Synopsis

Of

The Thesis Entitled

"Design, synthesis and biological evaluation of novel Janus Kinase Inhibitors for the treatment of inflammatory diseases"

To be submitted to The Maharaja Sayajirao University of Baroda

For the Degree

Of

DOCTOR OF PHILOSOPHY

In Chemistry

By

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Under guidance of

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To be submitted to The Maharaja Sayajirao University of Baroda

For the degree of DOCTOR OF PHILOSOPHY

Name of the student: Nandini Vasudevbhai Panchal

Faculty: Science

Subject: Chemistry

Name of Guide: Prof. Shubhangi S Soman (M.Sc. PhD)

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Zydus Research Centre

Ahmedabad

(Gujarat, India)

CHAPTER 1

Introduction

1.1. Inflammation

Inflammation is a defence mechanism in the body and plays a role in healing process. This mechanism recognizes damaged cells, irritants, and pathogens. When inflammation occurs, chemicals from white blood cells release into the blood or affected tissues. This release of chemicals increases the blood flow to the area of injury or infection and may result in redness and warmth. Some of the chemicals cause fluid to leak into your tissues, resulting in swelling. The inflammatory process may stimulate nerves and cause pain. (1)

1.1.1. Causes of inflammation

Many factors can lead to inflammation, such as: Infective agents like bacteria, viruses and their toxins, fungi, parasites. Immunological agents like cell-mediated and antigen antibody reactions. Physical agents like heat, cold, radiation, mechanical trauma. Chemical agents like organic and inorganic poisons. Inert materials such as foreign bodies. Nutritional and Tissue Necrosis.

1.1.2. Symptoms of inflammation

Symptoms of inflammation include: Rubor (redness), Tumor (swelling), Calor (heat), Dolor (pain) and loss of function.

1.1.3. Classification of inflammation

Inflammation can be classified in to two categories Acute and Chronic inflammation. Chronic inflammation can be again classified in to several diseases like inflammatory bowel diseases (IBD), Osteoarthritis (OA), Irritable bowel syndromes (IBS), Asthma, Gout, Rheumatoid arthritis (RA). Here in this research work we choose to work on the treatment of Rheumatoid arthritis. (2)

1.1.4. Current therapies for the treatment of RA

Treatment for RA mainly includes Non-steroidal anti-inflammatory drugs (NSAIDs), Biological treatments, Janus Kinase (JAK) inhibitors, Disease-modifying anti-rheumatic

drugs (DMARDs), Steroids, Surgery, Arthroscopy (remove inflamed joint tissue) and Joint replacement. (3)

All this treatments have their own side effects. The present scenario of non-availability of safe, cost effective and efficacious small molecule based on JAK inhibitors, prompted us to work for novel JAK inhibitors.

1.2. JAK inhibitor

Janus kinase (JAK) is a family of enzyme [JAK1, JAK2, JAK3 and Tyrosine kinase (TYK2)] are cytoplasmic protein tyrosine kinase, associated with various cytokine-mediated signal transduction pathways. Due to unique role of JAKs in the immune system, inhibition of JAKs emerged out as one of the most validated and attractive therapeutic target for the treatment of autoimmune disorders such as RA and other inflammatory diseases. (4)

1.2.1. The clinical significance for targeting JAK3

JAK3 is expressed in lymphoid cells, drives pro-inflammatory signalling cascades, inducing cytokine expression in the synovial fibroblasts, activated monocytes and macrophages. JAK3 pairs with the JAK1 and it is involved in common gamma chain (γc) cytokine (IL-2, IL-4, IL-9, IL-15AND IL-21) signalling pathways, which play important role in the T- cell differentiation, proliferation and survival. Selective JAK3 inhibition only deters common gamma chain receptors signalling and spare JAK1 dependent immune regulatory cytokines (IL-10, IL-27 and IL-35). Thus, JAK3 selective inhibitors are likely to offer a better efficacy to the safety ratio in the clinic for the treatment of chronic inflammatory disorders. (5)

1.2.2. Mechanism of action of JAK-STAT pathway

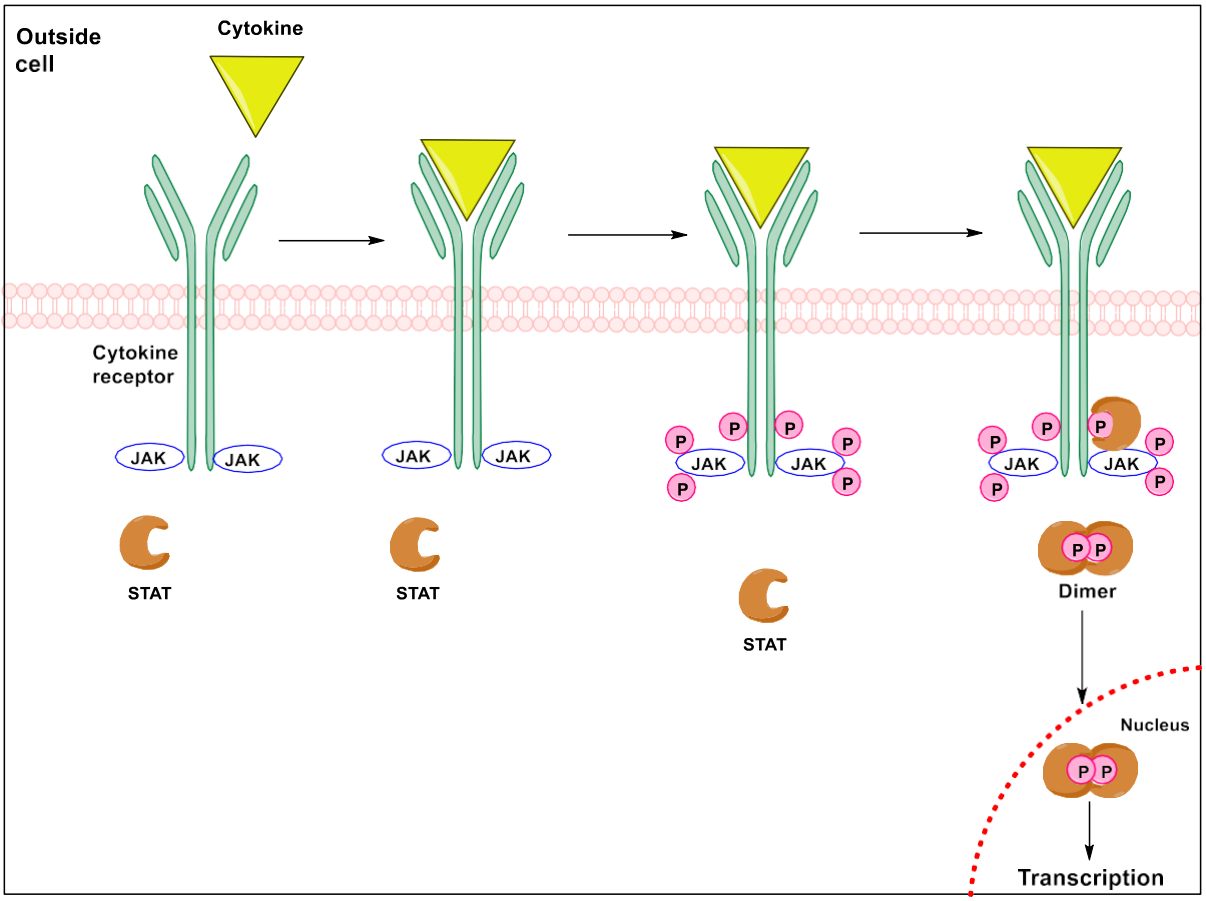


Figure 1. Mechanism of JAK-STAT pathway

JAK-STAT signalling pathway is made up of three main proteins: Cell-surface receptors, JAKs and STATs. The binding of different ligands (yellow triangle), generally cytokines, like interferons and interleukins, to cell-surface receptors, causes JAKs to add phosphate (pink circle) to the receptor. Then STAT proteins bind to the phosphates, and the STATs are phosphorylated by JAKs to form a dimer. Then after, dimerized STATs bind specific regulatory sequences to activate or repress transcription of target genes. (6)

1.2.3. Regulation of JAK-STAT pathway

There are various mechanism that cells have for regulating the amount of signal occurs. Three major groups of proteins that cells use to regulate this signalling pathways are protein inhibitors of activated STAT (PIAS), protein tyrosine phosphatases (PTPs) and suppressors of cytokine signalling (SOCS).(7)

- PIAS

This proteins add a marker, known as SUMO (small ubiquitin-like modifier), onto other proteins like JAKs and STATs, modifying their function.

A) Adding a SUMO group to STAT protein can block their phosphorylation, which prevents STAT entering the nucleus.

B) HDAC (histone deacetylase) recruitment can remove acetyl modifications on histones, lowering gene expression.

C) PIAS can also prevent STAT binding to DNA.

- PTPs

Adding phosphate groups on tyrosine is an important part of JAK-STAT signalling pathway function, removing this phosphate group can inhibit signalling. PTPs are tyrosine phosphate, so are able to remove these phosphate and prevent signalling.

- SOCS

SOCS can interact with numerous protein to form a protein complex, and this complex can cause the breakdown of JAKs and the receptors themselves, therefore inhibiting JAK-STAT signalling.

1.2.4. Pyrrolo pyrimidine and Pyrimidine based JAK inhibitors

Over the past decades, structurally diverse JAKs inhibitors were identified containing pyrrolo [2, 3-b] pyridine and Pyrimidine core as a promising scaffold (**Fig. 2**). (8)

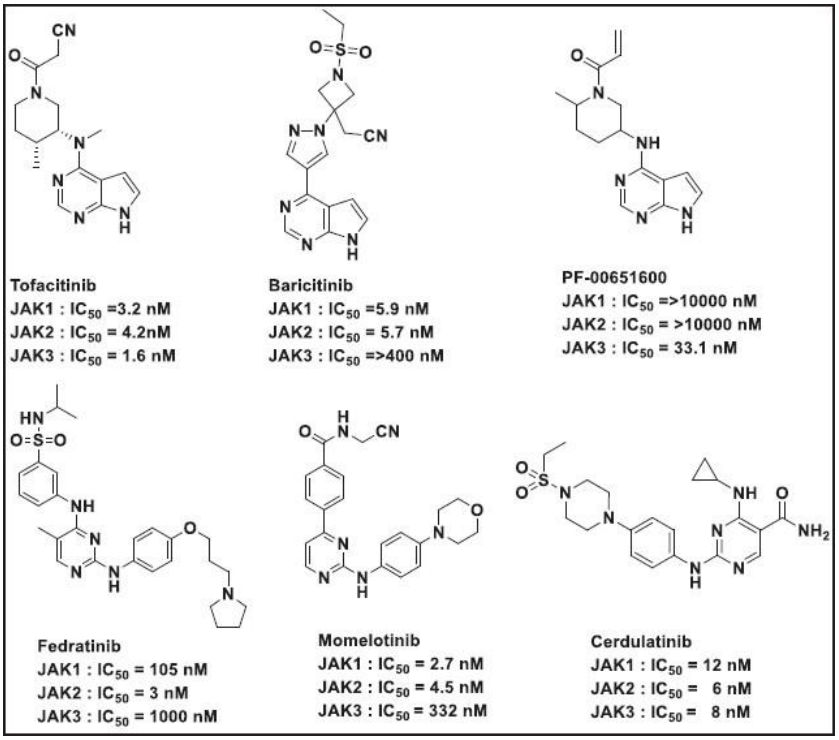


Figure 2. Structures of 1H-pyrrolo [2, 3-b] pyridine and Pyrimidine based JAK inhibitors

The Tofacitinib was discovered by Pfizer for RA. Baricitinib discovered by Incyte and Eli Lilly for moderate to severe RA treatment. (9) PF-00651600 an irreversible covalent inhibitor of JAK3, which is in the clinical trial for the treatment of inflammatory diseases. (10) Momelotinib discovered by Cytopia, currently under Phase-3 trials, for myelofibrosis treatment. (11) Fedratinib by TargeGen and Sanofi-Aventis is under Phase-2 clinical trials, for the treatment of myelofibrosis. (12) The reversible ATP-competitive dual SYK/JAK inhibitor, Cerdulatinib (Portola Pharmaceuticals) is in a Phase-2 trial for leukemia and lymphoma. All this drugs have their own side effects. (13)

Currently various pharma companies running JAK inhibitor programs, such as Portola Pharmaceuticals. Cerdulatinib molecule (With good IC_{50} value) of Portola Pharmaceuticals is in Phase 3. Here in this research work we select Cerdulatinib molecule as reference molecule for the treatment of RA as selective JAK3 inhibitor.

1.3. Objectives

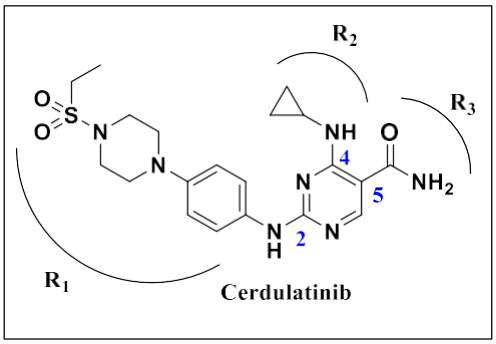
Our objective is designing of Novel, Orally active, selective JAK3 inhibitor as anti-inflammatory agent for the treatment of RA. Synthesis and Characterization of novel derivatives is carried out with modification at different positions of pyrimidine ring of Cerdulatinib to overcome existing side effects of currently available drugs. *In vitro* screening will be carried out using Fluorogenic substrate assay. The best compounds will be subjected for further biological activities. Molecular modelling of active compound will be done to understand its orientation across the different binding sites.

1.4. Design strategy

However, knowing the potential side effects associated with the JAKs isoforms inhibition, recently, more efforts are directed towards the development of isoform selective inhibitors, particularly JAK3 selective inhibitors, for the effective treatment of autoimmune disorders, such as RA. (14)

In this regard, our aim is to discover novel, potent and orally bioavailable JAK3 selective inhibitors, mainly by favouring the suitable interactions of the designed molecules, in the specificity and binding pocket, to achieve JAK3 selectivity. Considering the strong interaction of pyrimidine moiety with the ATP binding pocket of JAK enzymes, as a starting point, structural modifications were carried out in the dual SYK (Spleen tyrosine kinase)/JAK inhibitor, Cerdulatinib to improve the JAK3 selectivity. Structural modifications were carried

out in the Cerdulatinib (having pyrimidine moiety as a key pharmacophore). Three sets of compounds were designed. Our initial modifications is on the C2 position of pyrimidine ring (at R_1 position) in Cerdulatinib, with C4 cyclopropyl of Cerdulatinib altered with benzyl group (at R_2 position) led to the single digit nM potent compounds, with moderate isoform selectivity. In the second set modification were carried out on the C4-position (at R_2 position) to improve isoform selectivity and *in vivo* profile. And then modification were carried out on the C5-position (at R_3 position) of pyrimidine ring.



Cerdulatinib

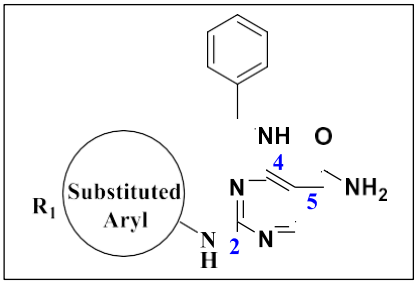
Herein, we report, design, synthesis and biological evaluation of novel 2, 4-diaminopyrimidine-5-carboxamides based JAK3 selective inhibitors.

Currently NSAID's, DMARD, Steroid and biological agents are available for the treatment of RA. Till today few small molecules like Tofacitinib is available for the treatment of RA. But Tofacitinib also has many side effects and limitations.

Presently there is none availability of safe, cost effective and efficacious small molecule based JAK inhibitors. To address these issues, in the next section, we have designed novel series of JAK inhibitors to develop next generation therapies for the treatment of RA.

CHAPTER 2

Design and synthesis of compounds modified at 2nd position of potent JAK inhibitor Cerdulatinib

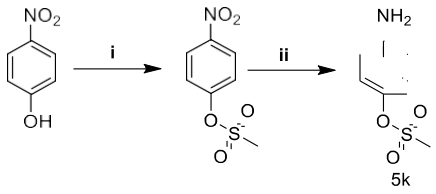


Chapter 2 is divided in three parts, In Part 1 synthesis of various intermediates for R_1 substitution. In Part 2, Synthesis of pyrimidine derivatives with the modification at 2nd position as shown in scheme-1 (compounds **5a-r**). All the compounds were characterized by using different spectral techniques like ^1H, ^{13}C NMR, IR, ESI-MS and UPLC analysis. In Part 3, *In vitro* JAK Inhibitory activity data of pyrimidine derivatives modified at 2nd position.

2.1. Synthesis of intermediates for R_1 Substitution

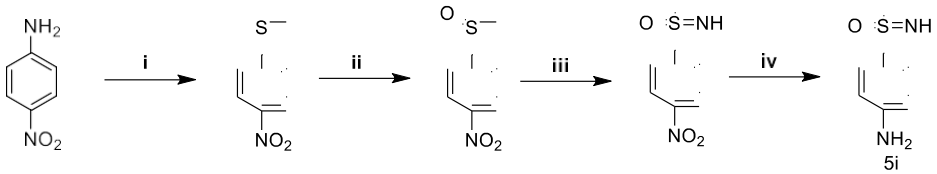
We prepared various intermediates. Initially we prepared para substituted intermediates as shown below.

- *Synthesis of intermediate **5 k** (Methane sulphonate)*



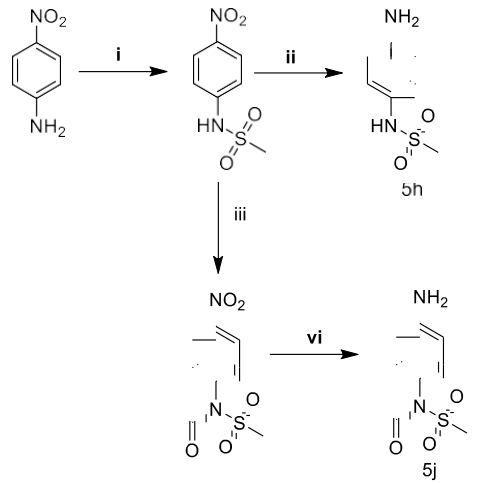
Reagents and conditions: (i) CH_3SO_2Cl, TEA, DCM, 30 °C, 2 h; (ii) Fe, NH_4Cl, Ethanol, reflux, 1 h

- *Synthesis of intermediate **5i** (Sulphoximine)*



Reagents and conditions: (i) Dimethyl disulfide, tert-Butyl Nitrile, ACN, 40 °C, 3 h; (ii) m-CPBA, $CHCl_3$, -10 °C, 20mins; (iii) NaN_3, H_2SO_4, $CHCl_3$, 40 °C, 3 h; (iv) Fe, NH_4Cl, Ethanol, reflux, 1 h

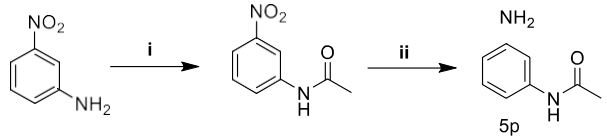
- *Synthesis of intermediate **5h** (methane sulfonamide) and **5i** (methane sulfonyl acetamide)*



Reagents and conditions: (i) CH_3SO_2Cl, TEA, DCM, 30 ^oC, 2 h; (ii) Fe,NH_4Cl, Ethanol, reflux, 1 h; (iii) CH_3COCl, TEA, DCM, 30 ^oC, 2 h; (iv) Fe,NH_4Cl, Ethanol, reflux, 1 h

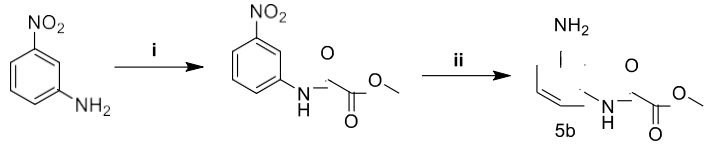
Then we prepared meta substituted intermediates as shown below.

- *Synthesis of intermediate **5p** (Acetamide)*



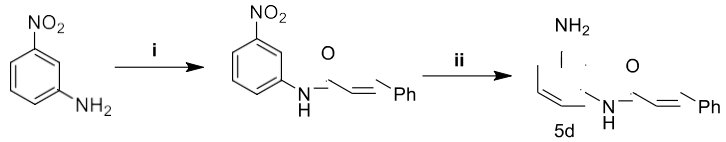
Reagents and conditions: (i) CH_3COCl, TEA, DCM, 30 ^oC, 2 h; (ii) Fe, NH_4Cl, Ethanol, reflux, 1 h

- *Synthesis of intermediate **5b** (Oxopropanamide)*



Reagents and conditions: (i) $CH_3OCOCOCl$, TEA, DCM, 30 ^oC, 2 h; (ii) Fe, NH_4Cl, Ethanol, reflux, 1 h

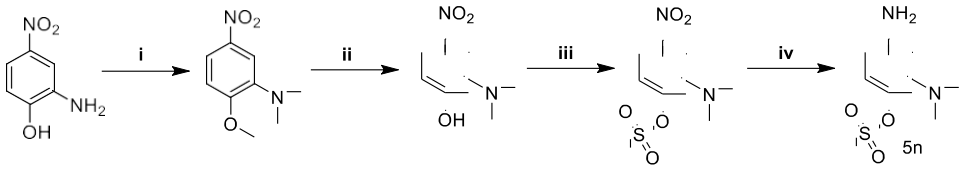
- *Synthesis of intermediate **5d** (Cinnamamide)*



Reagents and conditions: (i) PhCH=CHCOOH, EDC.HCl,HOBt, DMF, 30 ^oC, 2 h; (ii) Fe, NH_4Cl, Ethanol, reflux, 1 h

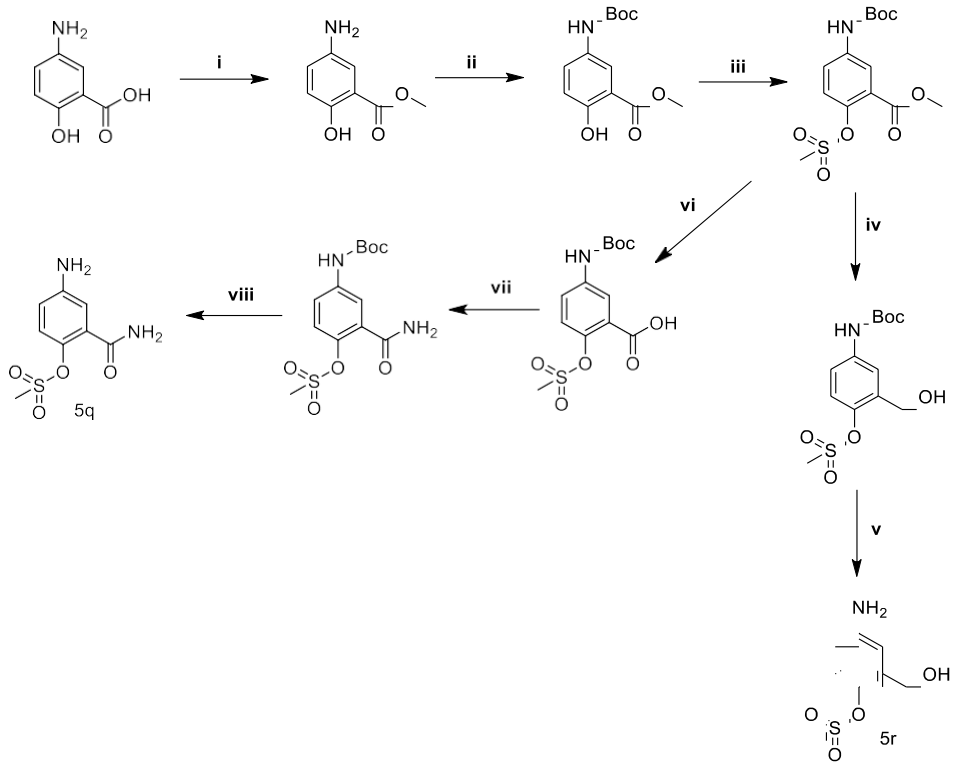
Preparation of di substituted intermediates as shown below.

- *Synthesis of intermediate **5n** (Dimethyl amine)*



Reagents and conditions: (i) CH_3I, K_2CO_3, DMF, 30 °C, 2 h; (ii) BBr_3, DCM, 30 °C, 2 h; (iii) CH_3SO_2Cl, TEA, DCM, 30 °C, 2 h; (iv) Fe, NH_4Cl, Ethanol, reflux, 1 h

- *Synthesis of intermediate **5q** (Carbamoyl) and **5r** (Hydroxyl methyl)*

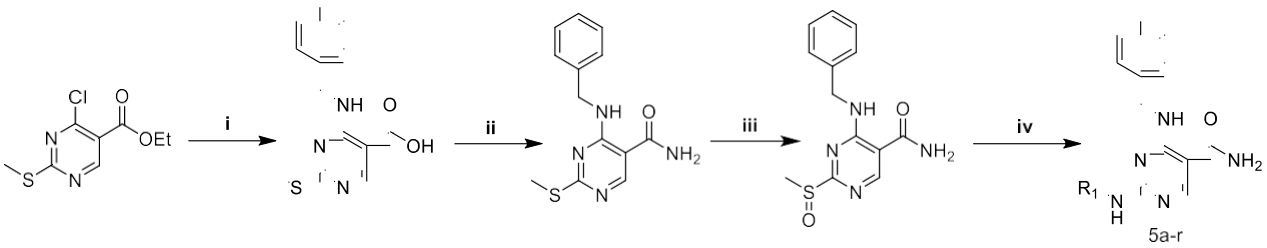


Reagents and conditions: (i) ClCOCOCl, DMF, MeOH, 60 °C, 2 h; (ii) $(BOC)_2O$, TEA, DCM, 30 °C, 4 h; (iii) CH_3SO_2Cl, TEA, DCM, 30 °C, 2 h; (iv) NaBH4, THF, 30 °C, 1 h; (v) TFA, DCM, 30 °C, 6 h; (vi) $LiOH.H_2O$, THF, 30 °C, 5 h; (vii) EDC.HCl, HOBt, aq NH_3, DMF, 30 °C, 2 h; (viii) TFA, DCM, 30 °C, 6 h

2.2. Synthesis of pyrimidine derivatives with the modification at 2nd position

Synthesis of 2, 4-diaminopyrimidine-5-carboxamides derivatives (**5a-r**, **11a-v** and **16a-o**) was carried out as depicted in Scheme 1, 2 and 3 respectively, following the modified literature procedure. (15)

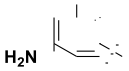
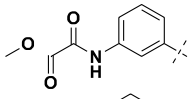
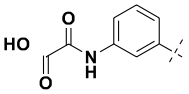
Scheme-1

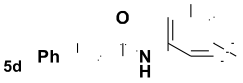
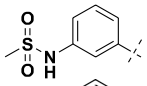
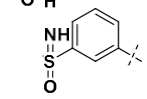


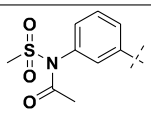
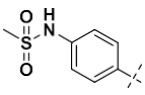
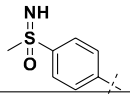
Reagents and conditions: (i) {a} Benzyl amine, DIPEA, Dioxane, 30 ^{o}C, 4 h; {b} $LiOH.H_2O$, 30 ^{o}C, 8 h; (ii) EDC.HCl, HOBt, aq NH_3, DMF, 30 ^{o}C, 2 h; (iii) m-CPBA, Dioxane:$CHCl_3$,-10 ^{o}C, 20 mins; (iv) R_1-NH_2, p-TSA, NMP, 110 ^{o}C, 1 h

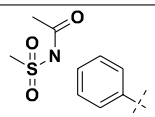
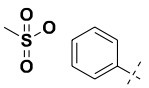
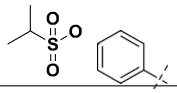
2.3. List of R_1 substituents at 2nd position of pyrimidine derivative

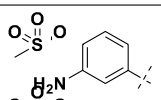
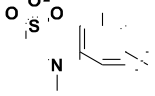
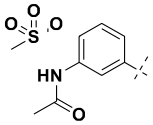
Substituents of compound **5a-r** are listed below.

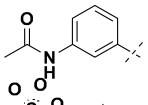
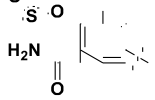
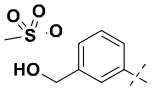
Compound	R_1
5a	
5b	
5c	

Compound	R_1
5d	
5e	
5f	

Compound	R_1
5g	
5h	
5i	

Compound	R_1
5j	
5k	
5l	

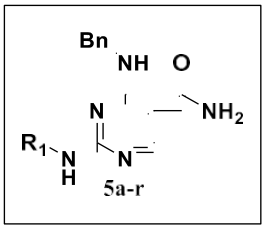
Compound	R_1
5m	
5n	
5o	

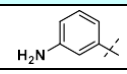
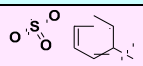
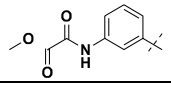
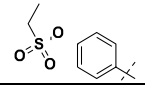
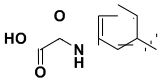
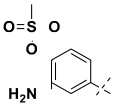
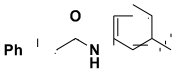
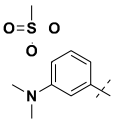
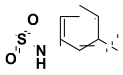
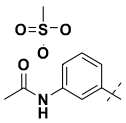
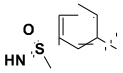
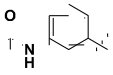
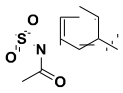
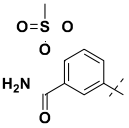
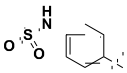
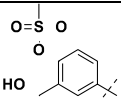
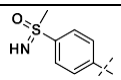
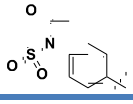
Compound	R_1
5p	
5q	
5r	

2.4. *In vitro* JAK Inhibitory activity data of pyrimidine derivatives modified at 2nd position

In vitro screening of compounds **5a-r** was carried out using Fluorogenic substrate assay and the IC_{50} values are mentioned below.

Table 1 Influence of modification at 2nd position of Pyrimidine moiety on JAK3 inhibitory activity (*In vitro*)



Sr.No.	Structure	IC_{50} (nM)	Sr.No.	Structure	IC_{50} (nM)
5a		12.6	5k		9.5
5b		33.4	5l		22.4
5c		53.7	5m		56
5d		178	5n		66
5e		122	5o		89
5f		136	5p		100
5g		132	5q		50
5h		12.4	5r		98
5i		48.4	Cerdulatinib		8
5j		10.2	Tofacitinib		1.6

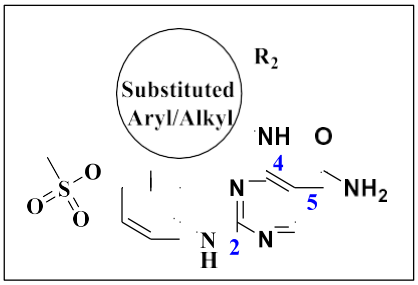
[a]All the data are shown as the mean for at least two experiments. [a]JAK3 inhibition (IC_{50}) determination using *in vitro* Fluorogenic substrate assays Kit from Millipore.

2.5. Conclusion

Modifications at 2nd position of pyrimidine of Cerdulatinib gave four compounds **5a, 5h, 5j** and **5k** which were equipotent as Cerdulatinib. Compound **5k** with IC_{50} : 9.5 nM was selected for further modification at 4th position in chapter 3 and detailed biological evaluation in chapter 5.

CHAPTER 3

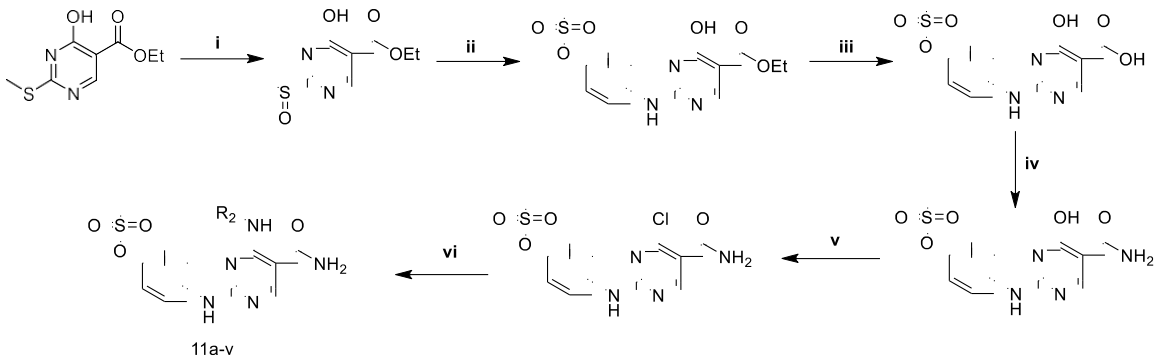
Design and synthesis of compounds modified at 4th position of potent JAK inhibitor Cerdulatinib



Chapter 3 is divided in two parts In Part 1, Synthesis of pyrimidine derivatives with the modification at 2nd position as shown in scheme-2 (compounds **11a-v**). All the compounds were characterized by using different spectral techniques like ^{1}H, ^{13}C NMR, IR, ESI-MS and UPLC analysis. In Part 2, *In vitro* JAK Inhibitory activity data of pyrimidine derivatives modified at 4th position.

3.1. Synthesis of pyrimidine derivatives with the modification at 4th position

Scheme-2



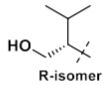
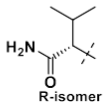
Reagents and conditions: (i) m-CPBA, Dioxane:$CHCl_3$,-10 ^{o}C, 20 mins; (ii) 4-aminophenyl methanesulfonate, p-TSA, NMP, 110 ^{o}C, 1 h; (iii) $LiOH.H_2O$, 30 ^{o}C, 8 h; (iv) EDC.HCl, HOBt, aq NH_3, DMF, 30 ^{o}C, 2 h; (v) $POCl_3$, DIPEA, Toluene,110 ^{o}C, 2 h; (vi) R_2-NH_2, DIPEA, Dioxane, 30 ^{o}C, 1 h

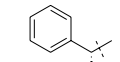
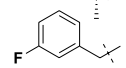
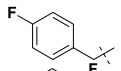
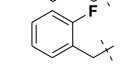
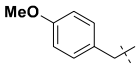
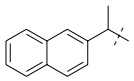
3.2. List of R_2 substituents at 4th position of pyrimidine derivative

Substituents of compound **11a-v** are listed below.

Compound	R_2
11a	H
11b	
11c	
11d	
11e	
11f	

Compound	R_2
11g	
11h	
11i	
11j	
11k	 Racemic

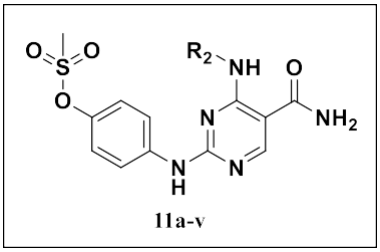
Compound	R_2
11l	 R-isomer
11m	 S-isomer
11n	 R-isomer
11o	 R-isomer
11p	 R-isomer

Compound	R_2
11q	
11r	
11s	
11t	
11u	
11v	

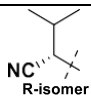
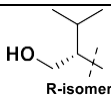
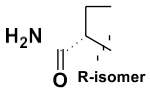
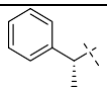
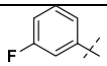
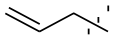
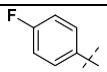
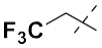
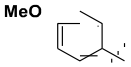
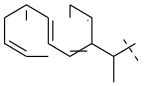
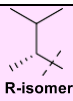
3.3. *In vitro* JAK Inhibitory activity data of pyrimidine derivatives modified at 4th position

In vitro screening of compounds **11a-v** was carried out using Fluorogenic substrate assay and the IC_{50} values are mentioned below.

Table 2 Influence of modification at C4 position of Pyrimidine moiety on JAK3 inhibitory activity (*In vitro*).



11a-v

Sr.No.	Structure	IC_{50} (nM)	Sr.No.	Structure	IC_{50} (nM)
11a		**300**	**11m**		**256**
11b		**49**	**11n**		**40**
11c		**37**	**11o**		**80**
11d		**33**	**11p**		**50**
11e		**79**	**11q**		**39**
11f		**12**	**11r**		**43.2**
11g		**150**	**11s**		**45.4**
11h		**110**	**11t**		**49.2**
11i		**9.8**	**11u**		**60**
11j		**25**	**11v**		**189**
11k		**20**	**Cerdulatinib**		**8**
11l		**1.7**	**Tofacitinib**		**1.6**

[a]All the data are shown as the mean for at least two experiments. [a]JAK3 inhibition (IC_{50}) determination using *in vitro* Fluorogenic substrate assays Kit from Millipore.

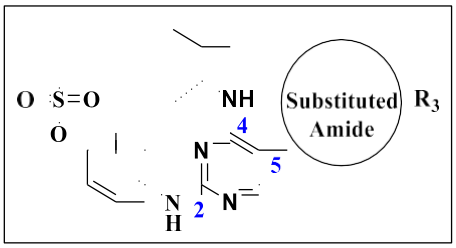
.

3.4. Conclusion

Modification at 4th position of pyrimidine ring of **5k** [IC_{50} : 9.5 nM] resulted in **11l** with IC_{50} : 1.7 nM (5 fold improvement) which is much better compare to Cerdulatinib [IC_{50} : 8.0 nM]. Interestingly compound **11l** showed activity as similar as another active drug Tofacitinib.

CHAPTER 4

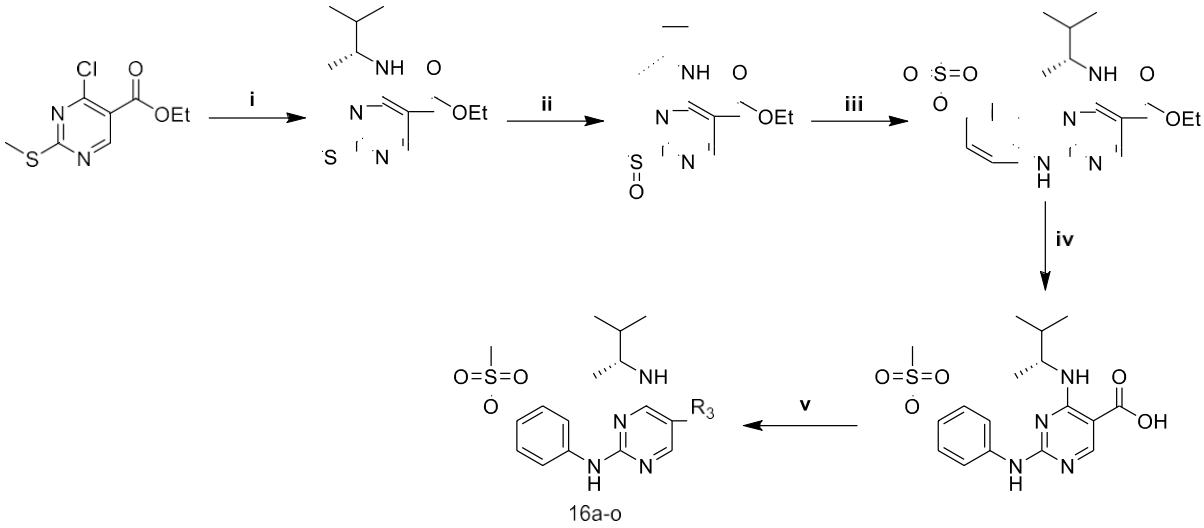
Design and synthesis of compounds modified at 5th position of potent JAK inhibitor Cerdulatinib



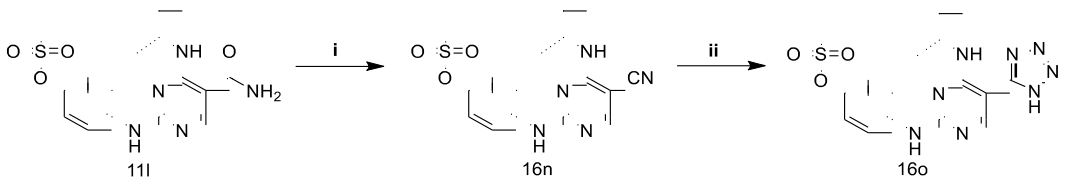
Chapter 4 is divided in two parts In Part 1, Synthesis of pyrimidine derivatives with the modification at 5th position as shown in scheme-3 (compounds **16a-o**). All the compounds were characterized by using different spectral techniques like ^{1}H NMR, IR, ESI-MS and UPLC analysis. In Part 2, *In vitro* JAK Inhibitory activity data of pyrimidine derivatives modified at 5th position.

4.1. Synthesis of pyrimidine derivatives with the modification at 5th position

Scheme-3



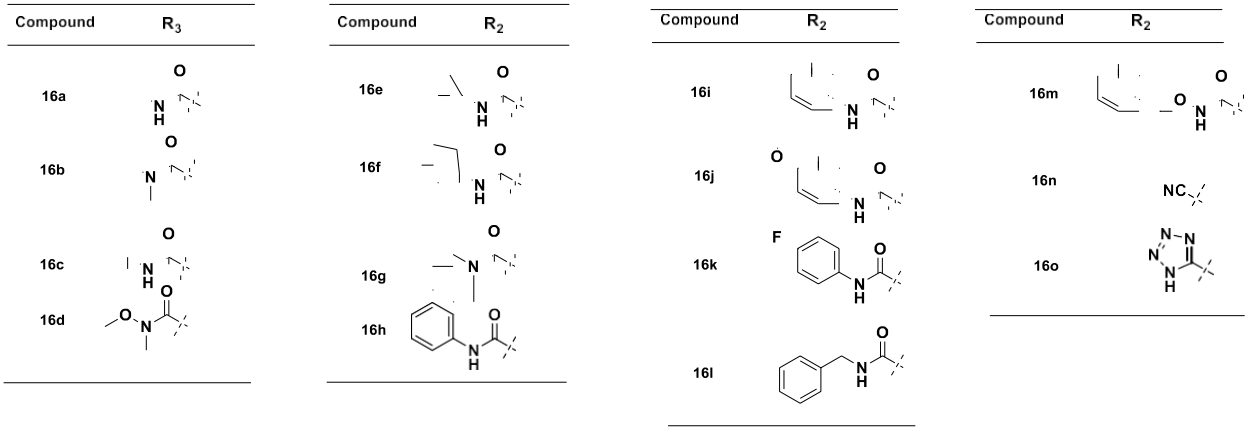
Reagents and conditions: (i) (R)-3-methylbutan-2-amine, DIPEA, Dioxane, 30 ^oC, 1 h; (ii) m-CPBA, Dioxane:$CHCl_3$,-10 ^oC, 20 mins; (iii) 4-aminophenyl methanesulfonate, p-TSA, NMP, 110 ^oC, 1 h; (iv) $LiOH.H_2O$, 30 ^oC, 8 h; (v) EDC.HCl, HOBt, aq NH_3, DMF, 30 ^oC, 2 h



Reagents and conditions: (i) TFAA, TEA, DCM, 30 ^{o}C, 2 h; (ii) NaN_3, NH_4Cl, DMF, 30 ^{o}C, 5 h

4.2. List of R_3 substituents at 5th position of pyrimidine derivative

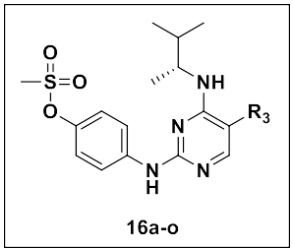
Substituents of compound **16a-o** are listed below.

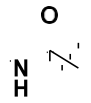
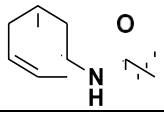
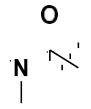
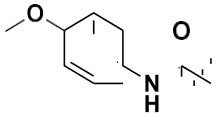
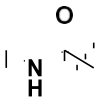
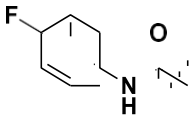
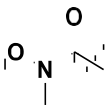
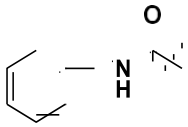
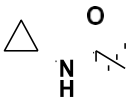
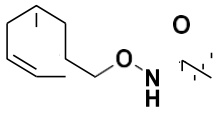
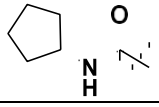
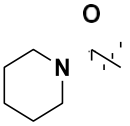
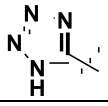
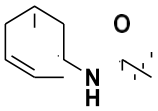


4.3. *In vitro* JAK Inhibitory activity data of pyrimidine derivatives modified at 5th position

In vitro screening of compounds **16a-o** was carried out using Fluorogenic substrate assay and the IC_{50} values are mentioned below.

Table 3 Influence of modification at C5 position of Pyrimidine moiety on JAK3 inhibitory activity (*In vitro*).



Sr.No.	Structure	IC_{50} (nM)	Sr.No.	Structure	IC_{50} (nM)
16a		**39**	**16i**		**140**
16b		**65**	**16j**		**170**
16c		**49**	**16k**		**102**
16d		**70**	**16l**		**258**
16e		**90**	**16m**		**300**
16f		**110**	**16n**		**250**
16g		**81**	**16o**		**189**
16h		**108**	**Cerdulatinib**		**8**
			Tofacitinib		**1.6**

[a]All the data are shown as the mean for at least two experiments. [a]JAK3 inhibition (IC_{50}) determination using *in vitro* Fluorogenic substrate assays Kit from Millipore.

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4.4. Conclusion

Modification at 5th position of compound **11l** resulted in loss of activity when substituents were other than amide group which shows that –$CONH_2$ group is essential for activity.

CHAPTER 5

Detailed biological evaluation of selected potent compounds (5k & 11l)

Chapter 5 include Kinome Selectivity study, JAKs Isoform Selectivity study, Potency and Selectivity determination study in human PBMC, *Ex vivo* study, CYP inhibition study, Pharmacokinetic study, *In vivo* study (AIA & CIA model), Safety pharmacology study and Docking study.

5.1. Kinome Selectivity Profile of 11l (*In-Vitro*)

In vitro kinase profiling study of **11l** was carried out at 1µM concentration, against Millipore panel of 170 purified kinases (n=2) and % inhibition was found to be < 20% at 1µM concentration, including key cysteine containing protein kinases, mainly from the TEC family (BMX, BTK, ITK, TXK, and TEC), ErbB family (EGFR, ERBB4, and ERBB2) and CLK2, MKK7β, PKG1α and Aurora kinase, see Table 4.

Table 4 Kinome Selectivity Profile of **11l**[a]

Kinase	%	Kinase	%	Kinase	%
ACK1	63	B-Raf	07	CDK2	10
PKA	13	MAPK1	13	CDK6	02
IR	00	PRK2	20	CDK7	03
Lck	19	IGF-1R	10	CDK9	00
Mer	31	JNK1α1	00	Plk3	03
KDR	00	JNK2α2	00	TAO1	32
SGK	05	PAK4	14	Aurora-A	75
DDR1	28	GCK	22	Aurora-B	89
Syk	00	Pim-1	66	MST1	19
Rsk2	23	Pim-2	05	TBK1	02
ZIPK	00	SAPK2a	01	TrkA	52
Src	00	SAPK2b	00	TrkB	60

ROCK-I	16	SAPK3	09	TrkC	22
ROCK-II	13	SAPK4	02	NEK2	02
FAK	29	MAPKAP-K2	02	Fms	04
STK33	09	Wee1	12	CaMK1	00
JAK3	99.2	MSK1	27	CaMKIIβ	18
Ret	12	Fyn	05	CaMKK2	00
ALK	02	Ab1	00	Lyn	00
ALK4	01	CDK1	09	MEK1	10
Flt1	02	FGFR1	22	PKG1α	79
Flt3	26	DYRK1A	05	GRK5	08
PDGFRα	00	DYRK1B	12	GSK3α	16
CK1	20	CLK2	85	GSK3β	00
MKK4	00	IKKα	02	PKBα	04
MKK6	29	IKKβ	00	PKCθ	17
MKK7β	77	IKKε	01	PKCξ	00

.[a]Values represent percent inhibition at 1 μM concentration, data are the mean of at least n=2 independent measurements. Lower numbers indicate stronger binding, where Negative control=DMSO (% inhibition=100%).

- Out of all kinome compound **11l** binds maximum with JAK3 kinome (99.2%).

5.2. JAKs Isoform Selectivity

Most potent compounds (**5k** and **11l**) were evaluated for their selectivity against JAK isoforms (JAK1, JAK2, JAK3 and TYK2). (16)

Table 5 *In vitro* isoform selectivity of compounds against JAK1, JAK2 and TYK2 enzymes.

Compound	IC_{50}[a] (nM)				Selectivity fold		
	JAK1[b]	JAK2[b]	JAK3[b]	TYK2[b]	JAK1/ JAK3	JAK2/ JAK3	TYK2/ JAK3
5k	18	42	9.5	45	2	4	5

11l	20	171	1.7	186	12	100	109
Cerdulatinib	15	7	8	5	2	1	<1
Tofacitinib	3	5	1.6	34	2	3	21

[a]The IC_{50} values are shown as the mean for at least two experiments. [b]JAK1, JAK2, JAK3, & TYK2 inhibitory assay Kit (Millipore) was used to screen the test compounds.

- As shown in Table 5, initial hit **5k** showed moderate selectivity (2 to 5 X) against JAK isoforms over JAK3. Compound **11l** (IC_{50}: 1.7 nM) demonstrated 12, 100, and 109 fold selectivity over JAK1, JAK2 and TYK2 respectively.
- Moreover, it was noted that selectivity of **11l** against all the three isoforms was higher than standard compounds. In general, it was observed that the potency and selectivity of diaminopyrimidine-5-carboxamides based JAK3 inhibitors can be modulated using suitable substituents at C2 and C4-position of pyrimidine ring.

5.3. JAK cellular assays using human peripheral blood mononuclear cells

To elucidate potency and selectivity profile in a cellular environment, compound **11l** was tested for the inhibition of phosphorylation of downstream signal (STAT proteins), in the human peripheral blood mononuclear cells (PBMCs). The different cellular stimuli were used to induce phosphorylation of STATs (pSTAT), either by dual JAK1/3 (IL-2 stimulus, pSTAT5), JAK2 (GM-CSF stimulus, pSTAT5), or with the PAN JAK1/JAK2/TYK2 (IL-6 stimulus, pSTAT3) stimuli To elucidate potency and selectivity profile in a cellular environment, compound 11i was tested for the inhibition of phosphorylation of downstream signal (STAT proteins), in the human peripheral blood mononuclear cells (PBMCs). The different cellular stimuli were used to induce phosphorylation of STATs (pSTAT), either by dual JAK1/3 (IL-2 stimulus, pSTAT5), JAK2 (GM-CSF stimulus, pSTAT5), or with the PAN JAK1/JAK2/TYK2 (IL-6 stimulus, pSTAT3) stimuli.

Table 6 Potency and Selectivity determination of **11l** in human PBMC.

JAKs involved	**Trigger**	**Readout**	**IC_{50}(nm)**		**Selectivity**	
			11l	**Tofacitinib**	**11l**	**Tofacitinib**
JAK1/JAK3	IL2	pSTAT5	22.16	25.22	_	_

JAK1/JAK2/ TYK2	IL6	pSTAT3	608	36.88	27.4	1.46
JAK2	GM-CSF	pSTAT5	511	210	23	8.32

IC_{50} values in hPBMC were determined by plotting the compound concentration vs the effect on the readouts, using flow cytometry (n = 2).

- As shown in Table 4, **11l** showed 27-fold selectivity for inhibition of the IL-2 (IC50: 22.16 nM) versus the IL-6 readout (IC_{50}: 608 nM) and a 23-fold selectivity for the inhibition of the GM-CSF (IC_{50}: 511 nM).
- Tofacitinib displayed similar potencies but lower selectivity than **11l** in the relevant pSTAT assays. Thus, compound **11l** showed preferential inhibition of JAK3 over JAK1, in the JAK/STAT signalling pathway, when assessed in the PBMC assay

5.4. CYP (Cytochrome P450) inhibition study (*In vitro*)

- Compound **11l** was also found to be devoid of CYP (<10% CYP inhibition at 10 µM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) and hERG liabilities (IC_{50}: > 10 µM).

5.5. *Ex vivo* study

- *Ex vivo*, compound **11l** evaluated for plasma protein binding studies (using mice, rat and human plasma) and liver microsomal stability studies (using immortalized mice, rats and human liver cell line).
- Compound **11l** showed 75 to 80% plasma protein binding and less than 10% metabolism at 30 minutes, in liver microsomal metabolic stability study.

5.6. Pharmacokinetic study (parameters of 5k, 11l and Tofacitinib in C57 mice)

A comparative single dose (3 mg/kg, po and 1 mg/kg, iv) PK profile of compounds **5k**, **11l** and Tofacitinib was evaluated in male C57BL/6J mice (n = 6) and the various PK parameters [Tmax, Cmax, $t_{1/2}$, Cl, Area under the curve (AUC) and %F] were recorded (Table 7).

Table 7 Pharmacokinetic study parameters of **5k**, **11l** and Tofacitinib in C57 mice

Compd	Tmax (h)	Cmax (ng/ml)	$t_{1/2}$ (h)	Cl (ml/min/kg), iv	AUC (0-α) h µg/ml	%F*

5k	0.25	146 ± 48	1.85 ± 0.43	40.37 ± 3.61	192 ± 56	15
11l	0.25	1737.95 ± 205	2.56 ± 0.45	11.59 ± 1.65	2104 ± 487	48
Tofacitinib	0.25	80.39± 13.86	1.32 ± 0.65	47.56 ± 3.95	208.2 ± 35.7	20

* Oral bioavailability (%F) was calculated wrt to iv AUC. Compounds **5k**, **11l** and Tofacitinib administered at 1 mg/kg dose, iv AUC (ng/ml): 412, 1459 and 350 respectively.

- In PK study, **5k** showed moderate AUC, due to high clearance, which resulted into overall low bioavailability (15%).
- Compound **11l** showed higher AUC (~10 fold, compared to std), extended $t_{1/2}$ (2.56 hr) and good oral bioavailability (%F: ~48 over std, 20%).
- Compound **11l** showed extended $t_{1/2}$ and higher AUC, which could be due to its low clearance compared to standard (11.59 vs 47.56 ml/min/kg, iv)

5.7. *In vivo* efficacy studies

★ *Anti-arthritic efficacy of test compounds in AIA (Adjuvant induced Arthritis) Rat Model*

Arthritis was induced in female Lewis rats by inoculation with Freund's complete adjuvant (CFA). (18)

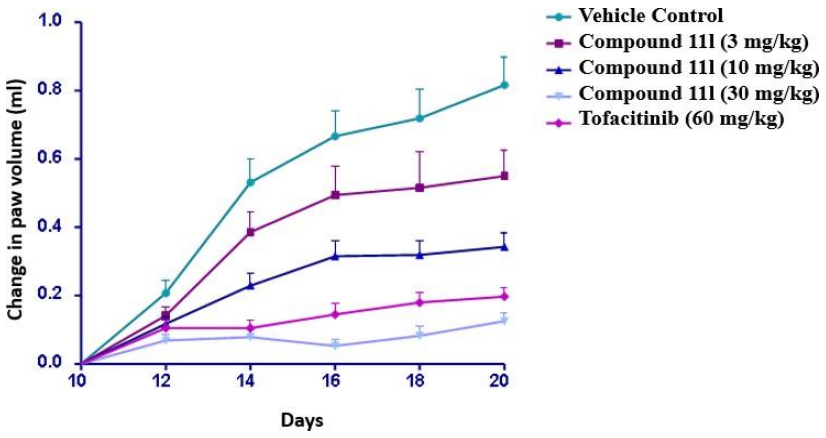


Figure 3a. Effect of Compound **11l** and Tofacitinib in AIA rat model

- As shown in the Fig.3a, standard and **11l** showed good reduction in the paw volume, compared to vehicle control (untreated group).
- Compound **11l** suppressed paw swelling in a dose-dependent manner (ED_{50}: 10 mg/kg) and at 30 mg/kg dose, efficacy was found to be comparable to that of standard (Tofacitinib, 60 mg/kg). Body weight was not significantly affected in rat in any treatment group compared with the vehicle control group.

★ *Anti-arthritic efficacy of test compounds in Collagen Induced Arthritis (CIA) mice model*

Arthritis was developed in male DBA1j mice, using collagen mixture and mice were recruited for the study once clinical signs were visible. (17)

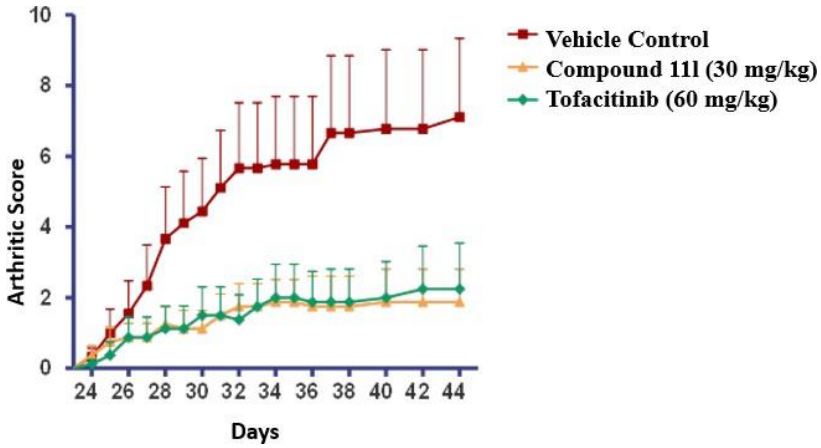


Figure 3b. Effect of Compound **11l** and Tofacitinib in CIA mice model

- As shown in the Fig.3b, standard and **11l** showed good reduction in the arthritic score, compared to vehicle control (untreated group).
- Two fold higher dose of a standard compound was used, considering more than two fold difference in the mice oral bioavailability.
- At 30 mg/kg dose, compound **11l** showed comparable activity to that of standard compound (dose 60 mg/kg).
- Body weights of the animals were also recorded 3 times a week as a measure of treatment related side effect. Body weight was not significantly affected in mice in any treatment group compared with the vehicle control group.

5.8. Safety pharmacology

Table 8 Hematological parameters and serum chemistry of compound **11l**

Parameters	Compound	
	Control	**11l**[a]
RBC (10^6 µl^{-1})	7.25 ± 0.19	8.35 ± 0.33
AST (U L^{-1})	146.88 ± 11.54	139.71 ± 9.50
TBILI (mg dL^{-1})	0.15.50 ± 0.05	0.18 ± 0.12
WBC (10^3 µl^{-1})	9.10 ± 0.35	8.99 ± 0.30
ALT (U L^{-1})	19.97 ± 1.55	20.69 ± 8.63
ALP (U L^{-1})	135.21 ± 5.78	120.80 ± 12.9

[a] Values expressed as mean ± SD: *n*=9, Male WR dose 100 mg kg^{-1}, po (bid), 14 days repeated dose toxicity study

- As shown in Table 8, the hematological parameters (WBC and RBC) of compounds **11l** were found to be comparable to that of control animals.
- Similarly, compound **11l** showed no significant changes in serum ALP, AST, ALT and TBILI (hepatotoxicity assessment parameters) as compared to the control group.

Table 9 Relative organ weights (%) after 14 days repeat dose treatment with compound **11l**

Organs	Compounds	
	Control (Vehicle)	**11l**[a] (100 mg kg^{-1}, po, bid)
Brain	0.730±0.028	0.690±0.03
Kidney	0.800±0.034	0.814±0.03
Heart	0.350±0.009	0.360±0.007
Spleen	0.250±0.006	0.243±0.01
Liver	3.598±0.15	3.660±0.078

[a] Values expressed as mean ± SD: *n*=9, Male WR, dose 100 mg kg^{-1}, po (bid), 14 days repeated dose toxicity study.

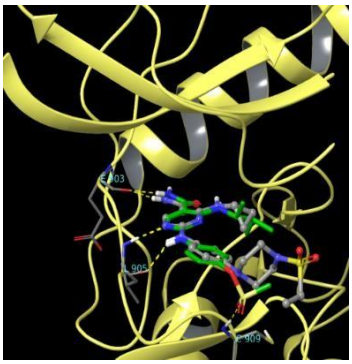
- To assess the safety profile of compound **11l**, repeat dose acute toxicity studies (14 days) was carried out in male Wistar rats (100 mg/kg, po, once daily,) and various

parameters such as gross pathology, clinical signs, body weight, organ weights, and serum chemistry/haematological changes were recorded.

- In general, daily oral administration of compounds **11l**, at 10X of ED_{50} dose, over a period of 2 weeks did not affect the survival of Wistar rats and also no adverse changes related to gross pathology, clinical signs, body weight and feed consumption were noticed as compared to control group.

5.9. Docking study

Multiple structures of JAK3, co-crystallized with various ligands, were analysed, and the structure with PDB ID 5W86 (solved at 2.6Å) was selected due to core similarity of the co-crystallized ligand and Cerdulatinib. (19). Docking study was done by using Glide Schroodinger software.



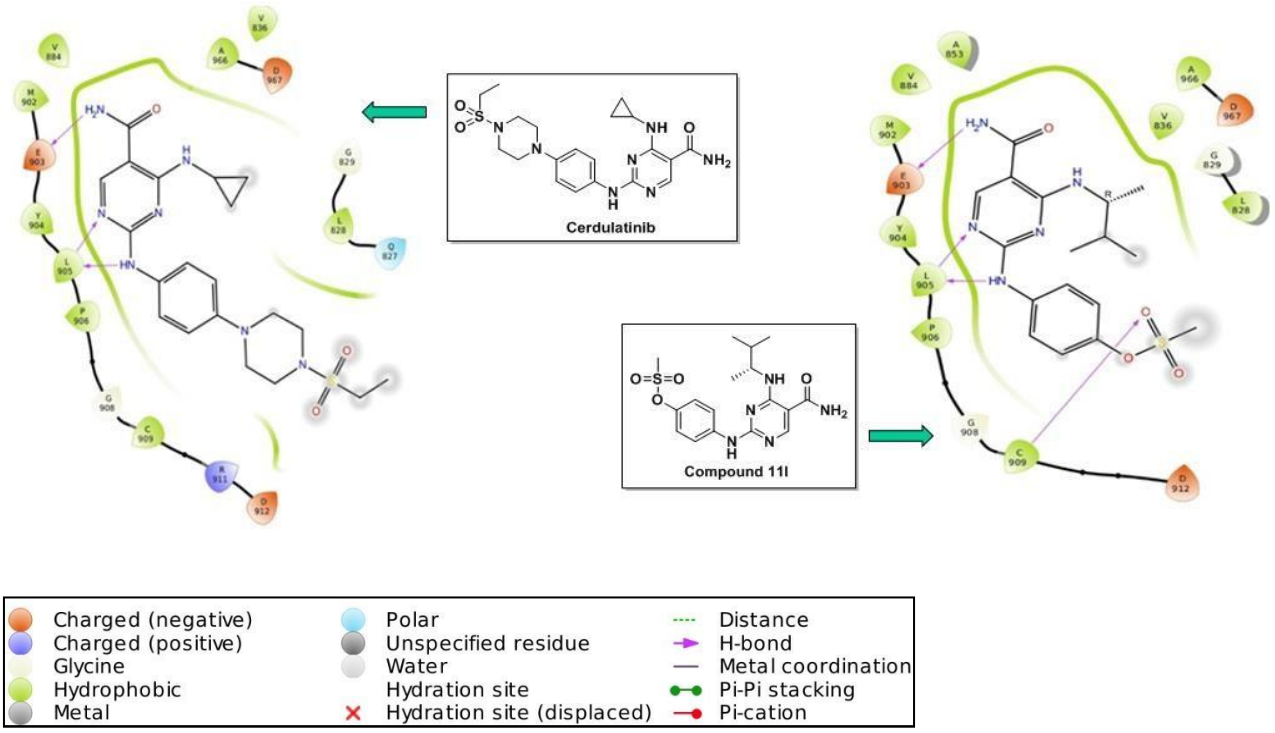


Figure 3. Docked pose of Cerdulatinib and **11l** within ATP binding site of JAK3 molecular surface (PDB ID: 5W86 @2.6Å).

- The docked poses of the core of Cerdulatinib and the co-crystallized ligand superimposed well.
- The potent and selective ligand **11l** was also observed to bind with similar interaction as that of Cerdulatinib.
- **11l** showed additional interaction with Cys909 through hydrogen bond between NH of Cys909 with oxygen of methyl sulfonate group, which was not observed with the Cerdulatinib.
- An additional interaction of **11l** with Cys909 might contribute towards its potent and selective JAK3 inhibitory activity.
- The docking score for Cerdulatinib and **11l** was found to be -8.6 and -9.9 kcal mol^{-1} respectively.

Summary and Conclusion

In summary, we synthesized and evaluated three different series of 2, 4-diaminopyrimidine-5-carboxamide derivatives as selective JAK3 inhibitors. Modifications at C2-position of pyrimidine ring led to an identification of a single digit nM potent JAK3 inhibitor (**5k**), with moderate isoform selectivity.

Further structure-activity relationship (SAR) studies on the C4-position of **5k** resulted in to the discovery of (R)-4-((5-carbamoyl-4-((3-methylbutan-2-yl) amino) pyrimidin-2-yl) amino) phenyl methane sulfonate (**11l**) with improvement in isoform selectivity.

It was also observed that suitable substituents on the C4-position contributed significantly towards improvement in the *in vivo* anti-arthritic activity (in a CIA mice and AIA rat models), which could be correlated with an improved oral bioavailability.

In repeat dose acute toxicity study, the most potent and selective compound **11l** showed no adverse changes related to gross pathology, clinical signs and liver toxicity.

Further modification at 5^{th} position of compound **11l** resulted in loss of activity when substituents were other than amide group which shows that $–CONH_2$ group is essential for activity.

All these data indicating that the new class JAK3 selective inhibitor could be viable therapeutic option for the effective management of rheumatoid arthritis.

References:

1. Medzhitov R. Origin and physiological roles of inflammation. *Nature*. **2008**, 454, 428–435. DOI: 10.1038/nature7201.
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