5. Detailed biological evaluations of selected potent JAK Inhibitors (41k & 48l)

As described in the previous Chapters, we designed and synthesized three Series of novel JAK inhibitors. This research led to identification of compound **41k** (IC_{50}: 9.5 nM) and compound **48l** (IC_{50}: 1.7 nM) which showed much better JAK3 inhibitory activity compare to cerdulatinib (IC_{50}: 8.0 nM) and also compounds **41k** and **48l** showed JAK3 inhibitory activity similar to that of Tofacitinib (IC_{50}: 1.7 nM). These two compounds were further selected for detailed biological evaluations. In Chapter-5 we described detailed profiling study of **41k** and **48l**, which includes:

a) Kinome selectivity study
b) JAKs isoform selectivity study
c) Potency and selectivity determination in human peripheral blood mononuclear cells (PBMC)
d) *Ex-vivo* study (CYP, hERG, Liver microsomal, Plasma protein binding)
e) Pharmacokinetic study
f) *In-vivo* study (AIA & CIA model)
g) Safety pharmacology study
h) Docking study.

5.1. Kinome Selectivity Profile of 48l (*In-Vitro*)

Among **41k** and **48l**, only **48l** selected for kinome selectivity profile as described in the pharmacokinetic section, **41k** showed inferior PK profile, also **41k** showed moderate isoform selectivity.

In-vitro kinase profiling study of compound **48l** 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 6**.

Table 6. *Kinome Selectivity Profile of* ***48l***[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%).

Overall, kinome selectivity profile clearly indicates that compound **48l** demonstrated greater JAK3 (99.2%) selectivity over other 170 kinase screened.

5.2. JAKs Isoform Selectivity

The most potent compounds (**41k** and **48l**) were evaluated for their selectivity against JAK isoforms (JAK1, JAK2, JAK3 and TYK2).

Protocol for JAKs isoform selectivity is described below.

Human JAK1, JAK2, and JAK3 kinase domains were purchased from Carna Biosciences, Inc. (Kobe, Japan), and the assay was performed, using a streptavidin-coated 96-well plate. The reaction mixture contained 15 mM Tris-HCl buffer (pH 7.5), 0.01% Tween 20, 2 mM DTT, 10 mM $MgCl_2$, 250 nM Biotin-Lyn-Substrate-2 (Peptide Institute, Inc., Osaka, Japan) and ATP. The final concentrations of ATP were 200, 10, and 8 μM for JAK1, JAK2, and JAK3, respectively. The test compounds were dissolved in DMSO and the reaction was initiated by adding the kinase domain, followed by incubation at room temperature for 1 hr. Kinase activity was measured as the rate of phosphorylation of BiotinLyn-Substrate-2, using HRP-conjugated anti-phosphotyrosine antibody (HRP-PY-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) with a phosphotyrosine-specific ELISA. The TYK2 kinase assay was performed with the ATP concentration modified to 10 μM. All experiments were performed in duplicate. The IC_{50} value of all compounds was calculated, using linear regression analysis. **[92]**

Table 7. *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
41k	18	42	9.5	45	2	4	5
48l	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 7**, initial hit **41k** showed moderate selectivity (2 to 5-fold) against JAK isoforms over JAK3. Compound **48l** (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 **48l** against all the three isoforms was found to be better than standard compounds (Tofacitinib and Cerdulatinib). Thus, potency and selectivity of diaminopyrimidine-5-carboxamides based JAK3 inhibitors can be modulated, using suitable substituents at C2, C4 and C5-positions of a pyrimidine ring.

5.3. JAK cellular assays using human peripheral blood mononuclear cells

To elucidate potency and selectivity profile in a cellular environment, compound **48l** was tested for the inhibition of phosphorylation of downstream signal (STAT proteins), in the human peripheral blood mononuclear cells (PBMCs). **[95]** 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. **[96]** PBMCs were collected from healthy volunteers (as per Zydus Research Centre, ethical committee protocol), in to sodium heparrin vacutainer tubes. After incubation with compound **48l** and Tofacitinib, at 37^{o}C, for 30 mins, blood was triggered with either recombinant human IL-6 (400 ng/mL; R&D Systems), IL-2 (20 ng/mL;

R&D System) 12GM-CSF (100 ng/mL; Pepro-Tech), or vehicle (PBS plus 0.1% [w/v] BSA), at 37°C, for 20 mins and treated with pre-warmed 13 lysis/fix buffer (BD Biosciences) to lyse RBCs and fix leukocytes. Cells were permeabilized with 100% MeOH and incubated with anti-pSTAT3 and anti-CD4 (IL-6-triggered samples), anti-pSTAT5 and anti-CD4 Abs (IL2-triggered samples), or anti-pSTAT5 Abs (GM-CSF-triggered sample; all Abs were from BD Biosciences), at 4°C, for 30 mins, washed once with PBS and analyzed on FACS Canto II flow cytometer. IC_{50} values were determined, using Prism software (Version 7, GraphPad), for Tofacitinib and **48l**, **Table 8**.

Table 8. *Potency and Selectivity determination of **48l** in human PBMC*

JAKs involved	Trigger	Readout	IC_{50}(nm)		Selectivity	
			48l	Tofacitinib	48l	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 8**, **48l** showed 27-fold selectivity for inhibition of the IL-2 (IC_{50}: 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 **48l** in the relevant pSTAT assays. Thus, compound **48l** showed preferential inhibition of JAK3 over JAK1, in the JAK/STAT signalling

pathway when assessed in the PBMC assay.

5.4. Ex-vivo studies: Liver microsomal study, CYP inhibition study and hERG inhibition study and plasma protein binding study

5.4.1. Liver microsomal study

To estimate microsomal stability, of compound **48l** against liver microsomes, test compound **48l** (0.2 or 1.0 μM) was incubated with male SD rat liver microsomes or male/female human liver microsomes (0.2 mg protein/mL) in the presence of NADPH (1 mM) and EDTA (0.1 mM), in phosphate buffer (100 mM), at 37°C. The percentage of compound remaining was determined by LC-MS/MS. Compound **48l** showed less than 10% metabolism at 30 mins, in the liver microsomal metabolic stability study. **[97]**

5.4.2. CYP inhibition study

For CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4 inhibition studies, Human liver microsomes (0.2 mg/ml), Testosterone (50 μM)/Dextromethorphan (5 μM) respectively, as probe substrate, potassium phosphate buffer (0.1 M; pH 7.4) and NADPH (1 mM) were incubated with different concentrations of compound **48l** @1, 10 and 100 μM concentrations), at 37^0C for, 10 mins, enzyme activity (% of control) was determined and IC_{50} values were calculated. Compound **48l** was found to be devoid of CYP (<10% CYP inhibition at 10 μM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and

CYP3A4). **[98, 99]**

5.4.3. hERG inhibition (Rb efflux assay) study

CHO (CHO-K1) cells stably expressing the hERG potassium channel were constructed and maintained in alpha-minimum essential medium (MEM) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% Geneticin in a humidified atmosphere at 37 ^{o}C, with 5% CO_2. On the day before experiments, stable clones were seeded at 6 X 10^4 cells/well, in 96-well Plate and cultured for 24 hr. The culture medium was exchanged to Rb loading medium and incubated for 1.5 hr. Medium was discarded, the plate was washed with pH 7.3 buffer, and high K^+ buffer (pH = 7.3) containing compound **48l** was added to initiate the Rb efflux through the potassium channel. After 10 mins incubation, the supernatant was transferred to a new plate (Plate [A] 0. The plate containing the cells was lysed with 1% Triton-X 100 in pH 7.3 buffer (Plate [B]). Rb in Plates (A) and (B) was detected using an Ion Channel Reader 8000 (Aurora Biomed Inc.), and the Rb efflux rate (RE) and the efflux inhibition rate were calculated using the following equations:

Rb efflux rate (RE) (%) = remained Rb (B)/total Rb (A+B) X 100

Efflux inhibition (%) = RE (test compound)

- RE (vehicle) / RE(positive control)
- RE (vehicle) X 100

The IC_{50} value of the efflux inhibition was calculated via nonlinear regression analysis. Compound **48l** was found to be devoid of hERG liabilities (IC_{50}: > 10 µM). **[100]**

5.4.4. Plasma protein binding study

An equilibrium dialysis method was used to determine the plasma fraction unbound (fu) values. Briefly, dialysis membranes (MWCO 12-14 K) and 96 well dialysis devices were assembled following the manufacturer's instructions (HT Dialysis, LLC, USA). Mice, rat and human plasma samples containing 1µmol/L compound **48l** with 1% DMSO were dialyzed against PBS, for 6 hr, in a humidified incubator (75% relative humidity; 5% CO_2/95% air), at 37^oC, at 450 RPM. Quadruplicates of binding were measured for compound **48l**. Samples were matrix-matched and quenched with cold acetonitrile, containing internal standard. The solution was centrifuged and the, supernatant was analyzed, using LC-MS/MS. Compound **48l** was evaluated for plasma protein binding studies (using mice, rat and human plasma) and it showed 75 to 80% plasma protein binding in mice, rat and human plasma. **[101]**

5.5. Pharmacokinetic study of 41k, 48l and Tofacitinib in C57 mice

A comparative single dose (3 mg/kg, po {oral administration} and 1 mg/kg, iv {intravenous}) PK profile of compounds **41k**, **48l** and Tofacitinib was evaluated in overnight fasted male C57BL/6J mice (n=6). Serial blood samples were collected in micro centrifuge tubes containing EDTA, at pre-dose, 0.15, 0.3, 0.5, 0.75, 1, 2, 4, 6, 8, 24 and 30 h post-dose after compounds administration. Approximately 0.3 ml of blood was collected at each time point and centrifuged at 4^oC. The obtained plasma was frozen, stored at -70^oC and the concentrations of compounds in plasma were determined by the LC-MS/MS (Shimadzu LC10AD, USA), using YMC hydrosphere C18 (2.0 x 50 mm, 3 µm) column (YMC Inc., USA). The pharmacokinetic

parameters, such as Tmax, $t_{1/2}$, Cmax, AUC and %F were calculated, using a non-compartmental model of WinNonlin software version 5.2.1. (**Table 9**). **[102]**

Table 9. *Pharmacokinetic study parameters of* ***41k, 48l*** *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*
41k	0.25	146 ± 48	1.85 ± 0.43	40.37 ± 3.61	192 ± 56	15
48l	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 **41k**, **48l** and Tofacitinib administered at 1 mg/kg dose, iv AUC (ng/ml): 412, 1459 and 350 respectively.

As shown in **Table 9**, in PK study, **41k** showed moderate area under the curve (AUC), due to high clearance (Cl), which resulted into overall low bioavailability (15%). Compound **48l** 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 **48l** 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/mins/kg, iv)

5.6. *In-vivo* efficacy studies with compound 48l and std (Tofacitinib)

The animal experiments were carried out in rats and mice, bred in the house. Animals were housed in groups of 6 animals per cage for a week in order to habituate them to vivarium conditions (25 ± 4^{o}C,

60-65% relative humidity, 12:12 hr light-dark cycle, with lights on at 7.30 am). All the animal experiments were carried out according to the internationally valid guidelines following approval by the 'Zydus Research Center, animal ethical committee'.

5.6.1. Anti-arthritic efficacy of test compounds in AIA (Adjuvant induced Arthritis) rat Model

The rat model of AIA has been widely used for the preclinical testing of numerous anti-arthritic agents. Arthritis was induced in female Lewis rats by inoculation with Freund's complete adjuvant (CFA). Briefly, on day 0, rats were anesthetized with a mixture of ketamine and xylazine (80:10 mg/kg, intraperitoneally) and then injected with 0.1 mL CFA 1 mg/mL of heat-inactivated Mycobacterium tuberculosis intra-dermally at the base of the tail. Treatment and group designations are as follows: vehicle (2% sodium carboxymethylcellulose (CMC)); 3, 10 and 30 mg/kg of compound **48l** and 60 mg/kg Tofacitinib was used as a positive control, which were administered once daily, by oral gavage, for 20 days. Paw edema determined by measuring changes in the paw volume, using a plethysmometer, on days 10, 12, 14, 16, 18 and 20, post- CFA injection. Statistical analysis was performed by one-way ANOVA (Dunnett's method, data represent the mean ± S.E.M., n = 8/group). **[103]**

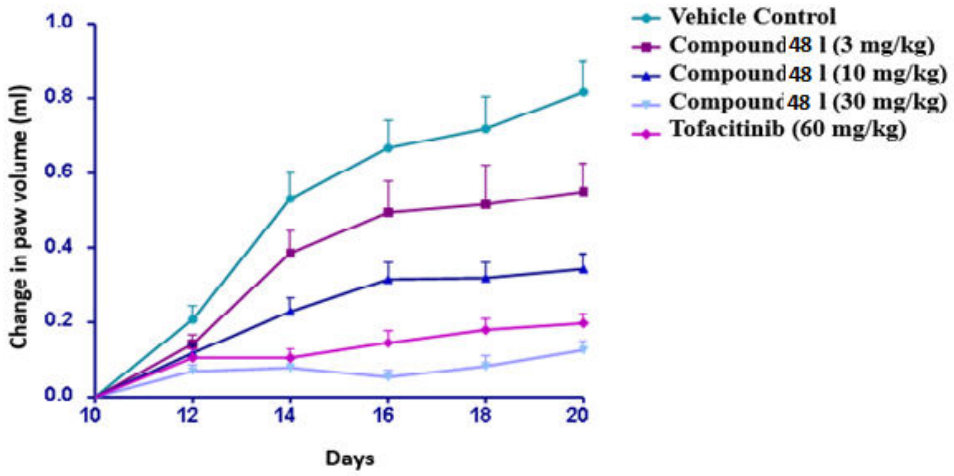

Figure 15. Effect of Compound **48l** and Tofacitinib in AIA rat model

As shown in the **Figure 15**, standard and **48l** showed good reduction in the paw volume, compared to vehicle control (untreated group). Compound **48l** 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 a rat in any treatment group compared with the vehicle control group.

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

The CIA study is a representative animal model of human rheumatoid arthritis. Following 7 days acclimation, male DBA1j (8 to 12-weeks old) mice were randomly assigned to groups according to body weight. Mice were immunized subcutaneously in the tail, using bovine type II collagen mix in complete Freund's adjuvant (CFA). Twenty-one days after the first immunization, mice were given a booster dose of collagen in incomplete Freund's adjuvant (IFA). Mice

were monitored every other day after the booster dose for the development of arthritis. Mice were recruited for the study once clinical signs were visible. Eight animals were assigned each of four groups [vehicle, Tofacitinib and two doses of test compound, **48l**] and treatment was continued for two weeks and percentage inhibition in clinical score is recorded as per graded score. The body weights of the animals were also recorded 3 times a week as a measure of treatment related side effect and paw thickness measured twice a week (**Figure 16**). The mice were scored in a blinded manner (0-12) for signs of arthritis in each paw according to the following scale: 0- no swelling or redness/normal paw; 1- swelling and/or redness in one digit; 2- swelling and/or redness in two or more digits; and 3- entire paw is swollen or red. The severity score was reported as the sum of all four paws for each mouse and severity was expressed as the average severity score for each group. **[104]**

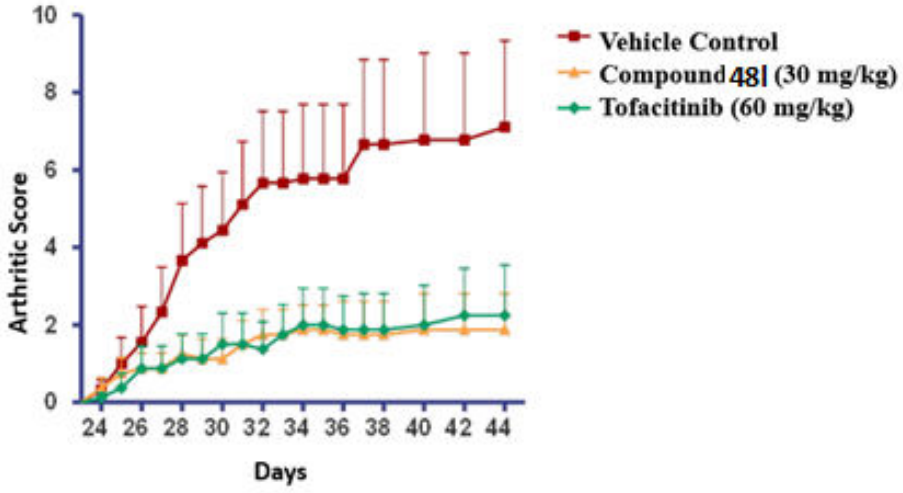

Figure 16. Effect of Compound **48l** and Tofacitinib in CIA mice model

As shown in the **Figure 16**, standard and **48l** 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 **48l** showed comparable activity to that of standard compound (dose 60 mg/kg). The body weights of the animals were also recorded 3 times a week as a measure of treatment related side effect. The body weight was not significantly affected in mice in any treatment group compared with the vehicle control group. Thus, improved PK of **48l** justifies it's potent *in-vivo* activity in the CIA mice model.

5.7. Safety pharmacology study of 48l

Repeated dose Toxicology study with compound **48l**

Repeated dose toxicity studies (14 days) of compound **48l** was carried out in male wistar rats (WR). Briefly, animals were divided into three groups (n=9), a control group and separate groups for test compounds. To each of the test groups, a daily oral dose of 100 mpk compound **48l** was administered, twice a day (bid), under fasted conditions for 14 days. After completion of the treatment period (14 days), animals were sacrificed and subjected to a complete necropsy examination and also changes in toxicological parameters, such as gross pathology, clinical signs, body weight, organ weights and serum chemistry/ hematological changes were recorded.

Table 10. *Relative organ weights (%) after 14 days repeat dose treatment with compound* ***48l***

Organs	Compounds	
	Control (Vehicle)	48l[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.

Daily oral administration of compounds **48l**, at 10X of ED_{50} dose (as per AIA rat model), 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 the control group. Compound **48l** treated groups showed no changes in the other key organs (heart, kidney, spleen and brain) weights.

To assure hematological parameter and serum biochemistry, rats were anesthetized 24hr post treatment with compound **48l** and blood samples were collected. The whole blood was centrifuged at 3000 rpm, using a centrifuge at 37°C for 15 mins and serum ALT, AST and ALP were assayed, using a diagnostic kit (Boehringer Mannheim).

Table 11. *Hematological parameters and serum chemistry of compound* ***48l***

Parameters	Compound	
	Control	48l[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})	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 11**, the hematological parameters white blood cell (WBC) and red blood cell (RBC) of compounds **48l** were found to be comparable to that of control animals. Similarly, compound **48l** showed no significant changes in serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine transaminase (ALT) and total bilirubin (TBILI) hepatotoxicity assessment parameters as compared to the control group. **[105]**

5.8. Docking study of compound 48l

Docking study of compound **48l** was carried out to understand its key interaction with JAK3 enzyme. Further it also helps us to understand SAR.

Experimental section for docking study:

Multiple structures of JAK3, co-crystallized with various ligands, were analyzed, and the structure with PDB ID 5W86 (solved at 2.6Å)

was selected due to core similarity of the co-crystallized ligand and Cerdulatinib. The protein was prepared, using protein preparation wizard of Schrodinger Suite 2018-4, at pH 7.4. Ligands were prepared, using Ligprep module at pH 7.0 ± 0.5, with default settings. Glide grid was used with default options for grid generation and Glide SP docking was used for docking simulation with default settings. The docked pose of Cerdulatinib and **48l** was analyzed with respect to docking score, docking pose and hydrogen bonding interactions with the key region residues of the JAK3 enzyme. **[106, 107]**

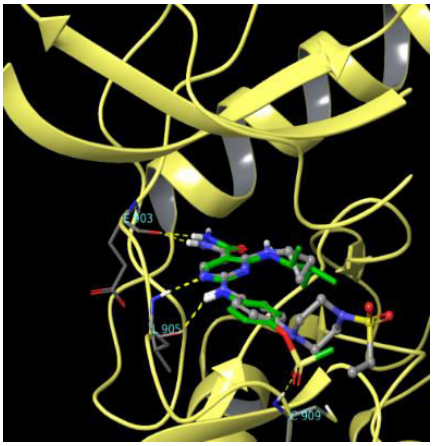

Figure 17. Docked pose of Cerdulatinib and **48l** superimposed

Cerdulatinib is shown in ball and stick model with grey carbon while **48l** is displayed in stick model with green carbon, yellow dashed lines show the H-bond interaction.

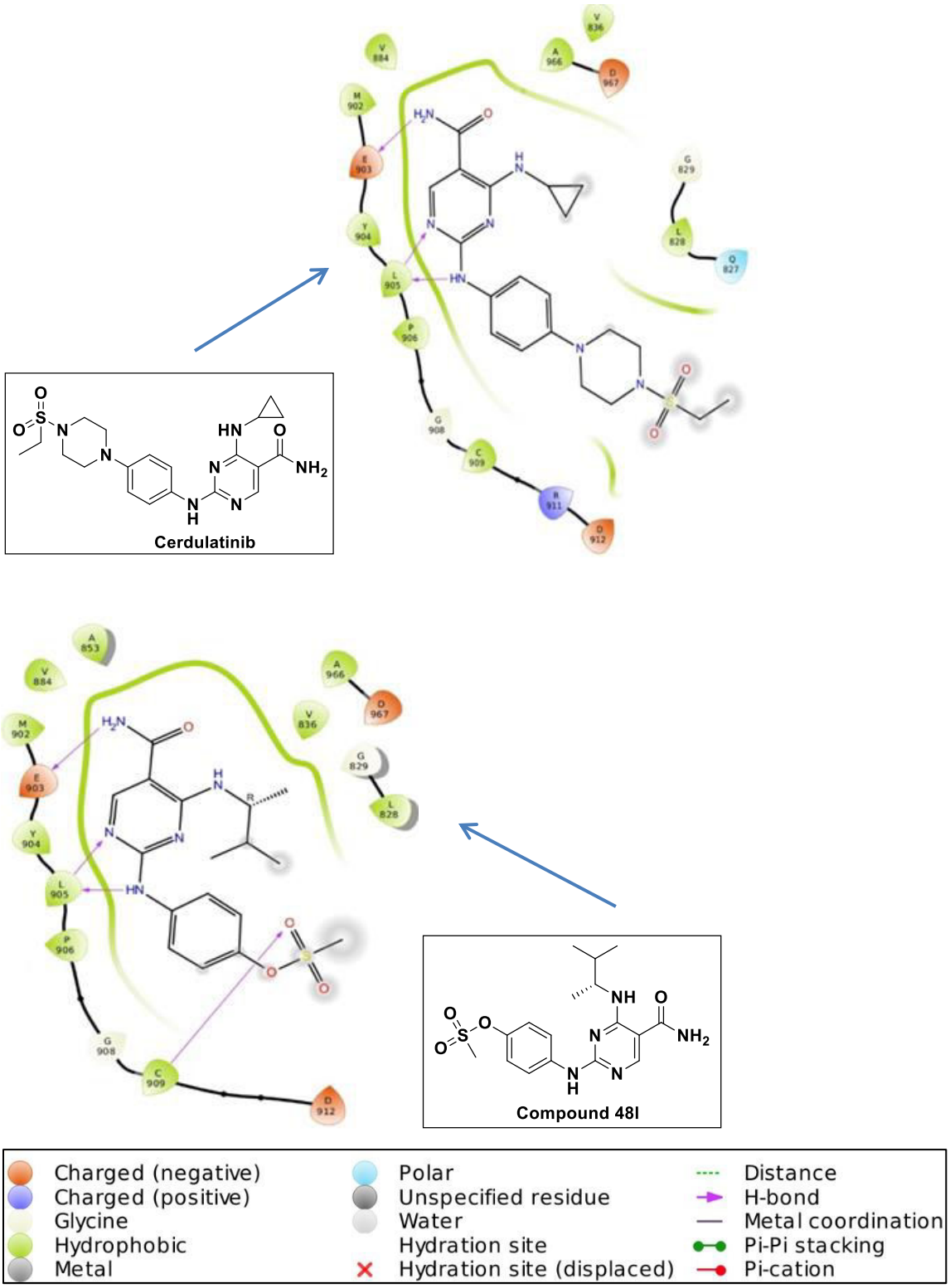

Figure 18. Docked pose of Cerdulatinib and **48l** within ATP binding site of JAK3 molecular surface (PDB ID: 5W86 @2.6Å).

Docking study results:

In the docking studies, Cerdulatinib binds in a similar orientation to that of 4, 6-diaminonicotinamide of the co-crystallized ligand 99YV; 4-(benzylamino)-6-9{4-[(1-methylpiperidin-4-yl)carbamoyl] phenyl} amino)pyridine-3-carboxamide), maintaining hydrogen bonding interactions with the hinge region of JAK3 (a 'classical' triad hinge binding interaction), **Figure 18** The docked poses of the core of Cerdulatinib and the co-crystallized ligand superimposed well. Compound **48l** showed similar interactions as that of Cerdulatinib, in the hinge region and superimposed very well with the core of Cerdulatinib. However, **48l** showed additional interaction with the Cys909 (hydrogen bond between NH of Cys909 with oxygen of methyl sulfonate group), which was not observed with the Cerdulatinib. Additional interactions of **48l** with the Cys909, in the catalytic domain of JAK3 enzyme likely to contribute towards its potent and selective JAK3 inhibitory activity. The docking score for Cerdulatinib and **48l** was found to be -8.6 and -9.9 $kcalmol^{-1}$ respectively.

5.9. Conclusion

In conclusion, compound **48l** showed better result in a detailed biological evaluation. Compound **48l** demonstrate greater JAK3 (99.2%) selectivity over other kinase. **48l** showed improved isoform selectivity in the biochemical assay. **48l** showed preferential inhibition of JAK3 over JAK1, in the JAK/STAT signaling pathway, when assessed in the PBMC assay. **48l** showed 75 to 80% plasma protein binding and less than 10% metabolism at 30 mins, in the liver microsomal metabolic stability study. **48l** was also found to be devoid of CYP

(<10% CYP inhibition) and hERG liabilities (IC_{50}: >10 μM). **48l** showed higher AUC, extended $t_{1/2}$, good oral bioavailability and low clearance compared to the standard.

In *in-vivo* in CIA and AIA models, at 30 mg/kg dose, compound **48l** showed comparable activity to that of the standard compound (Tofacitinib, 60 mg/kg). In the repeat dose acute toxicity study, compound **48l** showed no adverse changes related to gross pathology, clinical signs and liver toxicity. In docking studies **48l** showed additional interaction with Cys909 through a hydrogen bond between NH of Cys909 with oxygen of methyl sulfonate group, which was not observed with the Cerdulatinib. An additional interaction of **48l** with Cys909 might contribute towards its potent and selective JAK3 inhibitory activity. This detailed evaluation of compound **48l** indicates that the new class JAK3 selective inhibitor could be a viable therapeutic option for the effective management of rheumatoid arthritis.