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Thalidomide-based inhibitor for TNF-α: designing and *Insilico* evaluation

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Abstract

Background: Inflammatory diseases are the vast array of disorders caused by inflammation. During most inflammatory events, many cytokines expressions were modulated, and one such cytokine is tumor necrosis factor-alpha (TNF-α). TNF-α is mainly secreted by monocytes and macrophages. Notably, it has been proposed as a therapeutic target for several diseases. The anti-TNF biology approach is mainly based on monoclonal antibodies. The fusion protein and biosimilars are prevalent in treating inflammation for decades. Only a few small molecule inhibitors are available to inhibit the expression of TNF-α, and one such promising drug was thalidomide. Therefore, the study was carried out to design thalidomide-based small molecule inhibitors for TNF-α. The main objective of our study is to design thalidomide analogs to inhibit TNF-α using the *insilico* approach.

Results: Several thalidomide analogs were designed using chemsketch. After filtration of compounds through 'Lipinski rule of 5' by Molinspiration tool, as a result, five compounds were selected. All these compounds were subjected to molecular docking, and the study showed that all five compounds had good binding energy. However, based on ADMET predictions, two compounds (S3 and S5) were eliminated.

Conclusions: Our preliminary results suggest that S1, S2, S4 compounds showed potential ligand binding capacity with TNF-α and, interestingly, with limited or no toxicity. Our preliminary investigation and obtained results have fashioned more interest for further *in vitro* studies.

Keywords: Inflammatory diseases, TNF-α, *In-silico*, Small molecules, Cytokines thalidomide

Background

Inflammation is a response to tissue injury or infection. It is characterized in its acute phase by increasing vascular permeability by plasma extravasation, resulting in an accumulation of fluid, leucocytes, and mediators to the inflammatory site [1, 2]. Inflammatory diseases are the vast array of disorders and conditions caused by inflammation, including allergy, asthma, autoimmune diseases, coeliac disease, glomerulonephritis, hepatitis, inflammatory bowel disease, reperfusion injury, and transplant rejection, etc. [3]. During most of these events, many cytokines expressions were modulated, and one such cytokine is tumor necrosis factor alpha (TNF-α). TNF-α is among one of the pleiotropic cytokines, mainly secreted by monocytes and macrophages, TNF-α has been shown to play a pivotal role in orchestrating the cytokine cascade in many inflammatory diseases, and it is known to be "master-regulator" of inflammatory cytokine production. Notably, it has been proposed as a therapeutic target for several diseases [4]. TNF-α is a multidimensional cytokine protein that modulates various cellular functions, including survival, proliferation, and cell death [5]. The anti-TNF biology approach is mainly based on monoclonal antibodies (mAbs); the fusion protein and biosimilars are prevalent in treating inflammation for a decade. Small molecule inhibitors are likely to be cheaper to produce than mAbs or Ig-Fc-based fusion proteins [6]. Currently, few small molecule inhibitors have come to light. However, not even a single small

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molecule antagonist for TNF-α is released into the market. Therefore, the main objective of the present study is to design small molecules to inhibit TNF-α using the *insilico* method and evaluation of drug likeness.

Before designing a new molecule, thoroughly reviewed and studied all the existing small molecule drugs, inhibiting TNF-alpha directly and indirectly. After a thorough review, thalidomide was selected. Thalidomide derivative treats multiple diseases like scleroderma, Crohn's disease, and refractory multiple myeloma cutaneous lupus [7, 8]. Thalidomide (2-2,6-dioxo-3-piperidyl)isoindoline-1,3-dione) was a synthetic glutamic acid derivative and first synthesized by "Chemie Grunenthal" a German pharmaceutical company, in 1954 to treat morning sickness in pregnant women [9]. But due to its teratogenic effects, thalidomide was withdrawn from the world market [10]. Thalidomide was reintroduced to the market in 1998 and started clinical research to understand anti-inflammatory and immunomodulatory roles [11, 12]. Thalidomide was a well-recognized inhibitor of TNF-α expression [13, 14]. The overexpression of TNF-α leads to change in physiological conditions in various inflammatory diseases. Thalidomide blocks the production of TNF-α in many disease conditions without inducing immune suppression compared with glucocorticoid and cyclosporine [15]. The long-term objective of our study is to address the exiting demerits associated with thalidomide molecules for being used as drug molecule in the management of diseases. In the present study, novel thalidomide analogs were designed having better binding activities, improved drug potency and conversely reducing the side effects. The study also reports designed small molecule inhibitors have reduced teratogenic effect and more drug effectiveness with greater specificity. Further, our studies are under progress for *In vitro* validation.

Methods

Structure preparation of TNF-α
The crystal structure of TNF-α (PDB ID: 2az5) was downloaded from Protein Data Bank (PDB) [16]. Further, characterization and validation, stereochemistry, and structural geometry were accomplished using structure analysis and verification server (SAVES) (https://servicesn.mbi.ucla.edu/SAVES/).

Active site prediction of TNF-α
Active sites of targeted protein TNF-α were analyzed using PDBsum of RCSB server (www.ebi.ac.uk/pdbsum/) [17]. In this approach, the 2az5 protein structure was studied to identify amino acid residues, which were interacting with the inhibitory compound SPD-304 as revealed by PDBsum from the protein–ligand interaction map.

Referral drug selection
Referral drugs were selected through extensive literature survey and analyzed through TTD (Therapeutic Target Database), Uniprot, and drug bank databases. Only those drugs that are capable of inhibiting TNF-α associated with inflammatory diseases are selected for the study. In this approach, Thalidomide and SPD-304 were selected because they can inhibit the expression of TNF-α [18, 19].

Ligand preparation
Several analogs were designed manually based on the scaffold structure of Thalidomide using ACD (Advanced Chemistry Development)/ChemSketch [20] software and saved in mol format. The pharmacophore and pharmacokinetics properties of ligand molecules were studied using Hyperchem Professional 7.0 [21] and molinspiration (https://www.molinspiration.com/cgi-bin/properties). Further, the resultant ligand molecules were used for molecular docking studies.

Molecular docking
AutoDock Tool 1.5.6 program was used to perform molecular docking studies [22, 23]. A grid box was prepared to cover all the active sites/ligand binding sites of the protein. For running docking and molecular simulation, the Lamarckian genetic algorithm is used. Each simulation is carried out ten times, which ultimately yielded ten docking conformations. Further, during simulation, the best binding conformations are selected based on the least energy conformations.

ADMET analysis
ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) are calculated by using admetSAR (http://lmmd.ecust.edu.cn/admetsar/) web server [24]. It provides various parameters such as blood–brain barrier (BBB+), human intestinal absorption (HIA+), Caco-2 permeability (Caco2-), P-glycoprotein Substrate (logpGI), P-glycoprotein inhibitor (logGI), aqueous solubility (logs), and Probability of Caco-2 permeability (log Papp, cm/s) as well as toxicity also calculated as AMES toxicity, carcinogenicity and lethal dose 50 (LD50) in the rat.

Results

Structure preparation of TNF-α
The 3D structure of TNF-α with PDB ID: 2az5 was fetched from Protein Data Bank with a resolution of 3.0 Å and is ligand binded PDB structure. This was selected after inspecting all available TNF-α PDB structures. A Co-crystalized TNF-α trimer with a

ligand binded structure was not found and also having mutations in structures. The TNF-α dimer structure was found binded with ligand SPD-304. This structure provides active sites for *insilico* work. Using the discovery studio tool, the protein was prepared for molecular docking study by removing ligand and water molecules. SAVES online server predicts protein structure quality; in the Ramachandran plot, 89.6% of residues are in the favored region.

Active site prediction of TNF-α

PDBsum of RCSB server identified the active pockets of TNF-α molecule. The interacting amino acid residues with the ligand are selected as active sites by the ligplot shown in Fig. 1. The blue-colored chemical structure is a ligand molecule (SPD-304). The eyelids-like structures are active site amino acids.

Referral drug selection

Through literature survey thalidomide and SPD-304 potential inhibitors were selected for the study. Thalidomide indirectly inhibits the expression of TNF-α, and SPD-304 directly binds to TNF-α trimer and disassociating its subunits into dimer and monomer [16]. Those binding residues considered as active sites to build the grid box to study docking. The Referral drugs structures are shown in Fig. 2.

Ligand preparation

Several new molecules were designed based on the referral drug (Thalidomide), manually changing the functional groups, adding and deleting chemical bonds, benzene rings, etc., through ACD/ChemSketch. Canonical smiles and molecular formulas are also generated. These canonical smiles are used to predict the 'Lipinski rule of 5' [25] from molinspiration. Based on these rules, the designed compounds are filtered. As a result, a total
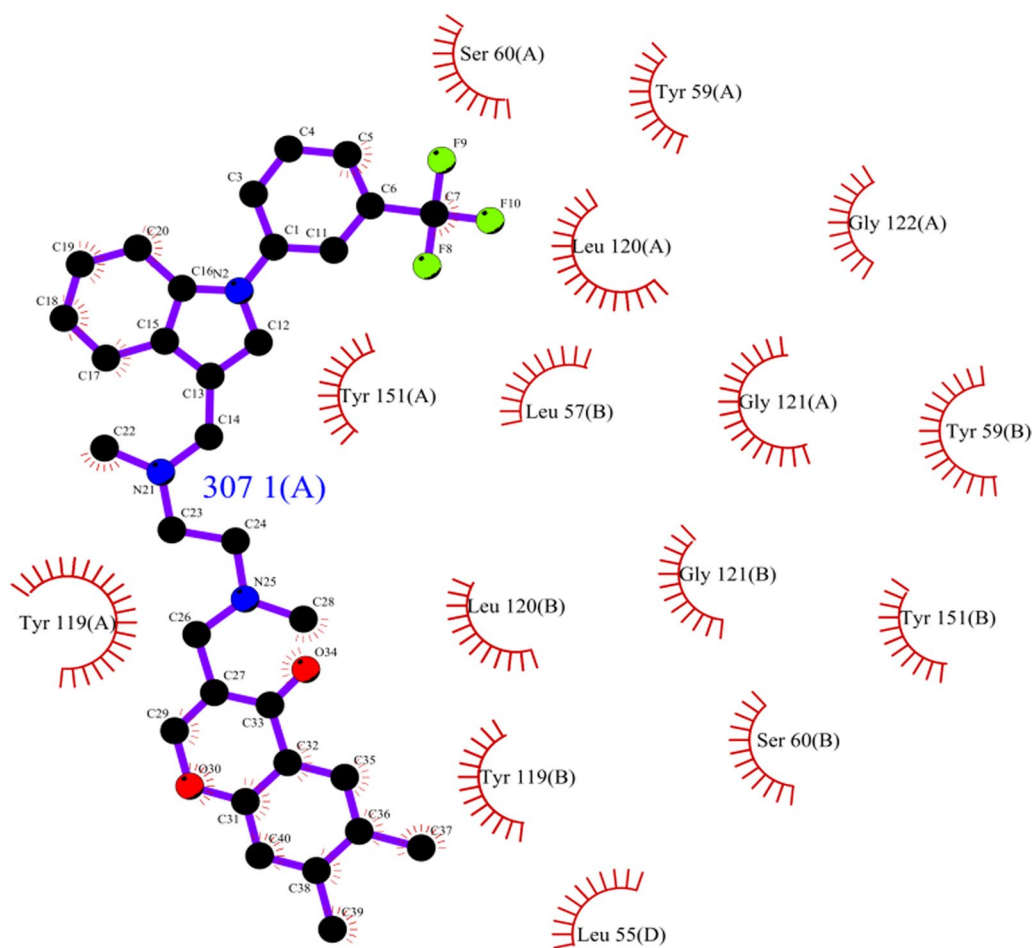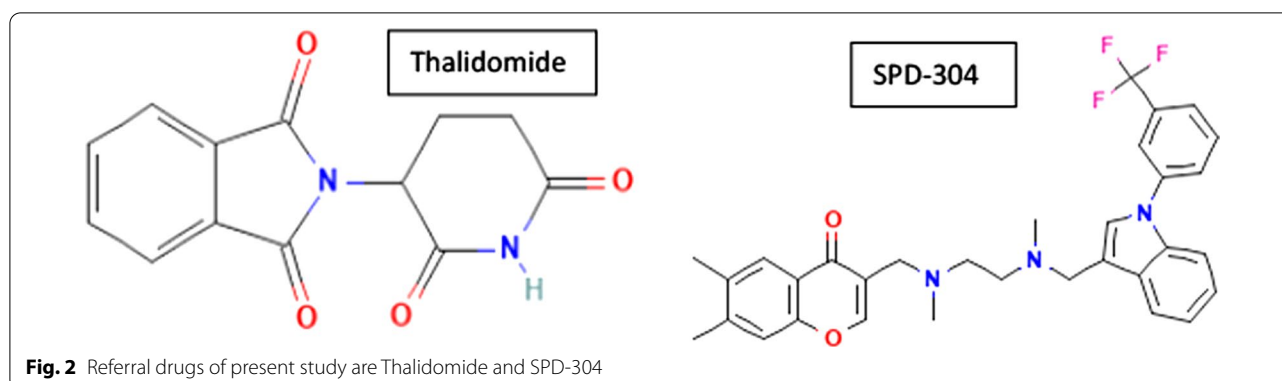

Fig. 1 Ligand (SPD-304) Protein (TNF-α) interaction plot from PDBsum

Fig. 2 Referral drugs of present study are Thalidomide and SPD-304

of five compounds were selected, which are shown in Table 1.

Molinspiration online tool was used to calculate 'Lipinski rule of 5' of ligand molecules, and it creates Pharmacophore properties of logarithm of octanol–water partitioning coefficient (LogP) −5 to 5, hydrogen bond Donor (HBD) ≤ 5, hydrogen bond acceptor (HBA) ≤ 10, number of rotatable bonds (NROTB) ≤ 12, molecular weight (MW) ≤ 500 Dalton, topological polar surface area (TPSA) ≤ 1000, and other properties like number of atoms (nAtoms), molecular volume (MV), number of violations of Lipinski's rule of five (nviolations). These values help to understand molecular descriptors of druglikeness shown in Table 2. The table illustrates that all five compounds obey the properties of the Lipinski rule of 5 with 0 violations. Both referral drugs had also undergone this step thalidomide shows 0 violations, but SPD-304 shows 2 violations. Further, new compounds are proceeding for the energy minimization step.

The energy minimizations of compounds are lead to optimize ligand molecules by hyperchem professional 7.0. [21] It was minimizing the energy of compounds by using AMBER (Assisted Model Building with Energy Refinement) force field. The energy minimization will change the molecular geometry to lower the compound's energy and yield a much more stable conformation. In minimization progression, it will search for a molecular structure in that energy will not modify with infinitesimal changes in geometry. This means that the derivative of energy regarding all Cartesian coordinates, called the gradient, is near zero. This is called a stationary point of potential energy surface [26]. Table 3 illustrated the values of before and after energy minimization. We can predict the stable structure conformation energy of all the five compounds from those values and the gradient values near zero. After energy minimization, these compounds are subjected to docking studies.
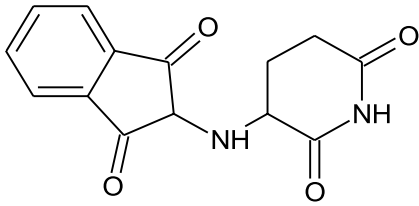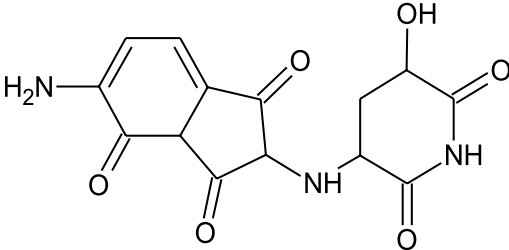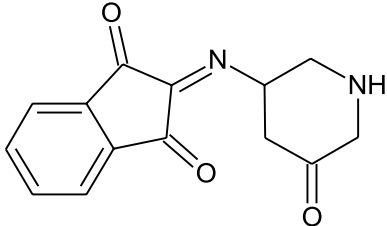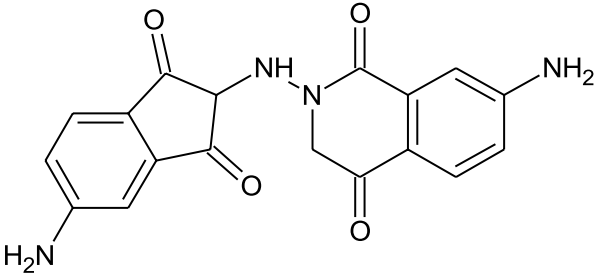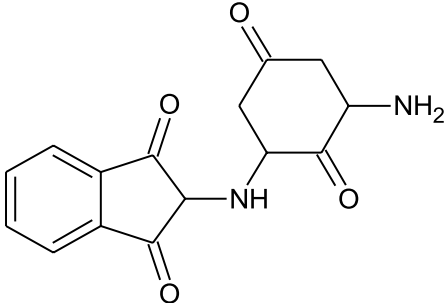
Docking result

The five thalidomide analogs and referral drug (SPD-304) were docked against the TNF-α cytokine to check the binding energy between them using Autodock 1.5.6. SPD-304 docking results demonstrated in Table 4 and Fig. 3. They show its binding mode with one hydrogen bond between Gly121 and binding energy −5.37 kcal/mol. The five compounds are showing good binding energy. However, S2, S3, S4, and S5 are showing hydrogen bond interactions. The S2 shows four hydrogen bond interactions between leu120, Gly121, Tyr151, and Ser60 with binding energy −6.24 kcal/mol, S3 shows two hydrogen bond interactions between Ser60 and Leu120 with binding energy −6.79 kcal/mol, S4 shows two hydrogen bond between Tyr119 and Lys98 with binding energy −7.53 kcal/mol, and S5 shows three hydrogen bond interaction with Ser60, Leu120, Tyr151 with binding energy −7.13 kcal/mol. Comparatively with referral drug, all five compounds that show less binding energy with more hydrogen bonds were detected. Docking results are illustrated in Table 4 and Fig. 4. Both three-dimensional (3D) and two-dimensional (2D) docked images illustrate the binding pose of the compounds in which the green dotted lines denotes hydrogen bond interactions between ligand and receptor.

ADMET analysis

Insilico ADMET prediction is a very important step in drug discovery, whereas the earlier detection of poor pharmacokinetics property and toxicity issues is very helpful for further, and it reduces the time of drug development process. The pharmacokinetic properties of the five compounds are shown in Table 5. The central nervous system (CNS) was concluded by the effectiveness of the blood/brain partition co-efficient. This concluded activity of CNS was determined from the −3 (inactive) to +1 (active) scale; all compounds are within the acceptable range. The blood–brain barrier plays an important role in managing homeostasis of the CNS, splitting

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Table 1 Designed analogs of thalidomide

Name	Molecular formula	Structure
S1	$C_{14}H_{12}N_2O_4$	
S2	$C_{14}H_{13}N_3O_6$	
S3	$C_{14}H_{12}N_2O_3$	
S4	$C_{18}H_{14}N_4O_4$	
S5	$C_{15}H_{14}N_2O_4$	

circulating blood from the brain [27]. Cell permeability Caco-2 range is from −1 (poor) to +1 (good), and all five compounds are within the range. The human intestinal absorption (HIA) of drug permeability and drug metabolism with intestinal membrane was calculated from 0 (poor) to 1 (great). All five compound are within the acceptable range of HIA. P-glycoprotein (log-PGI) substrate and non-inhibitor study are depending on drug–drug interaction within different tissues. This alters xenobiotics of effective reduction in drug absorption and intensifies the removal of drugs and moves into the liver and kidney [28]. The P-gp substrate range was −5 (poor)

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Table 2 Lipinski rule of 5 (RO5) properties predictions of 5 compounds

Ligand	Log P	TPSA	nAtoms	MW	HBA	HBD	NROTB	MV	nviolation
Thalidomide	0.34	85.25	19	258.23	6	1	1	213.91	0
SPD-304	6.36	41.62	40	547.62	5	0	9	491.08	2
S1	− 0.61	92.34	20	272.26	6	2	2	230.36	0
S2	− 3.92	155.66	23	319.27	9	5	2	258.08	0
S3	0.02	75.60	19	256.26	5	1	1	222.21	0
S4	− 0.95	135.59	26	350.33	8	5	2	291.10	0
S5	− 1.84	106.33	21	286.29	6	3	2	246.07	0

Table 3 Energy minimization values of new compounds

Ligand	Before energy minimization		After energy minimization		
	Energy	Gradient	Energy	Gradient	Total energy (kcal/mol)
S1	201.016724	64.778473	25.84063	0.095214	60.8144
S2	204.989288	62.958637	26.965887	0.081050	64.877
S3	199.691360	66.405952	27.837172	0.094678	61.4621
S4	235.531555	64.628952	30.998880	0.096454	74.0554
S5	200.725006	64.730629	25.581596	0.083197	61.5704

to + 1 (good), and the P-gp non-inhibitor range was 0 to 1. The aqueous solubility (PlogS) range was − 6.5 to − 0.5. P-gp of substrate, non-inhibitors, and PlogS are within range. The range of (logpapp) probability of Caco-2 cell permeability was − 1 to 1. Except S3, other componds are within the acceptable range. AMES toxicity test was to identify the compound is mutagenic or not. In this, S3 and S5 compounds are showing AMES toxic. All five compounds are noncarcinogenic and predicted lethal dose 50 (LD 50) in rats [29].
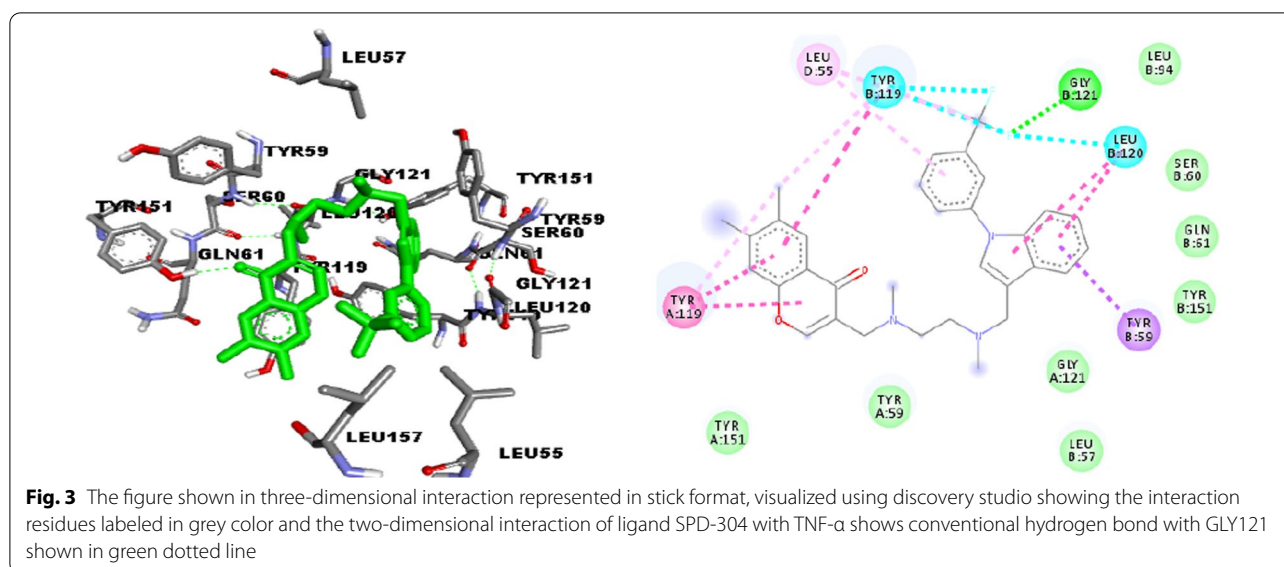
Discussion

Thalidomide was introduced as an inhibitor of TNF-α synthesis in 1991 [19], and it is used to treat numerous inflammatory diseases such as ulcerative colitis, ENL (Erythema Nodosum Leprosum), arthritis, Crohn's disease, multiple myeloma [30, 31]. Thalidomide can inhibit TNF-α through increasing mRNA degradation. Consequently, the TNF-α expression is reducing [18]. As a result, new analogs were synthesized and evaluated, aiming to enhance drug potency and reduce side effects, often referred to as IMiDs (Immunomodulatory imide drugs). Thalidomide's first analogs were produced in 1996 [32]. Thalidomide and its analogs have anti-proliferation, anti-angiogenic and anti-inflammatory activities [33]. Thalidomide analogs are 3,6'- dithiothalidomide [34], lenalidomide [35, 36], pomalidomide [35, 37], apremilast [38], adamantly phthalimidine and noradamantyl-type phthalimidine [39, 40], etc. These analogs indirectly inhibit the TNF-α as they will reduce TNF-α protein synthesis, and this interaction between analogs and TNF-α was studied through in vitro and in vivo methods. Thalidomide and its analogs are having teratogenic and neurotoxic effects [40, 41]. Because of the reported limitation of Thalidomide and its analogs, it is necessary to build new analogs of Thalidomide having less or no toxicity. In our study, we prepared different new Thalidomide analogs to behave like an antagonist for TNF-α. The direct inhibition can be accessed through the *insilico* method. Most of the existing Thalidomide and its analogs inhibit TNF-α production via binding to the IKK (IKB kinase) complex and several other proteins. In contrast, our designed Thalidomide analogs directly bind to TNF-α and bring the inhibition. Since the binding of Thalidomide to the IKK

Table 4 Molecular docking results of five new analogs and referral drug

Compounds	Rank	Run	Binding energy (Kcal/mol)	No. of H. bonds	Amino acids present in H. bond	Reference RMSD	Cluster RMSD
SPD-304	8	5	− 5.37	1	GLY121	83.14	0.0
S1	3	9	− 6.33	0	–	83.91	0.0
S2	6	9	− 6.24	4	Leu120, Gly121, Ser60,Tyr151	84.65	0.0
S3	2	1	− 6.79	2	Ser60, Leu120	83.60	0.0
S4	1	10	− 7.53	2	Tyr119, Lys98	85.05	1.56
S5	2	4	− 7.13	3	Ser60, Leu120, Tyr151	82.60	0.0

Fig. 3 The figure shown in three-dimensional interaction represented in stick format, visualized using discovery studio showing the interaction residues labeled in grey color and the two-dimensional interaction of ligand SPD-304 with TNF-α shows conventional hydrogen bond with GLY121 shown in green dotted line

complex and other proteins may indirectly inhibit several other molecular pathways and may result in unknown effects. Newly designed analogs have specificity for binding and inhibiting TNF-α. Most of these analyses were conducted using CADD (Computer-Aided Drug Design).

CADD is one of the important *insilico* methods for drug designing. It demonstrated that the drug designing tool is more efficient, saving time and money for the drug development process. The novelty of the present work is direct inhibition of TNF-α by *insilico* approach, and such inhibition is not being reported yet. In the present work, the *insilico* approach was adopted to build analogs of Thalidomide. TNF-α protein dimer structure (2AZ5) was retrieved from PDB, which binded to ligand SPD-304. These ligand binded residues are considered as active site amino acids. SPD-304 analogs were not generated because present study is to design analogs with main moiety, but SPD-304 contains toxic moiety of 3-alkylindole which is metabolized by cytochrome P450 enzymes via a dehydrogenation pathway same as potent pneumotoxin 3-methylindole, generating reactive electrophilic iminium material that could react with protein and DNA targets. Due to this reason, thalidomide was selected as referral drug for new compounds and thalidomide analogs were generated and filtered based on drug-likeness properties. Protein and ligands were proceeding to molecular docking. Many parameters were generated due to primarily binding energy, which shows affinity and strength of interaction between receptor and ligand. Lower binding energy will have stronger interaction. According to our molecular docking analysis, all the screened compounds show good binding energy, but S3 and S5

show AMES toxicity. The pharmacokinetic properties of ligands were analyzed earlier to reduce the further timeline burden to drug development. The finalized molecules S1, S2, and S4 show less binding energy, and S2, S4 are showing good binding energy with hydrogen bond interaction compared with SPD-304. These ligands are showing potent inhibitory activity against TNF-α. Further, in the future, these molecules will be chemically synthesized and subjected to *in vitro* validation studies on a cell-based model system.

Conclusions

To prevent inflammatory disease, the available TNF-α inhibitors are antibodies and fusion proteins used for decades. Still, small-molecule inhibitors are cheaper to produce as compared with antibodies. In the present work, we designed some new analogs of Thalidomide to inhibit TNF-α. The study shows that five compounds are showing good binding energy compared with SPD-304. Among the five, four compounds (S2, S3, S4, and S5) have hydrogen bond interactions 4, 2, 2, and 3, respectively; compared to referral drugs, these compounds have more hydrogen bond acceptors and hydrogen bond donor atoms in their structure due to this feature the compounds get more hydrogen bonds. Based on the ADMET prediction, the S1, S2, and S4 molecules have values within the acceptable range for all the parameters. This study suggests that S1, S2, and S4 compounds show highest potential with respect to referral drug SPD-304. S1, S2, and S4 show Binding energy -6.33, -6.24, and -7.53 kcal/mol, respectively
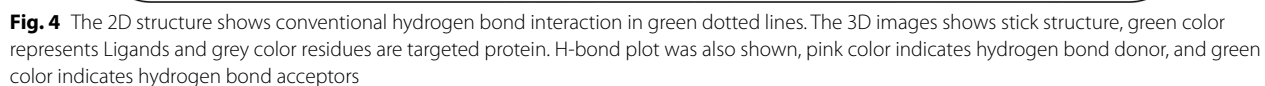
Fig. 4 The 2D structure shows conventional hydrogen bond interaction in green dotted lines. The 3D images shows stick structure, green color represents Ligands and grey color residues are targeted protein. H-bond plot was also shown, pink color indicates hydrogen bond donor, and green color indicates hydrogen bond acceptors

Table 5 ADMET property prediction of thalidomide-based analogs

Ligand	S1	S2	S3	S4	S5
BBB+	0.8156	0.8738	0.9581	0.9629	0.5070
Caco-2	0.6604	0.7177	0.5248	0.5537	0.6472
HIA	0.8277	0.8714	0.9953	0.9790	0.9704
logpGl(substrate)	0.6860	0.6852	0.6827	0.5732	0.6142
logpGl(non-inhibitor)	0.8127	0.9923	0.9051	0.9285	0.9213
PlogS	-2.8159	-2.6315	-3.0731	-3.1513	-3.8890
Logpapp	0.4276	0.1470	1.1293	0.6734	0.2822
AMES toxicity	Non AMES toxic	Non AMES toxic	AMES toxic	Non AMES toxic	AMES toxic
Carcinogenicity	Non-carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens
LD50 in rat	2.7649	2.4596	2.5553	2.1800	2.2751

(SPD-304: -5.37 kcal/mol). Hence, these molecules are carried forward for further *in vitro* validation studies.

Abbreviations

TNF-α: Tumor necrosis factor-alpha; ADMET: Absorption, distribution, metabolism, excretion, toxicity; admetSAR: ADMET structure activity relationship; mAbs: Monoclonal antibodies; Ig-Fc: Immunoglobulin-fragment crystallizable; PDB: Protein data bank; SAVES: Structure analysis and verification server; PDBsum: Pictorial database of 3D structures in the Protein Data Bank; RCSB: Research collaboratory for structural bioinformatics; TTD: Therapeutic target database; ACD: Advanced chemistry development; BBB: Blood–brain barrier; HIA: Human intestinal absorption; LD50: Lethal dose50; HBD: Hydrogen bond Donor; HBA: Hydrogen bond acceptor; MW: Molecular weight; NROTB: Number of rotatable bonds; TPSA: Topological polar surface area; nAtoms: Number of atoms; MV: Molecular volume; Nviolations: Number of violations; AMBER: Assisted model building with energy refinement; RMSD: Root mean square deviation; 3D: Three dimensional; 2D: Two dimensional; CNS: Central nervous system; ENL: Erythema nodosum leprosum; IMiDs: Immunomodulatory imide drugs; IKK: IKB kinase; CADD: Computer-aided drug design.

Authors' contributions

BS contributed to preparation of ligands and target and molecular docking analysis; SSC contributed to prediction of ADMET property of all the designed small molecule inhibitors; SSJ contributed to preparation of manuscript and proofreading work; BRL contributed to research supervisor, hypothesis design, guidance for the entire project and manuscript preparation. All authors have read and approved the final manuscript.

Availability of data and materials

Data and material are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author does not have any competing interest.

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