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MODELING AND DOCKING STUDIES OF SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION 4 (STAT4) PROTEIN INVOLVED IN CANCER

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ABSTRACT : In order to identify a better drug for cancer, Signal Transducers and Activators of Transcription 4 (STAT4) protein was selected as target. A three dimensional (3D) model of the STAT4 is generated based on the crystal structure of 1Y1U template by using Modeller software. With the aid of the molecular mechanics and molecular dynamics methods, the final model is obtained and is further assessed by Procheck and Verify 3D graph programs, which showed that the final refined model is reliable. With this model, a flexible docking study is performed with different drugs and the results indicate that Flurbiprofen is a more preferred inhibitor than other drugs, and that forms hydrogen bond with the STAT4 which is in good agreement with the experimental results. From the docking studies, we also suggest that MET3, ARG4, THR5 in STAT4 domain are three important residues in binding. The hydrogen bonding interactions play an important role for stability of the complex. Our results may be helpful for further experimental investigations

Key words: Cancer, Docking studies, Flurbiprofen, Modeling, Signal Transducers and Activators of Transcription Protein 4.

INTRODUCTION

Signal transducer and activator of transcription (STAT) family consists of six different transcription factors, which play an important role in cytokine signalling were first discovered by James Darnell in1993 (Shuai et al 1993, Levy et al 2002). STAT protein consists of a DNA-binding domain, a linker, an SH2 domain, and a trans activation domain (TAD). In TAD, tyrosine and serine phosphorylation sites are present that are needed for the activation of STAT. Although both serine and tyrosine phosphorylation of STAT4 are needed for full activation, evidence indicates that STAT4 contains a single serine phosphorylation site at position 727, which has no effect on its ability to bind to DNA. Among transcriptors STAT4 is important, as it plays a major role in tumorigenesis. STAT4 was discovered simultaneously by two different groups in 1994 (Zhong et al 1994, Akira .S et al 1994). Interleukin-6 (IL-6), leukemia inhibitory factor, oncostatin M, and the ciliary neurotrophic factor (CNTF) family of cytokines, mediate their signal through the gp130 protein which activates acute-phase response factor (Akira.S et al 1994). Engagement of cell-surface cytokine receptors activates the janus kinase (JAK) family of protein kinases, which in turn phosphorylates and activates latent cytoplasmic STAT4 protein to an active dimer capable of translocating to the nucleus and inducing transcription of specific target genes. Although four different members of the JAK family have been described (JAK1, JAK2, JAK3, and TYK2), JAK2 is one of the major mediators of STAT4 phosphorylation. Several other kinases have been implicated in the phosphorylation of STAT4, including members of the Src family (hck, src), Erb B1, Erb B2, anaplastic lymphoma kinase, protein kinase C (PKC), c-fes, gp130, and epithelial growth factor (EGF) receptor (Schreiner. S.J et al 2002, Ren. Z et al 2002, Garcia. R et al 2001, Zhang. Y et al 2000, Jain et al 1999, Sellers et al 1999, Nelson et al 1998).

Various studies also indicate that ERKs, 13JNK, 14p38 mitogen activated protein kinase and PKC9 participate in serine phosphorylation of STAT4.

Blockers of STAT include small peptides, oligonucleotides, (Nagel-wolfrum K.C et al 2004, Flowers et al 2005) and small molecules. Turkson et al. identified phosphotyrosyl peptides that block STAT4-mediated DNA-binding activity, gene regulation, and cell transformation (Turkson et al 2001). These include curcumin, (Bharti et al 2003, Chakravarthi et al 2006 resveratrol, (Wung et al 2005) curcurbitacin, (Blaskovich et al 2003) indirubin, (Nam. S et al 2005) piceatannol, (Su. L et al 2000) parthenolide, (Sobota R et al 2000) flavopiridol, (Lee et al 2006) magnolol, (Chen et al 2006) and epigallocatechin-3-gallate(Masuda et al 2001). How these agents suppress STAT activation is not fully understood. Curcumin, a well-established chemopreventive agent, has been shown to inhibit JAK2, (Natarajan et al 2002) Src, (Reddy. S et al 1994) Erb2, (Hong et al 1999) and EGFR, (Korutla et al 1994) all of which are implicated in STAT activation. Furthermore, curcumin has been shown to downregulate the expression of Bcl-xL, cyclin D1, VEGF, and TNF (Shishodia et al 2005) all of which are known to be regulated by STAT. A recent study by Kim et al. has shown that curcumin phosphorylates SHP-2, which in turn associates with JAK1 and JAK2, thus inhibiting initiation of the JAK–STAT pathway (Kim. H.Y et al 2003). Various small molecules that block STAT3 include 15-PGJ2, (Nikitakis et al 2002) platinum complex, (Turkson et al 2005) ethanol, (Chen. J et al 1999) sodium salicylate, (Wang. Z et al 2002) retinoic acid, (Zancai et al 2004) atiprimod, (Amit-Vazina et al 2005) PS-341, (Hideshima et al 2003) and statins. (Arnaud et al 2005). Several plant polyphenols have been identified that can suppress STAT activation. Thus, pharmacologically safe and effective therapeutic agents that can block constitutive or inducible activation of STAT4 have potential for efficacy in treatment of cancer. Given that growing evidence implicates a number of important STAT4 target genes in the formation of tumors, (Bromberg et al 2002) it seems logical to conclude that inhibition of STAT4 through pharmacological blockage of upstream molecules, such as Src and JAK may reduce tumor formation.

METHODOLOGY

3D model building

The initial model of Signal transducers and activators of transcription 4 (STAT4) protein was built by using homology-modeling methods and the MODELLER software; a program for comparative protein structure modeling optimally satisfying spatial restraints derived from the alignment and expressed as probability density functions (pdfs) for the features restrained. The pdfs restrain C^{α}-C^{α} distances, main-chain N-O distances, main-chain and side-chain dihedral angles. The 3D model of a protein is obtained by optimization of the molecular pdf such that the model violates the input restraints as little as possible. The molecular pdf is derived as a combination of pdfs restraining individual spatial features of the whole molecule. The optimization procedure is a variable target function method that applies the conjugate gradients algorithm to positions of all non-hydrogen atoms. The query sequence from Homo sapiens was submitted to domain fishing server Signal transducer and activator of transcription 4 prediction. The predicted domain was searched to find out the related protein structure to be used as a template by the BLAST (Basic Local Alignment Search Tool) program against PDB (Protein Databank). Sequence that showed maximum identity with high score and less e-value were aligned and was used as a reference structure to build a 3D model for Signal transducer and activator of transcription 4.

The co-ordinates for the structurally conserved regions (SCRs) for Signal transducer and activator of transcription 4 were assigned from the template using multiple sequence alignment, based on the Needleman-Wunsch algorithm. The structure having the least modeller objective function, obtained from the modeller was improved by molecular dynamics and equilibration methods using NAMD 2.5 software using CHARMM27 force field for lipids and proteins along with the TIP3P model for water. The energy of the structure was minimized with 1, 00, 00 steps. A cutoff of 12 Å (switching function starting at 10 Å) for van der Waals interactions was assumed. No periodic boundary conditions were included in this study.

An integration time step of 2 fs was used, permitting a multiple time-stepping algorithm to be employed in which interactions involving covalent bonds were computed every time step, short-range non bonded interactions were computed every two time steps, and long-range electrostatic forces were computed every four time steps. The pair list of the non bonded interaction was recalculated every ten time steps with a pair list distance of 13.5 Å. The short-range non bonded interactions were defined as van der Waals and electrostatics interactions between particles within 12 Å. A smoothing function was employed for the van der Waals interactions at a distance of 10 Å. CHARMM27 [force-field parameters were used in all simulations in this study. The equilibrated system was simulated for 1 ps with a 500 kcal/mol/Å2 restraint on the protein backbone under 1 atm constant pressure and 310 K constant temperature (NPT) and the Langevin damping coefficient was set to 5 ps unless otherwise stated. Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies. In this step, the quality of the initial model was improved. The final structure obtained was analyzed by Ramachandran's map using PROCHECK (Programs to check the Stereo chemical Quality of Protein Structures) and environment profile using ERRAT graph (Structure Evaluation server). This model was used for the identification of active site and for docking of the substrate with the enzyme.

Active site Identification

Active site of Signal transducer and activator of transcription 4 was identified using CASTp server. A new program, CASTp, for automatically locating and measuring protein pockets and cavities, is based on precise computational geometry methods, including alpha shape and discrete flow theory. CASTp identifies and measures pockets and pocket mouth openings, as well as cavities. The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings.

Docking method

Docking was carried out using GOLD (Genetic Optimization of Ligand Docking) software which is based on genetic algorithm (GA). This method allows as partial flexibility of protein and full flexibility of ligand. The compounds are docked to the active site of the STAT4. The interaction of these compounds with the active site residues are thoroughly studied using molecular mechanics calculations. The parameters used for GA were population size (100), selection pressure (1.1), number of operations (10,000), number of island (1) and niche size (2). Operator parameters for crossover, mutation and migration were set to 100, 100 and 10 respectively. Default cutoff values of 3.0 A° (dH-X) for hydrogen bonds and 6.0 A° for vanderwaals were employed. During docking, the default algorithm speed was selected and the ligand binding site in the STAT4 was defined within a 10 A° radius with the centroid as CE atom of LEU239. The number of poses for each inhibitor was set 100, and early termination was allowed if the top three bound conformations of a ligand were within 1.5A° RMSD. After docking, the individual binding poses of each drug were observed and their interactions with the protein were studied. The best and most energetically favorable conformation of each drug was selected.

Gold Score fitness function:

Gold Score performs a force field based scoring function and is made up of four components: 1. Protein-ligand hydrogen bond energy (external H-bond); 2. Protein-ligand vander Waals energy (external vdw); 3. Ligand internal vander Waals energy (internal vdw); 4. Ligand intramolecular hydrogen bond energy (internal- H-bond). The external vdw score is multiplied by a factor of 1.375 when the total fitness score is computed. This is an empirical correction to encourage protein-ligand hydrophobic contact. The fitness function has been optimized for the prediction of ligand binding positions.

GoldScore = S (hb_ext) + S (vdw_ext) + S (hb_int) + S (vdw_int)

Where S (hb_ext) is the protein-ligand hydrogen bond score, S (vdw_ext) is the protein-ligand van der Waals score, S (hb_int) is the score from intramolecular hydrogen bond in the ligand and S (vdw_int) is the score from intramolecular strain in the ligand.

RESULTS AND DISCUSSION

Homology Modeling of STAT4 Protein (Q14765)

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only two-reference proteins, including 1Y1U A (Chain A, Structure Of Unphosphorylated Stat5a) has a high level of sequence identity and the identity of the reference protein with the domain are 31%. Structurally conserved regions (SCRs) for the model and the template were determined by super imposition of the two structures and multiple sequence alignment. In the following study, we have chosen 1Y1U A as a reference structure for modeling domain. Coordinates from the reference protein (1Y1U A) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints. In the modeler we will get a 20 PDB out of which we select a least energy. All side chains of the model protein were set by rotamers. The final stable structure of the Signal transducer and activator of transcription 4 protein obtained is shown in Fig I.

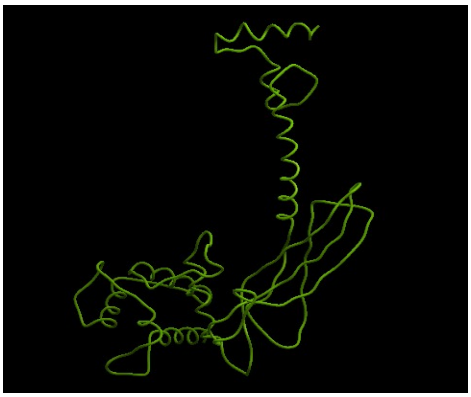

Fig I: 3D structure of STAT4 generated by Modeller7v7

By the help of SPDBV it is evident that Signal transducer and activator of transcription 4 domain has 7 helices and 10 sheets and it is shown in the Figure II.

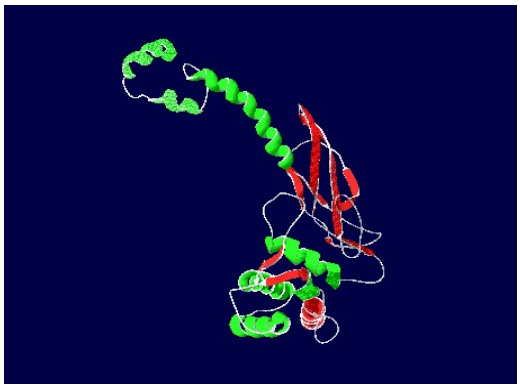

Fig II: Protein with helices and sheets

The structure having the least energy with low RMSD (Root Mean Square Deviation) which was obtained by the NAMD is in water molecule (TIP3) (Fig III).

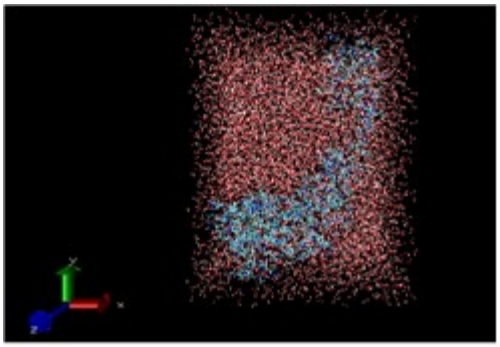

Fig III: STAT4 with water molecule (TIP3)

The final structure was further checked by verify 3D graph and the results have been shown in Fig IV. The overall scores indicates acceptable protein environment.

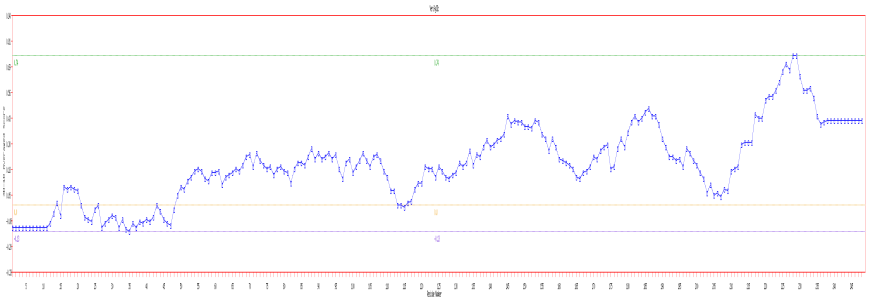

Fig IV: The 3D profiles verified results of Signal transducer and activator of transcription 4 model; overall quality score indicates residues are reasonably folded.

Validation of Domain

After the refinement process, validation of the model was carried out using Ramachandran plot calculations computed with the PROCHECK program. The π and ψ distributions of the Ramachandran plots of non-glycine, non-proline residues are summarized in Table I. The RMSD (Root Mean Square deviation) deviation for covalent bonds and covalent angles relative to the standard dictionary of Signal transducer and activator of transcription 4 was -3.27 and -0.65 Å. Altogether 89.7% of the residues of Signal transducer and activator of transcription 4 was in favored and allowed regions. The overall PROCHECK G-factor of Signal transducer and activator of transcription 4 was – 1.25 and verify3D environment profile was good (Fig V).

Table I: Ramachandran plot Analysis

Residues in most Favourable regions	89.7%
Residues in additional allowed regions	9.0%
Residues in generously allowed regions	1.3%
Residues in disallowed regions	0.0%
Total number of Residues	100.0%

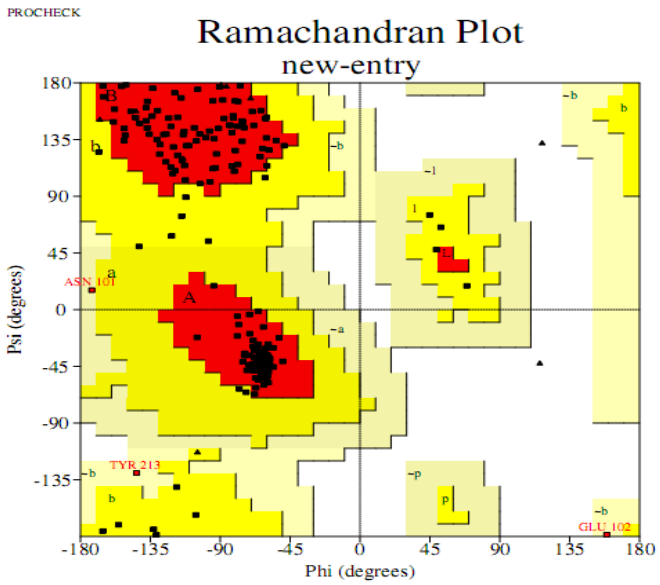

Figure V: Ramachandran Plot

Superimposition of 1Y1UA with Signal transducer and activator of transcription 4 domain

The structural super imposition of C trace of template and Signal transducer and activator of transcription 4 is shown in Fig VI. The weighted root mean square deviation of C α trace between the template and final refined models 1.84A°. This final refined model was used for the identification of active site and for docking of the substrate with the domain Signal transducer and activator of transcription 4.

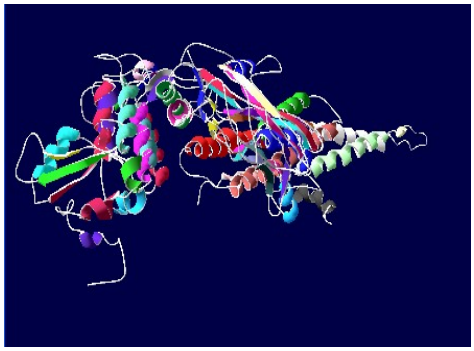

Fig VI: Super imposition of STAT4 and 1Y1U

Active site Identification of Signal transducer and activator of transcription 4 domain

After the final model was built, the possible binding sites of Signal transducer and activator of transcription 4 was searched based on the structural comparison of template and the model build and also with CASTp server and was shown in Figure VII. Since, Signal transducer and activator of transcription 4 and the 1Y1UA are well conserved in both sequence and structure; their biological function should be identical. It was found that secondary structures are highly conserved and the residues., ILE 153, LEU 179, VAL 182, MET 183, GLN 186, PHE 187, LEU 195, GLN 199, MET 202, LEU 203, PHE 223, TRY 236, TRP 238, LEU 239, GLU 240, ILE 242, LEU 243, ILE 246.

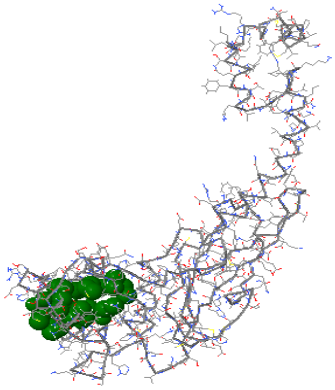

Fig VII: Active site of STAT4

The Ligand (inhibitor) molecules used for Docking studies

By modifying ligand molecules, 12 inhibitors are designed and they are listed below (Fig VIII- Fig XIX). The chemical properties of each drug are given in the Table II.

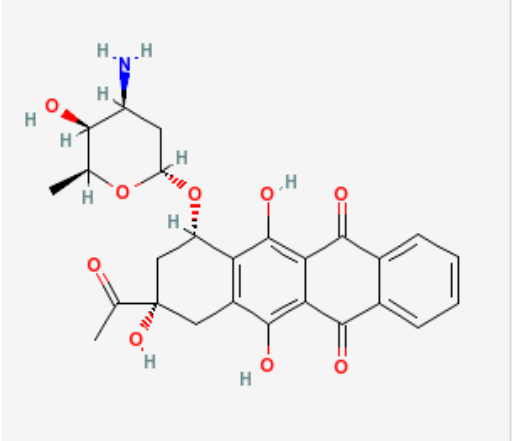

Fig VIII: Structure of Idarubicin

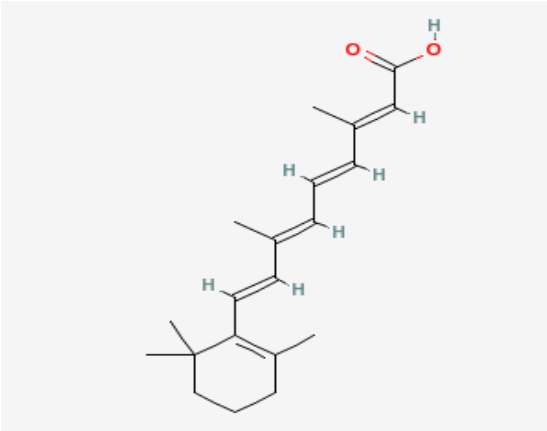

Fig IX: Structure of Tretinoin

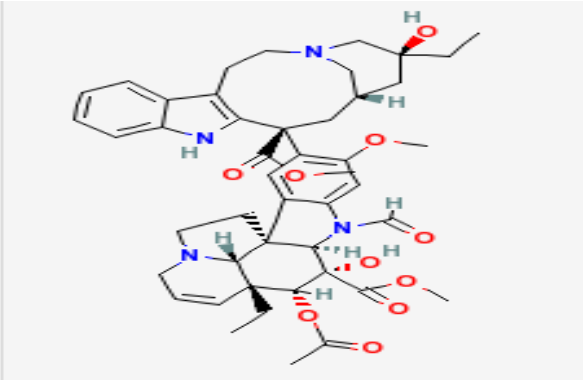

Fig X: Structure of Oncovin

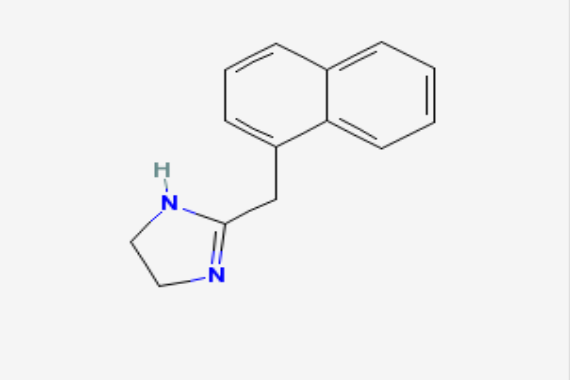

Fig XI: Structure of Naphazoline

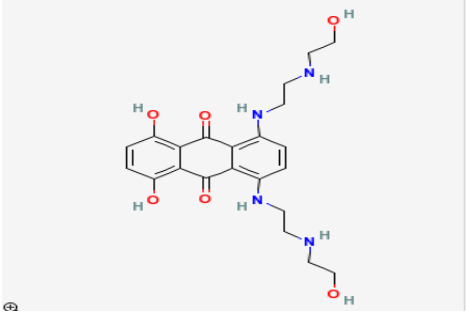

Fig XII: Structure of Mitoxantrone

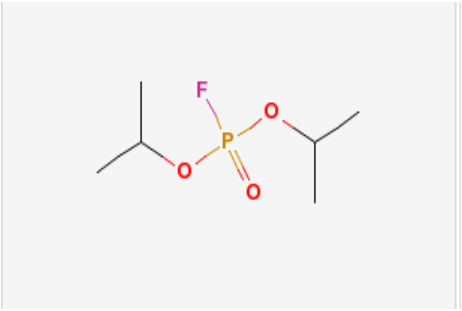

Fig XIII: Structure of Isoflurophate

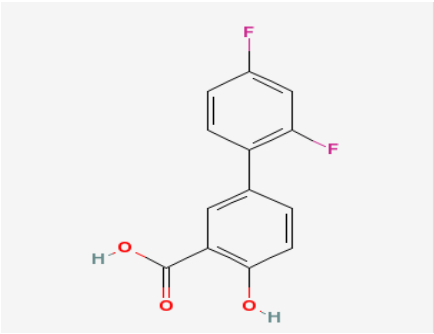

Fig XIV: Structure of Diflunisal

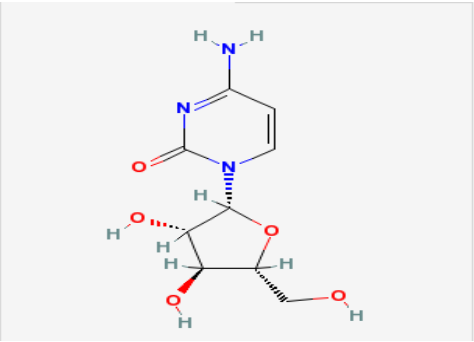

Fig XV: Structure of Cytosar

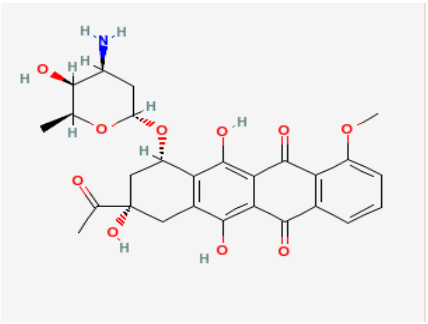

Fig XVI: Structure of Cerubidine

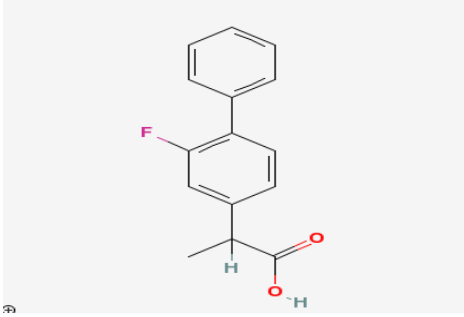

Fig XVII: Structure of Flurbiprofen

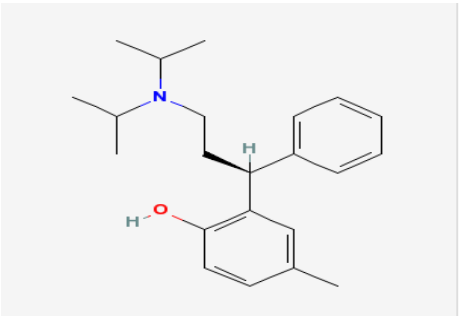

Fig XVIII: Structure of Tolterodine

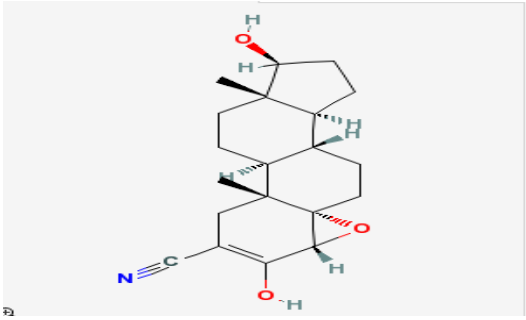

Fig XIX: Structure of Trilostane

Table II: The Chemical Properties these Structures are tabulated as follows

S.NO	Molecular formula	Formula weight	Molar refractivity cm^3	Index of refraction	Densityg/cm^3	Polarisability $10^{-24}cm^3$
1	$C_{26}H_{27}NO_9$	497.49388	123.61 ± 0.4	1.705 ± 0.03	1.56 ± 0.1	49.00 ± 0.5
2	$C_{20}H_{28}O_2$	300.43512	95.52 ± 0.3	1.556 ± 0.02	1.011 ± 0.06	37.87 ± 0.5
3	$C_{46}H_{56}N_4O_{10}$	824.95764	221.08 ± 0.4	1.677 ± 0.03	1.40 ± 0.1	87.64 ± 0.5
4	$C_{16}H_{20}N_2$	240.3434	75.91 ± 0.3	1.556 ± 0.02	1.018 ± 0.06	30.09 ± 0.5
5	$C_{22}H_{28}N_4O_6$	444.48092	119.70 ± 0.3	1.709 ± 0.02	1.450 ± 0.06	47.45 ± 0.5
6	$C_6H_{14}FO_3P$	184.1457252	40.29 ± 0.3	1.388 ± 0.02	1.079 ± 0.06	15.97 ± 0.5
7	$C_{13}H_8F_2O_3$	250.1976264	59.64 ± 0.3	1.601 ± 0.02	1.437 ± 0.06	23.64 ± 0.5
8	$C_9H_{13}N_3O_5$	243.21662	52.64 ± 0.5	1.756 ± 0.05	1.89 ± 0.1	20.86 ± 0.5
9	$C_{27}H_{29}NO_{10}$	527.51986	129.98 ± 0.4	1.691 ± 0.03	1.55 ± 0.1	51.52 ± 0.5
10	$C_{15}H_{13}FO_2$	244.2609232	66.58 ± 0.3	1.567 ± 002	1.199 ± 0.06	26.39 ± 0.5
11	$C_{21}H_{29}N$	295.46166	96.27 ± 0.3	1.534 ± 0.02	0.954 ± 0.06	38.16 ± 0.5
12	$C_{21}H_{31}NO_3$	345.47574	93.74 ± 0.4	1.586 ± 0.03	1.23 ± 0.1	37.16 ± 0.5

Docking of inhibitors with the active site of Signal transducer and activator of transcription 4

Docking of the inhibitors with Signal transducer and activator of transcription 4 was performed using GOLD 3.0.1, which is based on Genetic algorithimn. This program generates an ensemble of different rigid body orientations (poses) for each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site. Poses clashing with this 'bump map' are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function. We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a cocrystalized ligand by 4 Å. This dimension was considered here appropriate to allow, for instance, compounds larger than the cocrystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. The compounds used for docking was converted in 3D with SILVER. To this set, the substrate corresponding to the modeled protein were added (Fig XX- Fig XXXI). From the total docking scores, Flurbiprofen was identified as best inhibitor of STAT4 (Table III).

Table III: Docking results of the drugs with STAT4

S.NO.	Drug	Dockingvalue (K.Cal/mol)
1	Idarubicin	25.87
2	Tretinoin	9.37
3	Oncovin	13.42
4	Naphazoline	36.21
5	Mitoxantrone	1.42
6	Isoflurophate	24.01
7	Diflunisal	38.65
8	Cytosar	36.94
9	Cerubidine	24.19
10	**Flurbiprofen**	**39.66**
11	Tolterodine	4.45
12	Trilostane	10.35

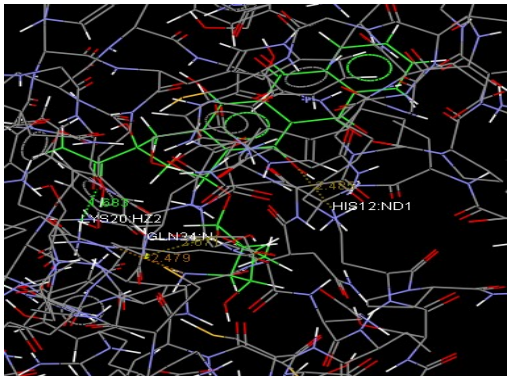

Fig XX: Docking of Idarubicin with STAT4

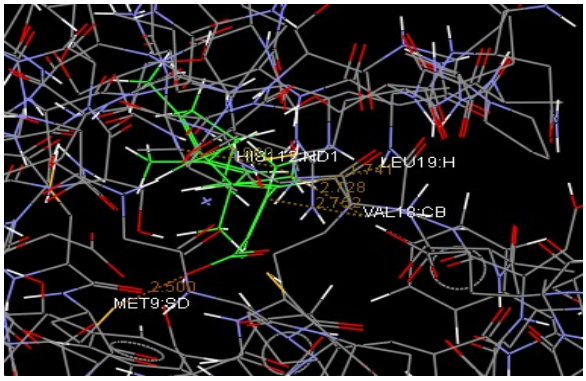

Fig XXI: Docking of Tretinoin with STAT4

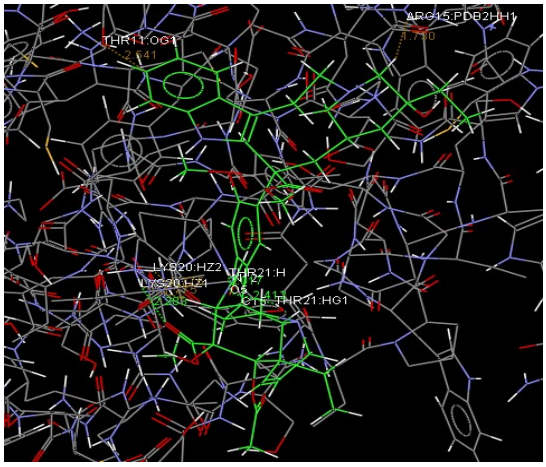

Fig XXII: Docking of Oncovin with STAT4

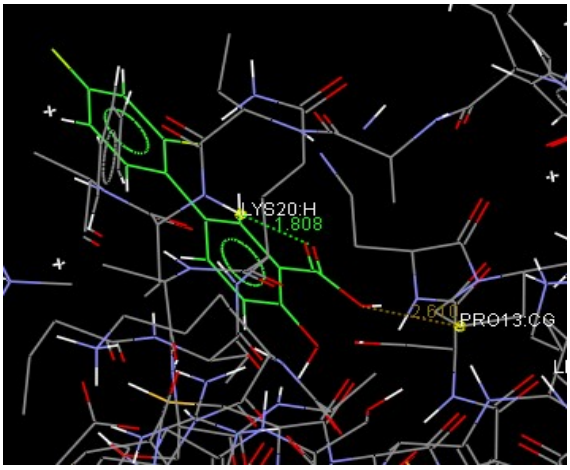

Fig XXIII: Docking of Naphazoline with STAT4

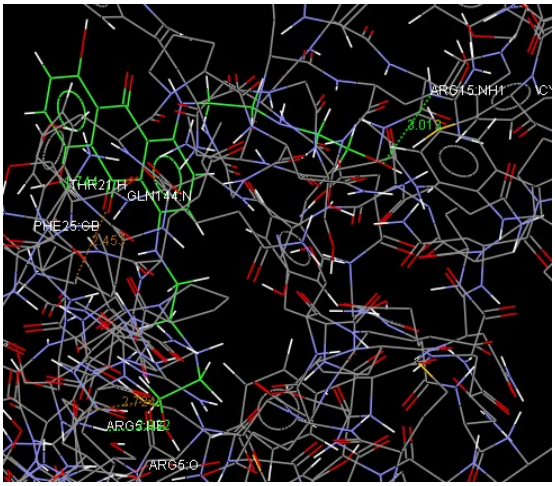

Fig XXIV: Docking of Mitoxantrone with STAT4

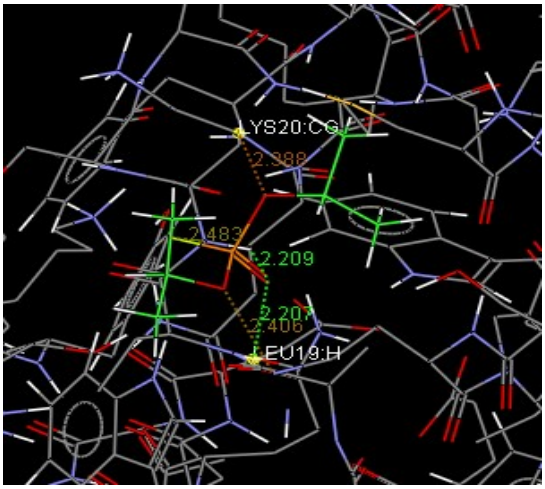

Fig XXV: Docking of Isoflurophate with STAT4

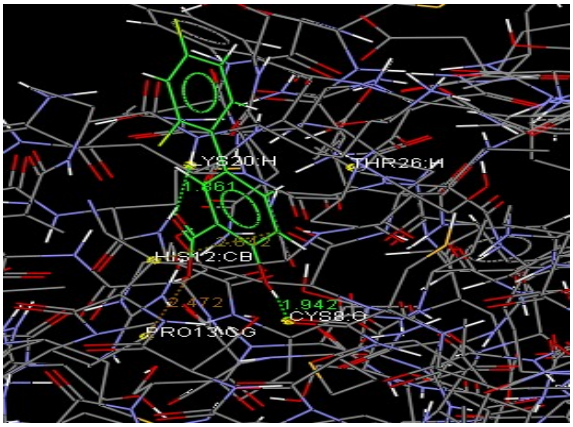

Fig XXVI: Docking of Diflunisal with STAT4

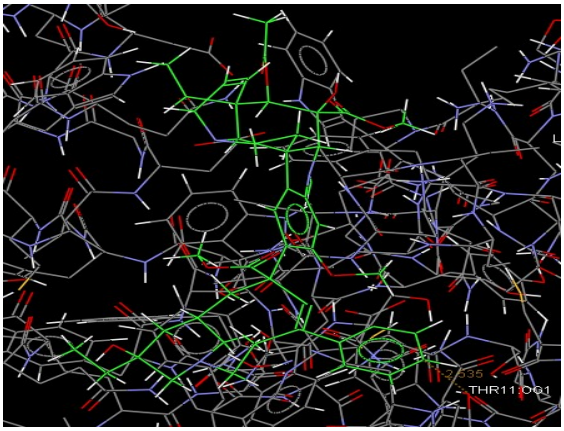

Fig XXVII: Docking of Cytosar with STAT4

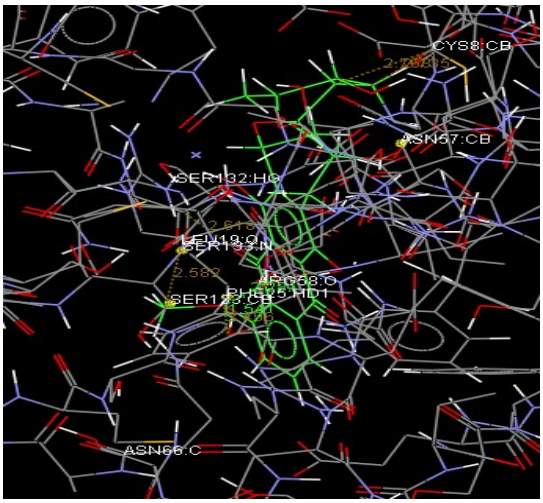

Fig XXVIII: Docking of Cerubidine with STAT4

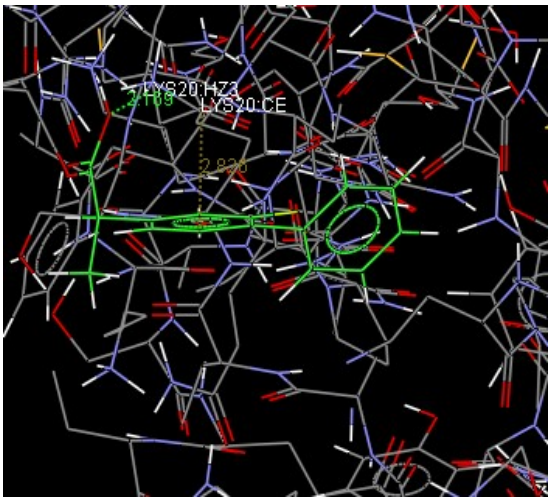

Fig XXIX: Docking of Flurbiprofen with STAT4

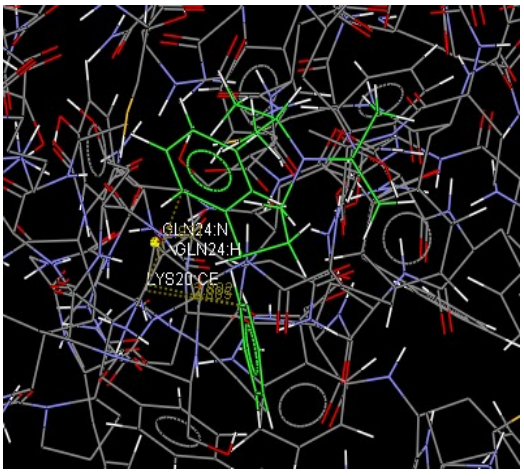

Fig XXX: Docking of Tolterodine with STAT4

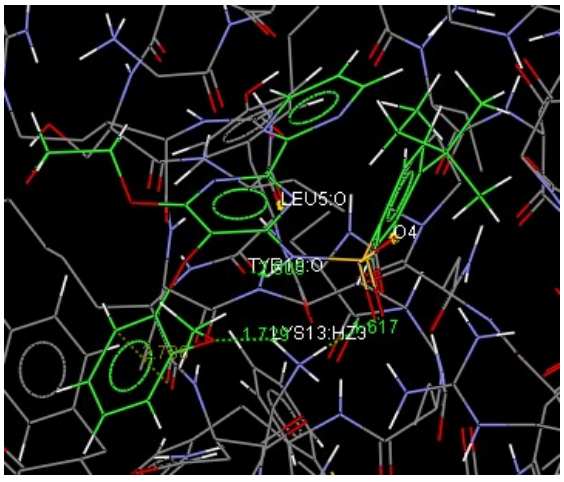

Fig XXXI: Docking of Trilostane with STAT4

CONCLUSION

STATs (signal transducers and activators of transcription) is a member of a recently identified family of transcription factors that activate gene transcription in response to a number of different cytokines in Homo sapiens. In this work, we have constructed a 3D model of STAT4 domain, using the MODELLER software and obtained a refined model after energy minimization. The final refined model was further assessed by ERRAT and PROCHECK program, and the results show that this model is reliable. The stable structure of STAT4 is further used for docking with modified ligand molecules. Docking results indicate that conserved amino-acid residues Signal transducer and activator of transcription 4 main play an important role in maintaining a functional conformation and are directly involved in donor substrate binding. The interaction between the domain and the inhibitors proposed in this study are useful for understanding the potential mechanism of domain and the inhibitor binding. As is well known, hydrogen bonds play important role for the structure and function of biological molecules. In this study it was found that, ILE 153, LEU 179, VAL 182, MET 183, GLN 186, PHE 187, LEU 195, GLN 199, MET 202, LEU 203, PHE 223, TRY 236, TRP 238, LEU 239, GLU 240, ILE 242, LEU 243, ILE 246 are important for strong hydrogen bonding interaction with the inhibitors. To the best of our knowledge MET1, MET3, ARG4, THR5 are conserved in this domain and may be important for structural integrity or maintaining the hydrophobicity of the inhibitor-binding pocket. The molecule Flurbiprofen showed best docking results with target protein.

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