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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

In vitro and in silico exploration of IL-2 inhibition by small drug-like molecules

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Abstract Interleukin-2 (IL-2) is an immunoregulatory cytokine produced by T lymphocytes in response to antigen. It is a potent growth and differentiation factor for several cell-types and is structurally related to the four-helix bundle family of cytokines. Here, we report IL-2 inhibitory potential and computational studies on different series of chalcones, benzothiazepines, semicarbazones, and dihydropyrimidines. These compounds were synthesized in wet lab and were then tested for their potency as IL-2 inhibitors through in vitro T cell proliferation, IL-2 cytokine production as well as their effect on oxidative burst. Compounds that showed significant suppressive activity were further evaluated for their cytotoxicity on normal two cell lines. Most of the chalcones were found to have a powerful inhibitory effect on T-lymphocytes proliferation and cytokine production. Among the aza heterocycles benzothiazepines, benzoxazepines, and benzodiazepinones were found to be the strongest IL-2 inhibitors. Molecular docking and MD simulation studies were carried out to correlate experimental and theoretical results whereby a good correlation was observed which indicated that computational studies could provide an alternate tool for the identification and designing of more potent IL-2 inhibitors.

Keywords IL-2 inhibitors · Chalcones and heterocycles · Molecular docking · MD simulation

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Introduction

Cytokines are small signaling protein molecules that are secreted by various cells and are used extensively in intercellular communication. The term "cytokine" has been used to refer immunomodulating agents, such as interleukins and interferons. Interleukin-2 (IL-2), which is one of the first cytokines identified, acts at the heart of the immune response (Smith, 1988). Mature IL-2, a secreted glycoprotein of 133 amino acids (15.5 kDa), is a single chain polypeptide produced by T cells in response to immune stimuli mediated by the T-cell receptor (TCR) and major histocompatibility complexes (MHC) I and II (Nelson and Willerford, 1998). Since IL-2 is central in immune and non-immune mechanisms of resistance to disease, it offers a large potential for therapeutic applications. Perhaps the best characterized of these is the use of IL-2 in the treatment of malignant neoplasms (Murakawa *et al.*, 1985). IL-2 has been suggested to participate in the pathogenesis of a variety of diseases. Chronic active hepatitis (Mule *et al.*, 1984), juvenile diabetes (Saxena *et al.*, 1986), rheumatoid arthritis (Zielasek *et al.*, 1990) multiple sclerosis (Phadke *et al.*,

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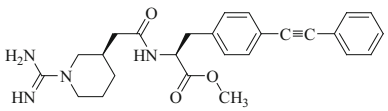
1986), allograft rejection (Trotter *et al.*, 1988), and lupus erythematous (Kirkman *et al.*, 1985) are among those diseases associated with aberrant IL-2 function. Certain immunodeficiencies have been directly correlated with a defect in the ability of T cells to secrete IL-2 (Pahwa *et al.*, 1989).

Most of the immunosuppressant drugs used in clinic today include agents that act at various points in the TCR-signaling pathway to block IL-2 production. These drugs include cyclosporin A (CsA), rapamycin FK506, and anti-IL-2Rα antibodies (anti-Tac) (Brown and Schreiber, 1996; Wiederrecht *et al.*, 1995; Waldmann and O'Shea, 1998; Michael and David, 2005). However, these drugs exhibit a plethora of unwanted side effects and significant toxicities associated with them (Tilley *et al.*, 1997).

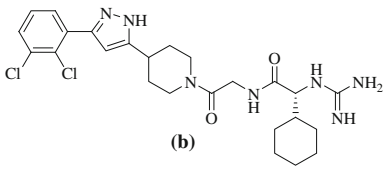
The first small molecule shown to inhibit the IL-2:IL-2Rα interaction was reported by Roche (Waldmann and O'Shea, 1998). Compound **a**, was an enantiomer-specific, competitive inhibitor of IL-2Rα with an $IC_{50} = 6$ μM.



(a)

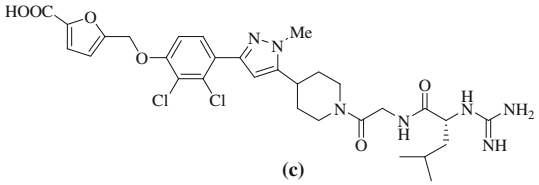
A fragment-minded approach was used to evolve compound **a** into a more potent and drug-like inhibitor.

Braisted *et al.* (2003) reported a compound (**b**, $IC_{50} = 6$ μM) that binds to IL-2, preventing its association with IL-2Ra .



(b)

Optimization of compound **b** included the introduction of a furanoic acid fragment onto the dichlorophenyl ring (**c**, $IC_{50} = 0.060$ μM) which offered a significant increase in activity.



(c)

In our recent paper, we isolated natural products from *Lindelofia stylosa* which has shown ability to modulate the immune response (Mesaik *et al.*, 2012).

In both lead identification and lead optimization processes there is an acute need for new organic small molecules. Organic synthesis has contributed substantially to the discovery of biologically active small molecules. In the development of new drugs, it is convenient to have a range of diverse chemical structures with the desired pharmacological activity.

Our research group has been engaged in target-based drug designing and has reported the synthesis of diverse classes of small drug-like compounds and quite a few have been identified as potential candidates for the inhibition of enzymes such as urease, cholinesterase etc. Moreover, the leads identified in different bioassays were subjected to molecular modeling and docking studies and an attempt was made to rationalize the results obtained in different bioassays. Based on these in silico studies, some predictions for the synthesis of new enzyme inhibitors with activity were also made (Ansari *et al.*, 2011; Nawaz *et al.*, 2008; Zaheer-ul-Haq *et al.*, 2010).

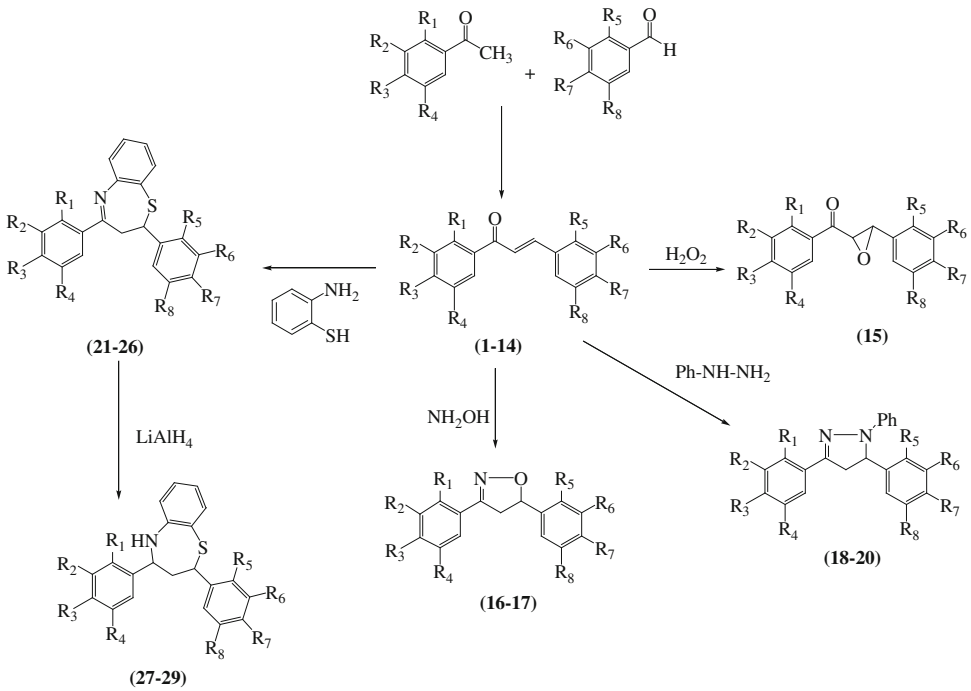
In the present study, we screened a library of 50 synthetic compounds belonging to six different classes both cyclic and alicyclic e.g., chalcones, oxazolines, pyrazolines, dihydropyrimidines, benzothiazepines, and semicarbazones which were synthesized and tested for their behavior as IL-2 inhibitors. Due to the immense biological applications of these compounds, herein we report a comparative study of IL-2 inhibitory potential of the these fifty compounds by conducting molecular docking and MD simulation studies using Molecular Operating Environment software (MOE 2011.10). These studies were carried out to develop a deeper understanding of inhibitory behavior of these compounds. Such information can be quite valuable in search of new immunomodulatory compounds.

Results and discussion

Chemistry

Chalcones **1**–**14** (Scheme 1) (Ansari *et al.*, 2005; Ahsan Ullah *et al.*, 2007) were synthesized by Claisen Schmidt condensation of different substituted aldehydes and different acetophenones. These chalcones were transformed into different classes of 5- or 7-member heterocycles by an [3 + 2] or [4 + 3] annulations, respectively. The epoxide 15 was synthesized by the reaction of a chalcone with hydrogen peroxide (Kee and Gavriilidis, 2007). Oxazolines (**16**–**17**) were synthesized by refluxing the corresponding chalcones with hydroxylamine as reported in literature earlier (Chandrasekhar *et al.*, 2002). Following the same protocol, pyrazolines (**18**–**20**) were prepared by the reaction of chalcones with phenylhydrazine (Sivakumar *et al.*, 2010; Baseer *et al.*, 2013). 2,3-Dihydrobenzothiazepines (**21**–**26**) were synthesized in solid phase earlier by the reaction of different chalcones with *O*-aminothiophenol (Ansari *et al.*, 2008a, b; Levai and

Scheme 1 Synthesis of chalcones and the derived azaheterocycles



No.	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1	H	OMe	OMe	OMe	H	Br	H
2	NH_2	H	H	H	H	Cl	H
3	H	H	H	H	H	H	F
4	H	H	H	H	H	H	Me
5	H	OH	H	H	H	NO_2	H
6	H	OH	H	H	OH	H	H
7	NH_2	H	H	H	H	H	OMe
8	NH_2	H	H	H	H	H	Br
9	H	H	NH_2	H	H	Cl	H
10	H	H	NH_2	H	OMe	H	H
11	H	H	NH_2	H	H	H	Me
12	H	OH	H	H	H	H	H
13	H	H	NH_2	H	H	NO_2	H
14	OH	H	H	H	H	H	OMe
15	OH	H	H	H	H	H	H
16	H	H	H	H	H	H	OMe
17	H	H	H	H	H	H	F
18	H	H	H	H	H	H	H
19	H	H	H	H	H	H	Br
20	H	OH	H	H	H	H	H
21	OH	OH	H	H	H	H	OMe
22	OH	OH	H	H	H	H	Br
23	OH	OH	H	H	H	H	NMe_2
24	H	OH	H	H	H	H	F
25	H	OH	H	H	H	H	Cl
26	H	OH	H	H	H	H	NO_2
27	OH	H	H	H	H	H	OMe
28	OH	H	H	H	H	H	F
29	OH	H	H	H	H	H	Cl

Szikszai, 2008). Tetrahydrobenzothiazepines (**27**–**29**) were synthesized by the reaction of 2,3-dihydrobenzothiazepines with lithium aluminium hydride. A diastereomeric mixture of tetrahydrobenzothiazepines was obtained which was separated through fractional crystallization in each case. Two diastereomers were obtained in different yield (Ansari *et al.*, 2005). XRD data of compound 29 has earlier been reported by our group (Parvez *et al.*, 2003).

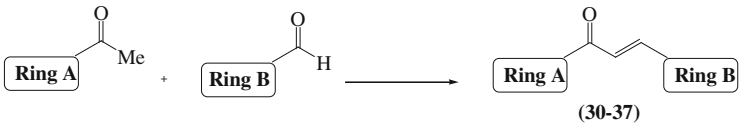
Chalcones **30–35** (Scheme 2) were synthesized by the same strategy leading to ferrocenylchalcones (Wu *et al.*, 2002) and biphenylchalcones (Thirunarayanan and Vanangamudi, 2007) using either acetylferrocenyl or acetylbiphenyl and different substituted arylaldehydes. Compound **36** was obtained by the reaction of acetophenone with 2-thiophenecarboxalde hyde while chalcone **37** was a result of condensation of 3-hydroxyacetophenone with 2-pyridinecarboxaldehyde (Ahsan Ullah *et al.*, 2007).

Benzodiazepinone **38–40** (Scheme 3) were synthesized by the reaction of *o*-phenylenediamine or 4-nitrophenylenediamine with crotonic acid as reported earlier by our group (Jafri *et al.*, 2012), while benzodiazepinone **42** was prepared by the reaction of 2,3-diaminopyridine and crotonic acid. Naphthodiazepinone **43** was the product of the reaction of 2,3-diamino naphthalene with crotonic acid.

3,4-Dihydropyrimidine-2-ones (**44–46**, Scheme 4) were synthesized by a modified multicomponent strategy developed by Biginelli by condensation of different arylaldehydes, urea, and dibenzoylmethane (Akbas and Furgan, 2008).

Synthesis of semicarbazones **47** and **48** was carried out by the reaction of different carbonyl compounds with semicarbazides. Semicarbazones **49** and **50** were prepared by the reaction of exocyclic carbonyl group of isatin and benzodiazepinone with semicarbazide, respectively, as reported earlier by our group (Jafri *et al.*, 2012) (Scheme 5).

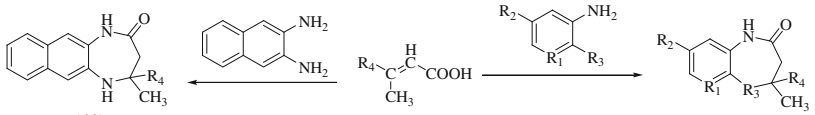
Scheme 2 Synthesis of chalcones derived from acetyl compounds



(30-37)

No.	Ring A	Ring B
30	2-Ferrocenyl	2-NO_2Ph
31	2-Ferrocenyl	4- NO_2Ph
32	2-Ferrocenyl	5- NO_2Ph
33	Biphenyl	2-OHPh
34	Biphenyl	3-OHPh
35	Biphenyl	3-OMePh
36	Phenyl	2-Thienyl
37	3-OHPh	2-Pyridyl

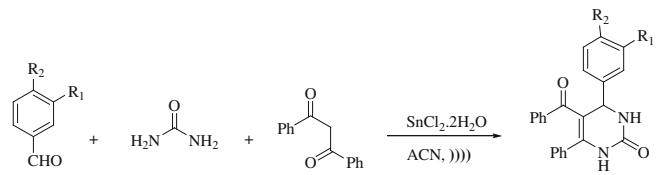
Scheme 3 Synthesis of heteroazepinones



(43) **(38-42)**

No.	R_1	R_2	R_3	R_4
38	CH	H	NH	H
39	CH	NH_2	NH	H
40	CH	NO_2	NH	Me
41	CH	H	O	H
42	N	H	NH	H
43	-	-	-	H

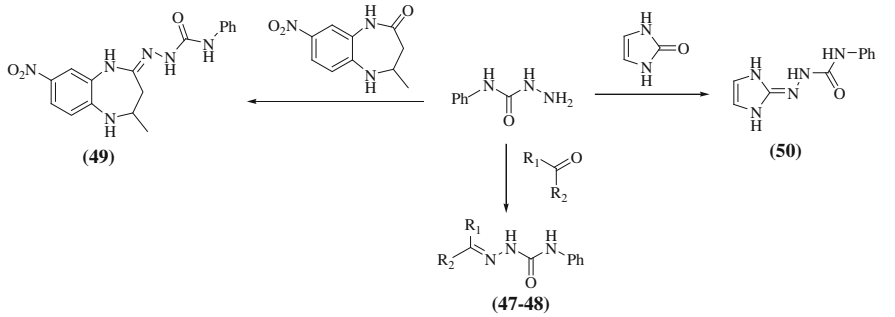
Scheme 4 Synthesis of 3,4-dihydroyrimidines (**44–46**)



(44-46)

No.	R_1	R_2
44	H	H
45	OMe	OH
46	H	OMe

Scheme 5 Synthesis of semicarbazones (**47–50**)



No.	R_1	R_2
47	3-OHPh	Me
48	4-OMePh	H

In vitro studies

The synthesized compounds **1–50** were tested for their potency as IL-2 inhibitors through in vitro T cell proliferation and IL-2 cytokine production assay as well as their effect on oxidative burst. Compounds that showed significant suppressive effect were further evaluated for their cytotoxicity on normal two cell lines. Most of the chalcones were found to have a powerful inhibitory effect on lymphocyte proliferation and cytokine production.

Effect of compounds on generation of reactive oxygen species (ROS)

The overproduction of ROS by phagocytes causes oxidative damage to membrane lipids, DNA, proteins, and lipoproteins. These reactions have functional consequences, which may be deleterious to cells and tissues. Thus, the inhibition of ROS production is a popular target for the attenuation of many inflammatory diseases (Shen *et al.*, 2002). In this study, we have tested 46 compounds out of 50 in the chemiluminescence assay. Five compounds (**6**, **12**, **26**, **39**, and **41**,) were found to be very potent inhibitors with an IC_{50} value of approximately 5 or less than 5 μg/mL. Compounds **11**, **17, 32**, **47**, and **40** showed an IC_{50} value of 12.4, 6.9, 15.8, 8.0, and 12.4, respectively. The results are summarized in Table 1.

Table 1 Effect of organic compounds (**1–50**) on ROS generation

No.	IC_{50} (μm/mL)
3	65.1 ± 34.6
4	31.0 ± 8.9
5	24.7 ± 3.8
6	2.9 ± 0.5
7	52.1 ± 12.9
9	24.4 ± 18.8
10	44.1 ± 9.8
11	12.4 ± 1.3
12	4.8 ± 0.1
13	17.0 ± 7.7
17	6.9 ± 0.5
26	0.2 ± 0.0
31	36.3 ± 0.1
32	15.8 ± 2.
36	72.2 ± 9.8
37	21.2 ± 2.7
39	4.5 ± 0.6
40	12.4 ± 2.9
41	2.3 ± 0.7
47	8.0 ± 0.8
48	5.3 ± 0.5

Results are expressed as mean ± SD of IC_{50} in μg/mL for triplicate runs of each parameter

Effect of compounds on T cell proliferation and IL-2 inhibition

Activated T cells play a central role in the regulation of immunity, and once activated, T cells may act as direct cytotoxic effectors or provide help for other cells that are important in the effector phase of the immune response. Here, T cells were activated in vitro by phytohemagglutinin (PHA) in the presence of three concentrations (0.5, 5.0 and 50 μg/mL) of each compound. Out of the 37 compounds tested, 21 compounds showed an interference with the PHA stimulated proliferation with an IC_{50} less than 20 μg/mL (Table 2).

As a growth factor for T cell, 23 compounds were subjected to screen their effect on IL-2 production. We found that the production of IL-2 by peripheral blood monocytes was very weak upon PHA activation. In order to achieve a better and higher production level of IL-2, T cells were activated by a combination of PHA and phorbol-myristate acetate (PMA) as it has been reported that PMA's

Table 2 Effect of compounds on T-cell proliferation and IL-2 inhibition

No.	T-cell proliferation IC_{50} (μg/mL)	IL-2 inhibition
3	15.7 ± 0.16	20.7 ± 0.6
4	7.8 ± 0.67	15.6 ± 0.2
5	0.7 ± 0.02	1.5 ± 0.0
6	2.1 ± 0.03	1.4 ± 0.2
8	17.0 ± 3.04	15.1 ± 0.2
9	<0.5	2.5 ± 0.1
10	3.0 ± 1.3	31.8 ± 0.3
11	3.2 ± 2.7	15.1 ± 0.2
12	8.2 ± 3.3	14.2 ± 0.1
13	1.9 ± 0.0	16.1 ± 1.6
15	11.0 ± 0.91	19.7 ± 0.4
17	21.9 ± 9.06	–
26	15.0 ± 2.8	2.3 ± 0.2
31	0.749 ± 0.4	13.5 ± 0.0
32	9.77 ± 0.2	15.2 ± 0.0
36	10.1 ± 2.03	26.7 ± 0.8
37	1.2 ± 0.04	5.0 ± 0.5
39	4.4 ± 1.2	13.0 ± 0.3
41	6.8 ± 0.4	14.9 ± 1.7
44	3.3 ± 1.0	–
45	11.1 ± 0.3	–
46	8.2 ± 0.6	–
48	10.5 ± 4.6	37.3 ± 6.1
49	25.5 ± 4.5	–

Results are expressed as mean ± SD of IC_{50} in μg/mL for triplicate runs of each parameter

mitogenic effect is additive to that of PHA, suggesting that PHA and PMA induce IL-2 receptor gene expression through different, complementary mechanisms. It was observed that when T cells were forced to produce high concentration of IL-2 using a combination of PHA and PMA, some compounds showed suppressive activity against T cells. Dihydropyrimidines failed to suppress the production of IL-2, where they showed $IC_{50} > 50$ μg/mL (Table 2). 13 compounds (**3**, **4**, **8**, **10**, **11**, **12**, **13**, **15**, **31**, **32**, **36**, **39,** and **41**) showed a suppressive activity with IC_{50} between 10 and 35 μg/mL. In addition five compounds (**5**, **6**, **9**, **26** and **37**) showed an IC_{50}value of 5 or < 5 μg/mL as presented in Table 3.

Cytotoxicity effect

In order to verify the suppressive activity of compounds that showed extreme potency against T-cell proliferation and IL-2 production, **5**, **6**, **9**, **26,** and **37** were tested for their cytotoxic effects. For this purpose, a set of two cytotoxicity experiments was developed using 3T3-L1 mouse embryo fibroblast and CC-1 rat Wistar hepatocyte cell-lines. Cells were incubated for 48 h with three concentrations of each compound. The activity of compounds **26** and **37** in exerting suppressive effect in vitro on T-cell proliferation and IL-2 production is clearly independent of any cytotoxic action as shown in Table 3. The toxicity or the IC_{50} of these two compounds on 3T3-L1 and CC-1 was found to be more than or nearly equal to the highest dose tested (20 μg/mL). The activity of compounds **5**, **6**, and **9** seems to be due to their cytotoxic action as their IC_{50} value for the growth of these cell lines ranges from 3.4 to16.7 μg/mL (Table 3).

Table 3 Cytotoxicity effect of synthesized compounds on cancer cell lines

No.	Cytotoxicity	
	3T3L-1 cells	CC-1 cells
5	3.4 ± 0.05	7.0 ± 0.3
6	5.3 ± 0.3	15.6 ± 2.4
9	9.5 ± 0.7	16.7 ± 0.69
26	16 ± 0.35	>20
37	20<	20<

In silico studies

Molecular docking

The binding modes of the compounds were predicted via molecular docking technique using MOE software, which revealed that all the compounds were accommodated well in the ligand binding site of IL-2. Initially, MOE docking performance was assessed by re-docking co-crystallized structure from PDB ID 1M48. The co-crystallized ligand, namely FRG (**1**) was docked into the active site of IL-2 to insure that the docking program regenerates the bound conformation correctly. The root-mean square deviation (RMSD) of the docked conformation to the experimental conformation was around 1.0.

The quality level of the docking complex was similar to that of the original crystal structure. This result suggested that methods applied for the docking simulation was sufficient for further analysis and could be accepted for virtual screening of IL-2 inhibitors. The important contact residues for compound **1** were Lys35, Arg38, Met39, and Phe42. Compound **1** can be divided into two components: a hydrophilic fragment that contained a piperidyl guanidine and a hydrophobic fragment containing a biaryl alkyne. The piperidyl guanidine moiety formed a two arene-cation bridge with Phe42 and Tyr45 and a side-chain donor linkage with carboxylate of Glu62. On the other hand the biaryl alkyne was well placed in the narrow channel

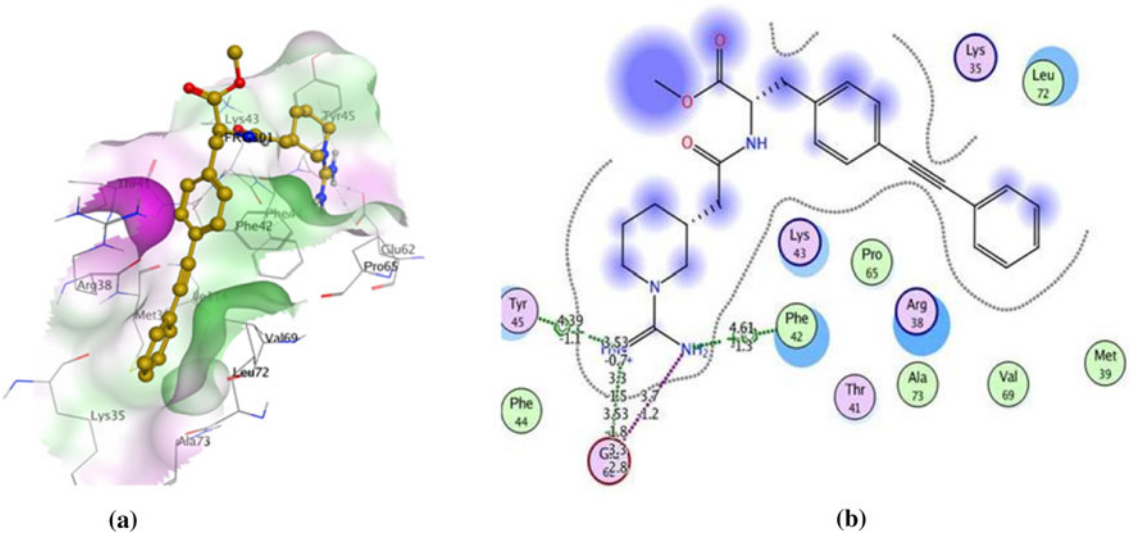


Fig. 1 Modeled mode of binding of FRG (**1**) in 1M48 active site. **a** 3D, **b** 2D

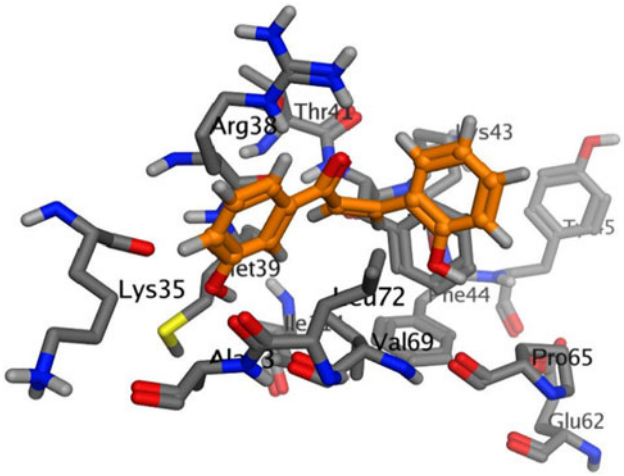


Fig. 2 Modeled mode of binding of chalcone **6** in 1M48 active site

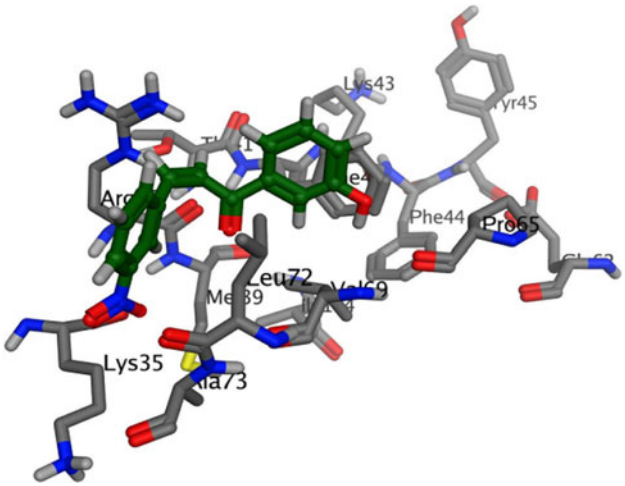


Fig. 3 Modeled mode of binding of compound **5** in the active site of 1M48

created by the hydrophobic side chains of Arg38, Met39, Phe42, Leu72, and Lys76 (Fig. 1).

All the compounds were docked into the active site of IL-2 using the above docking method to determine their most suitable docking conformations. The Active Site Finder tool of MOE was used to dock the reference and test compounds into the receptor binding site of IL-2. All the ligands that adopted almost identical position at the surface of the IL-2 protein comprise a hydrophobic cage of Arg38, Thr41, Phe42, Phe44, Lys43, Tyr45, Glu68, and Leu72.

The binding pattern of the most active compounds showed that they were involved in hydrogen bonding with three amino acids (Arg38, Thr41, Val69). The hydroxyl group of the most active compound i.e., clacone **6** (IC_{50} = 1.4 μM) was involved in arene–cation interactions with Leu72, Arg38 and also side chain acceptor interactions (2.74 Å) with Arg38 (Fig. 2).

The putative binding mode of compound **5** (IC_{50} = 1.5 μM) is shown in Fig. 3. The 4-NO_2 aryl ring interacted with Arg38 forming arene-H bond, while the oxygen of carbonyl group formed a hydrogen bonding with Arg38 as hydrogen-bond donor (HBD) at a distance of 2.77 Å.

Besides molecular docking and MD simulations, another tool helpful in drug designing is the prediction of properties of drug-like molecules using the Lipinski's rule of five (Ro5) which states four characteristics, H-bond donors and acceptors, molecular weight, and log *P* that are usually needed to make molecules more drug-like or orally active (Lipinski, 2000). For example, poor absorption or permeation of a drug is more likely when:

- There are more than 5 hydrogen-bond donors.
- There are more than 10 hydrogen-bond acceptors.
- Molecular weight is greater than 500.
- Log *P* is greater than 5.

Furthermore, molecules with a polar surface area of greater than 140 Å are usually believed to be poor at permeating cell membranes. Keeping in view the significance of these descriptors, the molecular descriptors of all the synthesized compounds as well as for the known IL-2 inhibitors were calculated and all the compounds were found to comply with the Ro5 cut-off limits (Figure S-1 in Supplementary data for known IL-2 inhibitors).

MD simulation studies

MD simulation studies were carried out on chalcone **6** identified as the lead of the library with $IC_{50} = 1.5$ μg/mL. We initiated our analysis of the MD results by plotting pressure, density, temperature, total energy, kinetic energy, and potential energy graphs from the output files. It was noticed that the temperature gradually increased and equilibrated reasonable by around 300 K. Similarly, changes in the pressure, kinetic energy, and potential energy were also stabilized at equilibrated values. Mass-weighted RMSD (only polypeptide backbone) calculated as a function of time indicated the stability of protein atoms during simulation. The system was converged at an average value of 1.64 Å. The black curve shows that a plateau is formed till 7,500 ps indicating the presence of a precise conformation followed by an increment in the RMSD value. This rise could be augmented by a change in the conformation which eventually came down at the end of simulation run time (Fig. 4a), while mass-weighted RMSF calculated as a function of time demonstrated the fluctuation of amino acid residues (Fig. 4b).

The amino acid residues that were involved in hydrogen bonding interactions (Arg38, Thr41 and Val69) had very less fluctuation (less than 0.9 Å), since they were involved in hydrogen bonding interactions. The carbonyl oxygen atom of Val69 formed a strong hydrogen bonding interaction with the hydroxyl group of chalcone **6**. As indicated in docking studies, the hydrogen bonding interaction became stronger during 2,400–2,600 ps. However, it attained an average interatomic distance around 3 Å. The Oγ atom of Thr41 formed a hydrogen bond with the hydroxyl group of chalcone **6**. This hydrogen bonding interaction weakened more than 5 Å during the same time when Val69 interaction was strong, making the chalcone-specific toward the active site region. During the whole simulation run time, Thr41 was no longer involved with the ligand. The behavior of $N\varepsilon_2$ of Arg38 in the sense of hydrogen bonding interaction with the carbonyl group of chalcone remained fairly the same as that of Thr41. But it became very weak till 1,250 ps, afterward the plot showed the same trend as observed with the Thr41. It might be suggested that any charged moiety should be present at that site to form a strong interaction with Arg38 (Fig. 5).

Conclusion

A 50 membered library of small drug-like molecules both acyclic and cyclic were synthesized and their in vitro IL-2 potential was evaluated. Chalcones **5** and **6** were found to be the lead structures with IC_{50} values 1.5 and 1.4 μg/mL, respectively. In silico molecular docking and MD simulation studies also supported the results of in vitro studies. All these compounds were found to obey Ro5 and it is anticipated that these compounds may be potential candidates for developing new immunomodulating agents.

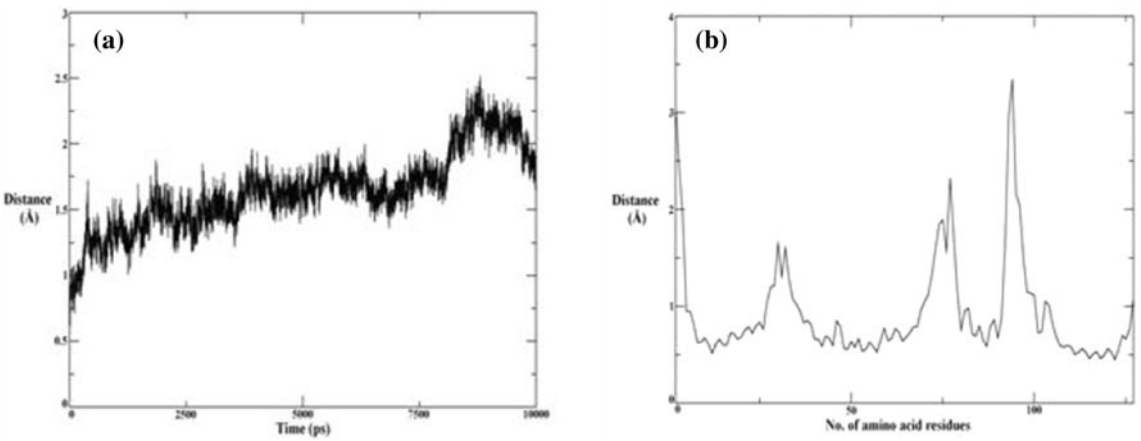


Fig. 4 **a** Mass-weighted RMSD (only polypeptide backbone) calculated as a function of time. **b** Mass-weighted RMSF (only polypeptide backbone) calculated as a function of time

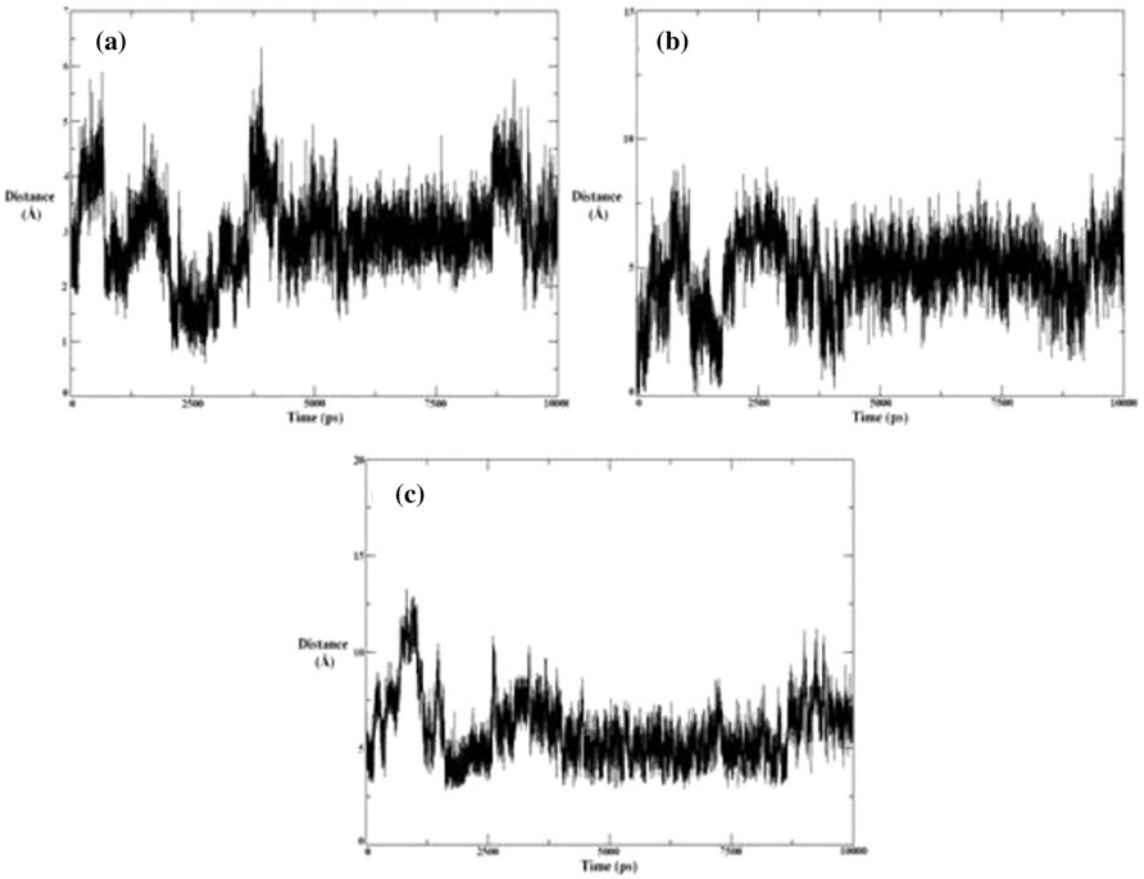


Fig. 5 Hydrogen bonding interactions between **a** carbonyl oxygen atom of Val69. **b** Oγ atom of Thr41 with the hydroxyl group of chalcone **6** and **c** $N\varepsilon_2$ of Arg38 with the carbonyl of chalcones, plotted as a function of time

Materials and methods

Chemistry

Synthesis and characterization of chalcones **1**, **9**, **10**, **11**, and **13** (Ansari *et al.*, 2005) and chalcones **3**–**6**, **12**, **14**, **36,** and **37** (Ahsan Ullah *et al.*, 2007; Ansari *et al.*, 2009) have been reported by our group. Furthermore, synthesis and characterization of pyrazolines **20** (Baseer *et al.*, 2013), 2,3-dihydro-1,5-benzothiazepines **21**–**26** (Ansari *et al.*, 2008a, b), and tetrahydrobenzothiazepines **27**–**29** (Ansari *et al.*, 2005; Parvez *et al.*, 2003) have also been reported by our group. Heteroazepinones **38**–**40**, semicarbazone**s 47**–**50** (Jafri *et al.*, 2012) and dihydropyrimidines **41**–**46** (Akbas and Furgan, 2008) have also been reported by our group.

2-Amino chalcones **2**, **7**, **8** (Yang *et al.*, 2000), chalcone epoxide **15** (Kee and Gavriilidis, 2007), oxazolines **16**, **17** (Sharma and Sharma, 2010), pyrazolines **18**, **19** (Sivakumar *et al.*, 2010), ferrocenyl chalcones **30**–**32** (Wu *et al.*, 2002) and biphenyl chalcones **33**–**35** (Thirunarayanan and Vanangamudi, 2007) have been synthesized in the lab and their physical constants agreed with the reported data. Characterization data of yet unreported heteroazepinones **41**–**43** has been given below.

*2-Methyl-2,3-dihydrobenzo[b][1,4]oxazepin-4(5H)-one (**41**)*

Yield 70 %, mp. 222 °C, ^{1}HNMR ($CDCl_3$): δ 7.28–7.89 (m, 4H, aryl-H), 8.75 (bs, NH), 3.75 (m, 1H, $J_{4,3a} = 4.2$ Hz, $J_{4,3b} = 4.2$ Hz, $J_{4,3b} = 7.2$ Hz, $J_{4,Me} = 6.0$ Hz, H-4), 2.42 (dd, 1H, $J_{3a,3b} = 12.6$ Hz, $J_{3a,4} = 4.4$ Hz, H-3a), 2.17 (dd, $J_{3b,3a} = 12.8$ Hz, $J_{3b,4} = 7.2$ Hz, H-3b), 1.37 (d, $J_{4,Me} = 6.0$ Hz, Me). ^{13}C NMR (75 MHz, DMSO-d_6): 20.4 (Me), 43.3 (CH_2), 55.4 (CH), 108–124 (Ar–C), 172.0 (C=O), GC–MS (*m*/*z*): 177 (M^+).

*4-Methyl-4,5-dihydro-1H-pyrido[2,3-b][1,4]diazepin-2(3H)-one (**42**)*

Yield 60 %, mp. 194 °C, ^{1}H NMR ($CDCl_3$): δ 7.94 (d, 1H, $J = 8.3$ Hz, H-9), 6.66 (dd, 1H, $J = 2.5$ Hz, H-8), 7.95 (d, $J = 2.5$ Hz, H-7), 3.02 (m, 1H, $J = 4.2$ Hz, $J = 7.2$ Hz, $J = 6.0$ Hz, H-4), 2.49 (dd, 1H, $J = 12.8$ Hz, $J = 4.2$ Hz

H-3a), 2.22 (dd, 1H, $J = 12.8$ Hz, H-3b). ^{13}C NMR (75 MHz, DMSO-d_6): 21.0 (Me), 42.7 (CH_2), 53.0 (CH), 106–142 (Ar–C), 170.0 (C=O), GC–MS (*m/z*): 177 (M^+).

*4-Methyl-4,5-dihydro-1H-naphtho[2,3-b][1,4]diazepin-2(3H)-one (**43**)*

Yield 60 %, mp. 240 °C, ^{1}HNMR ($CDCl_3$): δ 6.5–7.8 (m, aryl-H), 8.0 (s, 1H, NH), 3.02 (m, 1H, $J_{4,3a} = 4.2$ Hz, $J_{4,3b} = 7.2$ Hz, $J_4 = 6.0$ Hz, H-4), 2.55(dd, 1H, $J_{3a,3b} = 12.8$ Hz, $J_{3a,4} = 4.2$ Hz, H-3a), 2.3 (dd, 1H, $J_{3b,3a} = 12.8$ Hz, $J_{3b,4} = 7.2$ Hz, H-3b), 1.10 (d, 3H, $J_{4,Me} = 6.0$ Hz, Me). ^{13}C NMR (75 MHz, DMSO-d_6): δ 20.0(CH_3), 42.7 (CH_2), 53.0 (CH), 108–126 (Ar–C), 170.7 (C=O) GC–MS (*m/z*): 226 (M^+).

Biologic activities

T-cell proliferation assay

Fresh venous blood from healthy donor was mixed with equal volume of RPMI-1640 incomplete medium (Mediatech Inc., Herndon, VA, USA) containing 2 mM L-glutamine. The diluted blood was then layered onto lymphocyte separation medium (MP Biomedicals, Inc., Ohio, USA), and centrifuged for 20 min at 25 °C. The mononuclear cell layer was collected, washed with incomplete RPMI-1640 and centrifuged for 10 min and at 4 °C. The peripheral blood mononuclear cells (PBMNCs) were resuspended in RPMI-1640 containing 10 % fetal bovine serum of PAA laboratories GmbH, Pasching, Austria. In a 96-well plate (IWAKI, SciTech. DIV., Ashai Techno glass, Japan), 50 µL of cell suspension (2.5×10^6 cell/mL), 50 µL of PHA with a final concentration of 5 µg/mL, 50 µL supplemented RPMI-1640, and 50 µL of test compounds in a final concentration of 0.5, 5, and 50 µg/mL in triplicate were added. Plates were then incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air for 72 h. To each well, 0.5 µCi [methyl-^{3}H]-thymidine (Amersham Place Little Chalfont, Buckinghamshire, UK) was added for additional 18 h. Cells were harvested using Inotech cell harvester (Dottikon, Switzerland), and radioactive thymidine incorporation level was measured by Beckman coulter liquid scintillation counter (LS 6500, Fullerton, CA, USA).

IL-2 assay

The PBMNCs were cultured in a 96-well flat-bottomed plate (1.0×10^5 cell/well) in the presence or absence of three concentrations of test compounds (0.5, 5, and 50 µg/mL), and phytohemagglutinin PHA/PMA in a final concentration of 5 and 20 ng/mL. After an incubation period of 18 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air, supernatant was collected for IL-2 determination. IL-2 levels were measured using enzyme-linked immunosorbent assay (ELISA) development kit (R&D systems, Minneapolis, MN, USA). 96-well ELISA plates were coated with 4.0 µg/ml mouse anti-human IL-2, in phosphate buffered saline (PBS) at 7.4 pH. Then, 100 µL recombinant human IL-2 standards (R&D systems, Minneapolis, MN, USA) and culture supernatants samples were added. The plates were incubated for 2 h. at room temperature and washed three times with PBS, followed by the addition of 100 µL/well of biotinylated goat anti-human IL-2 and further incubated for 2 h at room temperature. Plates were again washed and 100 µL/well of streptavidin conjugated horse reddish peroxidase was added and incubated for additional 20 min at room temperature. After three final washes, 100 µL/well of the enzyme substrate solution of H_2O_2 and tetramethylbenzidine (1:1 v/v) was added and the color was allowed to develop at room temperature in the dark. Plates were then read at 450 nm in a plate reader (DIAReader GMBH, Wr. Neudorf, Austria). Results were analyzed using Microsoft Excel software.

Chemiluminscence assay (ROS)

In this assay, formation of the ROS in whole blood during the oxidative burst was measured by the luminol-enhanced chemiluminescence assay procedure (Waldmann and O'Shea, 1998; Michael and David, 2005). In brief, three concentrations of each compound (1.0, 10 and 100 µg/mL) were prepared in 25 µL of Hank's Buffered Salt Solution ($HBSS^{2+}$) in 96-well white flat-bottomed plate for a final incubation volume of 100 µL. Then, 25 µL of whole blood diluted 1:50 in suspension of $HBSS^{2+}$ with calcium chloride and magnesium sulfate from Sigma (St. Louis, USA) was added. Positive, negative control, and blank wells were included in the assay. Cells and compounds were incubated for 30 min at 37 °C, then 25 µL luminol (3-aminophthalhydrazide) purchased from Research Organics (Cleveland, OH, USA), was added into each well and 25 µL serum opsonized zymosan (*Saccharomyces cerevisiae* origin) purchased from Fluka, (Buchs, Switzerland) was added except for negative and blank wells. The ROS chemiluminescence kinetic was monitored with luminometer from Lab Systems Luminoskan, (Helsinki, Finland) for 50 min in the repeated scan mode. Peak and total integral chemiluminescence reading were expressed in the relative light unit (RLU).

Cytotoxicity assays using 3T3-L1 and CC-1 cell-lines and MTT

In vitro cytotoxicity assays were performed as described by Scholz *et al.* (1999) using the 3T3-L1 mouse embryo

fibroblast cell line (American Type Culture Collection (ATCC), Manassas, VA 20108, USA), and CC-1 cells, a rat Wistar hepatocyte cell line (European Collection of Cell Cultures, Salisbury, UK). The CC-1 cells were suspended in Minimum Essential Medium Eagle (MEM) supplemented with 10 % FBS, 2 mM glutamine, 1 % non-essential amino acids and, 20 mM HEPES, while the 3T3-L1 cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) formulated with 10 % FBS. Using flat-bottomed plates, both cell lines were plated at a concentration of 6×10^4 cells/mL and incubated for 24 h at 37 °C and 5 % CO_2 environment. After removal of medium, cells were challenged with three different concentrations (1.0, 5.0, and 20 µg/mL) of compounds in triplicate and were then further incubated for 48 h at 37 °C in CO_2 incubator. Following exposure to each compound, cells viability was assessed using 0.5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h followed by removal of supernatant and the addition of DMSO to solubilize the formazan complex. Plates were read at 540 nm after one minute shaking and readings were processed using MS Excel software. Results were expressed as mean ± SD of triplicate readings.

Computational studies

Molecular docking method

Docking studies were performed, using the Molecular Operating Environment by Chemical Computing Group Inc. (MOE 2011.10) on a Pentium 1.6 GHz workstation, 512 MB memory using the Windows Operating system. The data of high resolution crystal structure of IL-2 complex with its ligand FRG were obtained from Protein Data Bank (PDB entry code 1M48) (Arkin *et al.*, 2003). The study was carried out on B subunit of enzyme protein. The edited crystal structure after removing water molecules was imported into MOE and all hydrogen atoms were added to the structure with their standard geometry followed by their energy minimization using MOPAC 7.0. The resulting model was subjected to systematic conformational search at default parameters with RMS gradient of 0.01 kcal/mol using Site Finder (Ansari *et al.*, 2011). Enzymes were searched for their active sites and dummy atoms were created from the resulting alpha spheres. The backbone and residues were kept fixed and the energy minimization was performed. RMSD was used to compare the ligand between the predicted and its corresponding crystal structure. The resulting docked poses with RMSD less than 1.5 Å were clustered together. The lowest energy minimized pose was used for further analysis. Rule of five parameters (Ro5) were calculated by ligand property calculation function of MOE.

Molecular dynamics simulation method

Molecular dynamics simulations were carried out for IL-2 (PDB entry code 1M48) with the docked inhibitor **6** in an explicit solvent system using the AMBER 10.0 package. AMBER03 force field parameters were used to establish the potential of IL-2 and generalized AMBER force field (GAFF) parameters were used to generate the potentials of chalcone **6**. To ensure the electroneutrality of the system, one Na^+ ion was added in the modeled system followed by solvation coupled with TIP3P rectangular box around the solute unit. Overall, the system covered a dimension of $70.056 \times 77.104 \times 90.733$ Å^3 containing 37, 239 all-atoms (having 10, 425 water molecules inclusive). The solvated system was subjected to thorough energy minimizations before MD simulation. First, restrain minimization of water molecules was done while holding the solute fixed (5,000 steps using the steepest descent algorithm followed by 5,000 steps of conjugate gradient minimizations of the whole system). This was done to remove the steric conflicts between a protein and water molecules and also to relax the system. An unrestrained minimization was then carried out using the same procedure as for restrained minimization. Bond lengths involving hydrogen atoms were constrained with SHAKE (Ryckaert *et al.*, 1977) algorithm with harmonic restraints of 25 kcal/mol Å^2. The minimized system was first subjected to a gradual temperature increase from 0 to 300 K over 40 ps, and then equilibrated for 25 ps at 300 K. An unrestrained equilibration was also done for 100 ps followed by production runs of 10 ns. NPT ensemble system that is constant temperature (298 K) and constant pressure (1 atm) were controlled by the Berendsen coupling algorithm with a time constant for heat-bath coupling of 0.2 ps. The dielectric constant was set to 1.0. The cut-off distance was set to 9.0 Å. Long-range electrostatic calculations were carried out by particle mesh Ewald method (Essmann *et al.*, 1995). The PTRAJ module of AMBER package was used for the analysis of the subsequent trajectories. VMD (version-1.8.6) (Humphrey *et al.*, 1996) CHIMERA (Pettersen *et al.*, 2004) was also used to visualize the snapshots (structure changes obtained after the MD simulations). The MD simulation studies were calculated using the MPI SANDER module of AMBER 10 installed on cluster computing facility consisting of 10 nodes (equipped with Intel® Xeon® Quad™ core processor running under openSuSE LINUX OS).

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