In silico binding analysis of Withanolides with the Human GM-CSFR

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Abstract: Experimental studies have shown that Withanolides are group of pharmacologically active compounds (steroidal lactones), immunomodulatory agents mainly present in the leaves and roots of *Withania somnifera* plant. The present study is about virtual screening of Withanolide compounds to check for drug likeness by Lipinski's rule five and the screened compounds are allowed to binding with the human GM-CSFR, an immunomodulatory cytokine receptor expressed on dendritic cells. The binding pocket sites, the internal energy, the hydrogen bond interactions and the interacting amino acid residues of the human GM-CSFR with Withanolides were analyzed through molecular docking method. Among the Withanolides docked with human GM-CSFR, which is responsible for DCs survival, proliferation and differentiation, Withanolide A was identified to be a lead compound by binding with α subunit of GM-CSFR exhibiting a maximum Dock score of 28.07 and internal binding energy of -12.8 Kcal/mol. Levamisole as a standard immunomodulatory agent has shown maximum dock score of 28.639 and internal binding energy of -1.864 Kcal/mol. Withanolide A and Levamisole was docked with similar binding site amino acid, ARG302 of GM-CSFR. In addition, Withanolide A was also binding with LEU246 of GM-CSFR, as binding aminoacids predicted from PDBSUM.

Keywords: Withanolide A, *Withania somnifera*, Granulocyte Macrophage Colony Stimulating Factor, Levamisole.

INTRODUCTION

Dendritic cells (DCs) are part of the immune system (Janeway CA *et al*., 2001). Their main immunological function is to process protein antigens and present them to the T-cells and thus functioning as antigen presenting cells (Guermonprez *et al*., 2002). Dendritic cells are present in small quantities in tissues, mainly the skin, the inner lining of the nose, lungs, stomach and intestines. DC is also found to be in a lower percentage in the blood (Fearnley *et al*., 1999). Moreover, on contact with antigens, DC gets activated and transform from immature to matured state (Banchereau *et al*., 1998, Manickam A *et al*., 2011). Once activated, they migrate to the lymphoid tissues where they interact with T-cells and B-cells to initiate the adaptive immune response (Kasajima A *et al*., 2006). One of the most interesting recent developments has been the role of dendritic cells in immunotherapies (Wallack K *et al*., 2006). Although DCs comprise multiple subsets due to multiple differentiation, all are usually effective in antigen processing and presentation function. Monocyte-derived dendritic cells can be generated in-vitro from peripheral blood mononuclear cells (PBMCs). When the PBMCs are induced with granulocyte-macrophage colony stimulating factor (GM-CSF), some of these cells differentiate into immature dendritic cells (Abbas AK *et al*., 2003). Subsequent treatment with tumor necrosis factor differentiates the immature DC further into mature DCs (Prasad KV *et al*., 1997). Dendritic cells produce several cytokines, in a highly specific manner (Bruno C *et al*., 2000). The dendritic cells produce these cytokines via their communication with other cells in the body. This communication is based on direct cell-to-cell contact through cell surface proteins.

The GM-CSF ligand is a protein synthesized as a precursor of 144 AA, which included a hydrophobic secretory signal sequence at the amino terminal end. This cytokine stimulates the growth and differentiation of hematopoietic precursor cells form various lineages, including dendritic cells, granulocytes, macrophages, eosinophils, and erythrocytes. The GM-CSF receptor is a 400 AA protein and is a heterodimer of and a β subunit (Guido H *et al*., 2011). The β subunit is common to IL3, IL5 and GM-CSF receptors. The signaling GM-CSF receptor complex is a dodecamer of two head-to-head hexamers of two α, two β, and two ligand subunits (Barreda DR *et al*., 2004). Signaling is initiated by the cytoplasmic tyrosine kinase, Janus kinase2 (JAK2), which then acts on downstream proteins (Martinez MM *et al*., 2003, Hercus TR *et al*., 2009). The principle signaling modules activated inside the Janus Kinase/ Signal Transducer and activator of transcription (JAK/STAT) pathway, the Mitogen-Activated Protein Kinase (MAPK) pathway, and the phosphatidylinositol 3-kinase (PI3K) pathway (Jason SR *et al*., 2004). In addition, canonical NF-κB activation could occur secondary to the activation of signaling proteins, such as STAT5, PI3K, or PKβ, or depend on GM-CSF-induced secretion of other factors initiating canonical NF-κB activation, activation independent of such mechanisms has been reported.

Withania somnifera, commonly known as Ashwagandha, is an important medicinal plant that has been used for immunomodulation of dendritic cells. The major chemical constituents of the *Withania* genus, the withanolides, are a

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group of naturally occurring C28-steroidal lactone triterpenoids build on an intact or rearranged ergostane framework, in which C-22 and C-26 are approximately oxidized to form a six-membered lactone ring (Mohammad HM *et al*., 2009). In the present study, with anolides are primarily screened to identify a lead compound used for antigen presentation and processing of dendritic cells. If Withanolide can mimic the activity of GM-CSF, it holds the potential to trigger all critical mechanisms of the cell. Levamisole, an immunomodulator belonging to a class of synthetic Imidazothiazole derivatives is used as a standard for checking the binding properties similar to Withanolides.

The docking studies were studied using DS LigandFit, and results were analyzed from DS LigandScore of top-ranked poses from the binding sites of the Immunomodulatory molecule like GM-CSFR. LigandFit method employs a cavity detection algorithm (Venkatachalam CM *et al*., 2003) for detecting invaginations in the protein as candidate active site regions. The LigandFit module in Discovery Studio (DS) for Molecular Docking is used for secondarily screening of available 30 different withanolides recognizes steroidal lactone clusters, thus can calculates the scoring functions (Daisy P *et al*., 2009) including Dock Score, LigScore1, LigScore2, -PLP1 score, -PMF score, and Jain Score.

MATERIALS AND METHODS

Extraction of molecules from databases

Withanolide A (PubChem Id: CID 11294368), Withanolide B (PubChem Id: CID 14236711), 12-DeoxyWithastramonolide (Puchem Id: CID 11305931), Withanoside IV (Puchem Id: CID 71312551), Withanoside V (Puchem Id: CID 10700345) and, Withaferin A (Puchem Id: CID 265237) molecules were downloaded in SDF format from NCBI PUBCHEM Compound database and is shown in fig.1. Levamisole, an immunomodulatory agent and adjuvant was used as a standard for the Withanolides were downloaded from NCBI PUBCHEM compound database and is shown in fig. 2. Research Collaboratory for Structural Bioinformatics is an information portal and a resource house for many biological macromolecular structures. 3D structures of Human Immunomodulatory cytokine GM-CSFR complex and the bonding contacts of ligand and receptor of GM-CSF (Hansen G *et al*., 2008) was downloaded from RCSB PDB database and the binding sites were represented in fig. 3. The Universal Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data. The Uniprot Knowledgebase (UniProtKB) is the central access point for extensive curated protein information, including function, classification, and cross-reference. GM-CSF receptor and ligand protein sequences were retrieved for Human in FASTA format from UniprotKB databases represented in table 1. Preparation of the docking molecules and experiments were performed in Windows 2008 Server Operating System on the dual processor of 4 GB RAM, XEON Server X 3400.

Docking protein and ligand molecule

The DS LigandFit docking was performed by the first step that involves in identifying the cavity and selecting the region of the GM-CSFR protein as the binding site for docking, where the binding sites are verified from PDBSUM and UniProt databases. The second step identifies docking Withanolide ligands to a selected site and involves in employing three dimensional regular grids of points for estimating the interaction energy of the ligand with the protein during docking and with the flood-filling algorithm for cavity detection. After the initial placement of the ligand in the binding site, a rigid body minimization of the ligand performed using a steepest descent (SD) algorithm was 10 iterations, optionally followed by a Broyden-Fletcher-Goldfarb-Shanno (BFGS) Quasi-Newton minimization was 20 iterations. The maximum number of clusters per ligand and the Root Mean Square Deviation (RMSD) threshold used was 2.0 for the cluster and the maximum 10 poses retained was fixed in the LigandFit parameters explorer. The energy minimization was carried out by using the tool CHARMm force field potential energy function. A shape comparison filter is combined with a Monte Carlo conformational search for generating ligand poses consistent with the active site shape.

Analysis of docking results

The Withanolide A compound and the Human GM-CSFR receptor interaction necessary of immunomodulation expressed on Dendritic Cells were identified via docking and their relative stabilities were evaluated using their binding affinities. Thus binding sites were designed based on the ligands already present in the PDB file which were followed by site sphere definition for determining probe site radius. Here top ranked ligands were taken for binding affinity studies. The validation process consisted of two parts, usually by analyzing Hydrogen bond details of the top-ranked docked pose followed by prediction of binding energy between the docked ligands and the immunomodulatory cytokine molecule using various estimated scores calculated using Discovery studio (LigScore1, -PLP1, Jain, -PMF, Dock scores and Internal Energy scores) were taken for analysis (William NS *et al*., 2012, Grover A *et al*., 2012).

The score values include Ligscore1 (Protein-Ligand Affinity Energy) (Krammer A *et al*., 2005), PLP1 (Steric and H-bonding intermolecular function, Higher PLP scores indicate stronger receptor ligand binding (larger PKi values) (Muegge I *et al*., 1999), JAIN (sum of five interaction terms namely Lipophilic interactions, Polar attraction interactions, Polar repulsive interactions,

Table 1: Immunomodulatory Protein Information of *Homosapiens* from UniProt

S. No	Immunomodulatory Protein Name	Uniprot Accession No	Sequence Length (AA)	PDB Id	PDB Resolution (A°)
1	GM-CSFR	P15509	400 AA	3CXE	3.3
2	GM-CSFL	P04141	144 AA	1CSG	2.7

Table 2: Drug Likeness Properties of Withanolides from MolInspiration.

Properties	Withanolide A	Withanolide B	Withaferin A	12-Deoxy Withastramonolide
miLogP	3.95	4.86	3.625	3.624
Molecular Weight (g/mol)	470.6	454.6	470.6	470.6
Hydrogen Bond Donor	2	1	2	2
Hydrogen Bond Acceptor	6	5	6	6
Rotational Bonds	2	2	3	3
Molecular Formula	$C_{28}H_{38}O_6$	$C_{28}H_{38}O_5$	$C_{28}H_{38}O_6$	$C_{28}H_{38}O_6$
TPSA	96.36	76.13	96.36	96.36
Volume	441.8	433.77	442.09	442.09

Table 3: Summary of docking scores information of the top ranked poses of Withanolide compounds from DS2.5.5

S. No	Compound	LigScore1 (MM2)	-PLP1	Jain	-PMF	Dock Score (Gaussian)	I-E
1	Withanolide A	1.3	69.49	1.68	108.5	28.07	-12.8
2	Withanolide B	0.14	46.63	0.37	74.86	6.49	-5.7
3	12-Deoxy Withastramonolide	1.52	51.46	-0.45	108.3	20.43	-16.4
4	Withaferin A	1.3	22.16	-2.24	59.95	8.94	-6.22
5	Levamisole (Standard)	0.99	54.96	1.1	14.91	28.64	-1.86

Table 4: Hydrogen bond interactions of Withanolide A with Human Immunomodulatory Receptor molecules

Immunomodulatory Molecule	Chain: AA	Atom in AA	Position of AA	Withanolide A Position	Atom in Legend	Probe Site Radius (A°)
GM-CSFR	C	LEU	246	OH5	CO	7.9
GM-CSFR	C	ARG	302	H18	NH	7.9

Solvation of the protein and ligand, An entropy term for the ligand (Gehlhaar DK *et al*., 1995, Welch *et al*., 1996). PMF (developed based on statistical analysis of the 3D structures of protein-ligand complexes, scores were calculated by summing pairwise interaction terms over all interatomic pairs of the receptor-ligand complex, a higher score indicates a stronger receptor-ligand binding affinity) (Muegge I *et al*., 2006) and Dock Score (Candidate ligand poses are evaluated and prioritized according to the Dock Score function) (Kiwon Ok *et al*., 2013). The determination of the ligand binding affinity was calculated using the shape-based interaction energies of the ligand with the protein. The two scoring methodologies namely LigScore and PLP1 were used to estimate the ligand-binding energies. Larger score value indicated better ligand-binding affinity.

RESULTS

Methods have been developed to generate large numbers of DCs from monocytes in human (Romani N *et al*., 1996). Most of these protocols involve the use of growth factors such as granulocyte macrophage colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4), which can specifically promote DC generation. GM-CSF is renowned for its ability to maintain the homeostasis of all the cytokine signaling in the body especially on the antigen presenting cells (Van de Laar L *et al*., 2012). The GM-CSF receptor, first identified on cells of the myelomonocytic lineage by ligand-binding studies is a heterodimer that comprises a major binding subunit α with 2 chains (Gearing DP *et al*., 1989) and a major signaling subunit β with 2 chains (Hayashida K *et al*., 1990). The binding sites of ligands and receptors of Immunomodulatory cytokines of GM-CSF responsible for survival, differentiation and proliferation of dendritic cells were predicted from PDBSum. The Chain B in fig 3 shows the GM-CSFL and Chain C shows the GM-CSFR (Subunit alpha) having the bonding contacts in extra cellular membrane surfaces represented, labeled with AAs. Whereas, the Chain A shows the GM-CSF cytokine receptor subunit beta in GM-CSF-GM-CSFR complex. The GM-CSF receptor activation follows observation for other class I cytokine receptors that invoke receptor

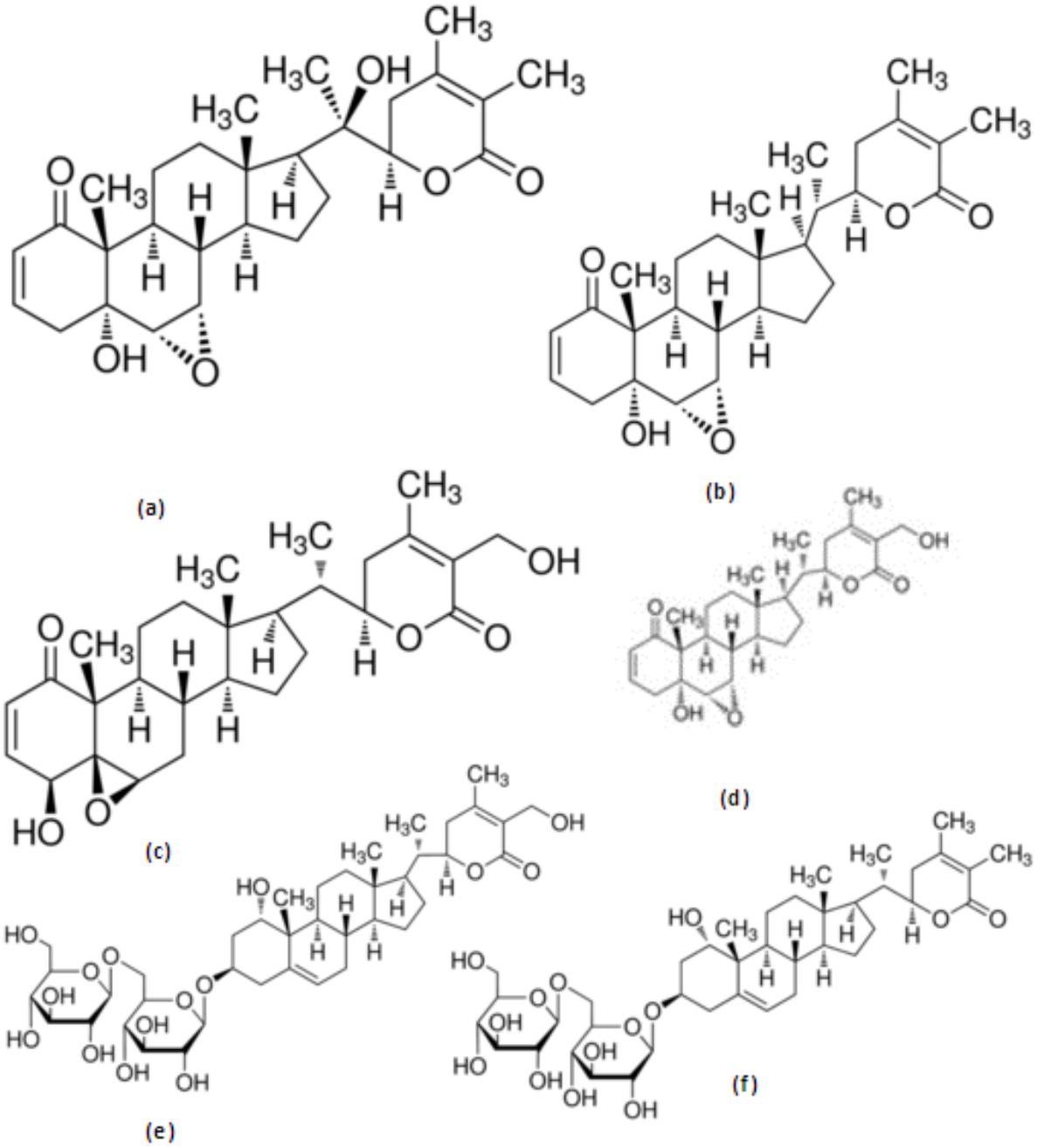

Fig. 1: Structure of Withanolides. (a) Withanolide A, (b) Withanolide B, (c) Withaferin A, (d) 12- Deoxy – Withastramonolide, (e) Withanoside IV, (f) Withanoside V

dimerization and tyrosine transphosphorylation of cytoplasmic domains (Schlessinger J *et al*., 2000). Although antibody therapeutics have been developed that block protein-protein interactions, no approved small-molecule drugs have yet been produced for this important target class. In the present study, the sub unit α of GM-CSF receptor is taken for binding studies with Withanolides from *Withania somnifera*. The elucidation of the structure of the GM-CSF receptor subunit alpha and its mechanism of activation has revealed distinct binding sites with GM-CSF ligand that may be targeted with modified cytokines (Timothy R.H *et al*., 2009) or small molecules like Withanolides. Not only the extracellular receptor component, but also the GM-CSF receptor intracellular signaling machinery may be amenable to therapeutic intervention.

According to US patent studies by Jadhav *et al*., 2010, maximum quantity of Withanolide compounds extracted in roots of *Withania somnifera* like Withanolide A, Withanolide B, 12-DeoxyWithastramonolide, Withanoside IV, Withanoside V and, Withaferin A from *Withania somnifera* were taken and were passed through primary screening for detecting drug likeness from MolInspiration. Only 4 compounds namely, Withanolide A, Withanolide B, 12-DeoxyWithastramonolide and Withaferin A has shown drug likeness properties shown in table 2. The other 2 compounds, Withanoside IV and Withanoside V were not subjected to molecular docking analysis has shown total number of 2 violations against Lipinski's rule five.

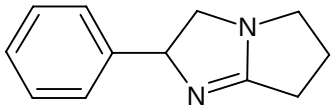

Fig. 2: Levamisole

The four Withanolide compounds selected after primary screening from MolInspiration were subjected to secondary screening by molecular docking studies with GM-CSFR (Subunit α). Withanolide A (IUPAC Name: (5|A,6|A,7|A,22R)-6,7-Epoxy-5,20,22-trihydroxy-1-oxo-ergosta-2,24-dien-26-oic acid -lactone, PubChem Compound Id: CHEMBL445041) shown in fig. 1 (a) was identified to be a lead compound because of maximum dock score and the same compound can serve as immunomodulatory agent with the GM-CSF receptor, which is needed for survival, proliferation and differentiation of Dendritic cells.

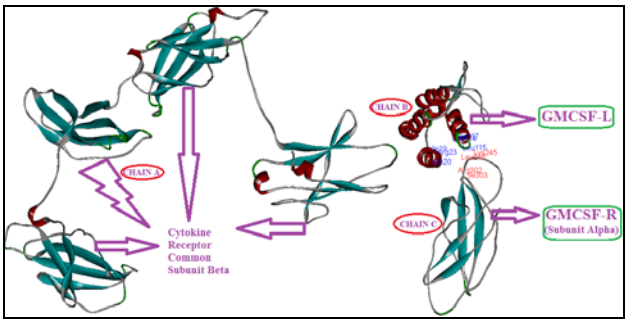

Fig. 3: 3D structure of GM-CSF Ligand-Receptor Complex from DS Visualizer

Withanolide A was docked with GM-CSFR (Subunit α) yielding maximum dock score of 28.071 and internal energy of -12.8 Kcal/mol, when compared with other Withanolides as shown in table 3. In present study, Levamisole, an immunomodulator and adjuvant (Sanagdol H *et al*., 2012) was used as a standard for Withanolides has docked with GM-CSFR (Subunit α) yielding maximum dock score of 28.64 and internal energy of -1.86 Kcal/mol. Thus, from Gaussian dock score in molecular docking studies has proved Withanolide A possess similar binding properties as that of Levamisole to serve as an immunomodulating agent.

The other docking scores like LigScore1, PLP1, Jain, PMF scores from DSLigand Score should be maximum to show the potential binding with Human GM-CSFR. Thus from the present study, Withanolide A exhibited with maximum scores, when compared with the other three Withanolides and Levamisole. Binding of the amino group of ARG302 present in extra cellular domain of GM-CSFR with methyl group of position18 with steroidal nucleus of Withanolide A and Binding of the carboxyl group of LEU246 present in extracellular domain of GM-CSFR with hydroxyl group of position5 with steroidal nucleus of Withanolide A was observed in fig. 4 (a) and the details of bonding are listed in table 4. Binding of the amino group of ARG302 of GM-CSFR (Subunit α) present in extra cellular region with Levamisole was also observed and was mentioned in fig. 4 (b).

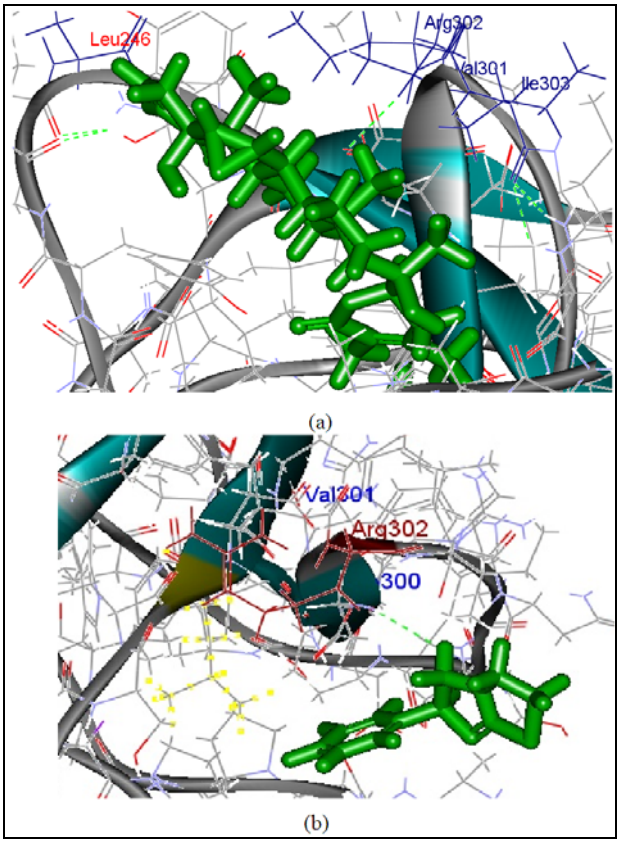

Fig. 4: (a) Binding sites of 3D structure of GM-CSFR (Subunit α) with Withanolide A from DS2.5.5 (b) Binding sites of 3D structure of GM-CSFR (Subunit α) with Levamisole from DS2.5.5

DISCUSSION

Maturation of DCs is essential to achieving appropriate immune responses, as it enhances surface expression of MHC and co-stimulatory molecules, which reflect

antigen-presenting capabilities, as well as the production of a variety of cytokines (Larsen *et al*., 1994; Mosca *et al*., 2000). Based on data from GM-CSF knockout animal models, GM-CSFRα - GM-CSFL site antagonists would be expected to be of clinical value in certain inflammatory conditions and autoimmune diseases such as Rheumatoid Arthritis (Niki Y *et al*., 2007, Praveen Kumar PK *et al*., 2013), multiple sclerosis (McQualter JL *et al*., 2001), type I diabetes and autoimmune glomerulonephritis (Timoshanko JR *et al*., 2005). Similar studies by Yogeswaran P *et al*., 2012 has proved the molecular docking studies of withanolides like Withaferin-A, Withanolide D from *Withania somnifera* bind to the active sites of COX-2 enzyme analyzed through energy scores, a target to cure Inflammation. The maximum dock score from the Withanolide A depicts Withaferin A and can be characterized to Levamisole in human monocyte-derived DC maturation. i.e Dendritic cell survival and differentiation were regulated by signaling via GM-CSF NF-κB pathway (Wipob S *et al*., 2010, Chen LY *et al*., 2008). Withanolide A, an active compound from *Withania somnifera* may mimic human GM-CSF Ligand in binding of GM-CSFR needed for Immunomodulation expressed on Dendritic cells, as these similar computational studies were proven for CD40L mimetic molecule binding by synthesized N-Benzhydryl benz amide from Vani V *et al*., 2008. It was also predicted that the compound Withanolide A can be used as an immunomodulatory agent and an adjuvant like Levamisole for Rheumatoid arthritis cure as similar studies proven by Runge LA *et al*., 1979 and Jadhav *et al*., 2010.

CONCLUSION

Docking software's have been frequently used in drug development to study the binding affinity of plant molecules to target proteins. The Protein-Ligand interaction plays a significant role in structural based drug designing for auto-immune diseases like Rheumatoid arthritis. Withanolide A along with 3 three more Withanolide compounds was taken for docking studies after primary screening of compounds by Lipinski's rule. The Gaussian dock scores were maximum and approximately similar for Withanolide A and Levamisole, as a standard for comparison for binding with GM-CSFR. Withanolide A binds with similar AA, ARG302 like Levamisole with α subunit of GM-CSFR which is predicted from PDBSUM. Thus, Withanolide A mimics GM-CSF ligand in binding the GM-CSF receptor, which stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including dendritic cells. Based on all the dock score values, it was predicted that the Withanolide A have similar and good binding affinities and mimic towards the specific immunomodulatory cytokine GM-CSF receptor molecule, expressed on dendritic cells. Thus, the plant compound Withanolide A can be used as the lead compound and can also be taken simultaneously along with Levamisole to yield synergetic effects for development of immunomodulatory drugs, which are applicable for inflammation, immunodeficiency and cancer.

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