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Article

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Design, Synthesis of Novel, Potent, Selective, Orally Bio-available Adenosine A_{2A} Receptor Antagonists and Their Biological Evaluation

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ABSTRACT:

Our initial structure-activity relationship studies on 7-methoxy-4-morpholino-benzothiazole derivatives featured by aryloxy-2-methylpropanamide moieties at the 2-position lead to identification of compound **25** as a potent and selective A_{2A} adenosine receptor (A_{2A}AdoR) antagonist with reasonable ADME and pharmacokinetic properties. However, poor intrinsic solubility and low to moderate oral bioavailability made this series unsuitable for further development. Further optimization using structure-based drug design approach resulted in discovery of potent and selective adenosine A_{2A} receptor antagonists bearing substituted 1-methylcyclohexyl-carboxamide groups at position 2 of the benzothiazole scaffold and endowed with better solubility and oral bioavailability. Compounds **41** and **49** demonstrated a number of positive attributes with respect to *in vitro* ADME properties. Both compounds displayed good pharmacokinetic properties with 63% and 61% oral bioavailability respectively, in rat. Further, compound **49** displayed oral efficacy in 6-OHDA lesioned rat model of Parkinson diseases.

INTRODUCTION

Adenosine, an endogenous purine nucleoside, and mediates its effect through four specific cell membrane receptors called adenosine receptors (AdoRs). These AdoRs, named A_1, A_{2A}, A_{2B}, and A_3 in human, belong to the G-protein-coupled receptors (GPCR) family.[1] AdoRs exhibit high sequence homology but differ in their affinity for adenosine and downstream signaling mechanism.[2-3] AdoRs have been implicated in several key physiological processes, and thus offer broad therapeutic potentials including cell growth, asthma, immune system, inflammatory diseases and central nervous system (CNS) disorders, etc.[4-6]

Adenosine A_{2A} receptor (A_{2A}AdoR) is a highly distributed receptor in the CNS. It is expressed at high levels in the nigrostriatum (basal ganglia), where it co-localizes with dopamine D2 receptors on striatopallidal output neurons.[7] Several pharmacological studies suggest that A_{2A}AdoR antagonists have potential for use in combination with existing therapies across multiple therapeutic area.[8] Parkinson's disease (PD) is a progressive neurodegenerative movement disorder affecting approximately 1% population over the age of 65. Unfortunately, current dopamine replacement therapies for PD suffer from poor long term control and undesirable side effects, mainly dyskinesia (involuntary movements). Antagonism of A_{2A}AdoR offers a non-dopaminergic approach to treat PD.[9] Stimulation of A_{2A}AdoR decreases the binding affinity of dopamine for dopamine D2 receptors[10] and elicits effects, opposite to dopamine D2 receptor activation at the level of second messenger system and early-gene expression.[11] Another potential disease modifying benefit of A_{2A}AdoR antagonism is the prevention of development of levodopa (L-DOPA) induced dyskinesias in PD as demonstrated in rodent and monkey studies.[12] A_{2A}AdoR are expressed on several immune cell types- T lymphocytes, dendritic cells, natural killer cells. A_{2A}AdoR activation on T cells and NK cells causes immunosuppression by reducing their proliferation, cytokine production and tumor killing activity.[13-14] In recent years, A_{2A}AdoR has shown exciting progress in the development of immunotherapy for the treatment of cancer[15] and multiple deals emphasize commercial potential of A_{2A}AdoR antagonists. Numerous research groups have devoted significant effort towards the discovery of selective A_{2A}AdoR antagonists for the treatment of Parkinson's disease. Some representative examples are shown in Figure 1.[16] A_{2A}AdoR antagonists are classified as xanthine and non-xanthine derivatives.[17-19] Numerous A_{2A}AdoR antagonists have reached phase I clinical trials and beyond. Among xanthine derivatives, **1** (Istradefylline, KW-6002, Kyowa Hakko Kirin

Co Ltd.), was launched in Japan for PD.[16] Several classes of non-xanthine A_{2A}AdoR antagonists have been reported in literature and they are either monocyclic,[20-21] bicyclic[22-23] or tricyclic core-based antagonists.[24-26] However, the discovery of A_{2A}AdoR antagonist with acceptable selectivity, physicochemical, and pharmacological properties has continued to remain challenging and consequently, majority of research has progressed towards identifying novel non-xanthine scaffolds. Hoffmann-La Roche has identified benzothiazole scaffold based **2** (SYN-115) [27-28] which is a potent, selective A_{2A}AdoR antagonist with desired ADME properties namely, and is currently in phase III clinical trials.

We initiated our research with the goal to discover a novel, potent and selective A_{2A}AdoR antagonist with desirable physicochemical, pharmacokinetic and pharmacological properties to provide therapeutic intervention for PD and cancer immunotherapy. Amongst A_{2A}AdoR antagonists with several structural types, benzothiazole and thiazolo[5,4-c]pyridine scaffold were chosen as starting point and new analogs were discovered subsequently (Figure 2). Herein, we describe the discovery and optimization of novel potent A_{2A}AdoR antagonist based on the chemical structure of **2**. After analyzing the available A_{2A} co-crystal structures with antagonists and docking pose of **2**, we decided to modify the most flexible part on right hand side of **2** with well tolerated and novel aryloxy-2-methylpropanamide or cyclohexane-carboxamide moieties (Figure 2). In the present study, a structure–activity relationship (SAR) was established via synthesis of analogues of **2** having urea linker replacement and various terminal substituents as shown in schemes 1 to 3.

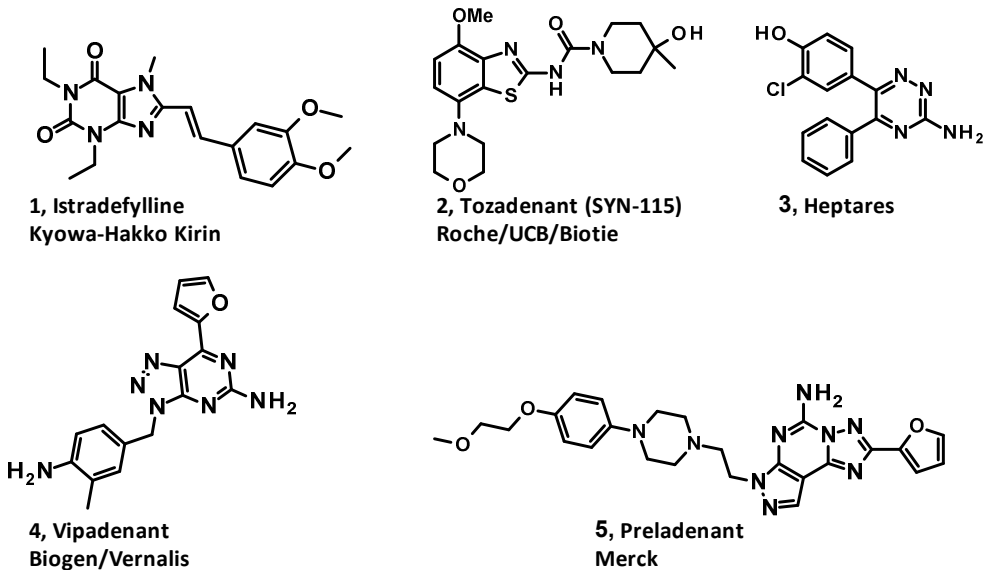


Figure 1. Representative examples of A_{2A}AdoR antagonists

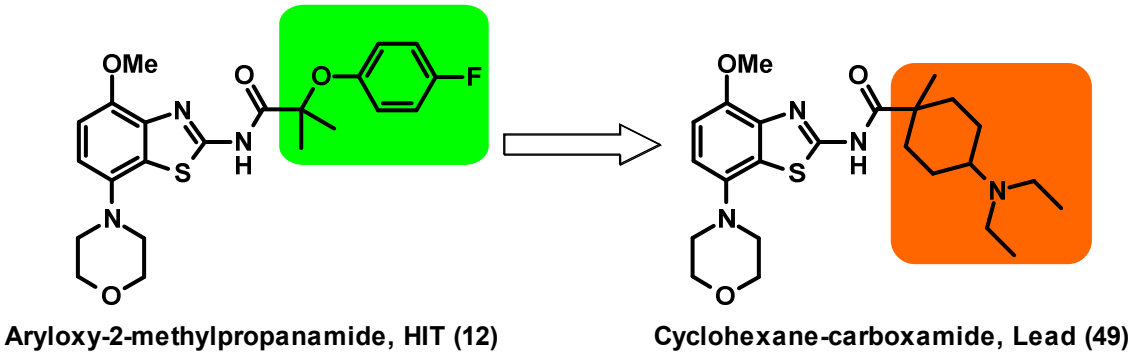
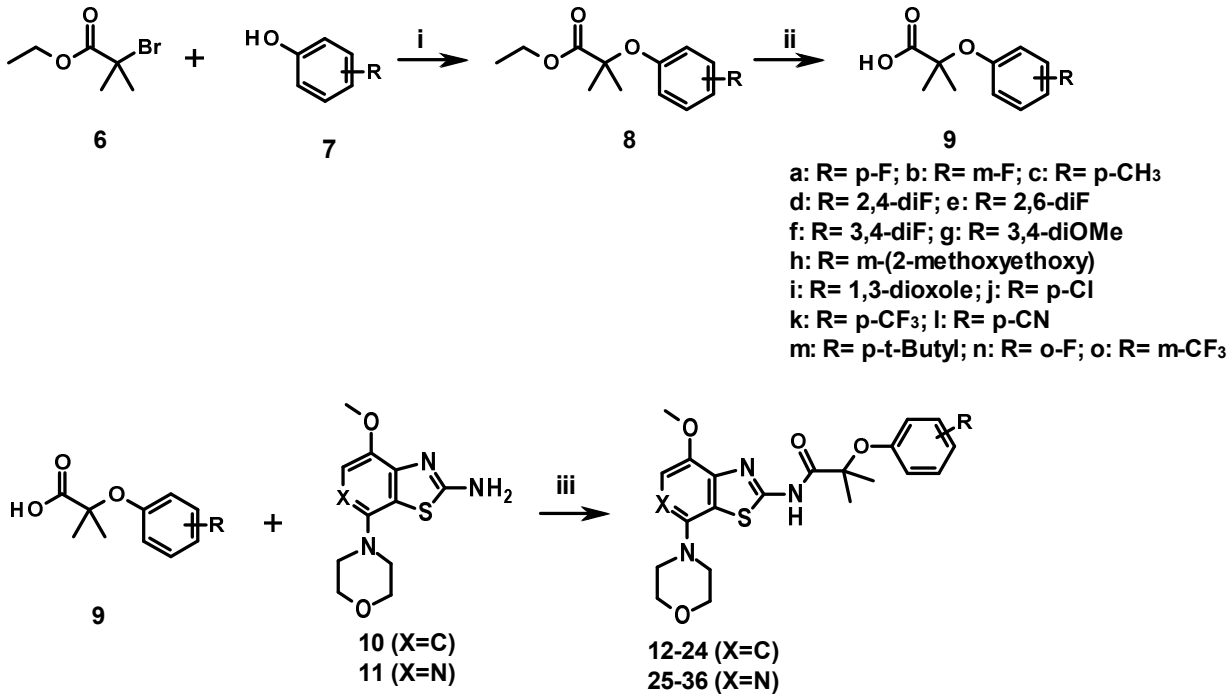


Figure 2. Identification of novel aryloxy-2-methylpropanamide and cyclohexane-carboxamide.

CHEMISTRY

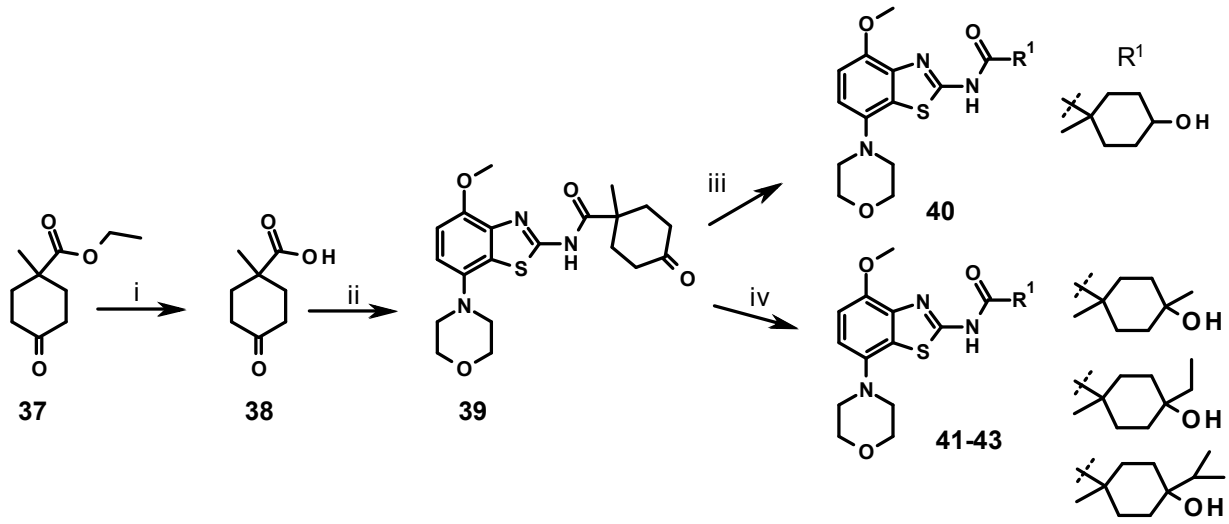
Scheme 1. Synthesis of **12-36**[a]



[a] Reagents and conditions: (i) K_2CO_3, DMF, rt, 16 h; (ii) NaOH, THF/MeOH/H_2O (3:2:1), 80 ^oC, 16 h; (iii) EDCI, HOBt, NMM, DMF, rt.

In scheme 1, substituted ethyl 2-methyl-2-phenoxy-propanoate derivatives (**8**) were prepared by reaction of ethyl-2-bromoisobutyrate (**6**) and phenol **(7)** in DMF at rt in presence of K_2CO_3. Hydrolysis of these variously substituted esters **8a-o**[29] under aqueous basic conditions yielded acid derivatives **9a-o**. Amide coupling of **9a-o** with **10**[27-28] and **11**[30] afforded final compounds **12-36** in overall good yields.

Scheme 2. Synthesis of **40-43**[a]



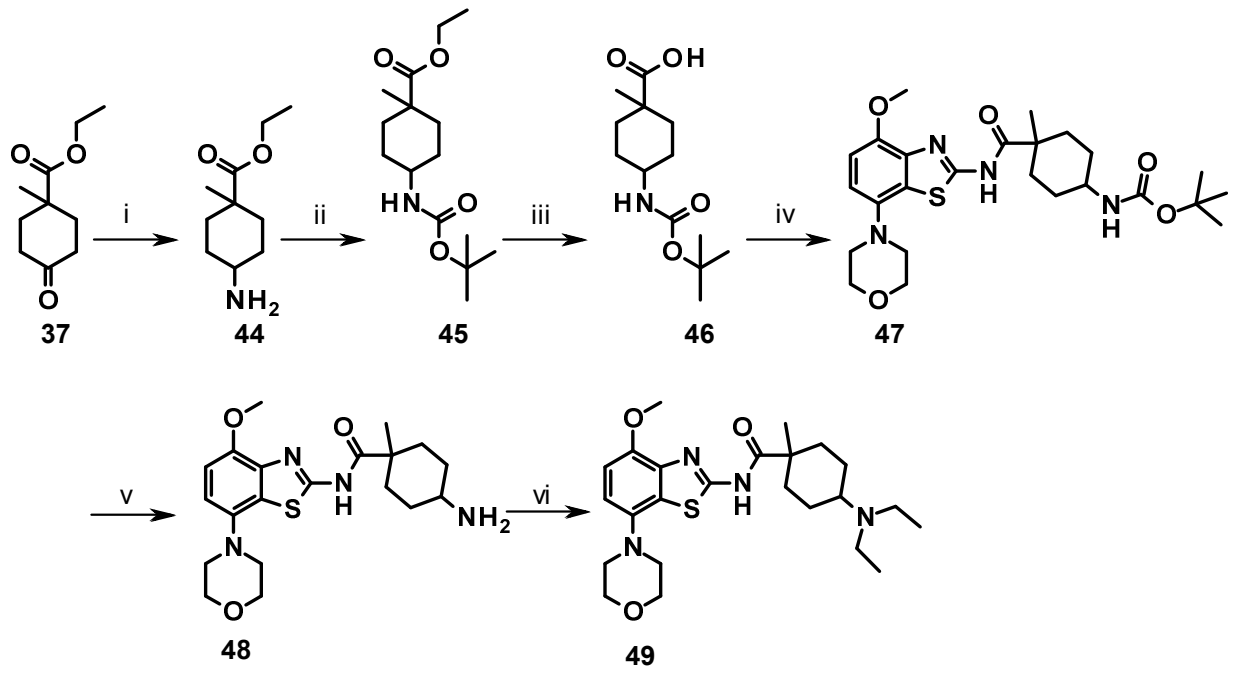
[a]Reagents and conditions: (i) $LiOH.H_2O$, THF/MeOH/H_2O (3:2:1), rt, 18 h; (ii) **10**, EDCI, HOBt, NMM, DMF, rt,18 h; (iii) $NaBH_4$, EtOH, 0 ^oC -rt, 3 h; (iv) **39**, appropriate Grignard reagents, dry THF, 0 ^oC - rt, 1h.

The synthesis of compounds **40-43** is outlined in scheme 2. Starting material **37**, prepared as described in literature[31] and its hydrolysis under aqueous basic conditions yielded **38**. Amide coupling of **38** with **10** afforded compound **39**. Reduction of **39** with $NaBH_4$ yielded final compound **40**, with terminal secondary hydroxyl group. Compound **39,** when treated with 1.2 molar equivalents of appropriate Grignard reagents gave corresponding tertiary hydroxyl derivatives as a mixture of isomers **41-43**.

Compound **49** was synthesized as illustrated in Scheme 3. Compound **37** upon treatment with methanolic ammonia followed by hydrogenation gave corresponding amine **44**,[32] which was further protected by boc-anhydride to yielded **45**. Hydrolysis of **45**, under aqueous basic conditions yielded **46**. Amide coupling of **46** with **10** using standard EDCI-HOBt method

afforded **47** which on treatment with methanolic HCl provided compound **48**. Finally, compound **49** (mixture of *cis* and *trans*) was prepared from compound **48** by reductive amination.

Scheme 3. Synthesis of **49**[a]



[a] Reagents and conditions: (i) Methanolic NH_3, H_2, 10 % Pd/C, rt, 24 h; (ii) Boc anhydride, Et_3N, MeOH, rt , 4 h; (iii) $LiOH.H_2O$, THF/MeOH/H_2O (3:2:1), 60 ^oC,18 h; (iv) **10**, EDCI, HOBt, NMM, DMF , rt, 14h; (v) methanolic HCl, DCM, rt 24 h; (vi) acetaldehyde, $NaCNBH_3$, MeOH, NaOAc, rt ,24 h.

RESULTS AND DISCUSSION

Biological data and structure–activity relationships

Initially, a new series of 2-substituted benzothiazole and thiazolo[5,4-c]pyridine derivatives were prepared with the objective of identifying novel, potent and selective A_{2A}AdoR antagonists with

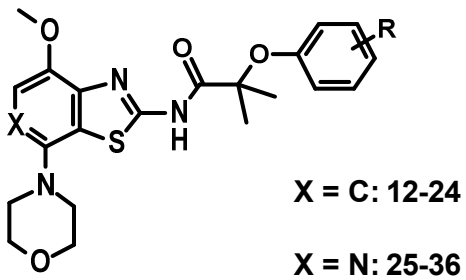
acceptable pharmacokinetic properties. For this purpose, urea linkage of **2** was replaced by aryloxy-2-methylpropanamide moieties. The compounds were first evaluated for affinity against A_{2A}AdoR and only compounds showing A_{2A} affinity <20 nM were evaluated for binding selectivy over A_1, A_{2B} and A_3 AdoR. The data obtained is listed in Table 1. Most of the aryloxy-2-methylpropanamide derivatives, **12-24** (except **21-24**) showed good binding affinity with K_i in the range of 2.8-22 nM for human A_{2A}AdoR (hA_{2A}). Compounds **25-36** (thiazolo[5,4-c]pyridine core) also exhibited good binding affinity with K_i in the range of 3.4-55 nM for human A_{2A}AdoR. In general, compounds containing small electron withdrawing groups like -F, -di-F or electron releasing groups such as -Me, and –OMe on phenyl ring (compound **12-20**) showed good binding affinity with K_i of <22 nM for A_{2A}AdoR.

However, compounds **21-23**, with larger electron withdrawing groups such as –Cl, $-CF_3$, -CN, showed modest binding affinity of 51, 76 and 58 nM, respectively, for A_{2A}AdoR. Compound **24** with more bulkier and hydrophobic *p*-t-butyl substitution had large negative effect on the affinity, and reduce binding K_i of 500 nM for A_{2A}AdoR. Compounds **34-36** with electron releasing group had moderate A_{2A}AdoR binding affinity of 42, 55 and 42 nM, respectively. Having identified compounds with good binding affinity, potent compounds with binding K_i< 20 nM for A_{2A}AdoR were tested for adenosine receptor subtype selectivity. Compounds **12-20**, **25**, **30**, **31** and **33** had moderate to good binding selectivity, while compound **26** showed poor selectivity and compound **32** showed moderate selectivity over A_1, A_{2B} and A_3AdoR

The potent and selective compounds, selected on the basis of binding affinity were subsequently assessed for stability in liver microsomes. Most of the compounds were metabolically stable in rat liver microsomes (RLM) and human liver microsomes (HLM) (Table 1). Compounds with very low nano-molar binding affinity (K_i <20 nM), good binding selectivity and metabolically

stable, were further tested for functional potency in cAMP assay using hA_{2A}-HEK293 cells (Table 1). Compound **12-18**, **20** and **25-26** showed excellent functional potency with K_i in the range of 0.95-8.3 nM. Compound **30-33** exhibited moderate functional potency with K_i in the range of 13.5-28 nM and were stable in RLM and HLM. To identify a lead, three potent, selective and metabolically stable compounds namely **12**, **17** and **25**, were shortlisted for further profiling. The data is summarized in Table 2 and 3.

Table 1. Affinity, potency, selectivity and microsomal stability of aryloxy-2-methylpropanamide derivatives



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Compound	R	Binding K_i (nM) hA_{2A}[a]	% Inhibition at 1 μM			Functional K_i (nM) hA_{2A}[b]	MR[c] (nmol/min/mg)	
			hA_1[a]	hA_{2B}[a]	hA_3[a]		RLM	HLM
12	*p*-F	13	16	0	11	4.2	0.04	0.02
13	*m*-F	2.8	52	36	20	0.95	0.1	0.08
14	*p*-CH_3	5.4	7	14	5	8.3	0.1	0.09
15	2,4-diF	7	33	0	21	2.6	0.07	0.11
16	2,6-diF	8	37	19	10	1.0	0.09	0.16
17	3,4-diF	6.4	38	34	28	2.4	0.09	0.07
18	3,4-diOMe	19	0	25	0	1.8	0.11	0.06

19	*m*-(2-methoxyethoxy)	22	34	38	20	nt	nt	nt
20	*m*-(1,3-benzodioxole	17	0	0	29	2.1	0.05	nt
21	*p*-Cl	51	nt	nt	nt	nt	nt	nt
22	*p*-CF_3	76	nt	nt	nt	nt	nt	nt
23	*p*-CN	58	nt	nt	nt	nt	nt	nt
24	*p*-*tert*-Butyl	500				nt	nt	nt
25	*p*-F	3.4	46	24	46	6.5	<0.04	<0.04
26	*m*-F	14	90	42	94	4.7	<0.02	<0.04
27	*o*-F	21	nt	nt	nt	nt	nt	nt
28	*p*-Cl	29	nt	nt	nt	nt	nt	nt
29	*m*-CF_3	24	nt	nt	nt	nt	nt	nt
30	2,4-diF	13	39	11	47	13.5	<0.01	<0.03
31	2,6-diF	15	33	42	48	26.5	0.05	0.11
32	3,4-diF	12	50	42	47	24	0.03	0.05
33	*m*-(2-methoxyethoxy)	14	38	23	41	28	0.05	0.1
34	*m*-(1,3-benzodioxole)	42	nt	nt	nt	nt	nt	nt
35	*p*-CH_3	55	nt	nt	nt	nt	nt	nt
36	*m*-OCH_3	42	nt	nt	nt	nt	nt	nt

nt- not tested. [a]Binding affinities were determined by using membrane preparations from HEK-293 cells overexpressing the relevant human AdoR isoform. All data points were evaluated in triplicates For K_i determination, 6 concentrations IC_{50} curves were plotted. [b]Compounds were evaluated at 12 concentrations, each data point in triplicates (n =2). The mean K_i from two independent experiments has been reported. [c]Metabolic Rate (MR) <0.1 nmol/min/mg is considered metabolically stable. RLM: Rat liver microsomes, HLM: Human liver microsomes.

All three compounds (**12**, **17** and **25**) showed high binding as well as functional selectivity over other AdoRs similar to that of **2** (Table 2). These compounds did not show any CYP liability

against major CYPs (data not shown) and hERG liability in binding assay (<5% inhibition at 3, 10 μM for compound **12** and **17**; <10 % inhibition at 10, 30 μM for compound **25**). Compound **25** was found to be stable in rat hepatocytes. Among these three compounds, **25** showed better pharmacokinetic (PK) profile compared to **12** and **17** with C_{max} of 1.1 μM, systemic exposure $AUC_{(0\text{-}t)}$ of 12 μM.h, $t_{1/2}$ of 5.4 h and oral bioavailability of 21% (Table 3).

Table 2. Binding affinity and functional potency of compounds **12**, **17**, **25** and **2**

Compound	Binding K_i (nM)				Functional K_i (nM)		
	hA_{2A}[a]	hA_1[a]	hA_{2B}[a]	hA_3[a]	hA_{2A}[b]	hA_1[b]	hA_{2B}[b]
12	13	5000	10000	5000	4.2	2000	nr
17	6.4	1100	5000	500	2.4	nr	nr
25	3.4	500	1400	400	6.5	nr	2000
2	11	1700	5000	5000	0.3	nt	nt

nt-not tested, nr- no response at highest tested concentration 10 μM. [a]Binding affinities were determined by using membrane preparations from HEK-293 cells overexpressing the relevant human AdoR isoform. Compounds were evaluated at 8 concentrations with each data point in triplicates (n = 2). [b]Compounds were evaluated at 12 concentrations, each data point in triplicates (n = 2). The mean K_i from two independent experiments has been reported.

Table 3. Pharmacokinetic profile[a] of compounds **12, 17** and 25 in male Wistar Rats

Compound	**12**		**17**		**25**	
Route of administration	IV	PO	IV	PO	IV	PO
Dose (mg/kg)	3	10	3	10	3	10
C_{max} (μM)	NA	0.19 ± 0.0	NA	1.33 ± 0.5	NA	1.1 ± 0.1
T_{max} (h)	NA	0.5 ± 0.0	NA	0.50 ± 0.0	NA	2.75 ± 0.9
$AUC_{0\text{-}t}$	7.6 ± 0.6	1.5 ± 0.25	10.7 ± 2.2	6.2 ± 1.7	16.4 ± 0.6	12 ± 1.3

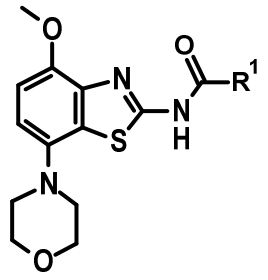
(μM.h)						
V_{ss} (L/Kg)	1.4 ± 0.2	NA	2.1 ± 0.44	NA	2.1 ± 0.3	NA
CL (mL/min/Kg)	15 ± 1.2	NA	10.2 ± 2.5	NA	6.6 ± 0.2	NA
$t_{1/2}$(h)	6.3 ± 0.6	NA	5.5 ± 0.6	NA	5.4 ± 1.0	NA
F (%)	NA	6	NA	17 ± 4.7	NA	21.7 ± 2.4

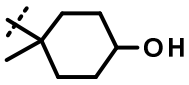
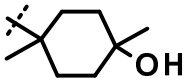
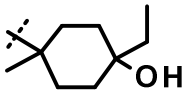
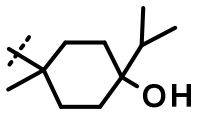
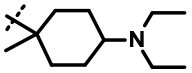
[a] Values indicate mean for n = 4. NA: Not applicable; Compound **12** : IV-NMP-10%, PEG300-15%, 20 % w/v HPß-CD in 0.1 M acetate buffer (pH 4.2) q.s; Compound **17**: IV-NMP-10%,Cr EL 10%, 0.1 M acetate buffer (pH 4.2) q.s; Compound **25**: Vehicle: IV-NMP-10%, Cr EL -10%, PEG-300- 10 %, 0.1 M Ammonium acetate buffer q.s (pH 4.2); Compound **12**, **17** and **25**: PO: Tween 80- 1%, 0.5% w/v NaCMC q.s.; NA: Not applicable

All three compounds from aryloxy-2-methylpropanamide series however showed poor oral bioavailability which could be due to poor solubility (aqueous solubility of compounds **12**, **17** and **25** are <6 μM at various pH such 2.1, 4 and 7.4). To overcome the issue of bioavailability and solubility of these compounds, we later assessed a series of cyclohexane-carboxamide derivatives, where a more rigid linker was employed and the phenyl ring was replaced by cyclohexane, thus reducing the potential stacking interaction. Further, compounds **40-43** were prepared with polar –OH group attached to cyclohexane ring and **49** containing tertiary amine with the intention to improve solubility. Compounds **40-43** (**41-43** are mixture of geometrical isomers) retained binding affinity (K_i = 2.1 - 25 nM) and functional potency (K_i = 0.2 - 2.1 nM) for A_{2A} AdoR (Table 4). Compounds **40**, **41** and **49** showed single digit nano-molar binding affinity with K_i value of 5.9 nM, 8.6 nM and 2.1 nM, respectively. These compounds (**40, 41**and **49**) also exhibited sub-nanomolar functional potency with K_i value of 1.1 nM, 0.25 nM and 0.20 nM, respectively. These compounds were further tested for AdoR binding selectivity. Compound **41** (with *tert*-hydroxy group) and **49** (with *tert*-amine group) showed high selectivity over A_1AdoR (>300 fold) while no response was seen in A_3AdoR. Both compounds showed moderate

to high selectivity over A_{2B}AdoR (>50 fold for **41** and >200 fold for **49**). Compound **40** however showed low binding selectivity over A_1AdoR (A_1/A_{2A}= 15 fold) and A_{2B}AdoR (A_{2B}/A_{2A}= 4 fold) but high selectivity over A_3AdoR. When tested for functional selectivity, compound **40** exhibited low selectivity over A_1AdoR (A_1/A_{2A}= 38 fold) and high selectivity over A_{2B}AdoR (A_{2B}/A_{2A}= 900 fold). Compounds **41** and **49** were further tested for functional selectivity and found to be highly selective against human A_1AdoR and A_{2B}AdoR (Table 4).

Table 4.- Affinity, potency and selectivity of cyclohexane-carboxamide derivatives



Compound	R^1	Binding K_i (nM)				Functional K_i (nM)		
		hA_{2A} [a]	hA_1 [a]	hA_{2B} [a]	hA_3 [a]	hA_{2A} [b]	hA_1 [b]	hA_{2B} [b]
40		5.9	90	23	nr	1.1	41.8	1000
41		8.6	2900	500	nr	0.25	nr	2000
42		25	nt	nt	nt	nt	nt	nt
43		17	nt	nt	nt	2.1	nt	nt
49		2.1	1200	500	nr	0.20	nr	650

nt- not tested; nr- no response at highest tested concentration 30 μM. [a]Binding affinities were determined by using membrane preparations from HEK-293 cells overexpressing the relevant human AdoR isoform. Compounds were evaluated at 8 concentrations with each data point in triplicates (n=2). [b]Compounds were evaluated in triplicates at 12 concentrations. The mean K_i from two independent experiments has been reported (n=2).

Based on the overall profile of best compounds **41** and **49** in terms of high binding as well as functional potency and selectivity, they were progressed for further profiling and the data has been summarized in Table 5. In general, this series of compounds showed high aqueous solubility across all pH. Among **41** and **49**, the latter compound (**49**) showed high aqueous solubility across all pH (Table 5). Both the compounds were metabolically stable in RLM and HLM as well as in rat hepatocytes. Compound **41** had high permeability towards absorptive direction (A-B) in Caco-2 cells, while compound **49** showed moderate permeability in the same. In addition, both compounds did not show any major CYP and hERG liability, and were not cytototoxic (IC_{50} >100 μM) to HepG2 cells. Serum protein binding of the compounds **41** and **49** were found to be 87% and 76%, respectively, as determined *in vitro* in spiked samples of rat. Compounds **41** and **49** were further evaluated for PK profile before proceeding for *in vivo* efficacy studies. A moderate systemic clearance (~60-65% of hepatic blood flow) was observed for these compounds, in accordance with low intrinsic clearance in rat hepatocytes (Table 6). Volume of distribution of compound **41** was found to be ~ 2 fold higher, while volume of distribution of compound **49** was approximately 8 fold higher than total body water normalized to body weight of rat. Furthermore, both compounds exhibited high oral bioavailability (F = 63%and 61% for compound **41** and **49**, respectively). Compound **49** had much better half life than compound **41**. Compound **49** displayed low brain penetration (brain/plasma AUC_{0-t} Ratio= 0.25 ± 0.08; Plasma AUC_{0-t} (μM.hr)= 4.7 ± 1.7; Brain AUC_{0-t} (μM.hr)= 1.1 ± 0.3; 10 mg/kg po, in Wistar rats, n=4).

Table 5. *In vitro* drug metabolism and pharmacokinetics (DMPK) of compound **41** and **49**

Compound	Aqueous Solubility at pH 2.1, 4.0 and 7.4(μM)	MR[a] (nmol/min/mg)		Rat hepatocyte $t_{1/2}$ (min); % parent remaining at 60 min	Caco-2 permeability (nm/sec) (A-B)	CYP P450 Inhibition (IC_{50}: μM; for 1A2, 2C9, 2C19, 2D6 and 3A4)	hERG; % Binding Inhibition at 10 and 30 μM
		RLM	HLM				
41	139, 111, 114	<0.04	<0.04	>60; 88	122	All >25	NI, 26
49	1840, 1807, 1586	0.01	0.03	>60; 89	37	All >50	13, 18

NI- No inhibition; [a]Metabolic Rate (MR) < 0.1 nmol/min/mg is considered metabolically stable. RLM: Rat liver microsomes, HLM: Human liver microsomes

Table 6. Phamacokinetic profile[a] of compounds **41** and **49** in male Wistar Rats

Compound	**41**		**49**	
Route of administration	IV	PO	IV	PO
Dose (mg/kg)	3	10	3	10
C_{max} (μM)	NA	5.4 ± 1.6	NA	2.02 ± 1.1
T_{max} (h)	NA	0.5 ± 0.0	NA	0.63
AUC_{0-t} (μM.h)	3.7 ± 0.5	7.8 ± 2.4	2.7 ± 0.2	5.5 ± 0.6
V_{ss} (L/Kg)	1.5 ± 0.3	NA	6.1 ± 0.6	NA
CL (mL/min/Kg)	32 ± 4.2	NA	37.2 ± 3.1	NA
$t_{1/2}$(h)	0.6 ± 0.07	NA	2.4 ± 0.3	NA
F (%)	NA	63 ± 19	NA	61.0 ± 7.0

[a]Values indicate mean for n = 4 Compound **41**: Vehicle: IV-NMP-10%, PEG-300- 15 %, 0.1 M Ammonium acetate buffer q.s (pH 4.2); Compound **49**: Vehicle: IV-NMP-10%, Cr EL -10%, 0.1 M Ammonium acetate buffer q.s (pH 4.2); Compound **41**, **49**: PO: Tween 80- 1%, 0.5% w/v NaCMC q.s. NA: Not applicable

MOLECULAR MODELING

Molecular modeling studies were carried out to understand the binding poses and intermolecular interactions of representative active molecules as well as to rationalize the observed SAR differences among the aryloxy-2-methylpropanamide series compounds. Molecular docking analysis of **2** in A_{2A}AdoR showed that it fits nicely in the adenosine binding site. Based on the docking model of **2**, we identified aryloxy-2-methylpropanamide and cyclohexane-carboxamide as suitable replacement for 4-hydroxy-methyl piperidine moiety as it can be altered easily without losing the key interactions and activity. Compounds **41-49** were obtained as a mixture containing both *cis* and *trans* geometric isomers. Molecular docking analysis of compounds **41** and **49** isomers revealed that both can fit nicely in the A_{2A} binding site in a similar binding orientation. However, compounds **41** *cis* isomer and **49** *trans* isomer were having better Glide score and more intermolecular interactions as compared to their other isomeric counterpart (Figure S1, Supplementary information). Therefore, we have compared only the superior geometric isomer of the compounds with **2**. Docking pose overlay of the representative active compounds (**12**, **41** and **49**) on **2** showed that compounds binding pose and orientation are similar to that of **2** (Figure 3). Furthermore, Glide docking score of the compounds **12** (-7.99 kcal/mol), **41** (-7.58 kcal/mol) and **49** (-6.89 kcal/mol) were also comparable to that of **2** (-7.58 kcal/mol).

In detail, binding model of **2** showed that benzothiazole core was buried in the hydrophobic pocket; had pi-pi interaction with Phe168 and hydrophobic interactions with Leu249 and Ile274. Methoxy group and carbonyl group of the compound formed hydrogen bonds with Asn253 and Tyr271 side chains, respectively. Morpholine group was surrounded by a few hydrophobic groups and the top portion 4-hydroxy-methyl piperidine was exposed to solvent front as shown

in Figure 3 and 4. Individual docking poses of compounds **12**, **41** and **49** showed that they retained the intermolecular interactions similar to that of **2** (Figure 4). Interestingly, these compounds made additional interactions viz. compound **12** dimethyl groups hydrophobically interacted with Tyr271 and Leu267; hydroxyl group of compound **41** at cyclochexyl position formed hydrogen bond with Glu169; compound **49,** *t*-nitrogen had cationic-pi interaction with His264. The 2D interaction diagram for all the four compounds is provided in supplementary Figure S1. We also attempted to justify the reason behind low potency of compound **24** using our docking model, which suggested that *p*-tert-butyl of phenoxy ring could be causing steric clashes with Lys153. The most plausible reason for modest/low potency of compounds **21-24** with larger electron withdrawing groups such as –Cl, -CF_3, -CN and –$C(CH_3)_3$ is due to their unfavorable interactions with the polar bulk solvent.

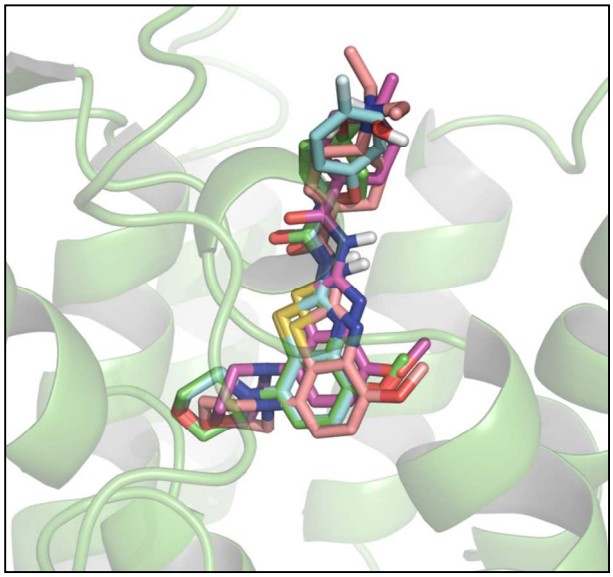


Figure 3. Overlay of Compound **12** (Cyan), **41** (magenta) and **49** (salmon) on reference compound **2** (green) in the A_{2A} receptor (shown in cartoon representation) ligand binding pocket.

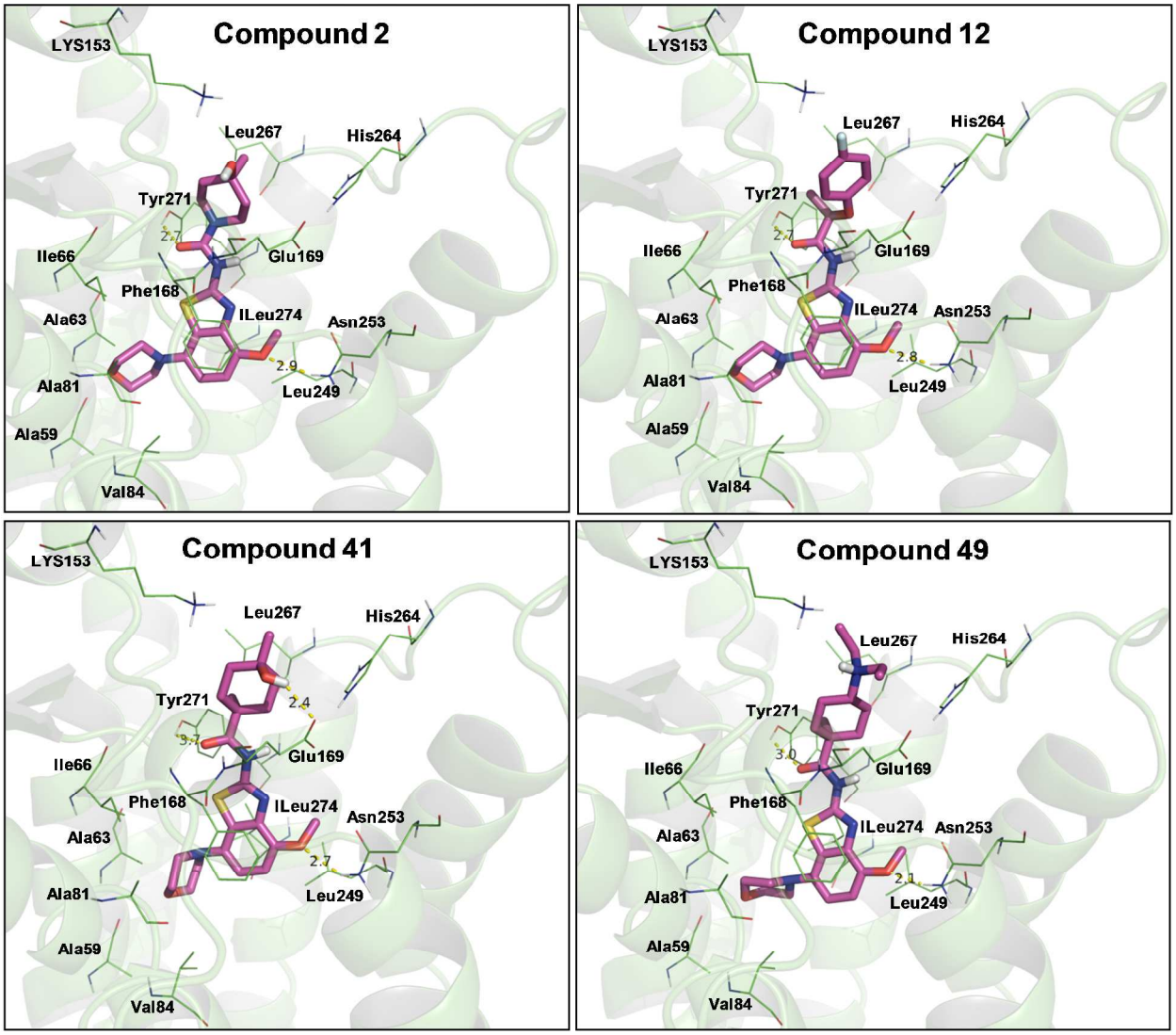


Figure 4. Docking poses of reference compound **2** and representative active compounds **12**, **41** and **49** in the A_{2A}AdoR. Ligands are shown in magenta color and stick representation, while residues are shown in green and line representation. Protein is shown in cartoon rendering in the background.

IN VIVO EFFICACY STUDY

Compound **49** was tested for efficacy in potentiation of levodopa-induced contralateral rotations in 6-OHDA (6-hydroxydopamine) lesioned rats. Compound **49,** at 30 and 100 mg/kg, p.o, was tested in L-dopa-induced rotations in 6-OHDA lesioned rats and showed dose dependent increase in contralateral rotations, post 60 min administration. The potentiation of L-Dopa induced contralateral rotations, measured upto 2 h, with compound **49** at 100 mg/kg, p.o. was significant when compared to L-DOPA (4 mg/kg, i.p.) alone. Compound **2** (30 mg/kg, p.o.) also showed significant effect on potentiation of L-DOPA induced contralateral rotations measured up to 2 h in 6-OHDA lesioned rats as compared with L-DOPA (4 mg/kg, i.p.) alone (Figure 5).

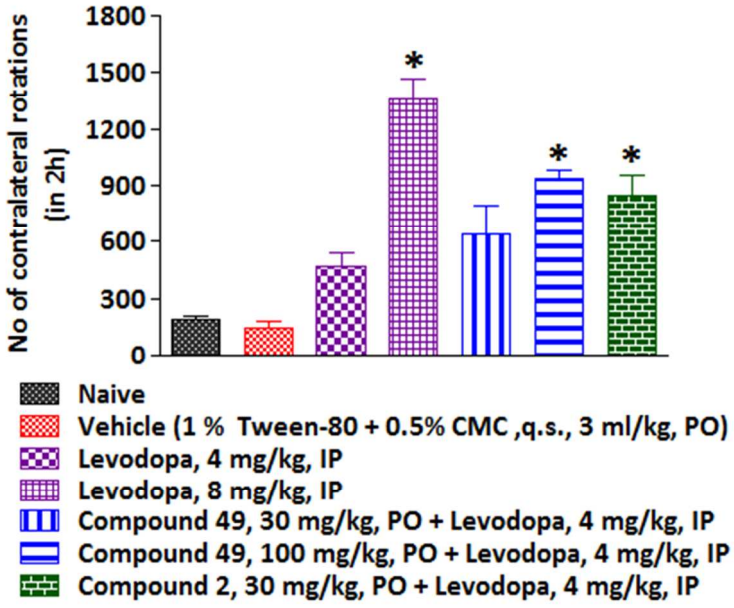


Figure 5. Effect of compound **49** on potentiation of levodopa-induced contralateral rotations in 6-OHDA lesioned rats. The data represent Mean ± SEM (n=6). *Significantly different as compared to L-DOPA, 4 mg/kg, IP alone group (*$P < 0.05$).

CONCLUSIONS

We have identified aryloxy-2-methylpropanamide and cyclohexane-carboxamide based compounds with good potency for A_{2A}AdoR and high selectivity against A_1, A_{2B} and A_3AdoR. These compounds showed molecular recognition interactions with A_{2A}AdoR similar to that of **2**. Several compounds showed low nano-molar activity with reasonable selectivity and ADME properties. Though the initial aryloxy-2-methylpropanamide series showed low to moderate oral-bioavailability; cyclohexane-carboxamides had better oral-bioavailability than former. Compound **49** was identified as a potent and selective A_{2A}AdoR antagonist with desirable physicochemical and drug-like properties, including high oral bioavailability. The compound on further profiling also showed *in vivo* efficacy in PD animal model where it potentiated the L-DOPA induced contralateral rotations significantly at 100 mg/kg, the compound can thus be considered as a potential lead for further exploration. Data from further optimization and evaluation of these series of compounds and their *in vivo* efficacy study will be reported in due course of time.

EXPERIMENTAL SECTION

General Chemistry - Commercial chemicals and solvents were of reagent grade and were used without further purification. Anhydrous solvents were used without further drying. The following abbreviations are used for reagent and solvents: DCM-dichloromethane; DMF-dimethyl formamide; DMSO-dimethyl sulfoxide; EtOAc-ethyl acetate; EtOH-Ethanol; and MeOH-methanol. EDCI- 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, HOBt-1-Hydroxybenzotriazole, NMM-N-Methylmorpholine Globe chemie silica gel (100-200 or 230-400 mesh) was used for column chromatography. Analtech thin layer chromatography plates (20 x 20 cm, 2000 microns) were used for preparative thin layer chromatography. Proton NMR (^{1}H NMR) spectra were recorded on a Varian 400 spectrometer (400 MHz). Solutions were typically prepared in either deuterated dimethyl sulfoxide (DMSO-d_6), deuterated methanol (CD_3OD) or deuterated chloroform ($CDCl_3$). Chemical shifts are reported in δ units (parts per million) downfield from tetramethylsilane and are assigned as: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q) and multiplets (m), and broad (br). Coupling constants (*J*) are reported in Hertz (Hz). Mass spectra (MS) were recorded on Agilent 6110. HPLC were recorded on Agilent RRLC using Eclipse XOB-C18 (250x4.6) mm Su column and 0.05 % fumaric acid (aq.) and acetonitrile as mobile phase with flow rate of 1ml/min at 30 °C for run time of 17 min and the HPLC purity is ≥95% unless otherwise stated.

General Procedure for the Synthesis of Compounds 12-36.

To a solution of substituted amines (**10** or **11**) (1 equiv) and intermediate **9** (1.3 equiv) in DMF (2 mL) were added 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.5 equiv), 1-hydroxybenzotriazole (1 equiv) and *N*-methylmorpholine (1.5 equiv). The reaction mixture

was stirred at room temperature for 14 h. The reaction mixture was quenched with water and the solid obtained was filtered. The crude product was purified by flash chromatography (100-200 silica gel, ethylacetate in hexane 0/100 to 50/100) to afford the products (**12-36**).

2-(4-Fluorophenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (12) . Off white solid; 23% yield; mp 178-179 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.67 (s, 1H), 7.07-7.12 (m, 2H), 6.91-6.94 (m, 4H), 3.83 (s, 3H), 3.79 (t, *J* = 4.4 Hz, 4H), 3.01 (t, *J* = 4.4 Hz, 4H), 1.57 (s, 6H); ^{13}C NMR (DMSO-d_6): δ 173.2, 157.7, 156.4, 150.6, 148.2, 140.0, 139.1, 126.7, 121.9, 115.7, 113.0, 108.1, 80.3, 66.5, 55.8, 51.4, 24.2; MS (*m*/*z*) 446.1$(M + H)^+$; HPLC 98%; HRMS ($C_{22}H_{25}FN_3O_4S$) calulated 446.1544 $(M + H)^+$; found 446.1541

2-(3-Fluorophenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (13). Off white solid; 23% yield; mp 170-171 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.75 (bs, 1H), 7.27-7.33 (m, 1H), 6.91-6.96 (m, 2H), 6.82-6.84 (m, 1H), 6.70-6.76 (m, 2H), 3.85 (s, 3H), 3.78 (t, *J* = 4.4 Hz, 4H), 3.02 (t, *J* = 4.4 Hz, 4H), 1.62 (s, 6H); MS (*m*/*z*) 446.1 $(M + H)^+$; HPLC 99%.

N-(4-Methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-2-(4-methylphenoxy)propanamide (14). Off white solid; 34% yield; mp 152-153 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.59 (bs, 1H), 7.05 (d, *J* = 8.4 Hz, 2H), 6.90-6.95 (m, 2H), 6.77 (d, *J* = 8.4 Hz, 2H), 3.85 (s, 3H), 3.78(t, *J* = 4.5 Hz, 4H), 3.02 (t, *J* = 4.4Hz, 4H), 2.20 (s, 3H), 1.56 (s, 6H). MS (*m*/*z*) 442.1$(M + H)^+$; HPLC 99%.

2-(2,4-Difluorophenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (15). Off white solid (55mg, 16%) mp 83-85 ^{o}C. ^{1}H NMR (DMSO-d_6): δ 12.68 (bs, 1H), 7.31-7.37 (m, 1H), 7.06-7.12 (m, 1H), 7.00-7.03 (m, 1H), 6.92-6.99 (m, 2H), 3.87 (s, 3H), 3.78 (t, *J* = 4.4 Hz, 4H), 3.03 (t, *J* = 4.4 Hz, 4H), 1.55 (s, 6H); MS (*m*/*z*) 464.1 $(M + H)^{+}$; HPLC 98%.

2-(2,6-Difluorophenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (16). Off white solid; 16% yield; mp 150-153 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.54 (bs, 1H), 7.13-7.20 (m, 3H), 6.91-6.97 (m, 2H), 3.88 (s, 3H), 3.79 (t, *J* = 4.4 Hz, 4H), 3.03 (t, *J* = 4.4 Hz, 4H), 1.55 (s, 6H); MS (*m*/*z*) 464.1 $(M + H)^{+}$; HPLC 95.66%.

2-(3,4-Difluorophenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (17). Off white solid; 26% yield; mp 158-159 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.69 (bs, 1H), 7.01-7.10 (m, 1H), 6.87-6.92 (m, 2H), 6.72-6.74 (m, 1H), 3.84 (s, 3H), 3.76 (t, *J* = 4.4 Hz, 4H), 3.00 (t, *J* = 4.4 Hz, 4H), 1.56 (s, 6H); ^{13}C NMR (DMSO-d_6): δ 172.8, 150.9, 149.0, 148.2, 145.3, 140.0, 139.0, 126.7, 117.3, 116.4, 113.1, 110.3, 108.1, 81.0, 66.5, 55.8, 51.4, 24.1; MS (*m*/*z*) 464.1 $(M + H)^{+}$ HPLC 100%.

2-(3,4-Dimethoxyphenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (18). White solid; 12% yield; mp 157-158 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.59 (s, 1H), 6.91-6.96 (m, 2H), 6.81 (d, *J* = 8.8 Hz, 1H), 6.67 (d, *J* = 2 Hz, 1H), 6.36-6.39 (m, 1H), 3.87 (s, 3H), 3.79 (t, *J* = 4.4 Hz, 4H), 3.67 (d, *J* = 3.6 Hz, 6H), 3.03 (t, *J* = 4.4 Hz, 4H), 1.55 (s, 6H); MS (*m*/*z*) 488.2 $(M + H)^{+}$; HPLC 99%.

2-[3-(2-Methoxyethoxy)phenoxy]-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (19). Off white solid; 15% yield; mp 130-132 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.72 (s, 1H), 7.12-7.16 (m, 1H), 6.91-6.96 (m, 2H), 6.57-6.59 (m, 1H), 6.42-6.44 (m, 2H),

3.99 (t, *J* = 4.4 Hz, 2H), 3.85 (s, 3H), 3.79 (t, *J* = 4.4 Hz, 4H), 3.58 (t, *J* = 4.8 Hz, 2H), 3.24 (s, 3H), 3.02 (t, *J* = 4 Hz, 4H), 1.60 (s, 6H); MS (*m*/*z*) 502.1 $(M + H)^+$; HPLC 100%.

2-(1,3-Benzodioxol-5-yloxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (20). White solid; 51% yield; mp 182-184 ^oC; ^1H NMR (DMSO-d_6): δ 12.59 (s, 1H) 6.90-6.95 (m, 2H), 6.78 (d, *J* = 8.8 Hz, 1H), 6.61 (d, *J* = 2.4Hz, 1H), 6.37 (dd, *J* = 8.8 Hz, 2.4 Hz, 1H), 5.95 (s, 2H), 3.85 (s, 3H), 3.77 (t, *J* = 4.4 Hz, 4H), 3.01 (t, *J* = 4.4 Hz, 4H), 1.51 (s, 6H); MS (*m*/*z*) 472.3 $(M + H)^+$; HPLC 100%.

2-(4-Chlorophenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (21). Off white solid; 26% yield; mp 178-178 ^oC. ^1H NMR (DMSO-d_6): δ 12.74 (s, 1H), 7.33 (d, *J* = 9.2 Hz, 2H), 6.89-6.96 (m, 4H), 3.85 (s, 3H), 3.79 (t, *J* = 4 Hz, 4H), 3.02 (t, *J* = 4.4 Hz, 4H), 1.60 (s, 6H); MS (*m*/*z*) 462.1 $(M + H)^+$; HPLC 98.65%.

N-(4-Methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-2-[4-(trifluoromethyl)phenoxy]propanamide (22). Off white solid; 21% yield; mp 102-103 ^oC. ^1H NMR (DMSO-d_6): δ 13.02 (bs, 1H), 7.86 (d, *J* = 8.8 Hz, 2H), 7.24 (d, *J* = 8.8 Hz, 2H), 7.14-7.17 (m, 2H), 4.07 (s, 3H), 4.02 (t, *J* = 4.4 Hz, 4H), 3.93 (t, *J* = 4.4 Hz, 4H), 1.90 (s, 6H); MS (*m*/*z*) 496.1$(M + H)^+$; HPLC 100%.

2-(4-Cyanophenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (23). Off white solid; 29% yield; mp 157-159 ^oC; ^1H NMR (DMSO-d_6): δ 12.84 (s, 1H), 7.74 (d, *J* = 8.8 Hz, 2H), 6.93-6.98 (m, 4H), 3.83 (s, 3H), 3.78 (bs, 4H), 3.02 (bs, 4H), 1.67 (s, 6H); MS (*m*/*z*) 453.0 $(M + H)^+$; HPLC 100%.

2-(4-Tert-butylphenoxy)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-2-methyl-propanamide (24). White solid; 23% yield; mp 229-230 ^oC; ^1H NMR (DMSO-d_6): δ 12.64 (s, 1H), 7.25 (d, *J* = 8.8 Hz, 2H), 6.89-6.94 (m, 2H), 6.76 (d, *J* = 8.8 Hz, 2H), 3.83 (s, 3H), 3.77 (t, *J*

= 4.4 Hz, 4H), 3.00 (t, *J* = 4.4 Hz, 4H), 1.56 (s, 6H), 1.19 (s, 9H); MS (*m*/*z*) 484.2 $(M + H)^+$; HPLC 100%.

2-(4-Fluorophenoxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl propanamide (25) . Off white solid; 38% yield; mp 280-281 ^oC;^1H NMR (DMSO-d_6): δ 13.06 (bs, 1H), 7.89 (s, 1H), 7.08-7.12 (m, 2H), 6.91-6.95 (m, 2H), 3.90 (s, 3H), 3.76 (t, *J* = 4.4 Hz, 4H), 3.22 (t, 4H), 1.55(s, 6H); ^{13}C NMR (DMSO-d_6): δ 174.2, 157.8, 150.3, 148.7, 143.4, 122.1, 116.9, 115.8, 80.3, 65.8, 57.0, 48.8, 24.1; MS (*m*/*z*) 447.3 $(M + H)^+$; HPLC 99%; HRMS ($C_{21}H_{24}FN_4O_4S$) calulated 447.1497 $(M + H)^+$; found 447.1492.

2-(3-Fluorophenoxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (26). Off white solid; 62% yield; mp 204-205 ^oC; ^1H NMR (DMSO-d_6): δ 13.12 (s, 1H), 7.91 (s, 1H), 7.28-7.34 (m, 1H), 6.86 (t, *J* = 8.4 Hz, 1H), 6.70-6.78 (m, 2H), 3.91 (s, 3H), 3.79 (bs, 4H), 3.36 (bs, 4H), 1.63 (s, 6H); MS (*m*/*z*) 447.1 $(M + H)^+$; HPLC 99%.

2-(2-Fluorophenoxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (27). Off white solid; 63% yield; mp 188-189 ^oC;. ^1H NMR (DMSO-d_6): δ 10.46 (bs, 1H), 7.87 (s, 1H), 7.08-7.16 (m, 4H), 4.04 (s, 3H), 3.90 (t, *J* = 4.4 Hz, 4H), 3.48 (t, *J* = 4.4 Hz, 4H), 1.60 (bs, 6H); MS (*m*/*z*) 447.2 $(M + H)^+$; HPLC 99%.

2-(4-Chlorophenoxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (28). Off white solid; 21% yield; mp 179-180 ^oC; ^1H NMR (DMSO-d_6): δ 13.30 (bs, 1H), 7.88 (s, 1H), 7.30 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 8.8 Hz, 2H), 3.89 (s, 3H), 3.76 (bs, 4H), 3.31 (bs, 4H), 1.58 (s, 6H); MS (*m*/*z*) 463.1 $(M + H)^+$; HPLC 97%.

N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-2-[3-(trifluoromethyl)phenoxy]propanamide (29). Off white solid; 60% yield; mp 210-212 ºC; ^1H NMR (DMSO-d_6): δ 13.33 (bs, 1H), 8.08 (s, 1H), 7.69 (bs, 1H), 7.56 (bs, 1H), 7.45 (bs, 1H),

7.35 (bs, 1H), 4.08 (s, 3H), 3.96 (bs, 4H), 3.51 (bs, 4H), 1.81 (s, 6H); MS (*m*/*z*) 497.2 $(M + H)^+$; HPLC 99%.

2-(2,4-Difluorophenoxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (30). Off white solid; 50% yield; mp 148-149 ^oC; ^1H NMR (DMSO-d_6): δ 10.42 (bs, 1H), 7.87 (s, 1H), 7.03-7.09 (m, 1H), 6.91-6.96 (m, 1H), 6.83-6.87 (m, 1H), 4.04 (s, 3H), 3.90 (t, *J* = 4.4 Hz, 4H), 3.48 (t, *J* = 4.4 Hz, 4H), 1.60 (bs, 6H); MS (*m*/*z*) 465.2 $(M + H)^+$; HPLC 95%.

2-(2,6-Difluorophenoxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (31). off white solid (85 mg, 62%) mp 188-189 ^oC. ^1H NMR (DMSO-d_6): δ 10.08 (bs, 1H), 7.87 (s, 1H), 6.60-6.64 (m, 1H), 6.48-6.51 (m, 2H), 4.03 (s, 3H), 3.91 (t, *J* = 4.4 Hz, 4H), 3.48 (t, *J* = 4.4 Hz, 4H), 1.67 (s, 6H); MS (*m*/*z*) 465.2 $(M + H)^+$ HPLC 99%.

2-(3,4-Difluorophenoxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (32). Off white solid; 8% yield; mp 169-170 ^oC; ^1H NMR (DMSO-d_6): δ 13.08 (bs, 1H), 7.91 (s, 1H), 7.35 (m, 1H), 7.07-7.13 (m, 1H), 6.75-6.77 (m, 1H), 3.92 (s, 3H), 3.78 (t, *J* = 4.0 Hz, 4H), 1.59 (s, 6H); MS (*m*/*z*) 465 $(M + H)^+$; HPLC 99%.

2-[3-(2-Methoxyethoxy)phenoxy]-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (33). Off white solid; 49% yield; mp 166-167 ^oC; ^1H NMR (DMSO-d_6): δ 13.07 (s, 1H), 7.91 (s, 1H), 7.15 (t, *J* = 8.0 Hz, 1H), 6.60 (d, *J* = 8.4 Hz, 1H), 6.42-6.45 (m, 2H), 4.00 (t, *J* = 4.4 Hz, 2H), 3.91 (s, 3H), 3.79 (bs, 4H), 3.59 (t, *J* = 4.4 Hz, 2H), 3.35 (bs, 4H), 3.25 (s, 3H), 1.61 (s, 6H); MS (*m*/*z*) 503.2 $(M + H)^+$; HPLC 99%.

2-(1,3-Benzodioxol-5-yloxy)-N-(7-methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-propanamide (34). Off white solid; 45% yield; mp 205-206 ^oC; ^1H NMR (DMSO-d_6): δ 12.99 (s, 1H), 7.92 (s, 1H), 6.80 (d, *J* = 8.4 Hz, 1H), 6.63 (d, *J* = 2.0 Hz, 1H), 6.39 (dd, *J* = 2.4

Hz, 8.8 Hz, 1H), 5.98 (s, 2H), 3.93 (s, 3H), 3.79 (bs, 4H), 3.35 (bs, 4H), 1.54 (s, 6H); MS (*m*/*z*) 473.2 $(M + H)^+$; HPLC 99%.

N-(7-Methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-methyl-2-(4-methylphenoxy)propanamide (35). Off white solid; 61% yield; mp 192-193 ^oC; ^1H NMR (DMSO-d_6): δ 13.05 (s, 1H), 7.91 (s, 1H), 7.07 (d, *J* = 8.0 Hz, 2H), 6.78 (d, *J* = 8.4 Hz, 2H), 3.91 (s, 3H), 3.79 (bs, 4H), 3.35 (bs, 4H), 2.21 (s, 3H), 1.57 (s, 6H); MS (*m*/*z*) 443.2 $(M + H)^+$; HPLC 99%.

N-(7-Methoxy-4-morpholino-thiazolo[5,4-c]pyridin-2-yl)-2-(3-methoxyphenoxy)-2-methyl-propanamide (36). Off white solid; 65% yield; mp 153-154 ^oC; ^1H NMR (DMSO-d_6): δ 10.27 (bs, 1H), 7.86 (s, 1H), 7.19 (t, *J* = 8.0 Hz, 1H), 6.69 (d, *J* = 8.8 Hz, 1H), 6.51-6.54 (m, 2H), 4.02 (s, 3H), 3.90 (t, *J* = 4.0 Hz, 4H), 3.79 (s, 3H), 3.47 (t, *J* = 4.0 Hz, 4H), 1.60 (s, 6H); MS (*m*/*z*) 459.2 $(M + H)^+$; HPLC 99%.

1-Methyl-4-oxo-cyclohexanecarboxylic acid (38).

To a solution of ethyl 1-methyl-4-oxo-cyclohexanecarboxylate (**37)** (1 g, 5.42 mmol) in 21 ml of THF: MeOH was added a solution of lithium hydroxide (0.65g, 15.49 mmol) in 4 ml of water. The reaction mixture was stirred at room temperature for 18 h. The reaction mixture was concentrated under reduced pressure. The residue was taken in water and washed with hexane. Aqueous layer was acidified up to pH 1-2 using 3 N HCl, Extracted with ethyl acetate. The organic layer dried (Na_2SO_4) and concentrated under reduced pressure to afford the title compound **38** (0.7 g, 83 %).

^1H NMR ($CDCl_3$): δ 2.33-2.54 (m, 7H), 1.68-1.76 (m, 2H), 1.38 (s, 3H).

N-(4-Methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1-methyl-4-oxo-cyclohexanecarboxamide (39).

To a solution of **38** (0.5 g, 3.2 mmol), **10** (0.605 g, 2.28 mmol) in 6 ml of DMF at 0 ^{o}C were added HOBt (0.43g, 3.2 mmol) and NMM (0.8 mL, 6.84 mmol). The reaction mixture was stirred at 0 ^{o}C for 15 mins. EDCI (0.87 g, 4.5 mmol) was then added to the reaction mixture and stirred at room temperature for 18 h. The reaction mixture was quenched by addition of water and extracted with ethyl acetate. The organic layer was separated and washed with water, brine, dried over Na_2SO_4. The organic layer was concentrated under reduced pressure. The resulting crude product was purified by column chromatography (230-400 silicagel, 96% CH_2Cl_2, 4 % MeOH) to afford the title compound **39** (0.45 g, 49%).

^{1}H NMR ($CDCl_3$): δ 9.21 (bs, 1H), 6.85-6.91 (m, 2H), 4.0 (s, 3H), 3.89-3.91(m, 4H), 3.11-3.13 (m, 4H), 2.38-2.57 (m, 6H), 1.86-1.92 (m, 2H), 1.44 (s, 3H); MS (*m*/*z*) 404.2 $(M + H)^+$

4-Hydroxy-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1-methyl-cyclohexanecarboxamide (40).

To a solution of **39** (0.09 g, 0.22 mmol) in 1 ml of ethanol at 0 ^{o}C was added sodium borohydride in portions and the reaction mixture stirred at room temperature for 3 h. The reaction mixture was quenched by addition of water and concentrated under reduced pressure. The residue was taken in ethyl acetate and washed with water, brine, dried over Na_2SO_4. The organic layer was concentrated under reduced pressure. The resulting crude product was purified by HPLC based purification to afford the title compound **40** as an off white solid (23 mg, 77%).

mp 111-113 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.18 (bs, 1H), 6.86-6.93 (m, 2H), 4.46 (d, J = 4.8 Hz, 1H), 3.85 (s, 3H), 3.76 (bs, 4H), 3.00 (bs, 4H), 2.26-2.31 (m, 1H), 1.89 (s, 1H), 1.66-1.68 (m, 3H), 1.19-1.22 (m, 4H), 1.17 (s, 3H); MS (*m*/*z*) 406.2 $(M + H)^+$; HPLC 99%.

General Procedure for the Synthesis of Compounds 41-43.

To a solution of **39** (0.074 mmol) in dry THF (2 ml) at 0 ^{o}C were added 2 to 3 eq. of desired Grignard reagents. The reaction mixture was stirred at 0 ^{o}C for 1h and then at room temperature for 1 h. The reaction mixture was then quenched with saturated ammonium chloride solution and extracted with ethyl acetate. The organic layer was separated and washed with water, brine, dried over Na_2SO_4. The organic layer was concentrated under reduced pressure. The resulting crude products were purified by HPLC based purification to afford the required compound.

4-Hydroxy-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1,4-dimethyl-cyclohexanecarboxamide (41). Off white solid; 8% yield; mp 138-14 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 12.13 (bs, 1H), 6.85-6.92 (m, 2H), 4.05 (s, 1H), 3.85 (s, 3H), 3.76 (t, *J* = 4.4 Hz, 4H), 3.00 (t, *J* = 4.4 Hz, 4H), 2.02-2.05 (m, 2H), 1.54-1.60 (m, 2H), 1.41-1.43 (m, 2H), 1.23-1.26 (m, 2H), 1.01(s, 3H); ^{13}C NMR (DMSO-d_6):. δ 176.4, 157.2, 148.1, 140.0, 139.0, 126.5, 112.6, 108.0, 66.5, 66.4, 55.8, 51.4, 42.5, 35.7, 30.6, 30.4; MS (*m*/*z*) 420.3 $(M + H)^+$; HPLC 100%; HRMS ($C_{21}H_{30}O_4N_3S$) calulated 420 $(M + H)^+$; found 420.1946.

4-Ethyl-4-hydroxy-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1-methyl-cyclohexanecarboxamide (42). Off white solid; 12% yield; mp 189-190 ^{o}C; ^{1}H NMR ($CDCl_3$): δ 6.82-6.87 (m, 2H), 3.97 (s, 3H), 3.89 (t, *J* = 4.4 Hz, 4H), 3.11 (t, *J* = 4.4 Hz, 4H), 2.15-2.19 (m, 3H), 1.52-1.62 (m, 7H), 1.32 (s, 3H),0.95 (t, *J* = 7.6 Hz, 3H); MS (*m*/*z*) 434.2 $(M + H)^+$; HPLC 98%.

4-Hydroxy-4-isopropyl-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1-methyl-cyclohexanecarboxamide (43). Light pink solid; 20% yield; mp 135-137 ^{o}C; ^{1}HNMR ($CDCl_3$): δ 6.83-6.88 (m, 2H), 4.00 (s, 3H), 3.89 (t, *J* = 4.4 Hz, 4H), 3.49 (s, 1H), 3.12 (t, *J* = 4.4 Hz, 4H),

2.03-2.12 (m, 2H), 1.77-1.84 (m, 3H), 1.44-1.54 (m, 4H), 1.30(s, 3H); MS (*m*/*z*) 448.2 $(M + H)^+$; HPLC 98%.

Ethyl 4-amino-1-methyl-cyclohexanecarboxylate (44).

To a solution of **37** (0.3 g, 1.62 mmol) in Methanolic ammonia (2 M, 15 ml) was added 10% Pd/C (0.3 g) at 0 ^oC and the reaction mixture stirred under hydrogen atmosphere for 24 h. The reaction mixture was then filtered over celite pad and catalyst washed with methanol. The filtrate obtained was concentrated under reduced pressure to afford **44** (0.3 g, 99%).

^1H NMR ($CDCl_3$): δ 4.09-4.17 (m, 2H), 2.62-2.80 (m, 1H), 2.16-2.22 (m, 1H), 1.96 (bs, 2H), 1.60-1.81 (m, 4H), 1.32-1.41(m, 1H), 1.14-1.27 (m, 8H); MS (*m*/*z*) 186.1 $(M + H)^+$.

Ethyl 4-(tert-butoxycarbonylamino)-1-methyl-cyclohexanecarboxylate (45).

To a solution of **44** (0.3 g, 1.62 mmol) in 5 ml of methanol was added triethylamine (0.3 ml, 2.10 mmol) and Boc anhydride. (0.35 g, 1.62 mmol). The reaction mixture was then stirred at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure. The residue was taken in CH_2Cl_2 and washed with H_2O and brine, dried over Na_2SO_4. The organic layer was concentrated under reduced pressure to afford **45** (0.42 g, 91%).

^1H NMR ($CDCl_3$): δ 4.34-4.50 (m, 1H), 4.09-4.15 (m, 2H), 3.38-3.51 (m, 1H), 2.16-2.20 (m, 1H), 1.76-1.88 (m, 3H), 1.54-1.63 (m, 2H), 1.42 (d, *J* = 5.6 Hz, 9H), 1.21-1.26 (m, 4H), 1.17 (bs, 1H); 1.12-1.15 (m, 3H), MS (*m*/*z*) 230.2 $(M -56)^+$.

4-(Tert-butoxycarbonylamino)-1-methyl-cyclohexanecarboxylic acid (46).

To a solution of **45** (0.41 g, 1.43mmol) in a mixture of THF:MeOH (18 ml) was added $LiOH.H_2O$ (0.18 g, 4.31 mmol) in water (4 ml). The reaction mixture was stirred at 50-60 ^oC for 18 h. The reaction mixture was concentrated under reduced pressure. The residue was taken in

water, acidified up to pH 2 using 3 N HCl, and extracted with ethyl acetate. The organic layer was separated and washed with water, brine, dried over Na_2SO_4. The organic layer was concentrated under reduced pressure to afford **46** (0.34 g, 92 %).

^{1}H NMR ($CDCl_3$): δ 4.34-4.50 (m, 1H), 4.09-4.15 (m, 2H), 3.38-3.51 (m, 1H), 2.16-2.20 (m, 1H), 1.76-1.88 (m, 3H); 1.54-1.63 (m, 2H); 1.42 (d, *J* = 5.6 Hz, 9H); 1.21-1.26 (m, 4H), 1.17 (bs, 1H), 1.12-1.15 (m, 3H); MS (*m*/*z*) 202.1 $(M-56)^+$.

Tert-butyl N-[4-[(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)carbamoyl]-4-methyl-cyclohexyl]carbamate (47).

To a solution of **46** (0.34 g, 1.28 mmol) and **10** (0.33 g, 1.28 mmol) in dry DMF (4ml), HOBt (0.17 g, 1.28 mmol) and NMM (0.42, 3.84 mmol) at 0 ^oC were added. The reaction mixture stirred at 0 ^oC for 15 min. EDCI (3.84 mmol) was then added to the reaction mixture and stirred at room temperature for 14 h. Water was added to the reaction mixture. The solid obtained was filtered and dried. The crude product obtained was purified by column chromatography (230-400 silicagel, 50% ethyl acetate: 50 % hexane) to afford the title compound to **47** (0.25 g, 34%).

^{1}H NMR ($CDCl_3$): δ 9.05 (bs, 1H), 6.85-6.88 (m, 2H), 4.51 (bs, 1H), 3.99 (s, 3 H), 3.88 (bs, 4H), 3.52 (bs, 1H), 3.11 (bs, 4H), 1.91-1.93 (m, 4H), 1.70-1.74 (m, 2H), 1.45 (s, 9H), 1.33 (s, 3H), 1.24-1.27 (m, 2H); MS (*m*/*z*) 505.3 $(M + H)^+$.

4-Amino-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1-methyl cyclohexane carboxamide (48).

To a solution of **47** (0.07 g, 0.138 mmol) in CH_2Cl_2 (3 mL) was added 3N methanolic HCl (5 ml). The reaction mixture was then stirred at room temperature for 14 h. The reaction mixture was concentrated under reduced pressure. The residue obtained was triturated with hexane and

dried to get pure 4-amino-N-(4-methoxy-7-morpholino-1, 3-benzothiazol-2-yl)-1-methyl-cyclohexanecarboxamide as HCl salt (0.05 g, 89%).

^{1}H NMR (DMSO-d_6): δ12.24 (bs, 1H), 7.83-7.87 (m, 2H), 6.88-6.95 (m, 2H), 3.87 (s, 3H), 3.78 (t, J = 4.4 Hz, 4H), 3.01 (t, J = 4.4 Hz, 4H), 3.04 (bs, 1H), 1.78-1.82 (m, 5H), 1.57-1.60 (m, 3H), 1.27 (s, 3H); MS (*m*/*z*) 405.2 $(M + H)^+$.

4-(Diethylamino)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1-methyl-cyclohexanecarboxamide (49).

To a solution of **48** (0.05g, 0.123 mmol) in methanol (1 mL) were added sodium acetate (0.021g, 0.258 mmol), catalytic amount of acetic acid, and acetaldehyde solution (0.07 g, 0.172 mmol). The reaction mixture was stirred at room temperature for 15 min and sodiumcyanoborohydride (0.014 g, 0.209 mmol) was added in single portion. The resulting reaction mixture was then stirred at room temperature for 48 h. It was then quenched by addition of water and extracted with ethyl acetate. The organic layer was separated and washed with water, brine, dried over Na_2SO_4. The organic layer was concentrated under reduced pressure. The resulting crude product was purified by HPLC based purification to afford the 4-(diethylamino)-N-(4-methoxy-7-morpholino-1,3-benzothiazol-2-yl)-1-methyl-cyclohexanecarboxamide as an off white solid, **49** (6 mg, 11%).

mp 113-116 ^{o}C; ^{1}HNMR ($CDCl_3$): δ 12.13 (bs, 1H), 6.86-6.94 (m, 2H), 3.99 (s, 3H), 3.89 (t, J = 4.0 Hz, 4H), 3.33 (bs, 2H), 3.14 (bs, 2H), 3.09 (t, J = 4.0 Hz, 4H), 2.11 (bs, 3H), 1.97 (bs, 5H), 1.42 (s, 3H), 1.39 (t, J = 6.8 Hz, 6H); ^{13}C NMR (DMSO-d_6): δ 177.5, 157.2, 148.1, 140.0, 139.1, 126.4, 112.6, 108.0, 66.5, 58.0, 55.8, 51.4, 43.2, 41.6, 31.9, 23.2, 19.5, 13.2

MS (*m*/*z*) 461.3 $(M + H)^+$; HPLC 96%; HRMS ($C_{24}H_{37}N_4O_3S$) calulated 461.2581 $(M + H)^+$; found 461.2582.

BIOLOGY

Radioligand Binding for Adenosine Receptors A_1, A_{2A}, A_{2B} and A_3.

Human adenosine receptor (A_1, A_{2A}, A_{2B} and A_3) cDNA was stably transfected into HEK-293 cells (referred to as HEK-A_1, HEK-A_{2A}, HEK-A_{2B}, HEK-A_3 cells). The HEK-293 cell was obtained from ATCC. The cells monolayer was washed with PBS once and harvested in a buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.4 (10 mM EDTA, 10 mM HEPES, pH 7.4 for HEK-A_3) at 1500 rpm for 5 min at room temperature. The cell pellet was incubated in sonication buffer containing 1 mM EDTA, 5 mM Tris, pH 7.4 (1 mM EDTA, 10 mM HEPES, pH-7.4 for HEK-A_3) for 10 mins at 4 ^{o}C followed by sonication on ice for 6 min. The lysate was centrifuged at 1000 *x g* for 10 min at 4 ^{o}C and the pellet was discarded. The supernatant was centrifuged at 49,000 *x g* for 45 min at 4 ^{o}C. The resultant protein pellet was resuspended in sonication buffer supplemented with 1 U/ml adenosine deaminase (ADA, Roche) and incubated for 30 min at room temperature with constant mixing. The protein was washed twice with same buffer at 49,000 *x g* for 45 min at 4 ^{o}C and the final protein was stored in 50 mM Tris, pH 7.4 supplemented with 1 U/mL ADA and 10 % sucrose (1 mM EDTA, 5 mM Tris, pH 7.4, 1 U/ml ADA and 10% sucrose for HEK-A3). The protein concentration was estimated by Bradford assay and aliquots were stored at -80 ^{o}C.

The binding affinity and selectivity of test compounds was determined using radioligand binding assays. DPCPX, **5** (Preladenant), 3-Ethyl-3,9-dihydro-1-propyl-8-[1-[[3-(trifluoromethyl)phenyl]methyl]-1H-pyrazol-4-yl]-1H-purine-2,6-dione (CVT-6883)[33] and 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191)[34] were used as internal standards. Competition radioligand binding assays were started by mixing 1 nM [^{3}H]-DPCPX (A_1), 1 nM [2-^{3}H]-4-(2-[7-amino-2-{2-

furyl}{1,2,4}triazolo{2,3-*a*}{1,3,5,}triazin-5-yl amino]ethyl)phenol ([^{3}H]ZM241385) (A_{2A}),[35] 1.6 nM radiolabelled N-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide ([^{3}H]-MRS-1754) (A_{2B})[36] or 2 nM [^{3}H]-HEMADO (A_3) with various concentrations of test compounds (and the respective membranes in assay buffer containing 50 mM Tris pH 7.4, 1 mM EDTA (A_1), 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 1 U/ml ADA (A_{2A}, A_3) or 50 mM Tris pH 6.5, 5 mM $MgCl_2$, 1 mM EDTA (A_{2B}) supplemented with 1 U/ml ADA. The assays were incubated at room temperature for 90 min with gentle agitation, stopped by filtration using a Harvester (Molecular Devices), and washed four times with ice-cold 50 mM Tris (pH 7.4). Nonspecific binding was determined in the presence of 100 μM NECA. The affinities of compounds (i.e., K_i values) were calculated using GraphPad software.

cAMP Assay for Adenosine Receptor.

The functional activity of test compounds on human A_{2A}AdoR and selectivity against adenosine receptor subtypes A_1 and A_{2B} was determined using HTRF based cAMP assay (Cisbio). Briefly, overnight seeded cultures (HEK-A_1, HEK-A_{2A}, HEK-A_{2B}) were treated with 1 U/ml ADA for 90 min at 37 ^oC and 5% CO_2. Cell suspensions were treated with increasing concentrations of test compounds for 15 min followed by treatment with agonists for 15 min (1nM CPA for HEK-A_1 and 70 nM NECA for HEK-A_{2B}) or 30 min (10 nM CGS-21680 for HEK-A_{2A}) at room temperature with continuous mixing in incomplete DMEM supplemented with 1U/ml ADA. Rolipram (20 μM) was included in the assay for A_1 and A_{2A} adenosine receptors. For functional activity of HEK-A_1, cell suspensions were further treated with forskolin for 30 min at room temperature with constant mixing. cAMP levels were quantified using a Flex Station III

(Molecular Devices) at an excitation maximum of 313 nm and emission maxima of 620 nm and 665 nm. Data was analyzed using GraphPad Prism to generate IC_{50} and K_i.

ANIMAL CARE, TISSUE ISOLATION AND *IN VIVO* PROTOCOLS:

All procedures described were reviewed and approved by the Institutional Animal Ethics committee (IAEC) constituted by Committee for the Purpose of Control and Supervision on Experiments (CPCSEA) on Animals, Govt. of India.

PHARMACOKINETIC EXPERIMENTS

General:

Male Wistar rats (weighing 230 ± 15g) were obtained from in-house breeding facility, Advinus Therapeutics Ltd., Pune, INDIA. The rats were grouped and housed in polycarbonate cages with not more than 3 rats per cage and maintained under standard laboratory conditions (temperature 25 ± 2 ^{o}C) with dark/ light cycle (12 h). Rats were maintained on T.2014C Global 14% protein rodent maintenance diets (Harlan, Teklad diet, USA) and water ad libitum. All procedures described were reviewed and approved by the Institutional Animal Ethics committee (IAEC) constituted by Committee for the Purpose of Control and Supervision on Experiments (CPCSEA) on Animals, Govt. of India.

Pharmacokinetic experiments were carried-out in male Wistar rats following Intravenous (IV) and Per oral (PO) administration. The animals were fasted overnight before the start of experimentation but had free access to water. For IV dosing, test compounds were dissolved in different vehicles and solution formulation (dose volume: 5 mL/kg and formulation strength: 0.6 mg/mL) was administered to each rat at a dose of 3 mg/kg via tail vein. For PO and tissue distribution studies (TDS), suspension formulation (1% Tween 80, 0.5 % NaCMC qs; Dose volume: 10 mL/kg and formulation strength 1 mg/mL) was administered to each rat at a dose of 10 mg/kg via oral gavage using oral feeding tube.

Study used serial sampling design (n=3/time point) with blood samples collected at 0.008 (IV only), 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post dose into labeled micro-centrifuge tubes containing K_2EDTA as an anticoagulant. Separately, blood and brain samples were collected (discrete sampling) at 0.5, 2, 4 and 8 h post oral dose administration for TDS. Tissue samples were washed with phosphate buffer saline (pH 7.4), blotted dry and weighed. All samples were stored below -70 °C until bioanalysis. Blood samples were immediately centrifuged at 6000 RPM for 5 min to separate the plasma.

Tissue samples were homogenized in phosphate buffer saline by homogenizer (Polytron PT 3100, Kinematica). Aliquot of plasma (100 µL) or tissue sample (300 µL) was precipitated by addition of 300 µL acetonitrile containing internal standard and vortex mixed for 1 min and centrifuged at 8000 RPM for 8 min. Finally, supernatant (100 µL) was collected from each test sample microcentrifuge tube and transferred into HPLC vials for LC/MS/MS to determine the concentrations in plasma. Calibration standards and quality control (separate weighing) samples were prepared in blank rat plasma or brain homogenates.

Data analysis:

Plasma pharmacokinetic parameters were calculated using the non-compartmental analysis tool of WinNonlin Professional software (Version 5.2.1). Pharmacokinetic parameters were determined from individual animals in each group. The area under the plasma concentration-time curve (AUC_{0-t} & AUC_{0-inf}), elimination half-life ($T_{1/2}$), clearance (CL) and volume of distribution (V_{ss}) were calculated from intravenous group. The peak plasma concentration (C_{max}), time to achieve C_{max} (T_{max}), area under the plasma concentration-time curve (AUC_{0-t} & AUC_{0-inf}), and oral bioavailability were calculated from the oral groups.

IN VIVO EFFICACY

Potentiation of L-Dopa-Induced Rotations in 6-OHDA induced circling behavior in nigrostriatal lesioned rats -

Animals

Male Wistar rats (295-305 g) were bred at Advinus Therapeutics Pune, India. The animals were housed with free access to food and water and kept in controlled environment (temperature of 22 ± 2 ºC and relative humidity of 55±5 % and 12:12 light: dark cycle).

The dose formulations were prepared freshly before administration to the respective group of animals. For the preparation of formulation, compound was weighed on weighing balance (Sartorius CPA 2245) and triturated in mortar with pestle. The compounds were mixed with 1% Tween 80 until completely miscible and 0.5% methyl cellulose (MC) was added to make up to required volume.

The animals were anesthetized by intraperitoneal administration of Ketamine (100 mg/kg, i.p.) and Xylazine (10 mg/kg, i.p.) and treated with desipramine (10 mg/kg, i.p.) 30 min before the 6-OHDA injection to block the uptake of toxin by noradrenergic terminals. The rats were subsequently placed in a stereotaxic frame, the skin over the skull was reflected, and a burr hole was drilled through the skull at the following stereotaxic coordinates: -2.2 posterior from bregma (anterior-posterior) and -1.5 lateral from bregma (medial-lateral). Subsequently, a total of 8 µg of 6-OHDA dissolved in 4 µl of saline containing 0.05% ascorbic acid was infused -7.8 mm ventral to the dura at a constant flow rate of 1 µl/min using a 36-gauge needle attached to an infusion pump. Two weeks after the lesion, the rats were administered Apomorphine 0.05 mg/kg, i.p. and selected on the basis of the number of full contralateral rotations quantified by an automated rotametry system during 1h testing period. The rats that made fewer than 100 complete rotations were not included in the subsequent studies.

Compound **49** and **2** were delivered 30 min before the delivery of benserazide. L-Dopa was delivered 30 min later and placed in the rotametry chambers. The number of contralateral rotations was recorded during a 2 h test.

Data were analyzed for statistical significance using the Graphpad Prism using one-way ANOVA and post hoc Dunnett's test.

MOLECULAR MODELING

Receptor and Ligand Structure Preparation

The X-ray crystallographic chimeric protein structure of A_{2A}AdoR with apocytochrome b (562) RIL in complex with small molecule antagonist ZM241385 (PDB ID: 4EIY, resolution 1.8 Å)[35] was considered for the purpose of molecular docking. Among the several other crystal structures in the Protein Data Bank (PDB), this structure was particularly selected due to its high resolution and better overall B-factor. A_{2A} GPCR structure was prepared by using the Protein Preparation Wizard tool implemented in Maestro interface (Maestro 10.0, Schrödinger, LLC, New York 2014) using the default parameters and standard protocol. Ligands were prepared using LigPrep module of Maestro interface by generating all possible low energy ionization and tautomeric states within the pH range 7.0 ± 2.0. For the minimization purpose in both, the preparation wizards OPLS_2005 force field was implemented.

GLIDE Docking

An interaction grid was generated for the receptor by using receptor grid generation wizard of GLIDE 6.5 by considering bound antagonist as the reference structure for defining the binding site. The centroid (XYZ coordinates: -0.62, 9.06, 16.85) of workspace ligand was used to generate the grid box. To set a docking protocol, the bound ligand was extracted from the

prepared enzyme structure. The atom and bond type of the extracted ligand were corrected using Ligprep, and was subjected to re-docking by implementing above mentioned grid parameters, and finally RMSD was determined.

After the validation of docking protocol, the set of prepared ligands were docked into the A_{2A} AdoR ligand binding site as defined above using Grid box by using GLIDE 6.5 at SP (Standard Precision) mode. The docking and scoring algorithms of GLIDE have been fully described elsewhere.[37-38] All docking calculations were performed using the OPLS_2005, while keeping all other parameters at their default value. The best docking pose was selected manually for each molecule on the basis of intermolecular interactions with the key residues and Glide docking score.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

Experimental procedures for synthesis of compounds **8a-o** and **9a-o** including NMR and LCMS data. Biological protocols for i*n vitro* ADME experiments. Docking poses of compounds **41** and **49** geometric isomers (Figure S1). 2D interaction diagrams for the docking poses of **2**, compound **12**, **41** and **49** (Figure S2).

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ABBREVIATIONS USED

EDCI, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBT, Hydroxybenzotriazole; NMM, N-Methylmorpholine; ADME, absorption, distribution, metabolism, and excretion; PK, Pharmacokinetics; DMPK, Drug metabolism and pharmacokinetics; 6-OHDA, 6-hydroxydopamine; L-DOPA, L-3,4-dihydroxyphenylalanine; PPB, rat plasma protein binding; hERG, Human ether-a-go-go-related gene; CYPs, Cytochromes P450; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine.

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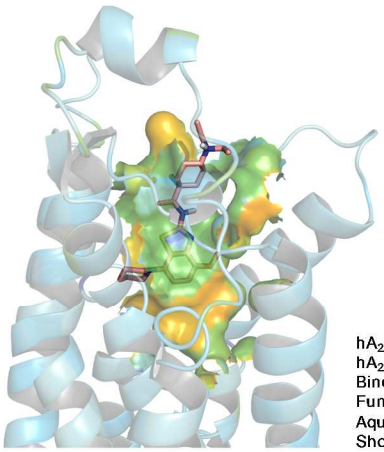
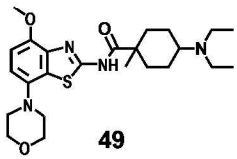
Table of Contents Graphic





49

hA_{2A} binding K_i = 2.1 nM
hA_{2A} functional K_i = 0.2 nM
Binding selectivity, A_1/A_{2A} = 570 fold
Functional selectivity, A_1/A_{2A} > 3000 fold
Aqueous solubility at pH 7.4 = 1586 μM
Showed oral efficacy in 6-OHDA lesioned rat