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HUMAN SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 (STAT3) PROTEIN MODELING AND DRUG DESIGNING FOR PSORIASIS

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Human Signal Transducer and Activator of Transcription 3 (STAT3) protein has been recently identified as a potential target for the treatment of psoriasis. However, three dimensional (3D) structure of this important protein for humans is not yet available. In order to design drug candidates for psoriasis 3D structure for human STAT3 protein was required. Therefore, an attempt has been made to predict the 3D structure of human STAT3 protein. Backbone conformation of the modeled structure was validated by PROCHECK and was found to be satisfactory. Overall quality factor given by ERRAT was found to be 92.55% and VERIFY3D profile confirmed good quality of modeled structure. The protein structure validation was followed by molecular dynamics simulations. Fludarabine has already been identified as an established drug which acts on human STAT1 protein and shares very high degree of homology with human STAT3. Hence, similarity with Fludarabine was selected as screening parameter. A huge library of ligands was generated from ZINC database using similarity value of 90%. The generated library was further screened on the basis of Lipinski rule of five to get 114 ligands. These ligands were subjected to docking studies. Our analysis provides insight into the structural properties of human STAT3 and defines its active sites. Moreover, ligand [5-(6-aminopurin-9-yl)-4-hydroxy-2 (phosphonooxymethyl) oxolan-3-yl] dihydrogen phosphate showed very good binding ability in comparison to Fludarabine. Toxicological studies found to be satisfactory. This study suggests that [5-(6-aminopurin-9-yl)-4-hydroxy-2 (phosphonooxymethyl) oxolan-3-yl] dihydrogen phosphate bears promises to be used as potential analogue in treatment of psoriasis.

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1. Introduction

Psoriasis is a skin disorder characterized by increased epidermal proliferation, incomplete differentiation, elongation and dilatation of superficial plexus of dermal capillaries. Moreover, it is a diverse inflammatory and immune cell infiltrate of the epidermis and papillary dermis. Numerous protein targets have been identified in various research studies for the treatment of psoriasis viz. Signal Transducer and Activator of Transcription 3 (STAT3), Want5, Endothelin-1, enzyme - alpha secretase, S100 proteins, p53, Serum Response Factor (SRF), Heat shock proteins 70 (HSP70), Bcl-x etc [1]. STAT3 is a protein involved in conveying extracellular signals to the nucleus. It is crucial to the development of the psoriasis [2]. STAT proteins transmit signals from

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cytokines or growth factors that have receptors on cell-surface which are associated with tyrosine kinase activity. Kinases, such as members of the Janus kinase family or SRC family, phosphorylate these receptors and provide docking sites for inactive STAT monomers, which in turn gets phosphorylated and forms activated dimers. Activated STATs progress to the nucleus. They involved in regulating numerous genes that control elementary biological process including apoptosis, cell proliferation and immune responses [3]. Blocking the function of STAT3 using antisense oligonucleotide inhibits the onset psoriasis and reverses established psoriatic lesions. Further analysis revealed a dual requirement of both activated STAT3 in keratinocytes as well as in T cells. It indicates that the pathogenesis of psoriasis is rooted in a co-operative process involving STAT3 regulated genes in both skin cells and the immune system [4]. Another study demonstrated that phosphatyrosyl peptides block STAT3 mediated DNA binding activity, gene regulation and cell transformation. Furthermore, to identify small molecular inhibitors of STAT3 the ability of its SH2 domain binding peptide PY*LKTK (Y* represents phosphorylation) to disrupt STAT3 activity in vitro has been investigated [5]. The presence of PY*LKTK, but not PYLKTK or PFLKTK, in nuclear extracts results in significant decrease in the levels of DNA binding activities of STAT3. In present study, for the first time human STAT3 protein structure modeling, validation, active site prediction, screening of potential ligands, their docking studies and toxicological predictions are demonstrated.

2. Material and methods

2.1 Human STAT3 protein modeling

Complete protein sequence of human STAT3 protein is available. However, validated 3D structure of it is not yet available in Protein Data Bank (PDB) [6]. Hence, the present exercise of developing the 3D model of human STAT3 was undertaken. In order to model the 3D structure of human STAT3 protein, its complete sequence was obtained from UniProtKB database (accession number P40763) and was used as template for comparative modeling approach. BLASTP [7] search was performed against PDB with the default parameters to find suitable templates for homology modeling. In order to ensure accuracy in modeling 3D structure, multiple templates were selected for this protein through BLAST against PDB (Protein Data Bank) database. The academic version of MODELLER9v7 [8] was used for 3D structure generation based on the information obtained from sequence alignment.

The MODELLER software uses probability density functions (PDFs) as the spatial restraints instead of energy [9-11]. The 3D model of a protein is obtained by optimization of the molecular pdf such that the model disobeys the input restraints as little as possible. The molecular pdf was derived as a combination of pdfs restraining individual spatial features of the whole molecule. Out of 20 models generated by MODELLER, the one with the best G-score of PROCHECK was selected for further studies [12].

2.2 Validation of modeled human STAT3 protein

Energy minimized refined model was subjected to validation on PROCHECK, VERIFY3D, WHATCHECK and ERRAT server. PROCHECK was used to analyze the stereo-chemical qualities of 3D model of STAT3 protein. VERIFY3D (a structure evaluation server) were used to check the residue profiles of the obtained three dimensional models. Quality of the model was evaluated for the environment profile using structure evaluation server ERRAT [13]. The final refined model was evaluated for its atomic contacts using the WhatIf program [14] to identify bad packing of side chain atoms or unusual residue contacts. This model was further subjected for molecular dynamics studies.

2.3 Molecular dynamics simulation of protein

Molecular dynamics are the computer simulation studies in which actual physical movements of the atoms in bio-molecular complexes are done. These interactions are important in simulation studies which mimic the natural body system. Thus, refinement and energy minimization of modeled protein was done by molecular dynamics using Groningen Machine for

Chemical Simulations (GROMACS) force fields [15]. In present study, GROMACS 4.0.6 software package [16] with GROMOCS 96 force field [17] and the flexible Simple Point Charge (SPC) water model was used for molecular dynamics simulations. The three-site models are very popular for molecular dynamics simulations because of their simplicity and computational efficiency. In three-site models there are three interaction sites, corresponding to the three atoms of the water molecule. In this model each atom gets assigned a point charge, and the oxygen atom also gets the Lennard-Jones parameters. Most models use a rigid geometry matching the known geometry of the water molecule. Net charge on the protein causes electrostatic repulsion in the protein so that was neutralized by adding 12 Na+ ions to the simulation system. This energy minimization leads to removal of bad contacts and overlapping regions in proteins. The initial structure was immersed in a periodic water box of cubic shape (0.2 nm thick). Electrostatic energy was calculated using the particle mesh Ewald method [18]. Cutoff distance for the calculation of the coulomb and van der Waals interaction was 1.0. The final MD calculations were performed for 20 ps. The results were analyzed using the standard software Pymol, provided by the GROMACS package.

Optimized Potentials for Liquid Simulations (OPLS-AA/L) force field adds sites in all atom models which allow more flexibility for charge distributions and torsional energetic over the atoms [19]. Hence, topology that is information about the bonds, angles and atoms of the protein was generated by OPLS-AA/L force field.

2.4 Active site prediction

After validation model was analyzed for the active site with Pocket finder and Q-SiteFinder online tools. Pocket Finder [20], a program for identifying and characterizing protein active sites, binding sites and functional residues located on protein surfaces was used to identify binding pockets of STAT3 protein. Pocket-Finder works by scanning probe of radius 1.6 angstroms along all gridlines of grid resolution 0.9 angstroms surrounding the protein. Cubic diagonals were also scanned by using this probe. Grid points are defined to be a part of a site when the probe is within range of protein atoms followed by free space followed by protein atoms. Q-SiteFinder uses the interaction energy between the protein and a simple van der Waals probe to locate energetically favorable binding sites. Energetically favorable probe sites were clustered according to their spatial proximity. Clusters were then ranked according to the sum of interaction energies for sites within each cluster.

2.5 Ligand library generation with target knowledge

In order to generate possible inhibitors of STAT3 protein, it was essential to identify potential lead molecule. STAT protein family contains different subtypes of proteins viz. STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6. There is high homology between STAT1 and STAT3 proteins as these proteins are phylogenetically linked [21]. By considering this fact Fludarabine was selected as lead molecule as its activity on STAT1 protein is already proved and reported in literature[22-26]. This molecule was obtained from Drug bank (accession id DB01073) (http://www.drugbank.ca/drugs/DB01073). This molecule was used as template to generate large ligands library from ZINC database similarity value of 90%. The ZINC database allows search by similarity value which is in multiple of 10% , if 100% is selected then there is very less probability of getting diverse structures with greater identity. Hence, 90% similarity value was chosen to maintain balance between diversity in ligands and their physicochemical properties. Moreover, by doing this one can avoid unwanted diversity among the screened ligands, which may occur due to selection of less similarity value. In addition, considering the Lipinski's rule of five as filtering criteria, obtained library was further screened to get specifically drug like molecules. Finally 114 ligands were screened out of ZINC database for further docking studies.

2.6 Protein- ligand docking studies

Docking studies were performed on the114 screened ligands from ZINC database on the modeled STAT3 protein with the help of DOCK6.4 tool.

AMBER99-based Lennard-Jones parameters for van der Waals energy potential were used for grid generation. This was required to compute the contact and electrostatic potentials for the

active site. Based on the docking score top scoring compounds were selected as possible inhibitors with reference to their dock score. The adopted methodology is summarized in following flowchart (figure1).

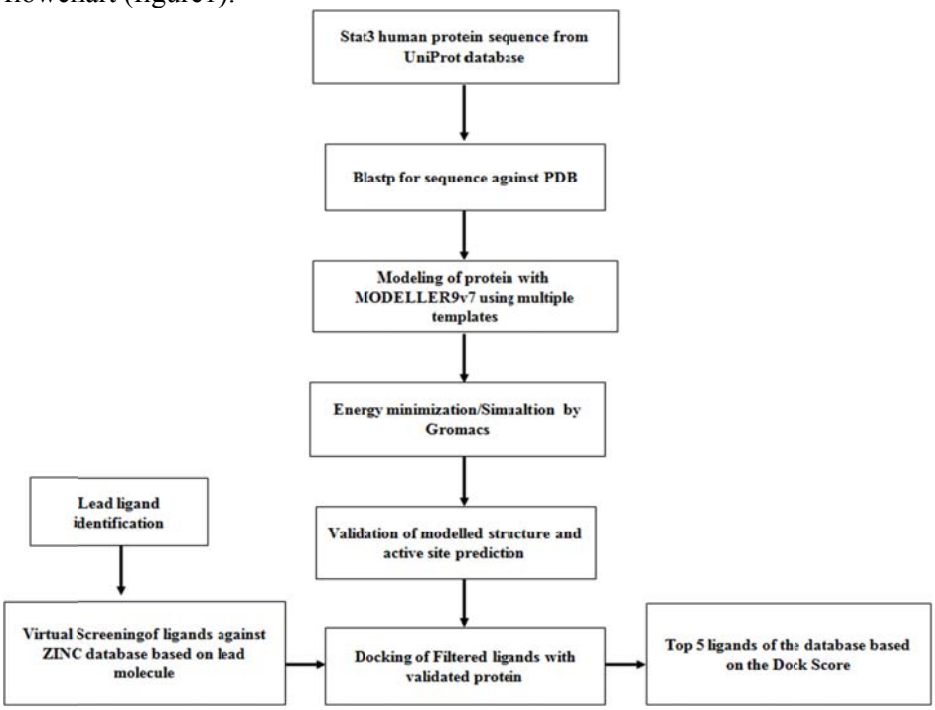


Fig 1 Flowchart demonstrating the adopted methodology

3. Results

3.1 Model building

The structure of STAT3 protein in the humans was determined by using homology modeling protocol. BLASTP search was performed against PDB with default parameters to get suitable templates for comparative modeling. Depending upon the maximum identity with high score, lower e-value and query coverage three templates 1bg1, 1yvl and 1bf5 were selected. Human STAT3 protein model was prepared by multiple alignment of the FASTA format of these target and template protein (figure 2).

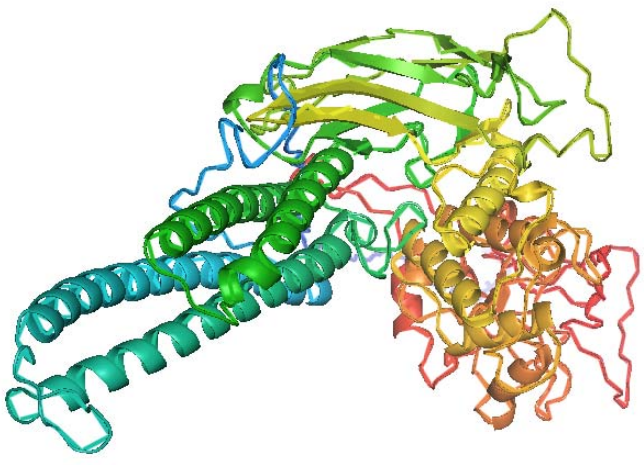


Fig. 2. Modeled human STAT3 protein structure using comparative modeling

3.2 Protein structure validation

The stereo chemical quality of the homology model of human STAT3 was analyzed using PROCHECK. It was found that the phi/psi angles of 86.0% residues fell in the most favored regions (Table 1). The model was also validated using ERRAT server as shown in graph (Figure 3). The quality factor of 92.55 suggests that the model has good quality as a score higher than 50 is acceptable for a reasonable model. High quality of model is also confirmed from VERIFY 3D server as 77.95% of residues of modeled protein showed a score higher than 0.22. Results of WHATCHECK also indicate about the correctness of the modeled structure (Table 2). Based on these results, it was ascertained that the obtained structure has reasonably good quality.

Table 1 Quality of main chain and side chain parameters of modeled human STAT3 proteins giving comparative account of values observed for modeled structure (parameter value) and values obtained for well refined structures at same resolution (typical values)

Chains	*Stereochemical parameter*	*No. of data points*	*Parameter value*	*Typical value*	*Band widths*	*No. of band widths from mean*
Main Chain Parameter	% residues in A, B, L	695	86.0	83.8	10.0	0.2
	Omega angle st. dev.	765	6.8	6.0	3.0	0.3
	Bad contacts / 100 residues	0	0.0	4.2	10.0	-0.4
	Zeta angle st. dev.	731	2.5	3.1	1.6	-0.4
	H-bond energy st. dev.	466	0.8	0.8	0.2	-0.1
	Overall G-factor	770	-0.2	-0.4	0.3	0.7
Side Chain Parameter	Chi-1 gauche minus st. dev.	102	12.0	18.1	6.5	-0.9
	Chi-1 trans st. dev.	256	12.7	19.0	5.3	-1.2
	Chi-1 gauche plus st. dev.	295	12.4	17.5	4.9	-1.0
	Chi-1 pooled st. dev.	653	13.0	18.2	4.8	-1.1
	Chi-2 trans st. dev.	230	14.8	20.4	5.0	-1.1
	Chi-1 gauche minus st. dev.	102	12.0	18.1	6.5	-0.9

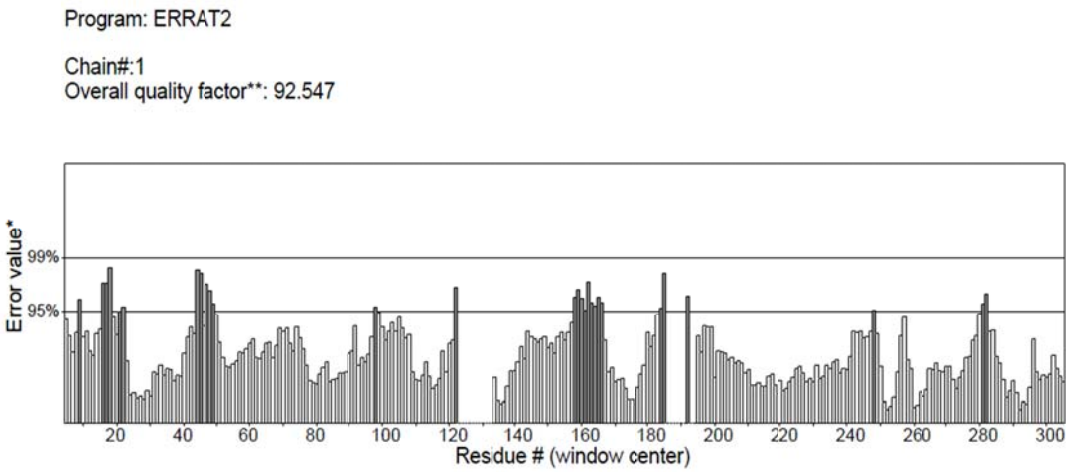


Fig. 3 The 3D profiles of human STAT3 protein verified using ERRAT server. Overall quality score indicates residues are reasonably folded.

Table 2 Quality indicators as calculated from WHATCHECK

Structure Z-scores		*RMS Z-scores*	
2nd generation packing quality	-1.849	Bond lengths	0.659
Ramachandran plot appearance	-3.422	Bond angles	1.135
chi-1/chi-2 rotamer normality	-2.416	Omega angle restraints	1.231
Backbone conformation	-8.381	Side chain planarity	1.668
		Improper dihedral distribution	1.544
		Inside/Outside distribution	1.080

3.3 Molecular dynamics simulation

The MD simulation of the modeled human STAT3 protein was performed and the resulted trajectory was analyzed to study the motional properties of the protein. Based on intrinsic dynamics, structural stability and improved relaxation of the modeled structure, the energy (Figure 3) of the energy minimized structure was also calculated. After simulation the average total energy score over time scale of 20 ps was stabilized at -3.6476e+06kJmol^{-1}. RMSD calculations demonstrated that the protein is flexible in nature and it can be concluded that there is presence of loop in the modeled protein. RMS fluctuation of Cα is presented as a function of residue numbers (figure 4). RMS fluctuation indicates the residue numbers 25, 195, 400, 539, 631, 701 and 744 of the protein. Amino acids present at these positions are Ser25, Val195, Asn400, Tyr539, Lys631, Ser701 and Ala744.

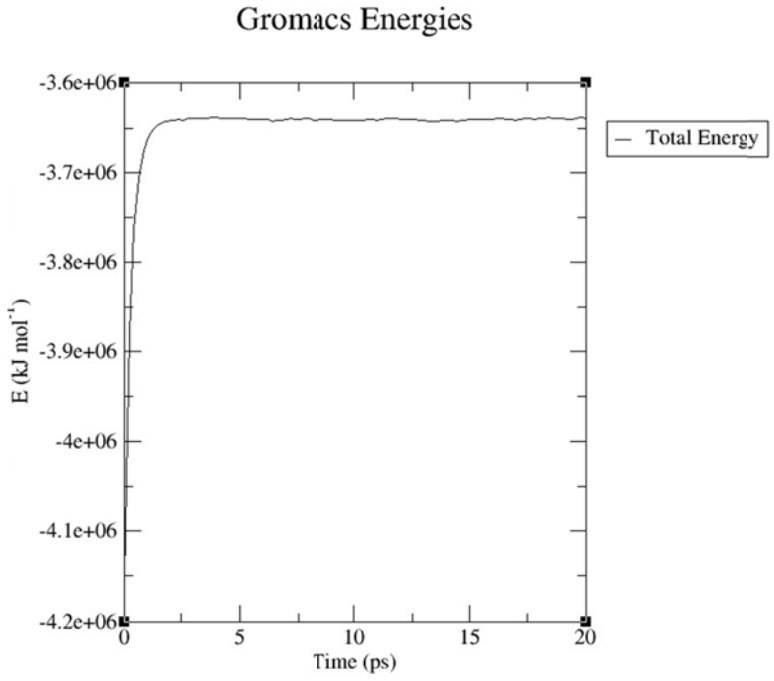


Fig 3 Calculated energy vs time plot for human STAT3 protein using GROMACS software. The x axis represents the simulation time in picoseconds. The y axis represents energy in kJ mol^{-1}.

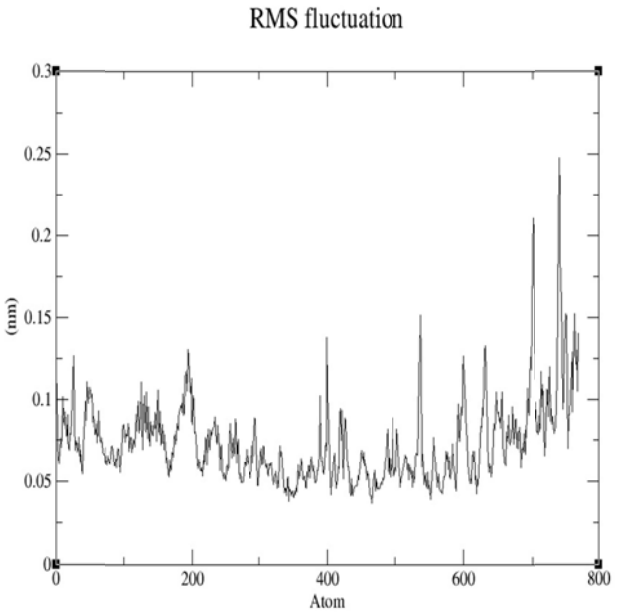


Fig 4 residue-wise RMS fluctuation profiles of Cα atoms of human STAT3 protein structure computed after stabilization of the RMSD trajectories. The x axis represents the residue number. The y axis represents RMS fluctuation in nm unit.

3.4 Active site identification of human STAT3 protein

After getting the final model, the possible binding sites of modeled human STAT3 were searched using the Pocket-Finder and Q-Sitefinder server. Pocket finder predicted Glu239, Asp242, Cys259, Leu260, Arg262, Leu263 and Trp266 residues in active site. Q-Sitefinder predicted Val136, Gln141, Arg246, Cys259, Arg262 and Leu263 residues. Residues forming bonds in top pose after docking were found to be Cys259, Arg262 and Leu263 (constituents of the active site) (Table 3).

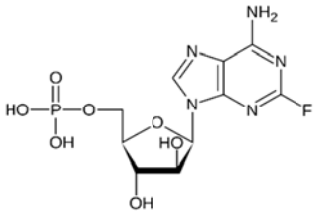
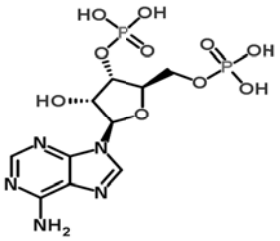
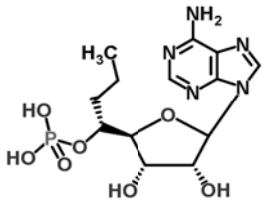
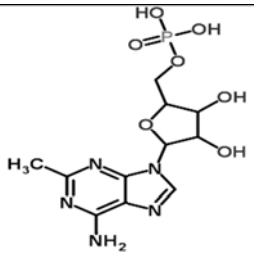
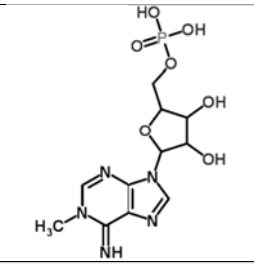
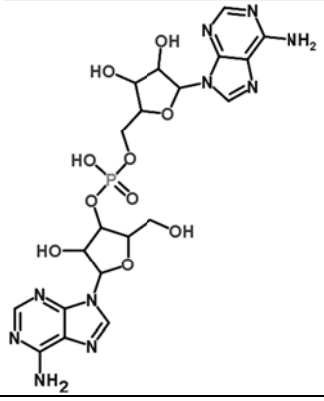
Table 3 Showing amino acids present in active site of STAT3 protein predicted by Pocket finder and Q-Sitefinder.

Cavity size	Qsite Finder	Pocket Finder	Residues forming bonds in top pose after docking
632 $Å^3$	Val136, Gln141, Arg246, Cys259, Arg262, Leu263	Glu239, Asp242, Cys259, Leu260, Arg262, Leu263, Trp266	Cys259, Arg262, Leu263

3.5 Protein-ligand interaction studies

Docking studies were performed using DOCK6.4 tool. Among 114 ligands docking scores of top 5 ligands along with their structures and IUPAC names are presented in **T**able 4. Ligand Zinc03869248 has better dock score among all the screened ligands including Fludarabin (figure 3).

Table 4 Showing the docked ligands, dock scores and structures of top five ligands among 114 screened molecules along with the Fludarabine.

Docked ligands	**Dock Scores**	**Structure of the ligands**
Fludarabine [(2R,3R,4S,5R)-5-(6-amino-2-fluoro-purin-9-yl)- 3,4-dihydroxy-oxolan-2-yl]methoxyphosphonic acid	-48.224	
Zinc03869248 [5-(6-aminopurin-9-yl)-4-hydroxy-2 (phosphonooxymethyl) oxolan-3-yl] dihydrogen phosphate	-55.254	
Zinc40738189 [(1R)-1-[(2S,3S,4R)-5-(6-aminopurin-9-yl)-3,4-dihydroxy-tetrahydrofuran-2-yl]butyl] dihydrogen phosphate	-52.870	
Zinc34920112 2-methyl-9-(5-O-phosphonopentofuranosyl)-9H-purin-6-amine	-50.985	
Zinc62088815 (6Z)-1-methyl-9-(5-O-phosphonopentofuranosyl)-1,9-dihydro-6H-purin-6-imine	-49.351	
Zinc04773655 Phosphoric acid 5-(6-amino-purin-9-yl)-3,4-dihydroxy-tetrahydro-furan-2-ylmethyl ester 5-(6-amino-purin-9-yl)-4-hydroxy-2-hydroxymethyl-tetrahydro-furan-3-yl ester	-48.649	

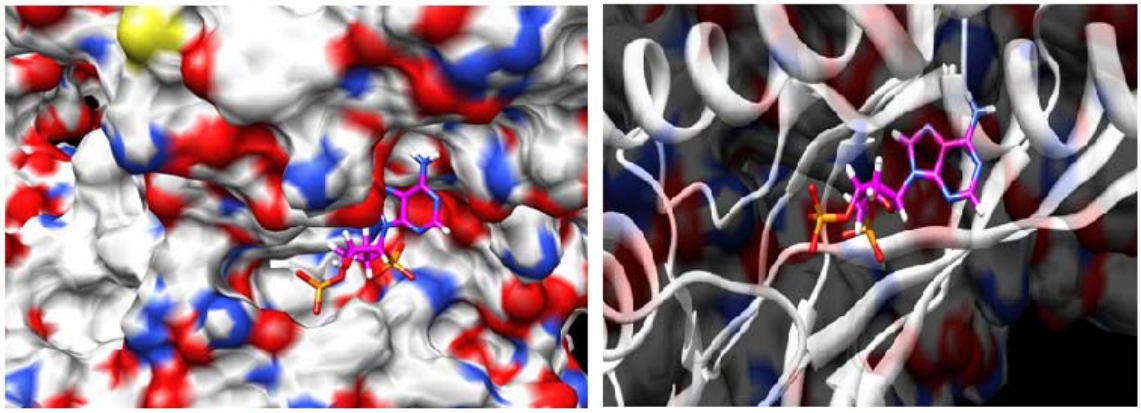


Fig 5 Binding pose of the top scoring ligand (ZINC03869248) in the active site of human STAT3 protein cavity. (a) Pose of top scoring ligand in binding pocket. (b) Transparent view of binding pose of ligand in cavity.

4. Discussion

The 3D structure of human STAT3 has not yet been determined as it is not present in PDB. Therefore, we built a model following comparative modeling approach to understand the structure of human STAT3 protein. This model was validated with four potential computational tools viz. PROCHEK, ERRAT, VARIFY 3D and WHATCHECK. Overall validation results confirmed that the modeled structure was reasonably good. Next step was to predict the active site of the modeled human STAT3. It was done with the help of Pocket Finder and Q-Sitefinder.

This protein structure derived from molecular dynamics simulations was used for docking studies. As discussed in section 2.5, human STAT3 and STAT1 proteins have high degree of homology and Fludarabine has potential binding properties with human STAT1 protein. In view of this fact a library of large number of compounds was generated from ZINC database by considering Fludarabin as lead molecule. To get drug like candidates this library was further screened on the basis of Lipinski rule of five. Finally 114 screened ligands were subjected to the docking studies. Among all 114 ligands top five have shown comparatively greater binding stability to the human modeled STAT3 protein. Among these five ligand [5-(6-aminopurin-9-yl)-4-hydroxy-2 (phosphonooxymethyl) oxolan-3-yl] dihydrogen phosphate (Zinc03869248) showed comparatively most stable complex with human STAT3 protein followed by [(1R)-1-[(2S,3S,4R)-5-(6-aminopurin-9-yl)-3,4-dihydroxy-tetrahydrofuran-2-yl]butyl] dihydrogen phosphate (Zinc40738189), 2-methyl-9-(5-O-phosphonopentofuranosyl)-9H-purin-6-amine (Zinc34920112), (6Z)-1-methyl-9-(5-O-phosphonopentofuranosyl)-1,9-dihydro-6H-purin-6-imine (Zinc62088815) and Phosphoric acid 5-(6-amino-purin-9-yl)-3,4-dihydroxy-tetrahydro-furan-2-ylmethyl ester 5-(6-amino-purin-9-yl)-4-hydroxy-2-hydroxymethyl-tetrahydro-furan-3-yl ester (Zinc04773655).

The toxicological studies of proposed drug candidates (Table 5) suggest encouraging results. While analyzing the toxicological outputs by TOPKAT version 3.1, it is obvious that drug candidates are showing comparative characteristics in almost all fronts under consideration with the drug Fludarabine. Among all five proposed drug candidates, ZINC03869248 ([5-(6-aminopurin-9-yl)-4-hydroxy-2 (phosphonooxymethyl) oxolan-3-yl] dihydrogen phosphate) has best binding energy with modeled human STAT3 protein. In addition, this drug candidate was found to be preeminent in terms of carcinogenic potency TD50 in mouse (137.789 mg/kg body weight/day). Furthermore, oral LD50 in rats for this drug candidate was found to be 0.154 g/kg body weight which is closest to drug Fludarabine (DB1073) in comparison to the rest of four drug candidates.

Table 5: Various critical properties of drug candidates derived from TOPKAT version 3.1

Compounds	Aerobic biodegradability	Ames mutagenicity	Developmental toxicity potential	Skin irritancy	Carcinogenic potency TD50 mouse (mg/kg body weight/day)	Rat Oral LD50 (g/kg body weight)
DB1073	Non-degradable	Non-mutagen	Non-toxic	Irritant	6.457	0.190
ZINC40738189	Non-degradable	Non-mutagen	Non-toxic	Irritant	31.725	0.709
ZINC34920112	Non-degradable	Non-mutagen	Non-toxic	Irritant	38.353	0.256
ZINC62088815	Non-degradable	Non-mutagen	Non-toxic	Irritant	40.328	0.608
ZINC04773655	Non-degradable	Non-mutagen	Non-toxic	Irritant	1.184	0.578
ZINC03869248	Non-degradable	Non-mutagen	Non-toxic	Irritant	137.789	0.154

5. Conclusion

The presented investigation provides insight into the structural properties of human STAT3 and defines the active binding sites. Docking studies confirmed that human STAT3 protein has reasonably good binding properties with considered drug candidates. Further investigations with these drug candidates may prove to be a novel therapeutic treatment for prevention, mitigation and cure of psoriasis.

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