Los *et al.*, 2007), or the inhibition of the VEGFR-2 in the intracellular region by blocking the ATP-binding site of the tyrosine kinase which is required to perform kinase activity (Underiner *et al.*, 2004; Schmidt *et al.*, 2008). The difficulties associated with the competitive inhibition of protein–protein interactions by small molecular weight compounds made targeting the catalytic site of kinases with ATP-competitive inhibitors is a more promising approach for drug intervention (Cochran *et al.*, 2000, Yang *et al*., 2008)

Taxifolin is a one of the the principal active component of *Larix gmelini* (Huang *et al*., 2005) and several other plants such as *Silybum marianum, Acacia sp., Rhododendron sp.* etc. Taxifolin (2-(3,4-dihydroxyphenyl)-2,3 dihydro- 3,5,7-trihydroxy-4H-benzopyran-4-one) is a dihydroflavonol with distinguished antioxidant activity compared to other antioxidants (Audron *et al.*, 2000; Bong-Sik *et al.*, 2000; Wang *et al.*, 2011). It can eliminate free radicals in the body, improve the impermeability of capillary vessels and recover their elasticity effectively. It is not embryotoxic and does not lead to malformations, hypersusceptibility or mutations (Wang *et al.*, 2011).

Therefore, an attempt was made to explore the potential of taxifolin to inhibit VEGFR-2 kinase. The stability of the binding complex was also demonstrated by molecular dynamics simulations. An analysis was also made to elucidate the probable molecular mechanism of action of taxifolin on the VEGFR-2 kinase.

COMPUTATIONAL METHOD

Molecular docking

AutoDock 4.0 suite was used as molecular-docking tool in order to carry out the docking simulations (Morris *et al.*, 1998). The crystal structure of VEGFR-2 kinase (pdb id 1YWN) obtained from RCSB protein data bank and the structures of ligands taxifolin generated from smile strings. Hydrogen atoms were added to VEGFR-2 kinase crystal structure using autodock program while all non polar hydrogen atoms were merged. Six bonds were made ‘‘active’’ or rotatable for the taxifolin. Lamarkian genetic algorithm was used as a search parameter which is based on adaptive local search. Short range vanderwaal and electrostatic interactions, hydrogen bonding, entropy losses were included for energy based autodock scoring function (Berendsen *et al.*, 2005; Sudhamalla *et al.*, 2010). The lamarkian GA parameters used in the study were: numbers of run, 30; population size, 150; maximum number of evals; 25000000, number of generation; 27000, rate of gene mutation; 0.02 and rate of cross over; 0.8. Blind docking is carried out using grid size 126, 126 and 126 along the X, Y and Z axes with 0.480 Å spacing. The grid center was set to 5.033 38.562 and 23.562 Å. RMS cluster tolerance was set to 2 Å. Semi-flexible docking was performed which includes a flexible ligand and a rigid receptor.

Molecular dynamic simulation in water

A 3800 ps MD simulation of the complex was carried out with the GROMACS4.5.4 package using the GROMOS96 43a1 force field (Van Gunsteren *et al.*, 1996; Lindah *et al.*, 2001). The lowest binding energy docking conformation generated by Autodock was taken as initial conformation for MD simulation. The topology parameters of VEGFR-2 were created by using the Gromacs program. The topology parameters of taxifolin were built by the Dundee PRODRG server. The complex was immersed in an octahedron box (488.82 nm^3) of extended simple point charge (SPC) water molecules (Van Gunsteren *et al.*, 1998). The solvated system was neutralized by adding 3 chloride ions. The entire system was composed of 46993 atoms (2855 atoms of VEGFR-2, 32 taxifolin, 3 Cl^- counterions and 44103 water atoms). To release conflicting contacts, energy minimization was performed using the steepest descent method of 1000 steps followed by the conjugate gradient method for 1000 steps. MD simulation studies consist of equilibration and production phases. In the first stage of equilibration, the solute (protein, counterions, and taxifolin) was fixed and the position-restrained dynamics simulation of the system, in which the atom positions of VEGFR-2 were restrained at 300 K for 300 ps. Finally, the full system was subjected to 3800 ps MD at 300 K temperature and 1 bar pressure. For analysis, the atom coordinates were recorded every 0.5 ps during the MD simulation.

RESULT AND DISCUSSION

Molecular docking

Taxifolin was found to bind at ATP-binding site of VEGFR-2 with lowest binding energy-8.30 Kcal/Mol (Fig. 1). Free energy of binding is calculated as a sum of four energy terms of intermolecular energy (vanderwaal, hydrogen bond, desolvation energy and electrostatic energy), total internal energy, torsional free energy and unbound system energy.

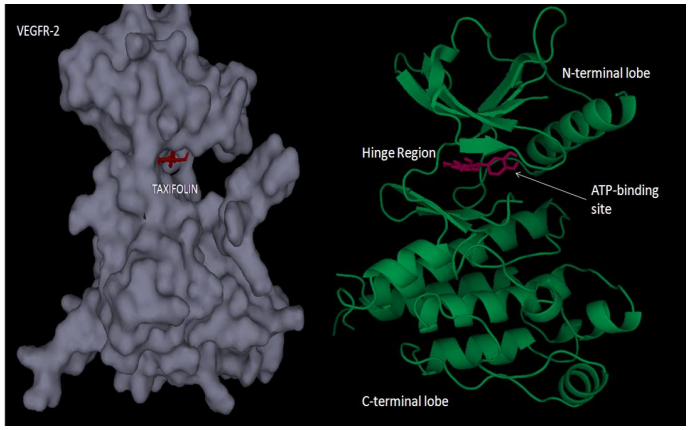

Fig. 1. Bindinding of taxifolin at ATP-binding site on VEGFR-2 kinase: surface view and ribbon structure.

The major interactions shown in the VEGFR-2 kinase-ATP-binding site are the important H-bonds with Leu 838 (2.86 Å), Glu 883 (2.77 Å, 2.69 Å), Glu 915 (2.72 Å) and Cys 917 (2.72 Å) (Fig. 2).

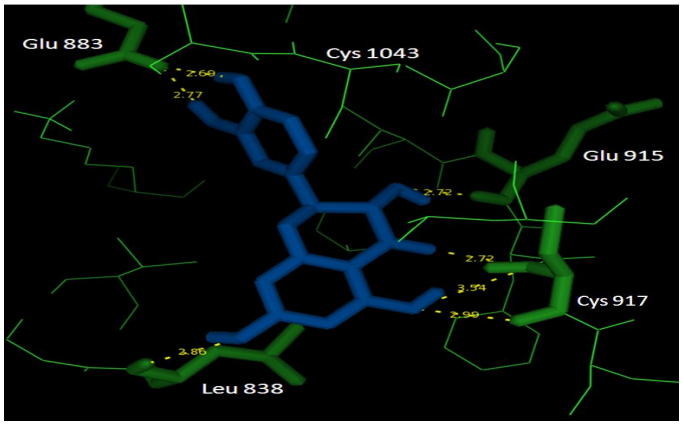

Fig. 2. Stereo view of H-bond pattern of taxifolin with ATP-binding pocket residues.

Large negative binding energies of VEGFR-2-taxifolin complex were obtained by Autodock 4.0 as is evident from Table 1. The groups involved in H-bonding were hydroxyl (hydrogen donar), and carbonyl (hydrogen acceptor) group of taxifolin and NH (hydrogen donar) of Cys 917, and oxygen atoms (hydrogen acceptor) of side chain or backbone of residues. These results evidenced that taxifolin has very high affinity for the ATP-binding site of VEGFR-2.

Table 1. Summary of molecular docking results obtained by Autodock.

Rank	Sub-Rank	Run	Binding Energy (Kcal/Mol)	Cluster RMSD	Reference RMSD
1	1	3	-8.32	0.00	34.37
1	2	12	-7.95	0.53	34.56
1	3	9	-7.11	1.73	34.79
2	1	4	-7.23	0.00	28.79
3	1	10	-6.62	0.00	45.26
3	2	13	-6.36	0.94	45.17
4	1	11	-6.46	0.00	32.74
4	2	8	-6.15	1.82	32.72
5	1	5	-6.37	0.00	40.66
6	1	6	-6.24	0.00	50.38
7	1	14	-6.15	0.00	47.39
8	1	15	-6.06	0.00	33.88
9	1	2	-5.91	0.00	47.77
10	1	7	-5.75	0.00	35.85
11	1	1	-5.57	0.00	57.18

Molecular dynamic simulation in water

The VEGFR-2 kinase-taxifolin complex with the binding energy of -8.32 kcal/mol obtained using Autodock (Table 1) was used for carrying out MD simulation. After MD simulation, we calculated RMSD trajectory of backbone of VEGFR-2 kinase and VEGFR-2 kinase-taxifolin complex at every 0.5 ps by using its initial structure as a reference. Fig. 3 shows that the RMSD trajectories were always less than 3.0 Å (0.3 nm) for the entire simulation suggesting the stability of simulation system.

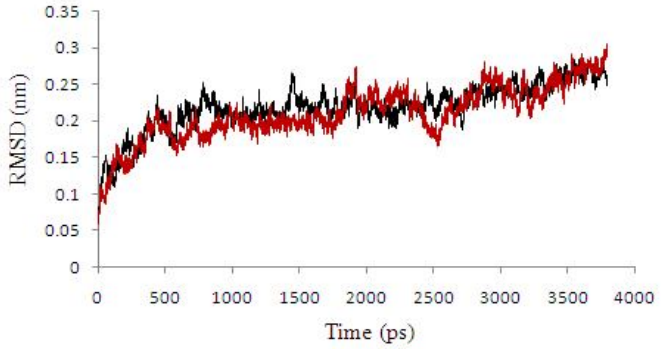

Fig. 3. Plot of root mean square deviation (RMSD) of backbone of VEGFR-2 unbound (red) and VEGFR-2-taxifolin complex (black). RMSDs were calculated using the initial structures as templates. The trajectories were captured every 0.5 ps until the simulation time reached 3800 ps.

The trajectories were stabilized after 500 ps till 3500 ps and thereafter slight increase was observed. In both cases no great difference in trajectory was found. RMSD of taxifolin was also calculated to investigate the stability of taxifolin in the ATP-binding pocket. Taxifolin RMSD was reached equilibrium after about 500 ps and remains constant with negligible fluctuation (Fig. 4).

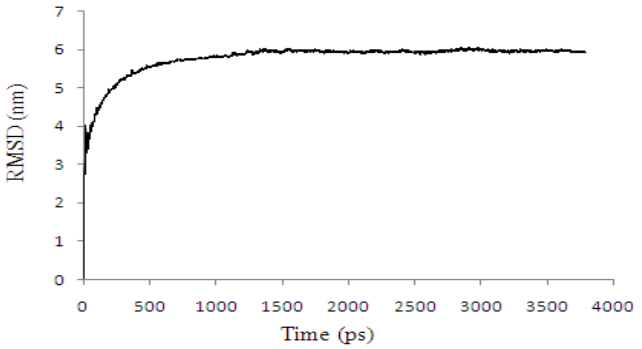

Fig. 4. Plot of root mean square deviation (RMSD) of taxifolin. The trajectories were captured every 0.5 ps until the simulation time reached 3800 ps.

This analysis indicates that taxifolin showed remarkable stability for ATP-binding pocket and potentially competes with ATP. Number of H-bonds (cut off 0.35 nm) which were formed during MD simulation between taxifolin and VEGFR-2 was also calculated. A variable profile was observed which fluctuate between 0 to 5 with an average value of 1.12 (Fig 5).

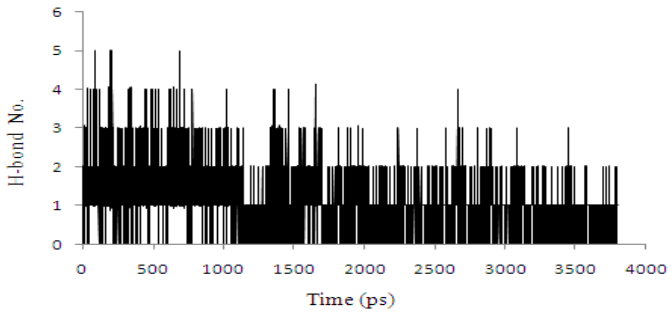

Fig. 5. Number of H-bonds formed during 3800 ps MD simulation.

Further to investigate the thermodynamic stability of complex during simulation, total energy and potential energy fluctuation were analyzed. All of these calculated properties of VEGFR-2 kinase-taxifolin complex showed very stable profile throughout the MD simulation. Total energy and potential energy was found to fluctuate about the average value of -518093 KJ/Mol and -638112 KJ/Mol respectively. Total energy and potential energy was found nearly similar for free and bound form (Fig. 6).

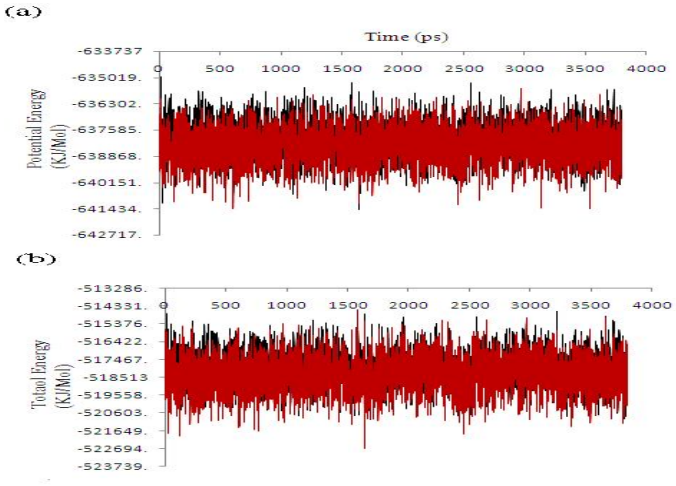

Fig. 6. Energy profile of VEGFR-2 unbound (red) and VEGFR-2-taxifolin complex (black) during 3800 ps MD simulation (a) potential energy, (b) total energy.

This indicates that taxifolin binding did not affect the thermodynamically stable DFG-out conformation (described later). These profiles clearly indicate the thermodynamic stability of the VEGFR-2 kinase-taxifolin complex during total time period of simulation. Furthermore, Radius of gyration of VEGFR-2 and VEGFR-2-taxifolin complex was analyzed to determine the effect of taxifolin on the folding of VEGFR-2. Rg value of complex was found slight higher than the unbound VEGFR-2 after 3800 ps (Fig. 7).

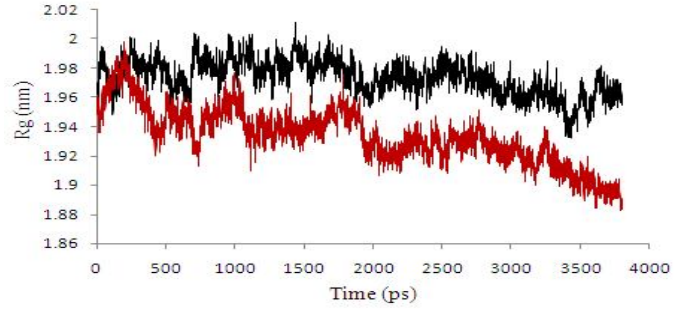

Fig. 7. Change in radius of gyration (Rg) of VEGFR-2 kinase unbound (red) and VEGFR-2-taxifolin complex (black) during 3800 ps MD simulation.

This observation indicate that taxifolin resist the folding of VEGFR-2 as the Rg value at 3800 ps remain nearly as at 0 ps while unbound VEGFR-2 showed decrease in Rg value. The ATP binding site of VEGFR-2 is mainly constituted of residues: Leu 868, Glu 883, Lys 885, Glu 915, Phe 916, Cys 917, Lys 918, Phe 919, Gly 920, Asn 921, Leu 926, Arg 927, Ser 1035, Asp 1044 and Lys 1053 (Lee *et al.*, 2010; Yang *et al.*, 2010).

To observe the fluctuations in ATP binding site (taxifolin binding site) we calculated the RMSF value of backbone atoms of VEGFR-2. Fig. 8 indicates that only few atoms of complex show higher fluctuation as compared to the unbound VEGFR-2.

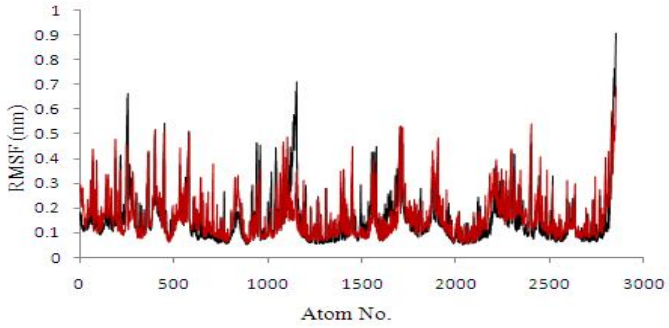

Fig. 8. Root mean square fluctuation (RMSF) of VEGFR-2 unbound (red) and VEGFR-2 kinase-taxifolin (black) atoms during 3800 ps MD simulation.

Additionally, RMSF of ATP-binding pocket residues was also analyzed to find out motion after binding with taxifolin (Fig 9).

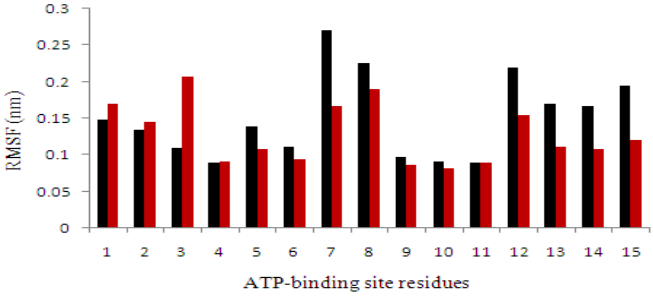

Fig. 9. Root mean square fluctuation (RMSF) of ATP-binding pocket residues (1-15: Leu 868, Glu 883, Lys 885, Glu 915, Phe 916, Cys 917, Lys 918, Phe 919, Gly 920, Asn 921, Leu 926, Arg 927, Ser 1035, Asp 1044 and Lys 1053) of VEGFR-2 unbound (red) and VEGFR-2 kinase-taxifolin (black) during MD simulation.

Most of the residues showed minor increase in fluctuation as compared to unbound form. These observations suggested that taxifolin remain stable in binding pocket. Residue Leu 868, Glu 883, Lys 885 showed decreased RMSF value which indicates that the motion of these residues was hindered by taxifolin. Increased RMSF of Lys 918, Phe 919, Asp 1044 and Lys 1053 indicate that these residues were involved in taxifolin stabilization in pocket along with Glu 915, Phe 916 and Cys 917 which show minor increase in RMSF value.

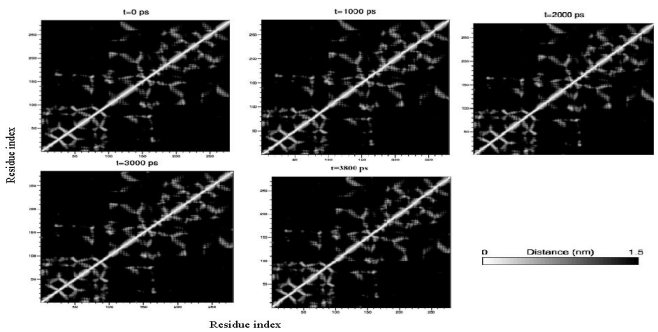

Fig.10. Minimum distance matrix at different time interval showing change in distance between residues.

Distance matrix analysis was used to determine change in the distance between the residues of protein. Fig. 10 shows distance matrix at different time interval of MD simulation which revealed that distance between the residues not affected significantly by binding of taxifolin. Time period of 3800 ps was found sufficient to achieve equilibrium. All the properties analyzed during MD simulation were found to favor each other further authenticate the success of MD simulation.

Earlier, there are mainly two types of inhibitor described for VEGFR-2: Type I inhibitors and Type II inhibitors. Type I inhibitors bind to the ATP binding site through the formation of hydrogen bonds to the 'hinge' region residues (link between N- and C-terminal lobes) and through hydrophobic interactions in and around the region as occupied by the adenine ring of ATP. These hydrogen bonds are similar to those normally formed by the exocyclic amino group of adenine. Type I inhibitors do not additionally require the DFG motif in the activation loop to adopt a 'DFG-out' conformation for binding (Traxler *et al.*, 1999; Liu *et al*., 2006). Type II inhibitors typically use the ATP binding site along with hydrogen bonding and hydrophobic interactions made possible by residues of the activation loop being folded away from the conformation required for ATP phosphate transfer. Type II kinase inhibitors occupy a hydrophobic site that is adjacent to the ATP binding pocket created by a unique conformation of the activation loop (DFG-out) in which the phenylalanine residue of the DFG motif moves from its position in the kinase active conformation. Although occupying the allosteric site is characteristic of type II inhibitors, they can also extend into the adenine region and form one or two hydrogen bonds with kinase hinge residues in a manner similar to that of type I inhibitors (Liu *et al.*, 2006). Analysis of molecular docking results and MD simulation poses at different time interval revealed that taxifolin is possibly acting as the Type I inhibitor. As described earlier taxifolin form H-bonds with hinge region residues: Leu 838, Glu 883, Glu 915 and Cys 917 (Fig. 2) and, additionally, found to form H-bond with activation loop residue Lys 1053 at different time interval of MD simulation. These interactions might lead to stable VEGFR-2 kinase (DFG-out conformation)-taxifolin complex during MD simulation (Fig. 11).

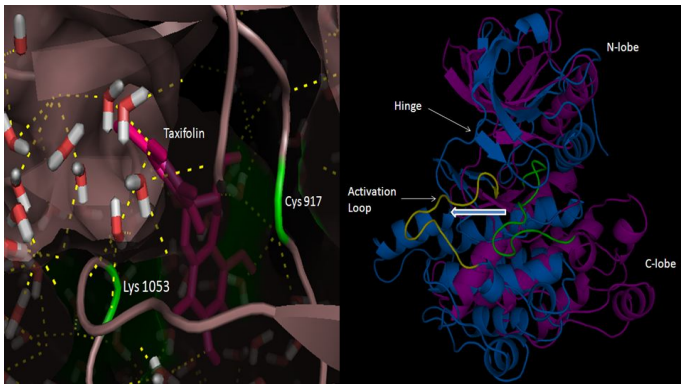

Fig. 11. Snapshot of system (VEGFR-2, taxifolin, water and counterions) at 3800 ps and conformational changes occurs in VEGFR-2 kinase during MD simulation (purple-0 ps, blue- 3800 ps, green-position of activation loop at 0 ps and yellow-position of activation loop at 3800 ps).

Type I inhibitors are known to stably interact with both DFG-in and DFG-out conformations. This property makes these inhibitors non-selective for kinases. To increase the selectivity for DFG-out conformation kinases, second-generation type II inhibitors came in existence. These inhibitors contain hybrid structure: Head region of type I inhibitor to form H-bond with hinge region residues and tail region contain moiety of type II inhibitor (Liu *et al.*, 2006). In the development of second-generation type II inhibitors, taxifolin may play a significant role by acting as the head region of these inhibitors. Molecular dynamic simulation analysis established that taxifolin form a stable complex with VEGFR-2 (DFG-out) and act as a potent type I competitive inhibitor which might leads to new dimension in understanding of VEGFR-2 kinase inhibition mediated cancer chemoprevention and development of second-generation type II inhibitors.

CONCLUSION

The VEGFR-2 kinase has been a promising target for cancer therapy. Specific inhibition of individual proteins or signalling pathways holds a great potential for subversion of cancers. Computational analysis in the present study provided a rationalization to the ability of naturally occurring taxifolin to alter the VEGF signaling pathway. Taxifolin was found to bind at the ATP-binding site on VEGFR-2 kinase with large value of binding energy and act as type I competitive inhibitor as revealed by molecular docking study. The thermodynamic stability of the binding was confirmed by molecular dynamic simulation by analyzing various parameters. These studies confirmed the potential role of taxifolin in cancer chemoprevention which should be further studied for modulation of other signaling pathways which play crucial role in cancer and development of second-generation type II inhibitors.

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