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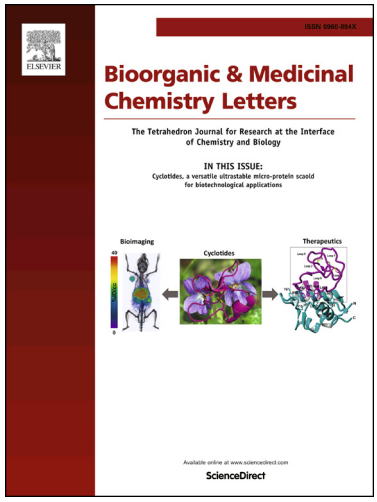

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Synthesis and *in-vitro* evaluations of 6-(hetero)-aryl-imidazo[1,2-*b*]pyridazine -3- sulfonamide's as an inhibitor of TNF-α production.

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Abstract

Tumor necrosis factor-α is an important pro-inflammatory cytokine having a key role in hosts defensive process of immune systems and its over expression led to a diverse range of inflammatory diseases such as Rheumatoid arthritis, Cronh's disease, psoriasis, etc. This paper describes our medicinal chemistry efforts on imidazo[1,2-*b*]pyridazine scaffold: design, synthesis and biological evaluation. By the introducing sulfonamide functionality at 3 positions and substituting 6 positions with (hetero)-aryl groups', a small library of compounds was prepared. All synthesized compounds were screened for lipopolysaccharide (LPS) mediated TNF-α production inhibitory activity. Biological data revealed that the majority of the compounds of this series showed moderate to potent TNF-α production inhibitory activity. Compound **5u** and **5v** are the most potent compounds from the series with activity of IC_{50} = 0.5 µM and 0.3 µM respectively. A short SAR demonstrates that 3-Sulfonyl-4-arylpiperidine-4-carbonitrile moiety on imidazo[1,2-*b*]pyridazine showed better activity compared to the 3-(4-aryllpiperazin-1-yl) sulfonyl) in hPBMC assay. The molecular modeling studies revealed that the potent TNF-α production inhibitory activity **5v** due to the extra stability of complex because of an extra pi-pi (π- π) stacking, hydrogen-bonding interactions.

Keywords

Imidazo [1,2-*b*]pyridazine, Sulfonamide, Tumor Necrosis Factor-Alpha (TNF-α), hPBMC (human peripheral blood mononuclear cells), RA (Rheumatoid arthritis).

Inflammation is self-protective process of the immune system to toxic stimuli, infection, tissue injury, etc. The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is having an important role in the body's defensive process of immune systems.[1,2] It was produced by the body as a natural physiological response to infections, tissue injury.[3-4] The excessive production of TNF-α led to the development of several inflammatory diseases such as rheumatoid arthritis (RA), psoriasis, inflammatory bowel disease, Crohn's disease and multiple sclerosis, etc.[4-10] An effective anti TNF biological therapies were available in the market, but the use of these biological agents was associated with several disadvantages.[10-15] A number of research groups devoted their considerable efforts to find out orally active small molecules as TNF-α production inhibitors with the same level of efficacy as biological agents without undesirable side effect; as a result, during the last few years quite a lot of classes of heterocyclic compound have been reported in literature as, TNF-α production inhibitor.[10, 16-17]

The structure of representative TNF-α inhibitor is illustrated in Figure1; compound (**I**) SPD304 having 1-(3-(trifluoromethyl)phenyl)-1*H*-indole moiety linked to a 6,7-dimethyl-4*H*-chromen-4-one by a dimethylethanamine spacer inhibits TNF-α production (IC_{50} = 22 μM),[18] the compound C87 (**II**) having thiazole and pyrazolidin-3-one core inhibits TNF-α production with IC_{50} = 9 μM[19] while the compound BIHC (**III**) having chromene and imidazole rings inhibits TNF-α production with IC_{50} = 16.5μM.[20] The rolipram (**IV**) is the most potent TNF-α production inhibitor ever reported with an IC_{50} = 0.2 μM.[21]

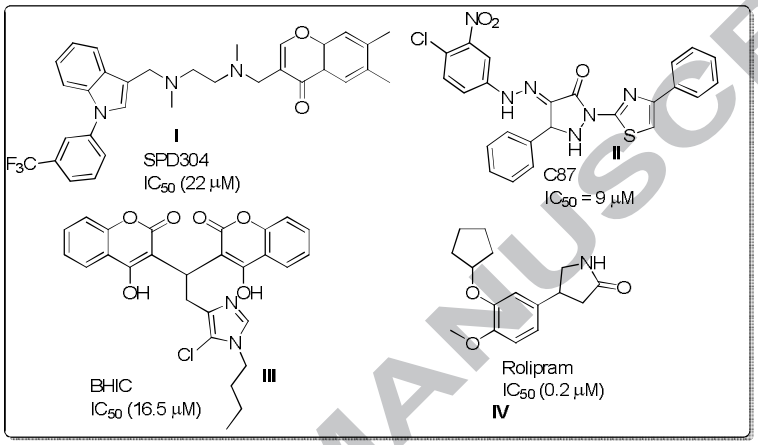


Figure 1. Molecular structure of representative TNF-α production inhibitor.

The imidazo[1,2-*b*]pyridazine have been identified as one of the promising scaffold in medicinal chemistry. Which have been known to possess a wide range of biological activities, including studied for treatment of inflammatory diseases such as, treatment of rheumatoid arthritis, JAK inhibitors (**VI**)[23], TNF-α inhibitors (**V**)[22, 25-26] treatment of dermal allergic inflammation[24] treatment of inflammatory diseases caused by dysfunctions of kinases,[27] etc. The compound **V** (**Figure 2**), reported by H. Shimizu et.al inhibits TNF-α production with IC_{50} = 1.7 μM.[22]

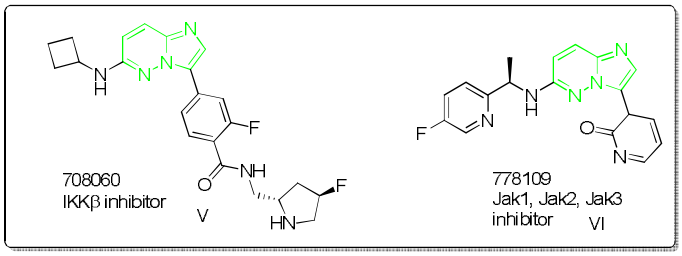


Figure 2. Molecular structure of representative developmental imidazo[1,2-*b*]pyridazine for treatment of rheumatoid arthritis (RA).

Similarly sulfonamide is an important pharmacophore having attractive pharmacological properties. It is present in many drug molecules from different therapeutic

category, such as antibacterial, diuretic, anti-viral, anti-inflammatory, anti-HIV, etc. More than 112 marketed drug molecules such as celecoxib, meloxicam, piroxicam, sulfasalazine, etc. have sulfonamide functionality.[28] Shen et. al. discovered the anti-TNF small molecule by the virtual screen (**IX**) also having sulfonamide motifs (**Figure 3**).[29] Further, upon additional SAR studies Shen and co-worker found out that most potent compound (**X**) contains two sulfonamide functional groups. They have reported on their investigations, the sulfonamide makes extra hydrogen bonds in the binding pocket of TNF with polar residues and the extra hydrogen-bonds increase TNF-α inhibition ability of compounds.

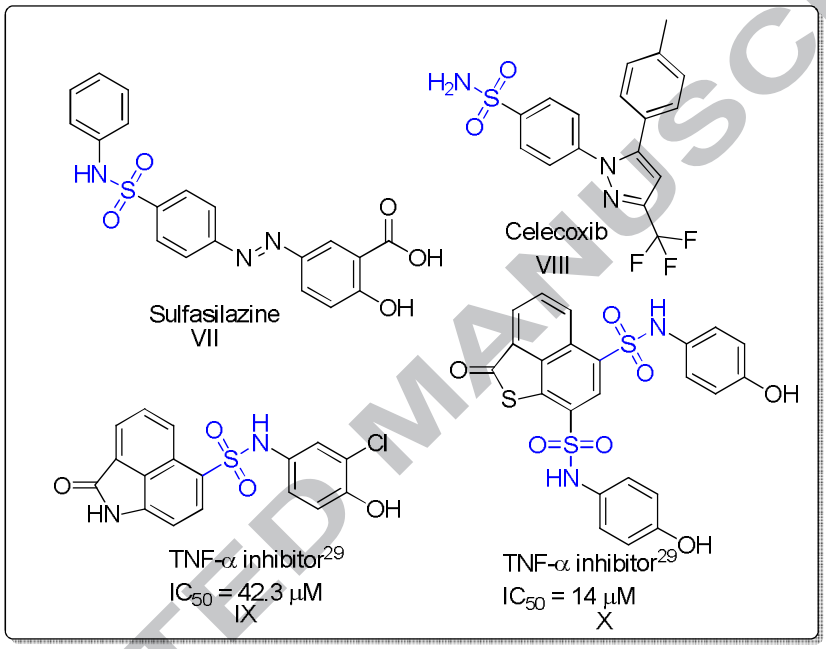


Figure 3. Molecular structure marketed sulfonamide derivatives for the treatment of inflammatory diseases and biologically active TNF-α inhibitor

In recent years we have been engaged in the design, synthesis of novel heterocyclic compounds and evaluate their TNF-α production inhibitory potential.[30] In persistent of our work and motivated by aforementioned literature, we envisioned our approach toward design and synthesis of 6-(hetero)-aryl-imidazo[1,2-*b*]pyridazine -3- sulfonamide's for evaluation of TNF-α production inhibitory potential. Therefore the single molecule which contains pharmacologically active sulfonamide functionality and biologically important imidazo[1,2-*b*]pyridazine core (**Figure 4**) could be more beneficial for inhibiting TNF- α production. We are reporting for the first time, 6-(hetero)-aryl-imidazo[1,2-*b*]pyridazine-3-sulfonamide derivatives as TNF-α production inhibitor.

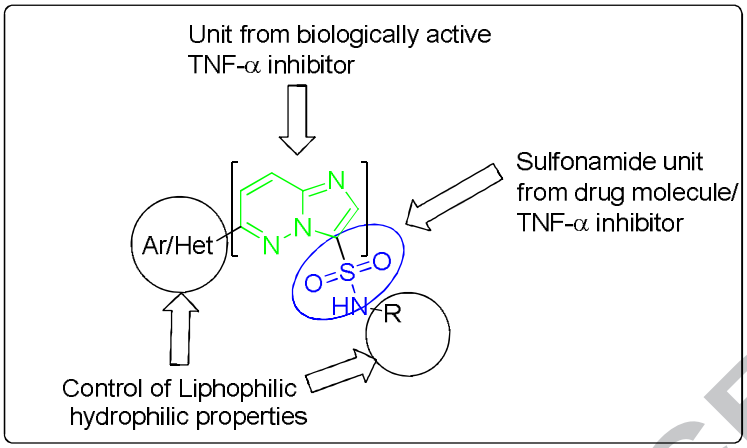
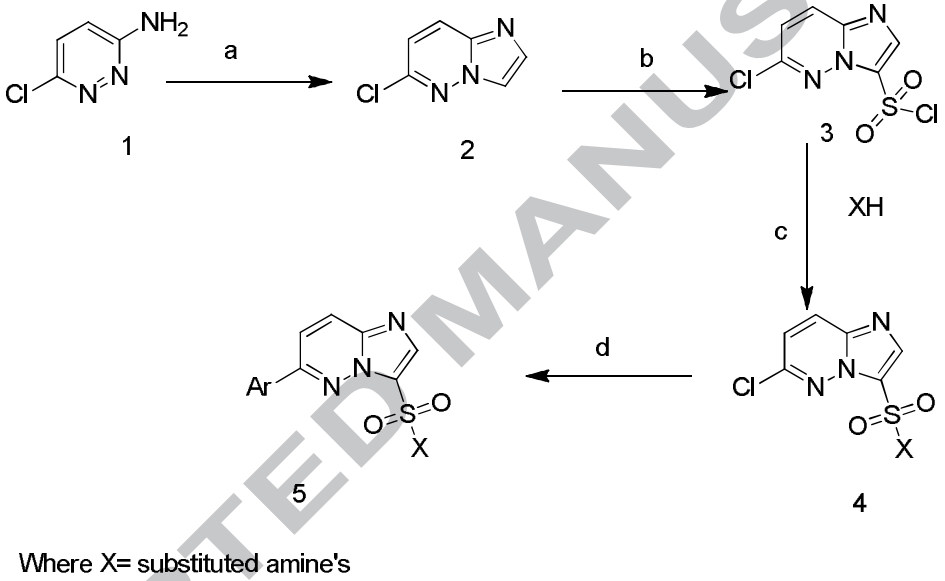


Figure 4. Designed Modification on imidazo[1,2-*b*]pyridazine core

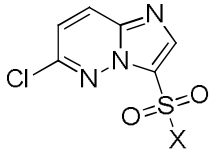


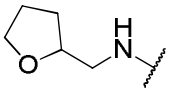
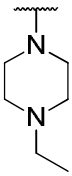
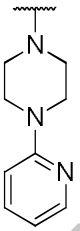
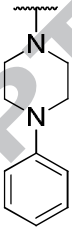
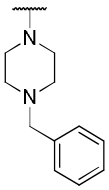
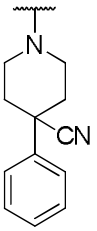
Scheme 1. Reagents and conditions: a) Chloroacetaldehyde dimethyl acetal, NaOAc, Conc. HCl, 60% aqueous ethanol, 80 °C, 16 h, 87%; (b) i) $ClSO_3H$, Ethylenedichloride, 80 - 85 °C, 3 h, ii) Cool to 50 °C, triethylamine, 10 min.; iii) $POCl_3$, 100 - 105 °C , 16 h, 35%; (c) Triethylamine, Ethylenedichloride, 25-30 °C, 1 - 3 h, 28 – 73%; (d) $ArB(OH)_2$/ArB(pin), K_2CO_3, $Pd(PPh_3)_2Cl_2$, DMF-H_2O, 120 °C, 3-5 h, 12 – 76%.

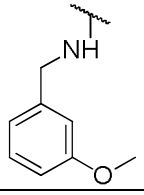
Syntheses of the designed derivatives of imidazo[1,2-*b*]pyridazine,**(5)** was achieved by the reaction sequence as depicted in Scheme 1**.** The reaction of 3-amino-6-chloropyridazine **(1)** with 2-chloro-1,1-dimethoxyethane in the presence of sodium acetate and concentrated hydrochloric acid in 60% aqueous ethanol at reflux produced core 6-chloroimidazo[1,2-*b*]pyridazine **(2)** as an off-white solid in 87% yield. The chlorosulfonation of the **2** to 6-chloroimidazo[1,2-*b*]pyridazine-3-sulphonylchloride **(3)** was achieved by sequential one pot reaction with chlorosulphonic and phosphorous oxychloride.[31] The reaction of 6-chloroimidazo[1,2-*b*]pyridazine-3-sulfonyl chloride (**3)** with suitable amine in the presence of triethylamine in ethylenedichloride produced sulfonamides **(4a-h)** in 29 – 73% yield. Finally, the Suzuki-Miyaura coupling of compound **4** with respective boronic acid or its pinacol ester in the presence of catalytic amount of bis-(triphenylphosphine)palladium(II) dichloride, potassium carbonate, and DMF-water provided desired inhibitors **5a-ag** in (12 - 76%) yield.

The purity of compounds synthesized was confirmed by TLC and HPLC. All newly synthesized compounds (Table 1, **4a-h** and Table 2, **5a** to **5ag**) were in full agreement with their spectral data ^{1}H-NMR and MS.

Table 1. Inhibition of TNF-α production by imidazo[1,2-*b*]pyridazines



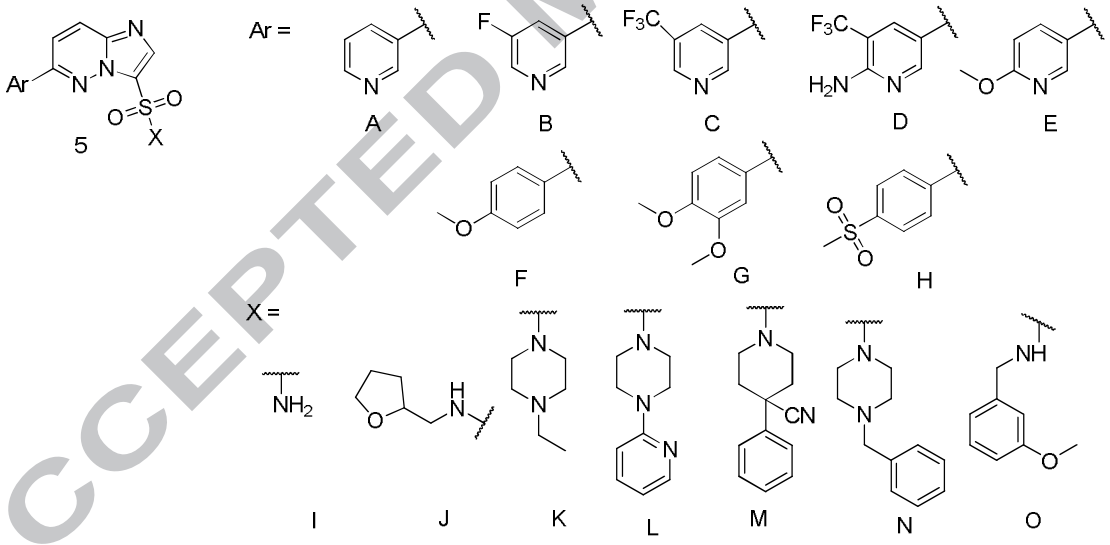
Sr. No.	Compound no.	X	TNF-α inhibition (IC_{50} in µM)[a]	Toxicity IC_{50} in µM	Docking Score	Glide energy (kcal/mol)
1	4a		28	>30	-6.052	-32.196
2	4b		30	>30	-5.895	-31.565
3	4c		>30	>30	-5.495	-26.117
4	4d		>30	>30	-5.551	-26.473
5	4e		30	>30	-5.944	-30.849
6	4f		30	>30	-5.759	-31.451
7	4g		25	>30	-6.099	-37.758

8	**4h**		>30	>30	-5.549	-28.137

[a] IC_{50}: the concentration that affords 50% inhibition of TNF-α, values are mean of three independent experiments. Assay conditions listed in the reference section, ref. no.34.

The TNF-α production inhibitory activity of the analogs of **4** was ascertained using LPS treated hPBMC assay in comparison with standard rolipram and activities are summarized in **Table 1**. The sulfonamide **4a**, **4b**, **4e**, **4f**, **4g** showed moderate TNF-α inhibitory activity ranging from 25 to 30 µM and other sulfonamides **4c**, **4d**, **4h** were inactive. The sulfonamide **4g** with 4-phenylpiperidine-4-carbonitrile moiety is the most active compound among the derivatives of **4**. The un-substituted sulfonamide **4a** showed TNF-α production inhibition with IC_{50} = 28 µM. **Table 1** depicts that, the designed sulfonamide derivatives of **4** showed moderate activity for TNF-α production inhibition.

Table 2. Inhibition of TNF-α production by imidazo[1,2-*b*]pyridazines**(5)**



Sr. No.	Compound No.	X	Ar	TNF-α inhibition (IC_{50} in µM)[a]	Toxicity IC_{50} in µM	Docking Score	Glide energy (kcal/mol)
01	**5a**	I	D	4	>30	-7.082	-43.711
02	**5b**	K	A	15	>30	-6.458	-34.981
03	**5c**	K	B	>30	>30	-5.740	-30.045
04	**5d**	K	C	9	>30	-6.695	-38.719
05	**5e**	K	E	20	>30	-6.355	-33.022
06	**5f**	K	F	20	>30	-6.342	-33.185
07	**5g**	K	H	15	>30	-6.413	-34.372

08	**5h**	N	A	18	>30	-6.396	-33.992
09	**5i)**	N	B	>30	>30	-5.597	-29.525
10	**5j**	N	H	12	>30	-6.526	-35.293
11	**5k**	L	A	11	>30	-6.652	-36.490
12	**5l**	L	B	28	>30	-6.321	-32.144
13	**5m**	L	C	11	>30	-6.621	-36.751
14	**5n**	L	D	11	>30	-6.632	-36.365
15	**5o**	L	E	>30	>30	-5.487	-29.725
16	**5p**	L	H	12	>30	-6.500	-34.7438
17	**5q**	M	A	7	>30	-6.853	-40.894
18	**5r**	M	B	>30	>30	-5.685	-29.985
19	**5s**	M	C	20	>30	-6.346	-33.606
20	**5t**	M	H	8	>30	-6.739	-39.912
21	**5u**	M	G	0.5	>30	-7.430	-46.240
22	**5v**	O	A	0.3	>30	-7.773	-48.595
23	**5w**	O	B	6	>30	-6.953	-41.034
24	**5x**	O	C	>30	>30	-5.711	-27.652
25	**5y**	O	D	>30	>30	-5.325	-25.439
26	**5z**	O	E	>30	>30	-5.266	-24.405
27	**5aa**	O	H	28	>30	-6.334	-32.384
28	**5ab**	J	A	4	>30	-7.064	-42.785
29	**5ac**	J	B	4	>30	-7.063	-42.741
30	**5ad**	J	C	>30	>30	-5.727	-27.296
31	**5ae**	J	E	6	>30	-7.045	-41.604
32	**5af**	J	F	4	>30	-7.025	-41.842
33	**5ag**	J	H	>30	>30	-5.411	-25.265
34	Rolipram			0.5 µM	>30	-8.669	-50.378

[a] IC_{50}: the concentration that affords 50% inhibition of TNF-α, values are mean of three independent experiments. Assay conditions listed in the reference section, ref. no.34.

The in-vitro potencies of the 6-(hetero-)aryl-imidazo[1,2-*b*]pyridazine -3-sulfonamide (**Table - 2**, entry 1 - 33) are illustrated in the table 2. To begin with, we planned to synthesize the compound bearing simple sulfonamide group and synthesized compound **5a** by Suzuki-Muryia coupling of **4a** with 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3-(trifluoromethyl)pyridin-2-amine. Compound **5a** showed TNF-α production inhibitory activity with IC_{50} = 4 µM with 7 fold improvement in activity over the parent **4a.** This results were inspiring to us and showed that designed modification have potential for TNF-α production inhibition. Further, we explored SAR by synthesizing compounds by modification of simple sulfonamides to substituted sulfonamides. For this purpose, we synthesized compounds with substituted piperazine, substituted piperidine, 3-methoxybenzylamine and (tetrahydrofuran-2-yl)methanamine. If we see the table 2, some interesting trends in SAR were observed with the nature of substituents on sulfonamide and type of substituent at 6 positions of imidazo[1,2-*b*]pyridazines. Among the derivatives **5b – 5g** having 4-

ethylpiperazine-1-sulfonamide: **5d** having 5-(trifluoromethyl)pyridine group showed 3 fold more activity (IC_{50} = 9 µM) over its chloro analog. Whereas upon changing substituent from 5-(trifluoromethyl)pyridine to less lipophilic 5-fluoropyridine (compound **5c**) loss in activity was observed. The rest of the analogs from this series (4-ethylpiperazine-1-sulfonamide) bearing, pyridine (**5b**), 6-methoxypyridine (**5e**), 4-methoxyphenyl (**5f**) and 4-methanesulfonylphenyl (**5g**) showed moderate activity (IC_{50} = 15 - 20 µM). Further, we synthesized compound with a more lipophilic benzyl group on piperazine. If we compare the activity of inhibitors bearing benzylpiperazine with 4-ethylpiperazine-1-sulfonamide derivatives, the activity is almost same with little difference (**Table-2**, compound **5h - j**). The compound **5h**, **5j** having 3-pyridyl and 4-methanesulfonylphenyl substitutions showed moderate activity for TNF-α inhibitions with IC_{50} = 18 and 12 µM respectively and the other compounds from this series were inactive. From the series of compounds **5k-p** with *N*-pyridylpiperazine substitutions, **5m** and **5n** showed moderate activity with IC_{50} = 11 µM, inhibitor **5p** having 4-(methylsulfonyl)phenyl substitutions showed IC_{50} = 12 µM and the compound **5l** with 5-fluoropyride derivative showed TNF- α inhibition with IC_{50} =28 µM. The compound **5o** having 6-methoxy-3-pyridyl substitution is inactive from the series. Most of the compounds from the piperazine series (**5b - p**) shows moderate activity. Further, we were curious to see the effect of changing piperazine to piperidine and we synthesized the compounds **5q – 5u** bearing 4-phenylpiperidine-4-carbonitrile. The compound **5u** with 3,4-dimethoxyphenyl substitutions showed promising TNF-α production inhibitory activity (IC_{50} = 0.5 µM). The **5u** is 50 fold more active over its chloro analog **4g**. This indicates the replacement of chloro with the lipophilic 3,4-dimethoxyphenyl is favorable for activity**.** The **5q,** with 3-pyridyl substitutions showed good TNF-α activity IC_{50} = 7 µM and the compound **5s** with 5-(trifluoromethyl)pyridine substitutions showed moderate activity (IC_{50} = 7 µM). All the compounds of this series bearing 4-phenylpiperidine-4-carbonitrile showed very well to moderate activity indicating piperidine substitutions are very well tolerated. Additionally, we explored the SAR by synthesizing compounds with 3-methoxybenzylamine and (tetrahydrofuran-2-yl)methanamine sulfonamide derivatives (Compound **5v - 5ag**). Compound **5v** with 3-pyridyl substitutions showed most potent TNF-α production inhibitory activity throughout the synthesized derivatives of **5** with IC_{50} = 0.3 µM. **5v** showed 100 fold improvement in activity over its chloro analog **4h**. **5w** showed moderate activity (IC_{50} = 6 µM) and the compound **5x, 5y, 5z** were inactive. Among (tetrahydrofuran-2-yl)methanamine sulfonamide derivatives **5ad, 5ag** were inactive, **5ab**, **5ac**, **5ae** and **5af** showed good activity (IC_{50} = 4 – 6 µM).

If we see the results illustrated in Table1 and Table 2, some interesting trends in SAR are observed with the nature and type of substitutions on sulfonamide and 6th position of imidazo[1,2-b]pyridazine. The replacement of chloro with (hetero)-aryl results in improvement in activity. However, in some cases there is no change or little change in activity is observed after replacement of chloro to corresponding (hetero)-aryl. Form the SAR one can conclude that the (hetero)-aryl group as well as the substitutions on sulfonamide have contributed towards the activity. 3-Sulfonyl-4-arylpiperidine-4-carbonitrile moiety on imidazo[1,2-*b*]pyridazines showed better activity compared to the 3-(4-aryllpiperazin-1-yl)sulfonyl) in hPBMC assay. Compound **5u** with substituted piperidine sulfonamide and 3,4-dimethoxyphenyl substitution at 6th position of imidazo[1,2-*b*]pyridazines inhibit production of TNF- α with IC_{50} = 0.5 µM. Also the compound **5v** having *N*-(3-methoxybenzyl)-sulfonamide with pyridine substitutions 6-positions of imidazo[1,2-*b*]pyridazines showed TNF-α production inhibition with IC_{50} = 0.3 µM. Compound **5u** and **5v** are the most potent compounds among the series. The more lipophilic 3-trifluoromethyl group on pyridine showed better activity rather than fluoro substituted pyridine. The 4-(methylsulfonyl)phenyl is well tolerated as compared to 4-methoxyphenyl or 6-methoxypyridine only exception in case of (tetrahydrofuran-2-yl)methanamine (inhibitors **5ae** and **5af** inhibits TNF-α production with IC_{50} 6 µM and 4 µM while the **5ag** bearing 4-(methylsulfonyl)phenyl is inactive).

We were further interested in understanding the binding mode of these inhibitors with binding pocket of TNF-α in complex. The molecular docking study was performed using Glide (Grid-Based Ligand Docking with Energetics)[32-33] module incorporated in the Schrödinger molecular modeling software (Schrödinger, Inc., USA, 2016) to investigate the binding modalities of sulfonamide analogues into the active site of toward possible targets comprising of TNF-α . The crystal structure of the target TNF-α in complex with its inhibitor (pdb code: 2az5) was retrieved from the Protein Data Bank (www.rcsb.org) and further optimized and minimized to obtain a low energy and structural correct target protein using the *Protein Preparation Wizard*. The protein structure was prepared for docking by adding essential hydrogen atoms, eliminating the cocrystallized water molecules (since none of them were found to be conserved in the ligand interaction) and assigning all atom force field (OPSL-2005) charges and atom types. Finally, the prepared structure was subjected to energy minimization until the average root mean square deviation of non-hydrogen atoms reached 0.3 Å in order to relieve the steric clashes among the residues due to addition of hydrogen atoms. The 3D structures of the ligands were sketched under investigation were sketched using the *build* panel in Maestro and refined using *LigPrep* module. This involved addition of

hydrogens, adjusting realistic bond lengths and bond angles, correction of chirality's and ionization states, generation of tautomer's, and ring conformations and assignment of partial charges to the structure using the OPLS-2005 force-field. Thereafter, each of them was subjected to energy minimization setting a RMSD cutoff of 0.001Å. After ensuring that the enzyme and ligands were in the correct form, target grid was generated using the position of the native ligand as the reference co-ordinates to define the active site for docking. The *Receptor Grid Generation* panel in Glide generates two cubical boxes having a common centroid to organize the calculations: a larger enclosing and a smaller binding box. With the non-covalently bound native ligand in place, the active site was defined by a box that has dimensions of 14x14x14Å centered on the centroid of native ligand in the crystal complex which was large enough to allow ligands to explore a large portion of the enzyme. All the sulfonamide molecules under study were docked into the binding site of TNF-α using the extra precision mode (XP) and Glide Score has been used to predict the binding affinity and ranking of the ligands. The Glide algorithm adopts a funnel approach which involves performing a systematic search of positions, orientations, and conformations of the ligand in the receptor binding site. The search begins with a random positioning of ligand in the active site and scoring phase that significantly limits the search space and reduces the number of poses to be selected for minimization on the precomputed OPLS-2005 van der Waals and electrostatic grids for the protein. The lowest-energy poses (5–10) obtained at this stage are then subjected to Monte Carlo simulations in which the ligand-receptor complex is subjected to a full molecular mechanics energy minimization (OPLS–AA force field) in order to optimize flexible side-chain residues of TNF-α that may interact with the ligands. Finally the energy minimized poses accepted are further rescored using the GlideScore function, which is a more sophisticated version of ChemScore.[32, 33] This force field includes additional terms accounting for solvation and repulsive interactions. The output file in terms of the docking poses of the ligands was visualized and quantitatively analyzed for the key elements of interaction with the target enzyme using the Maestro's Pose Viewer utility.

The molecular docking study revealed that all the sulfonamide analogues could optimally fit into the active site of TNF-α with varying degree of affinities adopting a very similar orientation and at co-ordinates close to that of the native ligand in the crystal structure. Their complexation was stabilized through a network of significant steric and electrostatic interactions. Their docking scores ranged from -5.266 to -7.773 while the minimum energy for the formation of the complex between the TNF-α and each of these analogues (Glide energy) was also observed to be negative, ranging from -24.405 kcal/mol to -48.595 kcal/mol

while the docking score and binding energy for the reference ligand-Rolipram **(Figure 1S)** (found in ESI section) was found to be -8.669 and -50.378 kcal/mol respectively. These findings are in agreement with the experimentally observed anti-inflammatory activity wherein the active compounds showed higher docking scores while those with relatively low inhibition were also predicted to have a lower score **(Table 2)**. A higher negative value for the docking score and the binding energy (Glide energy) signifies a good binding affinity of the ligand towards the target and vice versa. The binding energy represents the energy required for a ligand to cover the entire enzyme surface and its potential interactions with residues lining the active site.

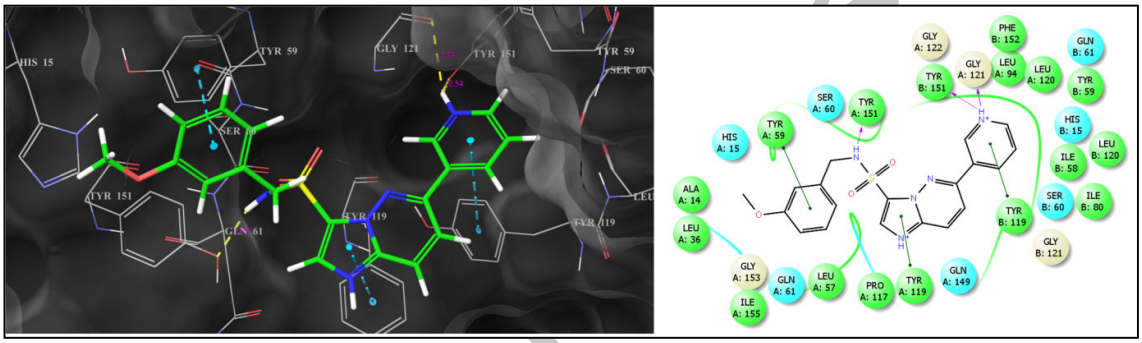


Figure 5. Binding mode of 5v into the active site of TNF-α (on right side: green lines signify pi-pi stacking interactions while the pink lines represent the hydrogen bonding interactions)

A detailed quantitative analysis of the per-residue interaction between the residue lining the active site of the TNF-α and these sulfonamide derivatives was carried out to identify the most significantly interacting the residues and the type of thermodynamic elements (bonded and non-bonded interactions) governing the binding of these molecules to the target. However, in order to maintain the brevity of text, this analysis is elaborated herein only for the most active compound **5v** while the results for the remaining active molecules (**5a, 5d, 5q, 5u, 5w and 5ab**) are summarized in Table 03 (found in ESI section) and their binding modes are provided in the supplementary material as **Figures (2S-7S)** (found in ESI section).

The lowest energy docked conformation of the most active compound 5v, having *N*-(3-methoxybenzyl)-sulfonamide with pyridine substitutions at 6th positions of imidazo[1,2-*b*]pyridazine, into the active site of TNF-α (**Figure 5**) showed that the inhibitor binds at the same co-ordinates as the native ligand and the reference ligand- Rolipram (**Figure 1S**) with an excellent binding affinity producing a docking score of -7.773 with a binding energy -48.595 kcal/mol. This higher binding affinity is attributed to the specific bonded and non-

bonded per-residue interactions with the residues lining the active site of TNF-α. A detailed analysis of the per-residual interactions revealed that the compound is stabilized within the active site TNF-α through an extensive network of significant van der Waals interactions with Tyr:A151(-3.735 kcal/mol), Tyr:A119(-7.651 kcal/mol), SerA60(-1.812 kcal/mol) through the imidazo[1,2-*b*]pyridazine scaffold. Similarly the 3-methoxy benzylsulfonamide component is also engaged in favorable van der Waals interactions with Gln:B61(-1.576 kcal/mol), Gln:A61(-2.886 kcal/mol), Tyr:A59(-6.049 kcal/mol) and His:A15(-2.091 kcal/mol) while pyridine ring substituted at the 6^{th} position of the scaffold showed van der Waals interactions with Tyr:B151(-1.922 kcal/mol), Leu:B120(-1.968 kcal/mol), Tyr:B119(-3.917), Ser:B60(-1.996 kcal/mol), Tyr:B59(-2.852 kcal/mol), Gly:A121(-2.643 kcal/mol), Leu:A120(-2.293 kcal/mol). The enhanced binding affinity of **5v** can also be attributed to favorable electrostatic contacts observed with Gln:A149(-3.867 kcal/mol), Gly:B148(-1.392 kcal/mol), Glu:A116(-4.865 kcal/mol), Ser:A60(-1.884 kcal/mol), Ile:A58(-1.146 kcal/mol) through imidazo[1,2-*b*]pyridazine scaffold; with Tyr:A151(-5.506 kcal/mol), Val:A62(-1.666 kcal/mol), Gln:A61(-7.236 kcal/mol) via the 3-methoxy benzylsulfonamide component and with Tyr:B151(-5.019 kcal/mol), Gly:B122(-1.564 kcal/mol), Leu:B120(-2.145 kcal/mol), Glu:B116(-5.990 kcal/mol), Gln:B61(-3.531 kcal/mol), Ser:B60(-3.761 kcal/mol), Gly:A122(-1.453 kcal/mol), Gly:A121(-3.549 kcal/mol) residues through the pyridine ring respectively. Three prominent pi-pi (π-π) stacking interaction was also observed between the 3-methoxy benzylsulfonamide and Tyr:A59(2.812Å); between the imidazole ring of imidazo[1,2-*b*]pyridazine scaffold and Tyr:A119 (2.816Å) and finally between the pyridine ring and Tyr:B119 (2.669Å) residues lining the active site. Furthermore the compound is also held in the active site through three crucial hydrogen bonding interactions: with Tyr:B151 (2.54Å) and Gly:A121((2.73Å) through the pyridine ring and with Tyr:A151(1.99) through the amino component of sulfonamide moiety. These types of the pi-pi (π- π) stacking and hydrogen-bonding interactions not only serve as an "anchor", determining the 3D orientation of the ligand in the active site but also facilitate the steric and electrostatic interactions adding to the stability of the enzyme-inhibitor complex. A similar binding mode and set of interactions was observed for the **5a, 5d, 5q, 5u, 5w** and **5ab** as well but decreasing gradually with their observed anti-inflammatory activity.

Overall, it is evident from the molecular docking simulations and specifically from the per-residue interaction analysis that these sulfonamide derivatives have a promising affinity for the TNF-α qualifying them as pertinent starting points for structure-based lead optimization.

In summary, we have designed modifications on imidazo[1,2-*b*]pyridazines by the introduction of the sulfonamide functionality and synthesized a small library of 6-(hetero)-aryl-imidazo[1,2-*b*]pyridazines-3-sulfonamide series of compounds of biological interests. Synthesized compounds were evaluated for TNF-α production inhibitory potential, results are presented in Table 1 and 2. The detailed SAR was described; from the SAR it was concluded that, the (hetero)-aryl group as well as the substitutions on sulfonamide have contributed towards the activity and the designed modification has the potential for TNF-α production inhibition, The compound **5u** and **5v** are the most potent compounds from the series with IC_{50} = 0.5 and 0.3μM respectively. Furthermore, the molecular docking study of the compounds was carried out for the better understanding of the drug-receptor interactions at the active site TNF-α. The docking studies of molecules shed some light on the binding modes, and subsequently the binding potency of the SAR molecules on to active site of TNF-α compared to the standard rolipram. The promising findings observed herein provides a good starting point for an extensive medicinal chemistry program, which may lead to further identification of more potent TNF-α production inhibitor.

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34. a) hPBMC assay (to check the cytotoxic effect of the compounds on mononuclear blood cells): Blood was collected from healthy donors. It was then diluted in the ratio 1:1 with sterile endotoxin free PBS (w/o calcium and magnesium). Blood was then loaded onto histopaque (by Sigma Chemicals) in a 50 mL centrifuge (15 mL histopaque and 30 mL of blood). These tubes were centrifuged at 1500 rpm for 40 min with acceleration and break at 1. With a pipette, the upper plasma layer is gently removed and discarded leaving approximately 1 cm of buffy coat above the interface. With a pipette, the interface layer containing the PBMCs was gently collected in another 50 mL centrifuge tube and diluted with PBS in the ratio 1:1. The cells were the spun at 1800 rpm for 10 min. The pellet obtained was broken and resuspended in PBS, and centrifuged at 1000 rpm for 10 min. Above step was repeated once again,

this time cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). The cells were plated in 96-well tissue culture plates at a concentration of 1 x 10^6 cells/ml. The viability, as determined by trypan blue dye exclusion, was uniformly ≥98%. After plating, different concentrations e.g. (0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 µM) of the compound dissolved in dimethylsulfoxide (DMSO) were added to the cells and incubated at 37 ^oC for 30 min in 5% CO_2 atmosphere. The final concentration of DMSO was maintained at 0.5%. The vehicle (0.5% DMSO) was used as control. Rolipram (300 µM) was used as a standard. The cells were then stimulated with lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8) at a final concentration of 1 µg/mL and the incubation was continued for 5 h. The culture plate was centrifuged (2500 rpm for 10 min) and the supernatants collected and stored at -70 ^oC. The amount of TNF-α in the supernatants were assayed using the ELISA protocol recommended by the manufacturers (OptiEIA ELISA sets, BD BioSciences, Pharmingen). The percentage inhibition was calculated by using pg/mL levels of Vehicle control by the formula. % inhibition= (X-Y)/ X *100, Where X = Mean cytokine level of Vehicle control, Y = Mean cytokine level of Test.

b) Evaluation of cytotoxicity: The cytotoxicity of the test sample in hPBMC assay was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium) staining. 40ul of MTS-PMA solution was added to the cells and incubated for 5-6 hours till the absorbance reaches 0.5. Absorbance was measured at 490 nm. Percent cytotoxicity was calculated by the equation: % Cytotoxicity = (A-B)/A x100, Where X = Mean Absorbance of Vehicle control, Y = Mean Absorbance of Test.

† Electronic Supplementary Information (ESI) available: Chemistry protocols and experimental data, scan spectral, Quantitative per-residue interaction analysis and Molecular docking images.

Synthesis and *in-vitro* evaluations of 6-(hetero)-aryl-imidazo[1,2-*b*]pyridazine -3- sulfonamide's as an inhibitor of TNF-α production.

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Graphical Abstract