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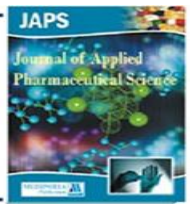

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Molecular Docking and Simulation studies of Farnesyl Trasnferase with the potential inhibitor Theflavin

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ABSTRACT

Ras proteins play crucial roles in cell growth regulation, signal transduction and proliferation. Abnormal Ras proteins caused by genetic mutations are more common in human cancers. Activation of Ras occurs by enzymatic attachment of Farnesyl moiety by Farnesyl transferase. It has been proven that Ftase is a potential anticancer protein target. In this study we perform a virtual screening using polyphenol derivative from various sources against Ftase and establish 'Theaflavin' an antioxidant polyphenol extracted from *Camellia sinensis* (tea), as a potential inhibitor. This conclusion is further supported by comparative studies of molecular docking and 10 nS molecular dynamics simulation with 'Tipifarnib' (Zarnestra) a preclinical Ftase inhibitor.

***Key words*:** Molecular Docking, Theflavin, Ras proteins.

INTRODUCTION

Farnesyl transferase belongs to the class of enzymes called prenyl transferase that transfers a prenyl group, here a farnesyl group to the substrate. The transfer of a 15-carbon farnesyl moiety to the Carboxyl end of Ras proteins by farnesyl transferase is called farnesylation. Such process converts Ras proteins into a functionally active proto-oncogene product (Crul *et al.*, 2001). Ras being the molecular switch in signal transduction, a blockade of Ras by farnesylation thereby blocks its attachment to membrane and interrupts Ras effector pathways, which has emerged as a strategic approach to the development of novel cancer therapies (Narsipur, 2003, Zhang, 1996). Preclinical Farnesyl Transferase Inhibitors (FTIs) such as, tipifarnib by Johnson & Johnson Pharmaceutical Research, lonafarnib by Merck & Co and FTI-277 by EMD Chemicals have been developed. Tipifarnib (Zarnestra) is being used as a comparative model for the study as it was the one which has entered into Phase-3 clinical trials, treated for Acute Myeloid Leukemia (AML).Apart from the synthetic FTIs there are some natural compounds like plant flavonoids acting as promising FTIs. One such compound is Theaflavin from *Camellia sinensis* (tea). Theaflavin has already been proven experimentally as anticancer compound that targets H-Ras pathway in cancer cells and cell lines (Sebti, 2000, Das, 2002, Manna, 2009). The present study also brings about the target and mechanism of action of Theaflavin's inhibitory properties in cancer.

Farnesyl Transferase

Farnesyltransferase is one of the three enzymes in the prenyltransferase group. Farnesyltransferase (FTase) adds a 15-carbon isoprenoid called a farnesyl group to proteins bearing a CAAX motif: a four-amino acid sequence at the carboxyl terminus of a protein. Farnesyltransferase's targets include members of the Ras superfamily of small GTP-binding proteins Farnesyltransferase posttranslationally-modifies proteins by adding an isoprenoid lipid called a farnesyl group to the -SH of the cysteine near the end of target proteins to form a thioether linkage. This process is called farnesylation (which is a type of prenylation), causing farnesylated proteins to become membrane-associated due to the hydrophobic nature of the farnesyl group. Most farnesylated proteins are involved in cellular signaling wherein membrane association is critical for function (Zhang, 1999, Reid, 2004, Eastman, 2006).

Farnesyltransferase structure and function

Farnesyltransferase has two subunits: a 48kDa alpha subunit and a 46kDa beta subunit. Both subunits are primarily composed of alpha helices. The α subunit is made of a double layer of paired alpha helices stacked in parallel, which wraps partly around the beta subunit like a blanket. The alpha helices of the β subunit form a barrel. The active site is formed by the center of the β subunit flanked by part of the α subunit. Farnesyltransferase coordinates a zinc cation on its β subunit at the lip of the active site. Farnesyltransferase has a hydrophobic binding pocket for farnesyl diphosphate, the lipid donor molecule. All farnesyltransferase substrates have a cysteine as their fourth-to-last residue. This cysteine engages in an SN2 type attack, coordinated by the zinc and a transient stabilizing magnesium ion on the farnesyl diphosphate, displacing the diphosphate. The product remains bound to farnesyltransferase until displaced by new substrates. The last three amino acids of the CaaX motif are removed later. There are four binding pockets in FTase, which accommodate the last four amino acids on the carboxyl-terminus of a protein. Only those with a suitable CaaX motif can bind ('C' is Cysteine, 'a' is an aliphatic amino acid, and 'X' is variable). The carboxyl-terminal amino acid (X) discriminates FTase's targets from those of the other prenyltransferases, allowing only six different amino acids to bind with any affinity. It has been shown that geranylgeranyltransferase can prenylate some of the substrates of Farnesyltransferase and vice versa. Farnesyltransferase's targets include members of the Ras superfamily of small GTP-binding proteins critical to cell cycle progression. For this reason, several FTase inhibitors are undergoing testing as anti-cancer agents. FTase inhibitors have shown efficacy as anti-parasitic agents, as well. FTase is also believed to play an important role in development of progeria and various forms of cancers (Beese, 2006, Long Stephen, 2002, Agarwal, 2009

Ras Proteins

The Ras superfamily is a protein superfamily of small GTPases. There are more than a hundred proteins in the Ras superfamily (Wennerberg, 2005) Based on structure, sequence and

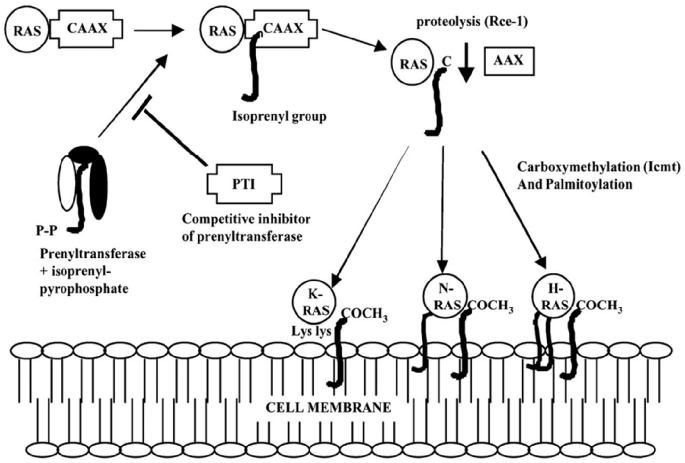

Fig:1 Pathway showing involvement of prenyl transferase (Ftase) in activation of RAS proteins.

functions, the RAS superfamily is divided into eight main families, each of which is further divided into subfamilies: Ras Rd, Rab, Rap, Ran, Rho, Rheb, Rit, Arf and Mir. Each subfamily shares the common core G domain, which provides essential GTPases and nucleotide exchange activity. The surrounding sequence helps determine the functional specificity of the small GTPase, for example the 'Insert Loop', common to the Rho subfamily, specifically contributes to binding to the effector protein such as IQGAP and WASP. The Ras family is generally responsible for cell proliferation, Rho for cell morphology, nuclear transport for Rannd vesicle transport for Rab and Arf (Goodsell, 1999).

The Ras Subfamily

The Ras subfamily (an abbreviation of RAt Sarcoma) is a protein subfamily of small GTPases that are involved in cellular sign al transduction, and is also used to designate gene subfamily of the genes encoding those proteins. Activation of Ras s ignalling causes cell growth, differentiation and survival Ras is the prototypical member of the Ras superfamily of proteins which are all related in structure and regulated diverse cell behaviours. Ras communicates signals from outside the cell to the nucleus; mutations in ras genes can permanently activate it and cause inappropriate transmission inside the cell, even in the absence of extracellular signals. Because these signals result in cell growth and division, dysregulated Ras signaling can ultimately leave to oncogenesis and cancer (Goodsell, 1999). Activating mutations in Ras are found in 20-25% of all human tumors and up to 90% in specific tumor types (Goodsell, 1999).

Theaflavin

Theaflavin (TF) and its derivatives, known collectively as theaflavins, are antioxidant polyphenols that are formed from flavan-3-ols such as in tea leaves during the enzymatic oxidation (called fermentation by the tea trade) of tea leaves, such as in black tea. Theaflavins are types of thearubigins, and are therefore reddish in color. Analogous compounds include EGCG in green tea, theaflavins are not found in green tea. Despite huge implications regarding health benefits, as of 2008, little is known about the

bioavailability of these compounds in black tea (Downward J., 2003), therefore it is not safe to assume that simply drinking black tea will bring out health benefits listed below as there is little known how well compounds are absorbed into the bloodstream. What is known is that EGCG will metabolize into some theaflavins in the liver.

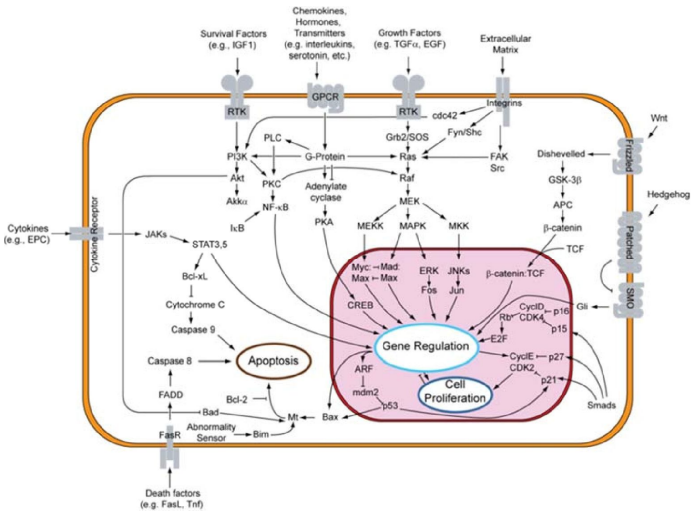

Fig: 2 Pathway showing involvement of RAS proteins in signal transduction and cell proliferation.

Theaflavin Role in Cancer

Theaflavins act on numerous points regulating cancer cell growth, survival, and metastasis, including effects at the DNA, RNA, and protein levels. Tea polyphenols including theaflavins are found to reduce angiogenesis (Liu S., 2005) which is implicated in non-liquid cancers, an area of intense current research, by decreasing vascular endothelial growth factor production and receptor phosphorylation.

Nitric oxide production was reduced by EGCG and black tea theaflavins by suppressing inducible nitric oxide synthase via blocking nuclear translocation of the NF-kappaB as a result of decreased IkappaB kinase activity.Metastasis is inhibited via effects on Urokinase and Matrix metalloproteinases (Liu S., 2005).

Three black tea theaflavins, namely theaflavin 3-O-gallate, theaflavin 3'-O-gallate, theaflavin 3,3'di-O-gallate and thearubigins as mimics the effects of insulin/IGF-1 action on mammalian FOXO1a, which is involved in longevity and aging. Furthermore, EGCG's effects on FOX01a are also due to its being converted to Thearubigins. Theaflavin inhibited tumor necrosis factor-alpha mediated Interleukin-8 gene expression, as measured by luciferase assay and Northern blot analysis, at concentrations of 10 and 30 microg/mL.

TF-2 inhibits colon cancer linked gene COX-2, even more than EGCG. TF-3 is a potent scavenger of superoxide, even more so than EGCG. Further investigations reveals that, Theaflavin-3'-monogallate (TFMG, TF-2) causes apoptosis in colon cancer cells (Maron DJ., 2003). Theaflavin-3,3'-digallate (TFDG, TF3) binds to gp41 of HIV as well as inhibits 3CLPro of severe acute respiratory syndrome (SARS) (Maron DJ., 2003). 3-Isotheaflavin-3-gallate (TF2B) inhibits 3CLPro of SARS (Maron DJ., 2003).

MATERIALS AND METHODOLOGY

PDB (http://www.rcsb.org)

The Protein Data Bank (PDB) is a repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids. (See also crystallographic database). The data, typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, are freely accessible on the Internet via the websites of its member organisations (PDBe, PDBj, and RCSB). The PDB is overseen by an organization called the Worldwide Protein Data Bank, wwPDB.

The PDB is a key resource in areas of structural biology, such as structural genomics. Most major scientific journals, and some funding agencies, such as the NIH in the USA, now require scientists to submit their structure data to the PDB. If the contents of the PDB are thought of as primary data, then there are hundreds of derived (i.e., secondary) databases that categorize the data differently. For example, both SCOP and CATH categorize structures according to type of structure and assumed evolutionary relations; GO categorize structures based on genes (Chia-Nan Chenl, 2005).

AutoDock(http://autodock.scripps.edu/)

AutoDock (Berman HM, 2008) is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. AutoDock is molecular modeling simulation software. Since 2009, it has been open source and is free for non-commercial usage. It is especially effective for Protein-ligand docking

AutoDock Vina(http://vina.scripps.edu/)

AutoDock Vina is a new open-source program for drug discovery, molecular docking and virtual screening, offering multi-core capability, high performance and enhanced accuracy and ease of use (Morris GM, 1998) AutoDock Vina has been designed and implemented by Dr. Oleg Trott in the Molecular Graphics Lab at The Scripps Research Institute.

Open Babel (The Open Source Chemistry Toolbox)

OpenBabel is free software, a chemical expert system mainly used for converting chemical file formats. Due to the strong relationship to informatics this program belongs more to the category cheminformatics than to molecular modeling.

PyRx(http://pyrx.scripps.edu/)

PyRx is a Virtual Screening software for Computational Drug Discovery that can be used to screen libraries of compounds against potential drug targets. PyRx enables Medicinal Chemists to run Virtual Screening from any platform and helps users in every step of this process - from data preparation to job submission and analysis of the results. While it is true that there is no magic button in the drug discovery process, PyRx includes docking wizard with easy-to-use user interface which makes it a valuable tool for Computer-Aided Drug Design. PyRx also includes chemical

spreadsheet-like functionality and powerful visualization engine that are essential for Rational Drug Design (Oleg Trott, 2010).

MGL Tools(http://mgltools.scripps.edu)

MGLTools is a software developed at the Molecular Graphics Laboratory (MGL) of The Scripps Research Institute for visualization and analysis of molecular structures (Oleg Trott, 2010).

AutoDock Tools

AutoDockTools is graphical front-end for setting up and running AutoDock - an automated docking software designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure.

Python Molecular Viewer (PMV)

PMV is a powerful molecular viewer that has a number of customizable features and comes with many pluggable commands ranging from displaying molecular surfaces to advanced volume rendering (Rajarshi Guha, 2006).

Antechamber

Antechamber is a set of auxiliary programs for molecular mechanic (MM) studies. This software package is devoted to solve the following problems during the MM calculations: (1) recognizing the atom type; (2) recognizing bond type; (2) judging the atomic equivalence; (3) generating residue topology file; (4) finding missing force field parameters and supplying reasonable and similar substitutes. As an accessory module in the AMBER 7 and AMBER 8 packages, antechamber can generate input automatically for most organic molecules in a database. The algorithms behind these manipulations may be useful outside the Amber family of programs as well (Wang J, 2001).

GROMACS

GROMACS is a versatile package to perform molecular dynamics, that is simulate the Newtonian equations of motion for systems with hundreds to millions of particles. It is primarily designed for biochemical molecules like proteins, lipids and nucleic acids that have a lot of complicated bonded interactions, but since GROMACS is extremely fast at calculating the non-bonded interactions (that usually dominate simulations) many groups are also using it for research on non-biological systems, e.g. polymers. GROMACS supports all the usual algorithms you expect from a modern molecular dynamics implementation, (check the online reference or manual for details), but there are also quite a few features that make it stand out from the competition (Erik Lindahl, 2001).

PyMol (http://www.pymol.org)

PyMOL is an open-source, user-sponsored, molecular visualization system created by Warren Lyford DeLano and commercialized by DeLano Scientific LLC, which is a private software company dedicated to creating useful tools that become universally accessible to scientific and educational communities. It can produce high quality 3D images of small molecules and biological macromolecules, such as proteins. According to the author, almost a quarter of all published images of 3D protein structures in the scientific literature were made using PyMOL.

PyMOL is one of a few open source visualization tools available for use in structural biology. The Py portion of the software's name refers to the fact that it extends, and is extensible by the Python programming language (www.pymol.org).

VMD (http://www.ks.uiuc.edu/Research/vmd/)

VMD is a molecular visualization program for displaying, animating, and analyzing large biomolecular systems using 3-D graphics and built-in scripting. VMD includes a Ramachandran plot plugin which plots Phi and Psi angles for a selected molecule. The angle plot automatically updates when simulation trajectory frames are advanced by hand, or when animated. Each plotted angle is individually selectable, and reports its segment ID, residue name, residue ID, Phi angle, and Psi angle, when selected. Ramachandran plots can be saved to a postscript file for inclusion into publications (Hsin J., 2008).

OpenMM

OpenMM is a library which provides tools for modern molecular modeling simulation. As a library it can be hooked into any code, allowing that code to do molecular modeling with minimal extra coding. Moreover, OpenMM has a strong emphasis on hardware acceleration, thus providing not just a consistent API, but much greater performance than what one could get from just about any other code available (Fredrichs MS, 2009)

CUDA

CUDA (an acronym for Compute Unified Device Architecture) is a parallel computing architecture developed by NVIDIA. CUDA is the computing engine in NVIDIA graphics processing units (GPUs) that is accessible to software developers through variants of industry standard programming languages. Programmers use 'C for CUDA' (C with NVIDIA extensions and certain restrictions), compiled through a PathScale Open64 C compiler[1] to code algorithms for execution on the GPU. CUDA architecture shares a range of computational interfaces with two competitors -the Khronos Group's Open Computing Language and Microsoft's DirectCompute. Third party wrappers are also available for Python, Perl, Fortran, Java, Ruby, Lua, MATLAB and IDL, and native support exists in Mathematica (McGlaun, 2008)

METHODOLOGY

Protein X-Ray structures and its active site analysis

The crystal structure of Human Farnesyl Transferase which is complexed with Farnesyl diphosphate and Tipifarnib (PDB code: 1SA4) (Reid TS., 2004) was selected as the model protein. The 2 subunits of Farnesyl transferase, a 48kDa alpha subunit and a 46kDa beta subunit are primarily composed of alpha helices. The α subunit is made of a double layer of paired alpha helices stacked in parallel, which wraps partly around the beta

subunit like a blanket. The alpha helices of the β subunit form a barrel. The active site is formed by two clefts that intersect the subunits. The active site comprises of a three heteroatom residues, Farnesyl-di-phosphate, Tipifarnib and Zinc II ion co-ordinatively complexed with Tipifarnib and Cystine 299 (B), Histidine 362 (B) and Aspartate 299 (B). In ideal conditions the Farnesyltransferasepost translationally-modifies proteins those with a suitable CaaX motif ('C' is Cysteine, 'a' is an aliphatic amino acid, and 'X' is variable) by adding an isoprenoid lipid farnesyl group to the -SH of the cysteine near the end of target proteins coordinated by the zinc ion to form a thioether linkage (Reid T, 2004, Beese, 2006).

Tipifarnib & water molecules are stripped off from the crystal structure while the Co-ordinated Zn II and Farnesyl-di-phosphate are retained as they provide structural basis for lead screening and to map the interactions with the critical active site molecules.

Molecular Docking

The results from the Autodock Vina virtual screening are shortlisted based on the lowest docking energy score along with the capability of the ligand making a chemical interaction with the Zn(II) ion. The shortlisted chemical compounds are then compared with Tipifarnib using a molecular docking program. Autodock 4.0 [32] is used for this study, adding a dimensionality to the screening; Autodock uses Lamarkian genetic algorithm (Huey R, 2007) as opposed to local search algorithm used in Vina, also Autodock 4.0 predicts the inhibition concentration (K_a) which brings about additional information along with energy values. The thus obtained docked complex is then mapped for the interacting residues in contrast with Tipifarnib using ligplot (Wallace AC, 1995)

Molecular Dynamics

To establish the stability and map the real-time interactions of the ligand molecules with the protein a 10 nS molecular dynamics simulation in an explicit water environment is performed using GROMACS (Hess B, 2008, Berendsen HJC, 1995), version 4.0.5 using OpenMM accelerated Molecular dynamics. OpenMM libraries allow carrying the simulation on current generation nVIDIA graphics processors which scales up speed and performance. The present study is carried on nVIDIA Geforce GTS450 Fermi architecture Graphics processing card with 192 CUDA computing cores with 1 GB DDR5 memory with a total speed of 601.34 GFlops (www.nvidia.com).

General Amber force field (Amber99sb) (Hornak V *et al.*, 2006) is used for all the molecules in the study. The forcefield parameters required for the hetero-molecules under study i.e. Tipifarnib, Theaflavin, Farnesyl-di-phosphate are computed using Antechamber software suite (Wang J, 2004, Wang J, 2006) of Amber tools version 1.4 and interconverted for the gromacs file type using ACPYPE software program (Sousa Da Silva (submitted).) the forcefield parameters for the protein is provided by the amber ports within the GROMACS 4.0 program. In order to create and maintain the covalent bonds formed by Zn(II) ion with the Cystine, Aspartate and Histidine a special specbond.dat file is handwritten and supplemented to the GROMACS program. The simulation is performed in 5 million leap frog integrator steps with a Reaction field electrostatics and Vander Waals interactions at 1nm cutoff in an explicit water environment using SPC (simple point charge) water system in an 11nm side length cubic box. The system is energy minimized and position restraints simulations using steepest decent integrator are done on a stepwise manner, Temperature coupled position restraints using V-rescale- a modified Berendsen thermostat algorithm at 300K for 50,000 steps followed by pressure coupling using Parrinello-Rahman algorithm at 1 bar for 50,000 steps.

Docking results

From the table 1, Theaflavin s how the lowest inhibition concentration of 85.16 fM mapping Interaction with the protein using Ligplot show identical interacting residues as that of Tipifarnib; in addition to that Teaflavin also makes addition al 3Hydrogen bonds to lysine 164(A), Tyrosine 93(B), Phenylalanine 360(B).

Table 1: Docking scores for the top 4 compounds from virtual screening.

Compound	Vina Score	Autodock Score	Inhibition Score
Tipifarnib (Reference)	-9.8 Kcal/mol	-15.05 Kcal/mol	9.32 pM
Theaflavin	-12.2Kcal/mol	-17.83Kcal/mol	85.16fm
cyanidin-3-O-rutinoside	-11.0Kcal/mol	-17.24Kcal/mol	2.29pM
Chebulic acid	-12.4Kcal/mol	-15.4Kcal/mol	4.08pM
Alpha-Hederin	-11.3Kcal/mol	-15.20Kcal/mol	6.4pM

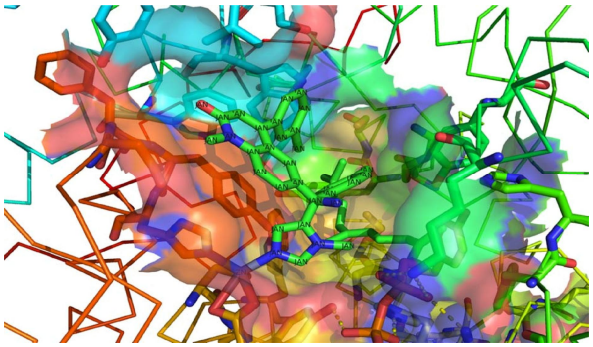

Fig 3: Tipifarnib (green) docked in the active site cleft between the 2 subunits of Ftas covalently bound with Zn (II).

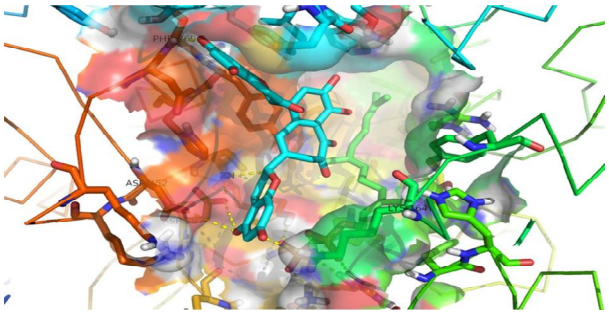

Fig 4: Docked theaflavin (cyan) with interacti ng residues lysine (164) and phenyl alanine (269) Aspa rtate (252) and Z n (ii) in background.

Molecular Dynamics study Theaflavin vs. Tipifarnib

A s theaflavin docked complex has the lowest in hibition concentration, it is subjected to molecular dynamics simulation for validation by com paring with that of Tipifarnib. The root mean square deviation (RMSD), the energy of the complex and the molecular interactions among them are taken into consideration to study the stability of the complex. Figure 3 shows the RMSD of theflavin comple x (shown in black) ranges from 0.8 A to 1.3 A which is similar to t at of Tipifarnib (shown in red). The total energy of the complex ranges from -1.55313e+06 to -1.53151e +06 during the simulation as shown in figure 6. As energy remains almost the same throughout the simulation with least RMSD shows that the binding subset is table even if the simulation time is longer.

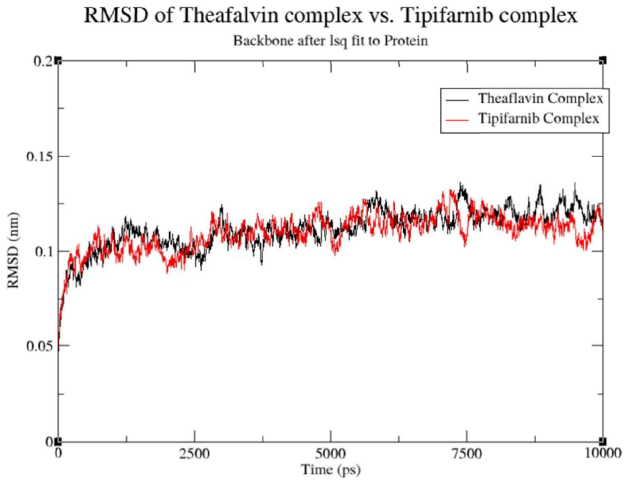

Fig 5: Graph showing th e RMSD of Theaflavin and Tipifarnib.

The structural ability of the ternary structure, theflavin complex is conferred by the interactions between the ligand and the protein. Apart from the protein-protein interactions between the two chains, there are hydrogen bonds and non-bonded interactions between protein and the ligand. When compared with the tipifarnib complex which is stabilized only by non-bonded interactions with amino acid residues and a covalent bonding with zinc ion, theaflavin complex has an addition 13 hydrogen bonds with NZ amino group of lyxijne 164(A), OH group of tyrosine 93(B) and amino N of phenyl alanine 360(B).

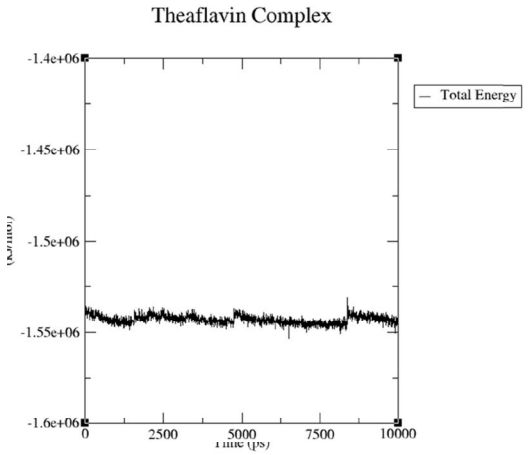

Fig 6: Graph showing the total energy for theaflavin complex.

The complex after undergoing energy minimization and position restraints forms an additional hydrogen bond with the OH-group of another Tyrosine 300 (B) apart from the previously formed bonds (Fig. 7). As the simulation begins, the fourth bond between Theaflavin and the Fpp 426(C) (Fig. 8) is formed replacing the Tyr 93(B). This bond remains stable throughout the simulation. The simulation proceeds and the Tyr 300(B) is replaced by Tyr 93(A) new bond formation with Tyr 361 is also seen during the 2nd nS. Apart from this there is bond shifting between the Lys 164 and Phe 360 between the 3rd and 5th nS.

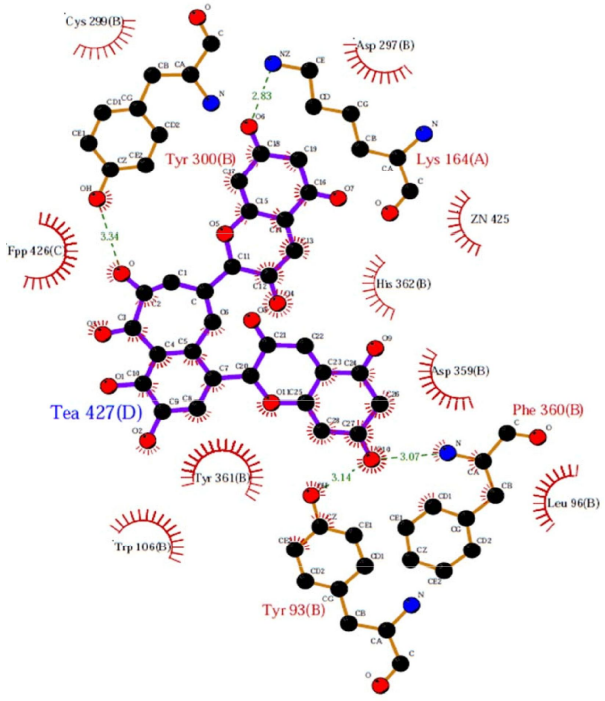

Fig 8 Ligplot showing the interactions between Theaflavin (TEA) and Ftase. Hydrogen bon ings are represented in gre n dashes with bond length; h ydrophobic interactions are represented in semi-circles w ith radial spikes pointing outward towards the inter acting residues.

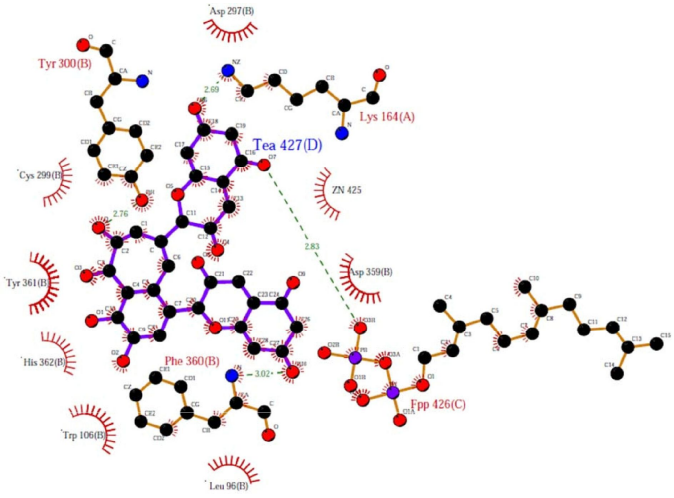

Fig 9: Ligplot for the theaflavin- FPP interaction.

Then after 5th nS, there is a stable bond formation with Tyr 361, Tyr 93, Phe 360 and Fpp 426 till the 10th S of the simulation (figure 9).

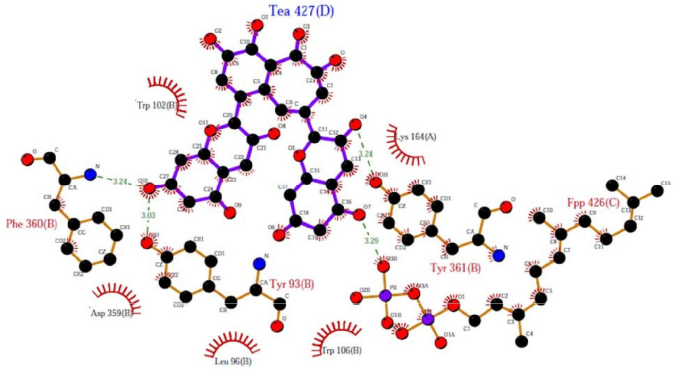

Fig 9: Theaflavin complex formin hydrogen bo nds with FPP, Phe 360 (B), T yr 93 (B) and Tyr 361 (B) during the 5th to 10th nS of simulation.

CONCLUSION

It has been observed from the dynamics study that the ternary complex of ftase with Theaflavin was stable throughout the simulation as compared with tiifarnib which can be supported by the RMSD and energy graphs obtained from the simulation. The MD and docking study with theaflavin and tipifarnib shows the identical interacting residues to the protein suggest the selectivity of theaflavin to farnesyl transferase also interaction with key Zn (II) ion which is the key residue in inhibition of ftase activity.

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