ORIGINAL RESEARCH

Design, synthesis and evaluation of amino-3,5-dicyanopyridines and thieno[2,3-b]pyridines as ligands of adenosine A$_1$ receptors for the potential treatment of epilepsy

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Abstract

Due to the implication of adenosine in seizure suppression, adenosine-based therapies such as adenosine receptor (AR) agonists have been investigated. This study aimed at investigating thieno[2,3-*b*]pyridine derivatives as non-nucleoside A$_1$ agonists that could be used in pharmaco-resistant epilepsy (PRE). Compound **7c** (thieno[2,3-*b*]pyridine derivative), displayed good binding affinity to the rA$_1$ AR ($K_i = 61.9$ nM). This could be a breakthrough for further investigation of this heterocyclic scaffold as potential ligand. In silico evaluation of this compound raised bioavailability concerns but performed well on drug-likeness tests. The effect of intramolecular cyclisation that occurs during synthesis of thieno[2,3-*b*]pyridines from the lead compounds, amino-3,5-dicyanopyridine derivatives (**6a-s**) in relation to AR binding was also evaluated. A significant loss of activity against rA$_1$/rA$_{2A}$ ARs with cyclisation was revealed. Amino-3,5-dicyanopyridines exhibited greater affinity towards rA$_1$ ARs ($K_i < 10$ nM) than rA$_{2A}$. Compound **6c** had the best rA$_1$ affinity ($K_i = 0.076$ nM). Novel compounds (**6d, 6k, 6l, 6m, 6n, 6o, 6p**) were highly selective towards rA$_1$ AR (K_i between 0.179 and 21.0 nM). Based on their high selectivity for A$_1$ ARs, amino-3,5-dicyanopyridines may be investigated further as AR ligands in PRE with the right structural optimisations and formulations.

Graphical Abstract

A decrease in rA$_1$ AR affinity is observed with intramolecular cyclisation that occurs during synthesis of thieno[2,3-*b*]pyridines (**7a, 7d, 7c**) from amino-3,5-dicyanopyridine derivatives (**6a, 6f, 6g**).

amino, 3,5-dicyanopyridine scaffold

6a: R^2 = 4-methoxyphenyl (**Ki = 139 nM**)
6f: R^2 = 4-hydroxyphenyl (**Ki = 48 nM**)
6g: R^2 = benzo[d][1,3]dioxol-5-yl (**Ki = 26.6 nM**)

thieno[2,3-b]pyridine scaffold

7a: R^2 = 4-methoxyphenyl (**Ki = 139 nM**)
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7c: R^2 = benzo[d][1,3]dioxol-5-yl (**Ki = 26.6 nM**)
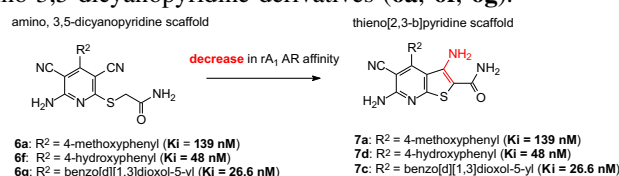
Keywords Amino-3,5-dicyanopyridines · Thieno[2,3-b]pyridines · Intramolecular cyclisation · Adenosine A$_1$/A$_{2A}$ receptors · Epilepsy

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Abbreviations

[^{3}H]DPCPX	1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine
[^{3}H]NECA	[^{3}H]-5′-*N*-ethylcarboxamidoadenosine
^{13}C	carbon-13
^{1}H	hydrogen-1 / protium
A$_1$ AR	adenosine A$_1$ receptor subtype
A$_{2A}$ AR	adenosine A$_{2A}$ receptor subtype
A$_{2B}$ AR	adenosine A$_{2B}$ receptor subtype
A$_3$ AR	adenosine A$_3$ receptor subtype

AEDs	antiepileptic drugs
ADME	absorption, distribution, metabolism, and excretion
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APCI	atmospheric pressure chemical ionisation
AR	adenosine receptor
BBB	blood-brain barrier
BOILED-Egg	Brain Or IntestinaL EstimateD permeation
$C=O$	carbonyl
Ca^{2+}	calcium ion
CADO	2-chloroadenosine
CCPA	2-chloro-N^6-cyclopentyladenosine
Ci	curie
Cl	chlorine
CNS	central nervous system
CPA	N^6-cyclopentyladenosine
CCPA	2-chloro-N6-cyclopentyladenosine
Csp^3	Fraction of carbon atoms in the sp3 hybridization
CYP	cytochrome P450 enzyme system
d	doublet
DCM	dichloromethane
dd	doublet of doublets
ddd	double double doublet
DMF	dimethylformamide
DMSO-d_6	deuterated dimethyl sulfoxide
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DSC	differential scanning calorimetry
EtOAc	ethyl acetate
EtOH	ethanol
Et_3N	triethylamine
F	fluorine
FeS	iron sulphide
GI	gastrointestinal
GPCR	G protein-coupled receptor
GTP	guanosine 5'-triphosphate
H	hydrogen
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
Hz	hertz
IC_{50}	half maximal inhibitory concentration
J	coupling constant
K_i	inhibition constant
KOH	potassium hydroxide
LogP	partition coefficient
m	multiplet
MeOH	methanol
$MgCl_2$	magnesium chloride
MHz	megahertz
MN	malononitrile

mp	melting point
MW	molecular weight
m/z	mass-to-charge ratio
N	nitrogen
NH_2	amino
nM	nano molar
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NWU	North-West University
OCH_3	methoxy
O	oxygen
OH	hydroxy
PAINS	pan assay interference compounds
PE	petroleum ether
P-gp	permeability glycoprotein
ppm (δ)	parts per million
PRE	pharmaco-resistant epilepsy
q	quartet
r	rat
Rf	retention factor
s	singlet
S	sulphur
SANS	South African National Standard
SAR	structure-activity relationships
SEM	standard error of the mean
SI	selectivity index
t	triplet
td	triple of doublets
TLC	thin layer chromatography
TMS	tetramethylsilane
TPSA	topological polar surface area
XLOGP3	LogP value of a compound using a logP of a reference compound

Introduction

Adenosine receptors (ARs) are a family of G protein-coupled receptors (GPCRs) with the nucleoside adenosine as endogenous agonist [1]. There are four known types of ARs, namely A_1, A_{2A}, A_{2B} and A_3 [2] which have been linked to both inhibition (A_1 and A_3) and activation (A_{2A} and A_{2B}) of adenylyl cyclase activity [3]. These receptors are widely expressed throughout all human body tissues and organs; such as the brain, heart, lung, liver, kidney, eye, joints, and blood cells [4]. ARs also play a role in various pathological conditions such as inflammatory diseases, ischaemia-reperfusion and neurodegenerative disorders [5], due to their broad spectrum of physiological and patho-physiological functions [6, 7]. All these physiological functions imply that ARs are potential drug targets for treatment of a variety of conditions such as asthma, neurodegenerative disorders, psychosis and anxiety, cardiac

ischaemic diseases, sleep disorders, cancer and many other pathophysiological states that are believed to be associated with changes of adenosine levels [8].

The ARs are far more abundant in the brain than in any other cell type or organ in mammals [9], where it has a role in mechanisms of seizure susceptibility, sleep induction, pain perception, respiration and others [10]. Adenosine levels in the brain extracellular space increase dramatically during enhanced nerve activity conditions, such as ischae-mia, seizures, or trauma to prevent neuronal injury [10]. The neuroprotective effects of adenosine may be due to stimu-lation of A_1 receptors and blockade of A_{2A} receptors [11]. Therefore, ARs are potential therapeutic targets for treat-ment of neurological [12] as well as neurodegenerative diseases including epilepsy [13].

Epilepsy is defined as a chronic neurological disorder characterised by recurrent, unprovoked seizures due to excessive discharge of cerebral neurons [14], which alter perception, consciousness, and motor activity. It affects about 50 million people worldwide, hence it is one of the most common neurological diseases globally [15]. Cur-rently there is no available cure for epilepsy. The current treatment of epilepsy consists of antiepileptic drugs (AEDs) (also known as anticonvulsants). These therapies are employed to control symptoms of the disease (i.e. sup-pression of seizures) [16].

Approximately one-third of epileptic patients on treat-ment remain poorly controlled [17]. Pharmaco-resistance epilepsy can be defined as failure to control seizures after introduction of two or three anticonvulsants that are suitable for the type of epilepsy, prescribed and taken at maximum daily therapeutic doses [18]. A strategy that prevents sei-zures in drug-resistant epilepsy would be an important therapeutic advance and altering purinergic signalling may be a viable option [19].

Adenosine is a long-known endogenous anticonvulsant substance that effectively inhibits excitatory transmission in the brain [20] through activation of A_1 ARs [3]. Firstly, the released adenosine binds to presynaptic A_1 receptors, which blocks the influx of Ca^{2+} through voltage-dependent cal-cium channels leading to inhibition of glutamate release, and hence, decreased excitation of postsynaptic glutamate receptors [11, 21]. Secondly, postsynaptic activation of A_1 receptors by adenosine opens potassium channels leading to K^+ efflux which results in resting membrane potential hyperpolarization rendering both ionotropic glutamate receptors (NMDA & AMPA) less responsive [22–24]. Both decreased neurotransmitter release and membrane potential hyperpolarization lead to decreased excitatory synaptic transmission and lower probability of seizure generation onset and propagation [21].

Therefore, adenosine receptor-based therapy—especially through A_1 AR activation—may provide therapeutic potential for patients who do not gain satisfactory seizure control with currently available AEDs [19, 21, 25].

Attempts have been made over the years to develop selective A_1 AR agonists that may be useful as anti-epileptic agents. Initially the approach for discovering AR agonists as antiepileptics has been restricted to modifica-tion of the physiological agonist adenosine [26], and justly, these adenosine derivatives represent the great majority of molecules developed and reported to date [27]. The development of these agonists has been limited by the essential requirement of the retention of the ribose moiety of adenosine for agonist activity [26, 28, 29]. Examples of adenosine derivatives include non-selective AR agonists such as 2-chloroadenosine (2-CADO) and A_1 AR selec-tive agonists such as 2-chloro-N6-cyclopentyladenosine (CCPA) [30].

However, the development of adenosine-based AR agonists as novel therapeutic agents has been limited by their pronounced peripheral side effects (mainly cardio-vascular effects such as bradycardia and hypotension) and central side effects (like sedation) [3, 6, 13] at doses that have relatively weak anticonvulsant and neuroprotective effects [3]. In addition, they exhibited low blood brain barrier permeability, and hence, limited use in the central nervous system (CNS) [6, 31]. Therefore, these drugs have not been pursued clinically [14].

The said limitations led to development of new strategies to produce potent and selective AR agonists with dominant CNS activity [14]. Non-nucleoside agonists provide an alternative set of compounds which are highly potent and selective for specific AR subtypes [28]. In this study thieno [2,3-b]pyridine derivatives were explored as alternative non-nucleoside A_1 AR agonists for the potential manage-ment of seizure disorders.

Thienopyridines as a class of heterocyclic compounds have attracted considerable interest due to their broad spectrum of biological activities [32]. The pharmacological potential of thienopyridine derivatives made these com-pounds a privileged scaffold in medicinal chemistry [33]. There are six isomeric thienopyridine structures, one of them being thieno[2,3-b]pyridine (Fig. 1) and its derivatives which have since attracted attention due to their antitumor, antibacterial [34], antiviral [35, 36], vasodilator and anti-hypertensive [37], antidiabetic [38], anti-inflammatory [39], antidermatophytic [40], antimalarial activities [41] in addi-tion to treatment of CNS disorders [42].

Despite their aforementioned promising biological activities, the thienopyridine core has only received scanty
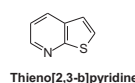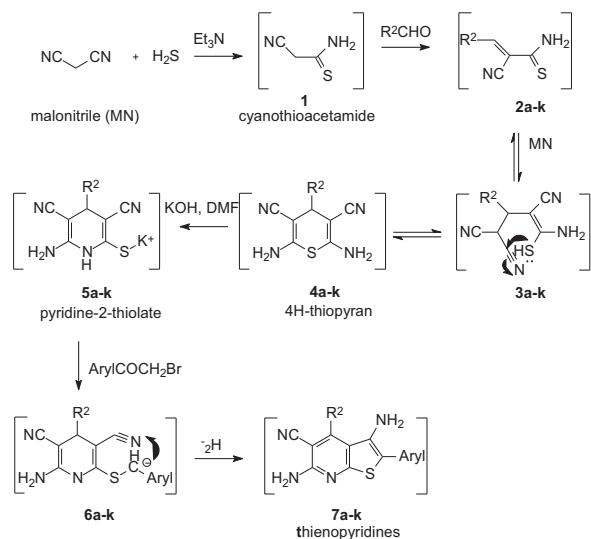

Thieno[2,3-b]pyridine

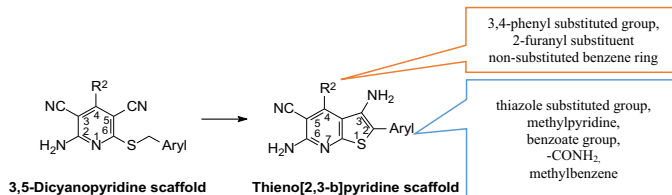**Fig. 1** Chemical structure of thieno[2,3-b]pyridine scaffold

Fig. 2 Synthesis of thieno[2,3-b] pyridine derivatives from lead compounds and modification on the thieno[2,3]pyridine scaffold

Scheme 1 Reaction route for preparation of target thieno[2,3-b]pyridine derivatives [44]

Scheme 2 Synthesis of thieno[2,3-b]pyridines from intermediates

attention as scaffold for the design of AR ligands. 3,5-Dicyanopyridine derivatives which serve as intermediates in the synthesis of thieno[2,3-b]pyridine derivatives, were themselves found to exhibit interesting affinity for ARs. Due to chemical similarity between the 3,5-dicyanopyridin core and the thieno[2,3-b]pyridine core, we envisaged that a suitably substituted thieno[2,3-b]pyridine core could lead to derivatives which may exhibit AR affinity. Notably, bicyclic scaffolds such as benzofurans [43], tetralones and indanones were previously associated with affinity for ARs.

The main aim of this research study was to design, synthesise, characterise, and evaluate novel and known amino-3,5-dicyanopyridines (intermediates) and thieno [2,3-b]pyridines (target compounds) as potent and selective A_1 AR agonists for the potential treatment of neurological conditions, such as epilepsy. Modifications at R_2 and the aryl position on the thieno[2,3-b]pyridines scaffold were influenced by the lead compounds amino-3,5-dicyanopyridine derivatives which displayed good affinity at A_1 AR (Fig. 2). The proposed modifications included thiophene ring closure (from lead compound) resulting in a fused 5-membered (thiophene) heterocyclic ring structure. Different functional groups were substituted at the meta and para positions of the 4-phenyl ring (R_2) and different aryl groups were substituted at position 2 (Fig. 2). The structure-activity relationship (SAR) of the

synthesised compounds were evaluated in relation to A_1 and A_{2A} AR affinity.
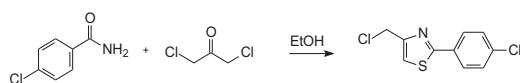
Results and discussion

Chemistry

The synthesis of the amino-3,5-dicyanopyridine derivatives (intermediates) **6a–6p** was done by multicomponent condensation of malononitrile (MN) with hydrogen sulphide, a corresponding aldehyde, and a suitable halide in the presence of trimethylamine (Et$_3$N) as catalyst [44]. As depicted in Scheme 1, initial addition of hydrogen sulphide to MN gives cyanothioacetamide (**1**) which reacts with the aldehyde according to a Knoevenagel condensation reaction to yield **2**. Further addition of MN results in **3** which undergoes chemoselective intramolecular cyclisation to 3,4-substituted phenyl-2,6-diamino-3,5-dicyano-4*H*-thiopyran (**4**). Recyclisation of the latter by the action of alkali (potassium hydroxide (KOH), dimethylformamide (DMF)) leads to pyridine-2-thiolate (**5**). The subsequent regioselective alkylation of **5** at the sulphur atom with a suitable halide results in a sulphide (**6**). According to the method adopted from [44], the sulphide was supposed to undergo intramolecular cyclisation in the presence of an alkali (KOH) to yield a thienopyridine (**7**)—a fused pyridine and thiophene ring heterocyclic compound—but all reactions except the one that yielded compound **7a**, did not go to completion. Instead, the method produced the intermediate compounds, namely amino-3,5-dicyanopyridine derivatives. Modifications such as increasing the KOH concentration and contact time with the reaction mixture were made without success to try and bring the reactions to completion. Otherwise ring closure reactions were performed to convert the synthesised intermediate compounds to thieno[2,3-b]pyridine derivatives using Scheme 2, where either 2–3 drops of KOH were added to a solution of amino-3,5-dicyanopyridines in DMF and then the reaction was left to stand for several hours [45]

or through heating a solution of amino-3,5-dicyanopyridines in ethanol (EtOH) containing KOH under reflux for 3 h [46]. Only 3 compounds, **7b–7d** were obtained through these attempts. Details of unsuccessful attempts have been summarised (see supplementary material).

From observation, only compounds with a carbonyl group at the aryl position managed to go to completion to thieno[2,3-b]pyridine derivatives (target compounds). This seems to be in line with the adopted method from [44, 45] since they used α-halo carbonyl compound as an alkylating agent to obtain thieno[2,3-b]pyridine derivatives in an one pot system. Most previously reported synthetic routes for thieno[2,3-b]pyridine derivatives involved the use of α-halo carbonyl compound as well [32, 47, 48]. It seems that the presence of a carbonyl compound at the aryl position has an influence on the intramolecular cyclisation of the intermediate compounds compared to aryl halides. This may be due to the fact that α-halo compounds are bifunctional since they can behave as both an electrophile and nucleophile in carbonyl condensation reactions. The target thieno[2,3-b] pyridines that were synthesised was based on intermediates with A_1 AR activity, hence the choice of halides used. Also, ring closure may have been accomplished with these compounds (**7a–7d**) due to presence of less bulky constituent (-CONH$_2$) at the aryl position as compared to other compounds with aromatic constituents at the same position. For Compounds **6q–6s** and **7a**, readily available cyanothioacetamide was used as starting material. One of the key starting materials for compounds **6q–6s**, 4-(chloromethyl)-2-(4-chlorophenyl)thiazole was synthesised by refluxing a mixture of 4-chlorobenzothioamide and 1,3-dichloroacetone in absolute EtOH for 2 h (Scheme 3) [49].

The test compounds were obtained in relatively poor yields (**6a**, **6c–l**, **6n–s** and **7a–d**: 11.8–66.4%; with the exception of **6b** and **6m**: >80%), purified by recrystallisation from a suitable solvent (either EtOH, methanol (MeOH) or hexane). The structure, molecular mass and purity of these compounds were verified by hydrogen-1 / protium (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra, mass spectroscopy and HPLC (see supplementary material). It should be noted that, protons on the NH$_2$-group (e.g., **6l**) and the OH-group (e.g., **6k**) are not always visible on a ^{1}H NMR spectrum as protons attached to a N-atom (or O-atom) are acidic, and thus, exchangeable [50]. Halogen-carbon bonds tend to cause splitting of ^{13}C NMR chemical shifts (e.g., **6p** and **7a**) due to deshielding by the F-atom on the directly bonded carbon nucleus [51] which results in multiple carbon peaks. This has the

potential of causing difficulty in interpreting ^{13}C NMR spectra of fluorinated organic compounds.

Biology

In vitro evaluation

Radioligand binding assays A total of 23 test compounds were synthesised (**6a–s** and **7a–d**); 7 of these compounds were novel (**6d** and **6k–p**), while 4 compounds (**7a–d**) have been synthesised before but have never been tested for AR affinity. The affinities of the test compounds **6a–s** and **7a–d** at rat (r) A_1 and A_{2A} ARs were determined by radioligand binding assays and are expressed as inhibition constant (K_i, nM) values (Table 1). All test compounds displayed specific binding values <20% at a maximum tested concentration of 100 μM (rA_1 screening), and therefore, all underwent full biological assay for determination of K_i values (nM). Compounds **6a–j** and **7a–d** displayed specific binding values <20% at a maximum tested concentration of 100 μM (rA_{2A} screening) and hence qualified for full rA_{2A} radioligand binding assay, unlike compounds **6k–s** with specific binding values >20%. The radioligand binding assays were validated with N_6-cyclopentyladenosine (CPA) (A_1 agonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (A_1 antagonist), istradefylline (A_{2A} antagonist) and caffeine (A_1/A_{2A} antagonist) as reference compounds and results were compared to literature values as shown by Table 1.

Structure-activity relationships (SAR) Modifications were made at the R^2 and aryl positions of the test compounds to assess how different substituents can influence both rA_1 and rA_{2A} ARs binding affinity as well as selectivity. As shown in Table 1, all test compounds displayed greater affinity toward the rA_1 than rA_{2A} AR. Compound **6c** had the best rA_1 AR affinity (K_i = 0.076 nM) of the present series. The latter compound together with **6b** displayed better rA_{2A} AR affinity than the other test compounds with K_i values of 48.3 nM (**6c**) and 48.0 nM (**6b**), respectively, but remain selective for the rA_1 AR. Comparing amino-3,5-dicyanopyridines (**6a–s**) and thieno[2,3-b]pyridines (**7a–d**), it is evident that there was a significant decrease in both rA_1 and rA_{2A} AR affinity from the open ring structures to the closed ring structures. The only thieno[2,3]pyridine derivative that showed moderately good rA_1 AR affinity is compound **7c** (r$A_1$$K_i$ = 61.9 nM). The general poor activity of thieno[2,3-b]pyridines relative to amino-3,5-dicyanopyridins suggest that the ring closure affects binding to the receptors, perhaps, due to steric hindrance. (This may be confirmed by molecular docking studies in the future.)

SAR for amino-3,5-dicyanopyrines (intermediates) For compounds **6a**, **6b**, **6l** and **6s**, the 4-methoxyphenyl group

Scheme 3 Synthesis of 4-(chloromethyl)-2-(4-chlorophenyl)thiazole

Table 1 K_i values of test compounds and reference compounds at rat A_1 and A_{2A} ARs

NC — R2 — CN / H2N — N — S — Aryl → NC — R2 — NH2 / H2N — N — S — Aryl

3,5-Dicyanopyridine derivatives **(6a-6s)** → Thieno[2,3-b]pyridine derivatives **(7a-7d)**

#	R2	aryl	$K_i \pm$ SEM (nM)[a] (Specific binding (%))[b]			GTP shift[e]	SI[f]
			rA_1[c] vs 1 nM [³H]DPCPX	rA_{2A}[d] vs 4 nM [³H]NECA	rA_1[c] + 0.1 mM GTP vs 1 nM [³H]DPCPX		
Amino-3,5-dicyanopyridines							
6a	4-methoxyphenyl	acetamide	139 ± 18.8[a]	1473 ± 256[a]	–	–	11
6b	4-methoxyphenyl	6-methylpyridin-2-yl	0.213 ± 0.019[a] (2.9)[g]	48.0 ± 11.1[a] (35)[g]	–	–	255
6c	3-methoxyphenyl	6-methylpyridin-2-yl	0.076 ± 0.002[a] (0.49)[g] (0.21)[h]	48.3 ± 10.1[a] (71)[g] (52)[h]	0.069 ± 0.006[a]	1	636
6d	3-methoxyphenyl	acetamide	10.3 ± 0.643[a]	1205 ± 367[a]	11.3 ± 0.663[a]	1	117
6e	4-hydroxyphenyl	acetamide	60.4 ± 3.83[a]	338 ± 79.1[a]	–	–	
6f	3-hydroxyphenyl	acetamide	48.0 ± 4.36[a]	751 ± 12.0[a]	–	–	16
6g	1,3-dihydroisobenzofuran	acetamide	26.6 ± 6.75[a]	429 ± 55.0[a]	–	–	16
6h	furan-2-yl	benzoic acid	7.54 ± 0.768[a] (4.12)[i]	(581)[i]	–	–	

Table 1 (continued)

3,5-Dicyanopyridine derivatives
(**6a-6s**)

Thieno[2,3-b]pyridine derivatives
(**7a-7d**)

#	R^2	aryl	$K_i \pm$ SEM (nM)[a] (Specific binding (%))[b]			GTP shift[e]	SI[f]
			rA_1[c] vs 1 nM [³H]DPCPX	rA_{2A}[d] vs 4 nM [³H]NECA	rA_1[c] + 0.1 mM GTP vs 1 nM [³H]DPCPX		
6i			4.57 ± 0.284[a]	634 ± 94.3[a]	–	–	139
6j			Not determined (3.5)[g]	20.6 ± 6.56[a] (15)[g]	–	–	
6k			8.82 ± 0.760[a]	(22)[b]	–	–	–
6l			21.0 ± 5.56[a]	(27)[b]	–	–	–
6m			0.179 ± 0.013[a]	(80)[b]	–	–	–
6n			0.831 ± 0.076[a]	(35)[b]	1.94 ± 0.509[a]	2	–
6o			1.64 ± 0.228[a]	(25)[b]	2.25 ± 0.159[a]	1	–
6p			0.430 ± 0.012[a]	(30)[b]	–	–	–

Table 1 (continued)

3,5-Dicyanopyridine derivatives
(6a-6s)

Thieno[2,3-b]pyridine derivatives
(7a-7d)

#	R^2	aryl	$K_i \pm$ SEM (nM)^a (Specific binding (%))^b			GTP shift^e	SI^f
			rA$_1$^{c} vs 1 nM [^{3}H]DPCPX	rA$_{2A}$^{d} vs 4 nM [^{3}H]NECA	rA$_1$^{c} + 0.1 mM GTP vs 1 nM [^{3}H]DPCPX		
6q			0.383 ± 0.069^a $(1.4)^j$	$(44)^b$	1.82 ± 0.582^a	5	–
6r			1.36 ± 0.040^a $(1.5)^j$	$(44)^b$	–	–	–
6s			4.06 ± 0.759^a $(5.0)^j$	$(27)^b$	–	–	–

Thieno[2,3-b]pyridines

#	R^2	aryl	rA$_1$^{c} vs 1 nM [^{3}H]DPCPX	rA$_{2A}$^{d} vs 4 nM [^{3}H]NECA	rA$_1$^{c} + 0.1 mM GTP vs 1 nM [^{3}H]DPCPX	GTP shift^e	SI^f
7a			1008 ± 58.3^a	308 ± 93.6^a			
7b			556 ± 28.1^a	561 ± 12.1^a	–	–	
7c			61.9 ± 2.11^a	1062 ± 126^a	145 ± 28.8^a	2	
7d			305 ± 15.3^a	162 ± 24.4^a	–	–	

Table 1 (continued)

3,5-Dicyanopyridine derivatives
(6a-6s)

Thieno[2,3-b]pyridine derivatives
(7a-7d)

#	R²	aryl	$K_i \pm$ SEM (nM)[a] (Specific binding (%))[b]			GTP shift[e]	SI[f]
			rA_1[c] vs 1 nM [³H]DPCPX	rA_{2A}[d] vs 4 nM [³H]NECA	rA_1[c] + 0.1 mM GTP vs 1 nM [³H]DPCPX		
Reference compounds							
CPA (A₁ agonist)			6.5 ± 0.4[a] (15.3)[k] (7.9)[l]	–	36.5 ± 2.28[a]	6	–
DPCPX (A₁ antagonist)			0.5 ± 0.1[a] (0.6)[k] (0.3)[m]	–	0.4 ± 0.032[a]	1	–
Istradefylline (A₂ₐ antagonist)			–	3 ± 0.9[a] (13; 2.2)[n] (11.1)[o]	–	–	–
Caffeine (A₁/A₂ₐ antagonist)			$52\,800 \pm 7\,400$[a] (44 000)[p] (41 000)[q] (26 000)[r]	$27\,800 \pm 5\,100$[a] (43 000)[q] (22 000)[r] (33 000)[k]	–	–	0.5

[a]Inhibition constant (K_i, nM) represented as the mean ± standard error of the mean (SEM), $n = 3$ samples

[b]Specific binding (%) of the radioligand at a maximum tested concentration of 100 μM is represented as the mean, $n = 2$ samples

[c]rA₁: rat whole brain membranes expressing adenosine A₁ receptor

[d]rA₂ₐ: rat striatal membranes expressing adenosine A₂ₐ receptor

[e]GTP shift calculated by dividing the K_i (nM) in the presence of 0. 100 μM GTP by the K_i (nM) in the absence of 100 μM GTP

[f]Selectivity index (SI) for the adenosine A₁ receptor subtype calculated by dividing the $rA_{2A}K_i$ (nM) by the rA_1K_i (nM)

[g]Literature value: human adenosine A₁ receptor and [³H]DPCPX; human adenosine A₂ₐ receptor and [³H]ZM241385 [56]

[h]Literature value: rat adenosine A₁ receptor and [³H]DPCPX; rat adenosine A₂ₐ receptor and [³H]ZM241385 [56]

[i]Literature value: human adenosine A₁ receptor and [³H]DPCPX; human adenosine A₂ₐ receptor and [³H]ZM241385 [79]

[j]Literature value: human adenosine A₁ receptor and [³H]DPCPX [57]

[k]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [26]

[l]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [75]

[m]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [52]

[n]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [80]

[o]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [60]

[p]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [62]

[q]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [63]

[r]Literature value: rat adenosine A₁ receptor and [³H]DPCPX [81]

was maintained at position R² and different functional groups were substituted at the aryl position. Compound **6b** with a methylpyridine substituent at the aryl position exhibited low nanomolar activity toward the rA₁ AR ($K_i = 0.213$ nM) as well as selectivity for the rA₁ AR over the rA₂ₐ AR (SI = 636). Affinity for the rA₁ and/or rA₂ₐ ARs decreased when introducing a 4-chlorophenylthiazole group (**6s**: rA₁$K_i = 4.06$ nM), benzoic acid substituent (**6l**: rA₁$K_i = 21.0$ nM) and a carbonyl containing substituent (**6a**: rA₁$K_i = 139$ nM) which displayed the lowest affinity for rA1 AR. In terms of selectivity, **6b** also showed affinity

toward the rA₂ₐ AR ($K_i = 48.0$ nM) while compounds **6l** and **6s** were more selective towards rA₁ ARs, as seen from the calculated SIs.

Replacing 4-methoxyphenyl with 3-methoxyphenyl at position R² while maintaining the same aryl functional groups as **6a**, **6b** and **6l** above was also explored. Comparison of compound **6d** to **6a** (aryl = -CONH₂) showed a significant increase in binding affinity towards rA₁ ARs (**6d**: rA₁$K_i = 10.3$ nM vs **6a**: rA₁$K_i = 139$ nM) but had no effect on rA₂ₐ AR affinity. In general, the presence of the 3-methoxyphenyl substituent resulted in increased

affinity for rA_1 ARs but had no influence on rA_{2A} AR affinity as shown by **6c** vs **6b** and **6o** vs **6l**. Again, the substituents at the aryl position had a similar effect on affinity as observed with the 4-methoxyphenyl containing compounds **6a**, **6l** and **6s** (in decreasing order of affinity: **6c** (methylpyridine) > **6o** (benzoic acid substituent) > **6d** (carbonyl containing substituent)). From these results it is evident that 3-methoxyphenyl is favoured over 4-methoxyphenyl in terms of rA_1 AR binding affinity.

For compounds **6e**, **6m**, **6n** and **6q**, 4-hydroxyphenyl was introduced at the R^2 position while maintaining almost all the same aryl groups mentioned earlier. Generally, these compounds displayed rA_1 AR affinity of 1 nM or smaller (except **6e**, aryl = $CONH_2$), with **6m** (aryl = -methylpyridine) being the best with $rA_1K_i = 0.179$ nM of these compounds. Comparing 4-methoxyphenyl and 4-hydroxyphenyl substitutions (**6a** vs **6e** and **6b** vs **6m**) showed that with the latter, rA_1 AR activity increased slightly. Looking at **6m** (aryl = methylpyridine) and **6n** (aryl = -methylbenzene), it appears that the introduction of a N-atom in compound **6m** had a positive influence in rA_1 AR affinity.

Replacing 4-hydroxyphenyl with 3-hydroxyphenyl at position R^2 was also studied (**6e** vs **6f** and **6m** vs **6j**), although a definite trend could not be observed with the limited data at hand. Comparison of **6f** (3-hydroxyphenyl) to its 4-methoxyphenyl substituted counterpart **6d** showed a four-fold decrease in rA_1 AR affinity, although rA_1 selectivity was maintained.

Comparison of **6a**, **6d**, **6e** and **6f** showed that the meta position is preferred to the para position whether OCH_3- or OH-group substitution is incorporated, and furthermore, it seems that a OCH_3-group is preferred to an OH-group.

Comparing **6l** and **6k** with 4-OCH_3 and 4-SCH_3 revealed that introducing a sulphur component increased binding affinity for compound **6k** ($K_i = 8.82$ nM) as compared to **6l** (21.2 nM).

Compounds, **6q**, **6r** and **6s** with the same (4-chlorophenyl)thiazole aryl substituent were also explored. All these compounds displayed rA_1 AR affinity but had no mentionable affinity for rA_{2A} ARs. Compound **6q** (R = 4-OCH_2-CH_2OH) had the best affinity of these compounds with $K_i = 0.383$ nM. SARs of amino-3,5-dicyanopyridines against rA_1 AR are summarised in Fig. 3.

SAR for thieno[2,3-b]pyridines (target compounds) Thieno[2,3-b]pyridine derivatives **7a–d** displayed poor affinity towards rA_1 ARs compared to their corresponding intermediate amino-3,5-dicyanopyridines (Fig. 4). These compounds all had a -$CONH_2$-group at the aryl position. The results indicate that ring closure from the intermediate open ring to fused ring structures decreased activity towards both rA_1 and rA_{2A} ARs. This corresponds with a study by [7] in

which intramolecular cyclisation of the 6-amino-3,5-dicyanopyridines, specifically BAY606583 (a potent A_{2B} receptor agonist) was evaluated. The study revealed that the bicyclic compound (thieno[2,3]pyridine derivative) that resulted after intramolecular cyclisation of BAY60 6586 bind none of the ARs suggesting that molecular stiffening decreases AR binding affinity.

GTP shift assays The type of binding affinities that test compounds **6c**, **6d**, **6n**, **6o**, **6q** and **7c** displayed at the rA_1 AR were determined through guanosine 5'-triphosphate (GTP) shift assays, as described in literature [52–54]. These test compounds were selected as they possessed the best rA_1 AR affinity among the investigated test compounds (Table 1). The theory of a GTP assay is that competition curve of an antagonist will be unaffected by GTP, thus resulting in a calculated GTP shift of approximately 1 [54]. Agonists' curves, on the other hand, will be shifted towards the right in the presence of GTP [55]. GTP shifts were calculated by dividing the rAK_i values of compounds reported in the presence of GTP by the rARK_i values obtained in the absence of GTP and the results are summarised in Table 1. Compounds **6c**, **6d** and **6o** behaved as antagonists (interestingly, all these compounds contained a 3-OCH_3 group at position R^2), while **6n**, **6q** and **7c** behaved as agonists (Fig. 5). Contradictory to the present results, Guo et al. [56] found **6c** to be a partial agonist and not an antagonist. Notably, Louvel and co-workers [57] also found **6q** to be a full agonist in accordance with the present results.

In silico evaluation

The physiochemical properties, pharmacokinetic profiles, drug-likeness and medicinal chemistry friendliness of compounds **6c**, **6d**, **6n**, **6o**, **6q** and **7c** were predicted through the free online web tool SwissADME (https://swissadme.ch). The prediction is based on the chemical structures of the compounds. The results are in the supplementary material.

The bioavailability radar (which takes in to account the physicochemical properties lipophilicity, size, polarity, solubility, flexibility and saturation) for compounds **6b**, **6d**, **6m**, **6o**, **6q** and **7c** may be seen in the supplementary material (Fig. S2). Almost all compounds fall within the optimal ranges of lipophilicity, size, solubility, and flexibility parameters except compound **6q** which exceeded the optimal size of the molecule (150–500 g/mol) since it has a molecular weight (MW) of 520.03 g/mol. All these compounds failed the saturation parameter since all have a lower fraction of carbon atoms in the sp3 hybridization (Csp3 > 0.25) and high polarity values (TPSA > 130 Å^2). These compounds are considered to be too polar with a low degree of saturation and consequently predicted not to be orally
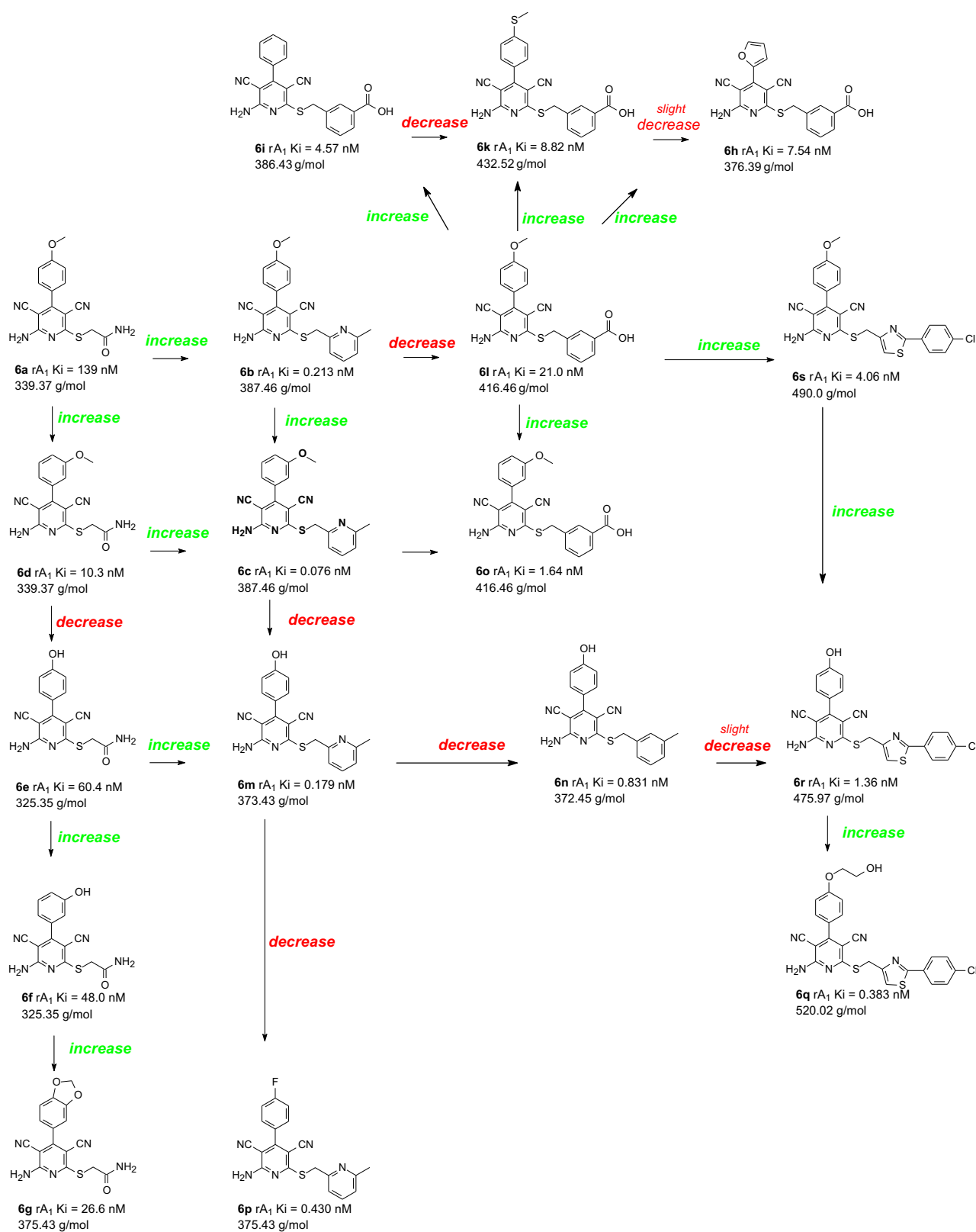
Fig. 3 Structure-activity relationship of amino-3,5-dicyanopyridines against rA_1 AR

Fig. 4 Structure-activity relationships of amino-3,5-dicyanopyridines (**6a**, **6f**, **6g**) vs thieno[2,3]pyridines (**7a**, **7c**, **7d**)
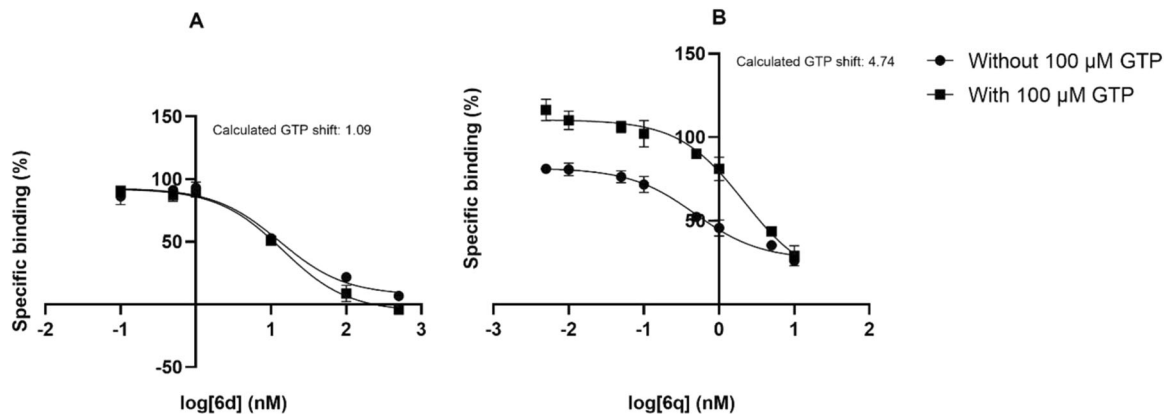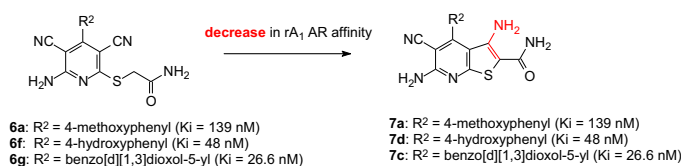

6a: R^2 = 4-methoxyphenyl (Ki = 139 nM)
6f: R^2 = 4-hydroxyphenyl (Ki = 48 nM)
6g: R^2 = benzo[d][1,3]dioxol-5-yl (Ki = 26.6 nM)

7a: R^2 = 4-methoxyphenyl (Ki = 139 nM)
7d: R^2 = 4-hydroxyphenyl (Ki = 48 nM)
7c: R^2 = benzo[d][1,3]dioxol-5-yl (Ki = 26.6 nM)

decrease in rA_1 AR affinity

Fig. 5 The binding curves of test compounds **6d** and **6q** in the presence and absence of 100 μM GTP using [^{3}H]DPCPX as radioligand in rat whole brain membranes expressing adenosine A_1 receptors as representative cases for adenosine A_1 receptor antagonistic and agonistic activity. **A** Calculated GTP shift: 1.09 (antagonist); **B** Calculated GTP shift: 4.74 (agonist)

bioavailable. The LogP value of a compound using a logP of a reference compound (XLOGP3) (<5.0) of compound **6q** slightly exceeded the limit (5.1) proving to be the most lipophilic.

In terms of water solubility (Log S), compound **6c**, **6m**, **6o** and **7c** are predicted to be moderately soluble to poorly soluble in water. Compound **6d** was predicted to be soluble to moderately soluble and compound **6q** was classified as poorly soluble. Poor water solubility of compound **6q** may be attributed to its high MW and the presence of lipophilic halogen (Cl) as part of the aryl substituent. Water solubility is the most important in terms of achieving desired drug concentration in systemic circulation for pharmacological response [58]. It must be understood that poorly water-soluble drugs have slow drug absorption leading to inadequate and variable bioavailability and gastrointestinal mucosal toxicity [58]. Solubility improvement techniques need to be employed for future formulation development especially for compound **6q** (capadenoson), since any drug to be absorbed must be present in an aqueous solution at the site of absorption.

The BOILED-Egg predictive model allows evaluation of passive gastrointestinal (GI) absorption and brain penetration (BBB). Compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c** are all predicted to have low GI absorption and no blood brain barrier (BBB) permeability, probably because of a high topological polar surface area (TPSA) (>130), although some sources recommend TSPA < 140Å^2 (e.g. **6c**) to be adequate for high probability of good intestinal permeability

[59]. This may also be attributed to the high polarity of these compounds. Interestingly, a study by [60, 61] indicated that compound **6q** (capadenoson), showed hints of CNS effects in humans. Compound **6c** as well has been considered to possess high BBB permeability by [56, 62, 63] despite this prediction. The prediction of permeability glycoprotein (P-gp) substrate indicates that only compound **6q** can be actively effluxed by P-gp while compounds **6c**, **6m**, **6o** and **7c** are not substrates of this efflux mechanism. The potential interaction of compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c** with cytochromes P450 (CYP) isoenzymes was also evaluated. This is important for determination of drug-drug interactions and adverse effects due to low drug clearance leading to accumulation of the drug [50, 51]. Generally, all the compounds are inhibitors of CYP isoforms (CYP1A2, CYP2C19, CYP2C9, CYP3A4) with a few exceptions, but they did not affect CYP2D6 except compound **6q**.

SwissADME also provides qualitative assessment of drug-likeness which predicts a molecule's chance to be classified as an oral drug candidate [64] by implementing different rule-based filters [65–69]. Additionally, compounds **6c**, **6d**, **6m**, **6o**, **6q** and **7c** all had a bioavailability score of 0.55 (the probability that a compound will have >0% bioavailability in rat or measurable Caco-2 permeability) [64].

The medical chemistry friendliness of compounds was assessed by identifying pan assay interference compounds (PAINS) [70] and structural alerts [71]. All compounds

passed both PAINS and Brenk tests as no alerts were raised. This means that these compounds may not affect any bioassays [72] and generally have good pharmacokinetics properties with an acceptable toxic level [73]. Interestingly, only one compound (**6d**) passed the lead-likeness test, and hence, can be used as a lead compound in drug discovery processes. Compounds **6c**, **6m**, **6o**, **6q** and **7c** all had higher MW (>350) as well as high partition coefficient (log*P*) values (XLOGP: >3.5). Structural optimisation for these chemical scaffolds is needed, most probably by decreasing size, polarity and/or lipophilicity.

Conclusion

The aim of this study was to investigate use of amino-3,5-dicyanopyridine and thieno[2,3-*b*]pyridine derivatives as potential AR agonists. A total of 23 test compounds were synthesised (**6a–s** and **7a–d**) and 7 of these were novel (**6d** and **6k–p**), while 4 compounds (**7a–d**) have been synthesised before but have never been tested for AR affinity.

Overall, amino-3,5-dicyanopyridine displayed superior activity towards rA_1 ARs compared to thieno[2,3]pyridines. The general poor activity of thieno[2,3-b]pyridines suggest that the intramolecular cyclisation results in molecular stiffening or rigidity which negatively affects binding to the receptors, perhaps, due to steric hindrance. On the R^2 substitution, it was observed that 3- and 4-methoxyphenyl groups favoured rA_1 AR binding compared to their 3- and 4-hydroxyphenyl counterparts. Looking at the aryl substitution, the methylpyridine substituent displayed the overall best rA_1 AR affinity. Novel compounds (**6d**, **6k**, **6l**, **6m**, **6n**, **6o** and **6p**) proved to be highly selective with low nanomolar rA_1 AR affinity (K_i values between 0.179 nM and 21.0 nM). The only thieno[2,3-*b*]pyridine derivative that displayed moderately good rA_1 AR activity ($K_i =$ 61.9 nM) has been investigated as a TGF-β receptor kinase inhibitor for the treatment of tumours and now AR affinity may be included.

Compounds **6n**, **6q** and **7c** acted as potent, highly selective agonists at A_1 ARs; however, compounds **6c**, **6d** and **6o** (notably all containing a 3-OCH$_3$ group at position R^2) behaved as rA_1 antagonists.

Upon in silico evaluation, the SwissADME profiles of the test compounds raised concern about their bioavailability; therefore, it may be advisable to confirm BBB permeation via in vitro evaluation of promising test compounds before further structure optimisation.

The high affinity and selectivity for the rA_1 AR displayed by the amino-3,5-dicyanopyridine scaffold showed that, if correctly modified, it may produce highly potent AR ligands which can be used in development of treatment for epilepsy.

Experimental

Chemistry

Materials and methods

Unless otherwise noted, all starting materials and solvents were purchased from commercial manufacturers (Sigma-Aldrich and AmBeed) and used without further purification. Thin layer chromatography (TLC) silica gel 60 F254 aluminium sheets from Merck was used to monitor reaction progress. Melting points (mp) were determined on a Buchi M-545 melting point apparatus. Mp for compounds **6h**, **6i**, **6j**, **7c** and **7b** were obtained through differential scanning calorimetry (DSC) analysis using Mettler Toledo analyser. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 and 151 megahertz (MHz) respectively, using DMSO-d6 (deuterated dimethyl sulfoxide) as solvent and tetramethylsilane (TMS - Si(CH$_3$)$_4$) as reference. Chemical shifts were reported in parts per million (ppm) in relation to the solvent peak (DMSO-d6: residual CH$_3$ at 2.50 ppm for ^1H NMR and 39.52 ppm for ^{13}C NMR). Spin multiplicities were indicated as follows: singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), triplet of doublets (td), double double doublet (ddd) and multiplet (m). Coupling constant (J) values were reported in Hertz (Hz). High-resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF-Q II mass spectrometer in atmospheric chemical ionisation (APCI) mode. High-performance liquid chromatography (HPLC) analyses were done on Shimadzu Nexera-i LC-2040C 3D Plus HPLC system to determine the purity of test compounds.

Synthesis of test compounds

General procedure for the synthesis of **6a–6p**

2-((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)acetamide (6a) Three drops of trimethylamine were added to a solution of MN (0.629 ml, 10 mmol) in 20 mL of EtOH, while stirring with magnetic stirrer. The reaction mixture was cooled to 10 °C and H$_2$S generated by reaction between iron sulphide (FeS) (12.081 g) and hydrochloric acid (HCl) (90 ml) was passed through the mixture for 24 h to produce cyanothioacetamide (**1**). The reaction mixture was then stirred for 15–20 min before adding 3-methoxybenzaldehyde (1.217 ml, 10 mmol) while stirring at room temperature to produce **2a**. After obtaining a homogeneous mixture, more MN (0.629 ml, 10 mmol) was added and the mixture stirred until it became homogeneous again. It was then left to stand at room temperature for 12–14 h to obtain 2,6-diamino-4-methoxy-4H-thiopyran-

3,5-dicarbonitrile (**4a**). The mixture was then diluted with an equal volume of DMF and 10% aqueous KOH (5.6 mL, 10 mmol) and left to stand for 24 h to produce potassium 6-amino-3,5-dicyano-4-methoxy-1,4-dihydropyridine-2-thio-late (**5a**). 2-bromoacetamide (10 mmol, 1.382 g) was added to the mixture and continuously stirred for 3 h, after which 10% aqueous KOH (5.6 mL, 10 mmol) was added again. After 2–4 h, ice was added and the resulting precipitate was filtered off, washed with distilled H_2O, EtOH and hexane, dried (30 °C) and recrystallised from MeOH to yield the title compound **6a** as whitish powder (0.988 g, 29.1%): Rf: 0.77 (DCM/PE/EtOAc 10:1:1); mp: 237.2–238.0 °C; ^1H NMR (600 MHz, DMSO) δ 7.93 (s, 2H), 7.49 (d, $J = 8.7$ Hz, 2H), 7.46 (s, 1H), 7.19 (s, 1H), 7.12 (d, $J = 8.7$ Hz, 2H), 3.89 (s, 2H), 3.85 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C,-<u>C</u>O), 166.1 (C, C-6), 160.8 (C, C-2), 159.6 (C, C-4'), 158.0 (C, C-4), 130.1 (C, C1'), 125.7 (CH, C-2',C-6'), 115.3 (CH, C-3', C-5'), 115.3 (C, CN), 114.1 (C, CN), 93.3 (C, C-5), 85.9 (C, C-3), 55.3 (CH$_3$, O<u>C</u>H$_3$), 33.3 (CH$_2$, S-<u>C</u>H$_2$-); APCI-HRMS *m/z*: calculated for $C_{16}H_{14}N_5O_2S$ [M + H] + 340.0863, found 340.0871; Purity (HPLC, λ = 280): 100%

2-amino-4-(4-methoxyphenyl)-6-(((6-methylpyridin-2-yl) methyl)thio)pyridine-3,5-dicarbonitrile (6b)

Prepared as for **6a** from 4-methoxybenzaldehyde (1.217 ml, 10 mmol) and 2-(bromomethyl)-6-methylpyridine (1.862 g, 10 mmol) to yield **6b** which was recrystallised from MeOH as white flakes (3.418 g, 88.2%): Rf: 0.77 (PE:EtOAc 1:1); mp: 168.5–171.9 °C; ^1H NMR (600 MHz, DMSO) δ 8.00 (s, 2H), 7.63 (t, $J = 7.6$ Hz, 1H), 7.48 (d, $J = 8.7$ Hz, 2H), 7.42 (d, $J = 7.6$ Hz, 1H), 7.12 (dd, $J = 24.1, 8.1$ Hz, 3H), 4.56 (s, 2H), 3.84 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.1 (C, C-6), 160.8 (C, C-2), 159.6 (C, C-4'), 158.1 (C, C-4), 157.7 (C, C-2″), 155.73 (C, C-6″), 137.0 (CH, C-4″), 130.1 (C, C-1'), 125.8 (CH, C-2', C-6'), 121.8 (CH, C-5″), 120.6 (CH, C-3″), 115.4 (CH, C-3', C-5'), 115.4 (C, CN), 114.0 (C, CN), 93.2 (C, C-5), 85.9(C. C-3), 55.3 (CH$_3$, O<u>C</u>H$_3$), 35.3 (CH$_2$, -S<u>C</u>H$_2$-), 23.9 (CH$_3$, -<u>methyl</u>pyridine); APCI- HRMS *m/z*: calculated for $C_{21}H_{18}N_5OS$ [M + H] + 388.1227, found 388.1208; Purity (HPLC, λ = 254): 100%

2-amino-4-(3-methoxyphenyl)-6-(((6-methylpyridin-2-yl) methyl)thio)pyridine-3,5-dicarbonitrile (6c)

Prepared as for **6a** from MN (0.315 ml, 5 mmol), 3-methoxybenzaldehyde (5 mmol, 0.608 ml) and 2-(bromomethyl)-6-methylpyridine (0.933 g, 5 mmol) to yield **6c** which was recrystallised from MeOH as white solid (0.505 g, 26.1%): Rf: 0.64 (DCM/PE/ EtOAc 10:1:1); mp: 175.5–176.7 °C; ^1H NMR (600 MHz, DMSO) δ 8.05 (s, 2H), 7.63 (t, $J = 7.7$ Hz, 1H), 7.50–7.40 (m, 2H), 7.17–7.08 (m, 3H), 7.07–7.05 (m, 1H), 4.57 (s, 2H), 3.80 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.1 (C, C-6), 159.5 (C, C-2), 159.0 (C, C-3'), 158.1 (C,

C-4), 157.7 (C, C-2″), 155.7 (C, C-6″), 137.0 (C, C-1'), 135.1 (CH, C-4″), 129.9 (CH, C-5'), 121.8 (CH, C-3″), 120.6 (CH, C-5″), 120.4 (CH, C-6'), 115.8 (CH, C-4'), 115.1 (CH, C-2'), 115.0 (C, CN), 114.0 (C, CN), 93.2 (C, C-5), 86.0 (C, C-3), 55.3 (CH$_3$, O<u>C</u>H$_3$), 35.3 (CH$_2$, -S<u>C</u>H$_2$-), 23.9 (CH$_3$, <u>methyl</u>pyridine). APCI-HRMS *m/z*: calculated for $C_{21}H_{18}N_5OS$ [M + H] + 388.1227, found 388.1208; Purity (HPLC, λ = 254): 100%

2-((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl) thio)acetamide (6d)

Prepared as for **6c** from 3-methoxybenzaldehyde (5 mmol, 0.608 ml) and 2-bromoacetamide (0.691 g, 5 mmol) to yield **6d** which was recrystallised from MeOH as yellowish powder (0.537 g, 31.6%): Rf: 0.78 (DCM/PE/EtOAc 10:1:1); mp: 232.3–237.0 °C; ^1H NMR (600 MHz, DMSO) δ 7.98 (s, 2H), 7.53–7.42 (m, 2H), 7.20 (s, 1H), 7.14–7.09 (m, 2H), 7.08–7.04 (m, 1H), 3.89 (s, 2H), 3.81 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C, CO), 166.0 (C, C-6), 159.4 (C-C2), 159.0 (C, C-3'), 158.1 (C, C-4), 135.1 (C, C-1'), 129.9 (CH, C-5'), 120.4 (CH, C-6'), 115.8 (C, C-4'), 115.1 (C, CN), 115.0 (CH, C-2'), 114.0, (C, CN) 93.3 (C, C-5), 86.0 (C, C-5), 55.3 (CH$_3$, O<u>C</u>H$_3$), 33.3 (CH$_2$, -S-<u>C</u>H$_2$-); APCI-HRMS *m/z*: calculated for $C_{16}H_{14}N_5O_2S$ [M + H] + 340.0863, found 340.0844; Purity (HPLC, λ = 254): 100%

2-((6-amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-yl) thio)acetamide (6e)

Prepared as for **6c** from 4-hydroxybenzaldehyde (0.614 g, 5 mmol) and 2-bromoacetamide (0.692 g, 5 mmol) to yield compound **6e** which was recrystallised from MeOH as cream white powder (0.650 g, 40.0%): Rf: 0.15 (EtAOc only); mp: 267.6–268.0 °C; ^1H NMR (600 MHz, DMSO) δ 10.03 (s, 1H), 7.89 (s, 2H), 7.46 (s, 1H), 7.40–7.34 (m, 2H), 7.19 (s, 1H), 6.95–6.89 (m, 2H), 3.88 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 168.9 (C, CO), 166.1 (C, C-6), 159.7 (C, C-2), 159.4 (C, C-4), 158.3 (C, C-4'), 130.1 (C, C-1'), 124.1 (CH, C-2', C-6'), 115.4 (CH, C-3', C-5'), 115.4 (C, CN, 115.4 (C, CN), 93.2 (C, C-5), 85.8 (C, C-3), 33.3 (CH$_2$, -S<u>C</u>H$_2$-); APCI-HRMS *m/z*: calculated for $C_{15}H_{12}N_5O_2S$ [M + H] + 326.0706, found 326.0688; Purity (HPLC, λ = 254): 100%

2-((6-amino-3,5-dicyano-4-(3-hydroxyphenyl)pyridin-2-yl) thio)acetamide (6f)

Prepared as for **6c** from 3-hydroxybenzaldehyde (0.612 g, 5 mmol) and 2-bromoacetamide (0.690 g, 5 mmol) to yield **6f** which was recrystallised from ethanol as light brown solid (0.735 g, 45.2%): Rf: 0.28 (DCM/MeOH: 10:1); mp: 248.1–248.4 °C; ^1H NMR (600 MHz, DMSO) δ 9.83 (s, 1H), 7.95 (d, $J = 6.9$ Hz, 2H), 7.47 (s, 1H), 7.35 (t, $J = 7.9$ Hz, 1H), 7.20 (s, 1H), 6.95 (ddd, $J = 8.2, 2.4, 0.8$ Hz, 1H), 6.92–6.88 (m, 1H), 6.88–6.85 (m, 1H), 3.89 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C, CO), 166.1 (C,

C-6), 159.5 (C, C-2), 158.3 (C, C-4), 157.3 (C, C-3'), 135.0 (C, C-1'), 129.9 (CH, C-5'), 118.8 (CH, C-6'), 117.2 (CH, C-4'), 115.1 (CH, C-2'), 115.0 (C, CN), 115.0 (C, CN), 93.2 (C, C-5), 85.9 (C, C-3), 33.3 (CH$_2$, -S$\underline{C}$H$_2$-); APCI-HRMS m/z: calculated for C$_{15}$H$_{12}$N$_5$O$_2$S [M + H] + 326.0706, found 326.0689; Purity (HPLC, λ = 254): 100%

2-(((6-amino-4-(benzo[d][1,3]dioxol-5-yl)-3,5-dicyanopyridin-2-yl)thio)acetamide (6g)

Prepared as for **6c** from piperonaldehyde (0.752 g, 5 mmol) and 2-bromoacetamide (0.690 g, 5 mmol) to yield **6g** which was recrystallised from MeOH as light orange solid (0.682 g, 38.6%): Rf: 0.40 (DCM/MeOH: 10:1); mp: 244.5–245.1 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.93 (s, 2H), 7.46 (s, 1H), 7.19 (s, 1H), 7.14 (d, J = 1.8 Hz, 1H), 7.10 (d, J = 8.0 Hz, 1H), 7.02 (dd, J = 8.0, 1.8 Hz, 1H), 6.15 (s, 2H), 3.88 (s, 2H).; ^{13}C NMR (151 MHz, DMSO) δ 168.8 (C, CO), 166.0 (C, C-6), 159.5 (C, C-2), 157.9 (C, C-4), 148.9 (C, C-3',C-4'), 147.3 (C, C-1'), 127.2 (C, C-6'), 122.9 (CH, C-2'), 115.2 (CH, C-5'), 108.8, (C, CN) 108.5 (C, CN), 101.7 (CH$_2$, C at dioxol), 93.4 (C, C-5), 86.1 (C, C-3), 33.3 (CH$_2$, -S$\underline{C}$H$_2$-); APCI-HRMS m/z: calculated for C$_{16}$H$_{12}$N$_5$O$_3$S [M + H] + 354.0655, found 354.0635; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzoic acid (6h)

Prepared as for **6c** from furan-2-carbaldehyde (0.414 g, 5 mmol) and 3-(bromomethyl)benzoic acid (1.081 g, 5 mmol) to yield **6h** which was recrystallised from acetone as cream white powder (0.341 g, 18.1%):Rf: 0.38 (DCM:MeOH 10:1); mp: 279.59 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.10–8.03 (m, 2H), 7.86 (d, J = 6.3 Hz, 2H), 7.57 (d, J = 7.0 Hz, 1H), 7.37 (d, J = 3.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 6.81 (dd, J = 3.6, 1.7 Hz, 1H), 4.50 (s, 2H).; ^{13}C NMR (151 MHz, DMSO) δ 167.4 (C, COOH), 160.1 (C, C-6), 146.5 (C, C-2), 145.1 (C, C-4, C-2'), 143.7 9CH, C-5', 136.5 (C-3'), 127.7 (C, C-4'', C-1''), 116.3 (CH, C-5'',C-6'', C-2''), 115.7 (C, CN), 115.7 C, CN), 112.8 (CH, C-3', C-4'), 89.2 (C, C-5), 81.6 (C, C-3), 33.4 (CH$_2$, -S$\underline{C}$H$_2$); APCI-HRMS m/z: calculated for C$_{19}$H$_{13}$N$_4$O$_3$S [M + H] + 377.0703, found 377.0687; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-phenylpyridin-2-yl)thio)methyl)benzoic acid (6i)

Prepared as for **6c** from benzaldehyde (0.460 ml, 5 mmol) and 3-(bromomethyl)benzoic acid (1.083 g, 5 mmol) to yield **6i** which was recrystallised from hexane as white fluffy solid (0.601 g, 31.1%): Rf: 0.89 (DCM:MeOH 10:1); mp: 200 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.01 (s, 2H), 7.78 (d, J = 7.6 Hz, 1H), 7.58–7.49 (m, 5H), 7.44 (d, J = 7.5 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 4.51 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.4 (C-$\underline{C}$OOH), 159.5 (C, C-6), 158.3 (C, C-2), 135.7 (C, C-4), 133.9 (C, C-3''), 130.3 (C, C-1'), 130.2 (CH, C-4''), 128.6

(C, C-1''), 128.3 (CH, C-3', C-5', C-4', C2'') 128.0 (CH, C-5'', C-6''), 127.1 (CH, C-2', C-4'), 115.1 (C, $\underline{C}$N), 115.1 (C-$\underline{C}$N), 93.2 (C, C-5), 85.9 (C, C-3), 33.6 (C, -S$\underline{C}$H$_2$); APCI-HRMS m/z: calculated for C$_{21}$H$_{15}$N$_4$O$_2$S [M + H] + 354.0655, found 354.0635; Purity (HPLC, λ = 254): 100%

2-amino-4-(3-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6j)

Prepared as for **6c** from 3-hydroxybenzaldehyde (0.610 g, 5 mmol) and 2-(bromomethyl)-6-methylpyridine (0.933 g, 5 mmol) to yield **6j** which was recrystallised from MeOH as light yellow powder (0.580 g, 31.1%): Rf: 0.73 (DCM/MeOH 10:1); mp: 231.22 °C; ^{1}H NMR (600 MHz, DMSO) δ 9.82 (s, 1H), 8.04 (s, 2H), 7.63 (t, J = 7.7 Hz, 1H), 7.43 (d, J = 7.7 Hz, 1H), 7.34 (t, J = 7.9 Hz, 1H), 7.15 (d, J = 7.7 Hz, 1H), 6.94 (ddd, J = 8.2, 2.4, 0.7 Hz, 1H), 6.91–6.83 (m, 2H), 4.56 (s, 2H), 2.46 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.1 (C, C-6), 159.5 (C, C-2), 158.4 (C, C-4), 157.7 (C, C-2''), 157.3 (C, C-6''), 155.7 (C, C-3'), 137.0 (C, C-1'), 135.0 (CH, C-4''), 129.8 (CH, C-5'), 121.8 (CH, C-3''), 120.6 (CH, C-5''), 118.8 (C, C-6'), 117.2 (CH,C-4'), 115.1 (CH, C-2'), 115.0 (C, CN), 115.0 (C, CN), 93.1, (C, C-5) 85.8 (C, C-3), 35.4 (CH$_2$, -S$\underline{C}$H$_2$-), 23.9 (CH$_3$, $\underline{\text{Methyl}}$pyridine); APCI-HRMS m/z: calculated for C$_{20}$H$_{16}$N$_5$OS [M + H] + 374.1070, found 354.1063; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(4-(methylthio)phenyl)pyridin-2-yl)thio)methyl)benzoic acid (6k)

Prepared as for **6c** from 4-(methylthio)benzaldehyde (0.664 ml, 5 mmol) and 3-(bromomethyl)benzoic acid (1.080 g, 5 mmol) to yield **6k** which was recrystallised from MeOH as yellowish powder (0.580 g, 31.1%): Rf: 0.69 (DCM/PE/EtOAc 10:1:1); mp: 193.2–193.3 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.05 (s, 1H), 7.83 (d, J = 7.6 Hz, 1H), 7.73 (d, J = 7.5 Hz, 1H), 7.47 (dd, J = 6.1, 4.2 Hz, 2H), 7.44–7.35 (m, 3H), 4.57 (s, 2H), 2.53 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.01 (C, $\underline{C}$OOH), 163.8 (C, C-6), 159.6 (C, C-2), 157.9 (C, C-4), 141.7 (C, C-3'', C-4'), 130.1 (C, C-4'), 129.8 (C, C-4''), 129.1 (C, C-1''), 128.3 (CH, C-2''), 128.2 (CH, C-5'', C-6''), 125.2 (CH, C-2', C-3', C-5', C-6'), 115.4 (C, CN, CN), 93.1 (C, C-5), 85.9 (C, C-3), 32.9 (CH$_2$, -S$\underline{C}$H$_2$-), 14.0 (CH$_3$, SCH$_3$). APCI-HRMS m/z: calculated for C$_{22}$H$_{17}$N$_4$O$_2$S$_2$ [M + H] + 3433.0787, found 433.0784; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio)methyl)benzoic acid (6l)

Prepared as for **6c** from 4-methoxybenzaldehyde (0.608 ml, 5 mmol) and 3-(bromomethyl)benzoic acid (1.080 g, 5 mmol) to yield **6l** which was recrystallised from MeOH as white solid (0.311 g, 14.9%): Rf: 0.97 (DCM/PE/EtOAc 10:1:1); mp: 226.5–226.6 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.07 (s, 1H), 7.87 (d, J = 7.2 Hz, 1H), 7.57 (d, J = 6.7 Hz, 1H), 7.47

(d, $J = 8.7$ Hz, 2H), 7.28 (t, $J = 7.6$ Hz, 1H), 7.08 (d, $J = 8.8$ Hz, 2H), 4.50 (s, 2H), 3.82 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.3 (C, COOH), 160.8 (C, C-6), 159.7 (C, C-2), 158.1 (C, C-4'), 136.6 (C, C-4), 130.4, (C, C-3″) 130.3 (CH, C-4″), 128.4 (C, C-1″, C-1'), (128.4 (CH, C-2', C-6'), 127.7 (CH, C-2″), 125.8 (CH, C-5″, C-6″), 115.6 (CH, C-3', C-5'), 115.5 (C, CN), 114.1, (C, CN), 93.2 (C, C-5), 85.9 (C, C-3), 55.4 (CH$_3$, OCH$_3$), 33.3 (CH$_2$, -SCH$_2$-); APCI-HRMS m/z: calculated for C$_{22}$H$_{17}$N$_4$O$_3$S [M + H] + 417.1016, found 417.1009; Purity (HPLC, λ = 254): 100%

2-amino-4-(4-hydroxyphenyl)-6-(((6-methylpyridin-2-yl) methyl)thio)pyridine-3,5-dicarbonitrile (6 m)

Prepared as for 6c from 4-hydroxybenzaldehyde (0.611 g, 5 mmol) and 2-(bromomethyl)-6-methylpyridine (0.932 g, 5 mmol) to yield 6m which was recrystallised from MeOH as creamy white powder (1.611 g, 86.3%): Rf: 0.68 (PE:EtOAC 1:1); mp: 190.8–191.7 °C; ^{1}H NMR (600 MHz, DMSO) δ 10.09 (s, 1H), 7.63 (t, $J = 7.7$ Hz, 1H), 7.43 (d, $J = 7.6$ Hz, 1H), 7.41–7.33 (m, 2H), 7.14 (d, $J = 7.7$ Hz, 1H), 6.94–6.87 (m, 2H), 4.54 (s, 2H), 2.45 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.2 (C, C-6), 159.8 (C, C-2), 159.5 (C, C-4), 158.4 (C, C-4'), 157.8 (C, C-2″), 155.9 (C, C-6″), 137.1 (CH, C-4″), 130.3 (C, C-1'), 124.1 (CH, C-2', C-6'), 121.9 (CH, C-3″), 120.8 (CH, C-5″), 115.7 (CH, C-3', C-5'), 115.6 (C, CN), 115.4 (C, CN), 93.1 (C, C-5), 85.8 (C, C-3), 35.4 (CH$_2$, -SCH$_2$-), 24.0 (CH$_3$, Methylpyridine); APCI-HRMS m/z: calculated for C$_{20}$H$_{16}$N$_5$OS [M + H] + 374.1070, found 374.1061; Purity (HPLC, λ = 254): 100%

2-amino-4-(4-hydroxyphenyl)-6-((3-methylbenzyl)thio)pyridine-3,5-dicarbonitrile (6n)

Prepared as for 6c from 4-hydroxybenzaldehyde (0.612 g, 5 mmol) and 1-(bromomethyl)-3-methylbenzene (0.678 ml, 5 mmol) to yield 6n which was recrystallised from MeOH as light yellowish powder (0.532 g, 28.6%): Rf: 0.75 (PE/EtOAC 1:1); mp: 224.9–226.1 °C;^{1}H NMR (600 MHz, DMSO) δ 10.06 (s, 1H), 7.39–7.33 (m, 2H), 7.33–7.26 (m, 2H), 7.20 (t, $J = 7.6$ Hz, 1H), 7.06 (d, $J = 7.5$ Hz, 1H), 6.93–6.85 (m, 2H), 4.45 (s, 2H), 2.28 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.3 (C, C-6), 159.7 (C, C-2), 159.4 (C, C-4), 158.4 (C, C-4'), 137.6 (C, C-1″), 137.3 (C, C-3″), 130.3 (C, C-1'), 129.9 (CH, C-2', C-6'), 128.3 (CH, C-5″), 127.9 (CH, C-4″), 126.4 (CH, C-2″), 124.2 (CH, C-6″), 115.6 (CH,C-3', C-5'), 115.6 (C, CN), 115.4 (C, CN), 93.1 (C, C-5), 85.7 (C, C-3), 33.2 (CH$_2$, -S-CH$_2$-), 20.9 (CH$_3$, Methylbenzyl); APCI-HRMS m/z: calculated for C$_{21}$H$_{17}$N$_4$OS [M + H] + 373.1118, found 373.1111; Purity (HPLC, λ = 254): 100%

3-(((6-amino-3,5-dicyano-4-(3-methoxyphenyl)pyridin-2-yl) thio)methyl)benzoic acid (6o)

Prepared as for 6c from 3-methoxybenzaldehyde (0.608 ml, 5 mmol) and 3-

(bromomethyl)benzoic acid (1.083 g, 5 mmol) to yield 6o which was recrystallised from MeOH as cream white powder (0.490 g, 23.5%):Rf: 0.70 (DCM/PE/EtOAc 10:1:1); mp: 240.0–241.1 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.04 (s, 1H), 7.83 (d, $J = 7.7$ Hz, 1H), 7.69 (d, $J = 7.6$ Hz, 1H), 7.48–7.42 (m, 1H), 7.37 (t, $J = 7.7$ Hz, 1H), 7.14–7.04 (m, 3H), 4.57 (s, 2H), 3.79 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.0 (C, COOH), 159.5 (C, C-6), 159.0 (C, C-2), 158.2 (C, C-3'), 137.5 (C, C-4), 135.2 (C, C-1', C-3″), 132.5 (C,C-4″), 130.1 (C, C-5', C-1″), 130.0 (CH, C-2″), 128.2 (CH, C-5″, C6″), 120.5 (CH, C-6'), 115.9 (CH, C-4'), 115.2 (C, CN), 115.2 (C, CN), 114.0 (CH, C-2'), 93.3 (C, C-5), 86.1 (C, C-3), 55.3 (CH$_3$, OCH$_3$), 32.9 (CH$_2$, -SCH$_2$-); APCI-HRMS m/z: calculated for C$_{22}$H$_{16}$N$_4$O$_3$S [M + H] + 417.1016, found 417.1012; Purity (HPLC, λ = 254): 100%

2-amino-4-(4-fluorophenyl)-6-(((6-methylpyridin-2-yl) methyl)thio)pyridine-3,5-dicarbonitrile (6p)

Prepared as for 6c from 4-fluorobenzaldehyde (0.536 ml, 5 mmol) and 2-(bromomethyl)-6-methylpyridine (0.932 g, 5 mmol) to yield 6o which was recrystallised from MeOH as white powder (0.222 g, 11.8%): Rf: 0.84 (PE:EtOAC 1:1); mp: 216.8–216.9 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.33 (s, 2H), 7.62 (dt, $J = 8.9$, 6.5 Hz, 3H), 7.48–7.36 (m, 3H), 7.14 (d, $J = 7.7$ Hz, 1H), 4.55 (s, 2H), 2.45 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.2 C, C-6), 164.0 (C, C-2), 162.3 (C, C-4), 159.5 (C, C-2″), 157.8 (C, C-6″), 157.5 (CH, C-5″), 155.8 (CH, C-3″), 137.1 (CH, C-4″), 131.1 (d, $J = 8.8$ Hz,C, C-1'), 131.1 (C, C-6'), 130.3 (d, $J = 3.0$ Hz, CH, C-2', C-6'), 121.9 (C, CN), 120.8 (C, CN), 115.8 (d, $J = 22.0$ Hz, C, C-4'), 115.2 (d, $J = 11.2$ Hz, CH, C-3', C-5'), 93.3 (C, C-5), 86.1 (C, C-5), 35.4 (CH$_2$, -SCH$_2$-), 24.0 (CH$_3$, Methylpyridine); APCI-HRMS m/z: calculated for C$_{20}$H$_{15}$FN$_5$S [M + H] + 376.1027, found 376.1039; Purity (HPLC, λ = 254): 100% General procedure for the synthesis of 6q–6s and 7a:

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-(2-hydroxyethoxy)phenyl)pyridine-3,5-dicarbonitrile (6q)

Cyanothioacetamide (0.503 g, 5 mmol) was dissolved in 10 ml EtOH. The reaction mixture was then stirred for 15–20 min before adding 4-(2-hydroxyethoxy)benzaldehyde (0.696 ml, 5 mmol) while stirring at room temperature. After obtaining a homogeneous mixture, MN (0.315 ml, 5 mmol) was added and the mixture stirred until it became homogeneous again, then left to stand at room temperature for 12–14 h. The mixture was then diluted with an equal volume of DMF and 10% aqueous KOH (2.8 ml, 5 mmol) and left to stand for 24 h. 4-(Chloromethyl)-2-(4-chlorophenyl)thiazole (1.221 g, 5 mmol) was added to the mixture and continuously stirred for 3 h, after which 10% aqueous KOH (5.6 mL, 10 mmol) was added again. After 2–4 h, ice was added and the resulting precipitate was

filtered off, washed with distilled H_2O, EtOH and hexane, dried (30 °C) and recrystallised from MeOH to yield the title compound **6q** as light brown powder (1.355 g, 52.1%): Rf: 0.82 (DCM/PE/EtAc 10:1:1); mp: 162.1–163.5 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.97–7.92 (m, 2H), 7.89 (s, 1H), 7.59–7.53 (m, 2H), 7.50–7.42 (m, 2H), 7.15–7.02 (m, 2H), 4.86 (t, $J = 5.5$ Hz, 1H), 4.64 (s, 2H), 4.08 (t, $J = 5.0$ Hz, 2H), 3.74 (dd, $J = 10.1, 5.2$ Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 165.8 (C, C-2″), 165.6 (C, C-6), 160.3 (C, C-2), 159.7 (C, C-4'), 158.1 (C, C-4), 152.4 (C, C-4″), 134.8 (C, C-1‴), 131.6 (C, C-4‴), 130.1 (C, C-1'), 129.2 (CH, C-3‴, C5‴), 127.7 (CH, C-2', C-6'), 125.7 (CH, C-2‴, C-6‴), 118.7 (CH, C-5″), 115.3 (CH, C-3', C-5'), 115.3 (C, CN), 114.5 (C, CN), 93.4 (C, C-5), 85.9 (C, C-3), 69.7(CH$_2$, O$\underline{C}$H$_2$CH$_2$OH), 59.4 (CH$_2$, OCH$_2$$\underline{C}H_2$OH), 29.2 (CH$_2$, -S$\underline{C}H_2$-); APCI-HRMS *m/z*: calculated for $C_{25}H_{19}ClN_5O_2S_2$ [M + H] + 520.0663, found 520.0651; Purity (HPLC, λ = 254): 100%

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-hydroxyphenyl)pyridine-3,5-dicarbonitrile (6r)

Prepared as for **6q** from 4-hydroxybenzaldehyde (0.612 g, 5 mmol) and 4-(chloromethyl)-2-(4-chlorophenyl)thiazole (1.220 g, 5 mmol) to yield **6r** which was recrystallised from MeOH as light pink powder (1.203 g, 50.6%): Rf: 0.81(DCM/PE/EtAc 10:1:1); mp: 233.8–234.6 °C; ^{1}H NMR (600 MHz, DMSO) δ 10.06 (s, 1H), 7.97–7.92 (m, 2H), 7.90 (s, 1H), 7.58–7.52 (m, 2H), 7.39–7.33 (m, 2H), 6.92–6.87 (m, 2H), 4.63 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 165.9 (C, C-2″), 165.6 (C, C-6), 159.8 (C, C-2), 159.4 (C, C-4), 158.4 (C, C-4'), 152.5 (C, C-4″), 134.8 (C, C-1‴), 131.6 (C, C-4‴), 130.2 (C, C-1'), 129.3 (CH, C-2', C-6'), 127.8 (CH, C-2‴, C-6‴), 124.1 (CH, C-3‴, C-5‴), 118.8 (CH, C-3', C-5'), 115.6 (CH, C-5″), 115.5 (C, CN), 115.4 (C, CN), 93.3 (C, C-5), 85.8 (C, C-3), 29.3 (CH$_2$, -S$\underline{C}$H$_2$-); APCI-HRMS *m/z*: calculated for $C_{23}H_{15}ClN_5OS_2$ [M + H] + 476.0401, found 476.0386; Purity (HPLC, λ = 254): 100%

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (6 s)

Prepared as for **6q** from 4-methoxybenzaldehyde (0.608 ml, 5 mmol) and 4-(chloromethyl)-2-(4-chlorophenyl)thiazole (1.222 g, 5 mmol) to yield **6s** which was recrystallised from MeOH as cream white solid (0.850 g, 34.9%): Rf: 0.72 (DCM/PE/EtAc 10:1:1); mp: 238.2–240.4 °C; ^{1}H NMR (600 MHz, DMSO) δ 8.06 (s, 2H), 7.96–7.92 (m, 2H), 7.89 (s, 1H), 7.58–7.53 (m, 2H), 7.50–7.46 (m, 2H), 7.13–7.08 (m, 2H), 4.64 (s, 2H), 3.83 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 165.8 (C, C-2″), 165.6 (C, C-6), 160.8 (C, C-2), 159.7 (C, C-4'), 158.0 (C, C-4), 152.4 (C, C-4″), 134.8 (C, C-1‴), 131.6 (C, C-4‴), 130.1 (C, C-1'), 129.2 (CH, C-2', C-6'), 127.7 (CH, C-‴3, C-5‴), 125.7 (CH, C-2‴, C-6‴), 118.7 (CH, C-5″), 115.4 (CH, C-3', C-5'), 115.3 (C, CN), 114.0 (C, CN), 93.4 (C, C-5), 85.9 (C, C-3), 55.3 (CH$_3$, O$\underline{C}$H$_3$), 29.3 (CH$_2$, -S$\underline{C}$H$_2$-); APCI-HRMS *m/z*: calculated for $C_{24}H_{17}ClN_5OS_2$ [M + H] + 490.0558, found 490.0538; Purity (HPLC, λ = 254): 100%

3,6-diamino-5-cyano-4-(4-fluorophenyl)thieno[2,3-b]pyridine-2-carboxamide (7a)

Prepared as for **6q** from 4-fluorobenzaldehyde (0.536 ml, 5 mmol) and 2-bromoacetamide (0.693 g, 5 mmol) to yield compound **7a** which was recrystallised from MeOH as yellow to greenish solid (0.687 g, 42.0%): Rf: 0.77 (DCM/PE/EtAc 10:1:1); mp: 257.5–260.7 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.72–7.53 (m, 2H), 7.52–7.39 (m, 2H), 7.33 (s, 2H), 6.99 (s, 2H), 5.63 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.7 (C, CO), 163.6 (C, C-6), 163.4 (C, between position 1 & 7 of thienopyridine), 162.0 (C, C-4), 158.3 (C, C-3), 151.3 (C, C-2), 146.1 (C, C-1'), 130.60 (d, $J = 8.5$ Hz, C-2',C-6'), 129.91 (d, $J = 3.1$ Hz), C-3', C-5'), 116.22–115.68 ((m), C-4'), 114.2 (C, between position 3 & 4 of thienopyridine), 93.3 (C, CN), 90.4 (C-C5); APCI-HRMS *m/z*: calculated for $C_{15}H_{11}FN_5OS$ [M + H] + 328.0663, found 328.0652; Purity (HPLC, λ = 254): 100% General procedure for synthesis of **7b** and **7c**

3,6-diamino-5-cyano-4-(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (7b)

Prepared by dissolving 2-((6-amino-3,5-dicyano-4-(4-methoxyphenyl)pyridin-2-yl)thio) acetamid (**6a**) (0.203 g, 0.598 mmol) in 10 ml DMF. Two or three drops of 10% aqueous KOH was then added to the solution. The reaction mixture was maintained at room temperature for 24 h. Product was precipitated by adding ice. The resulting precipitate was filtered off, washed with distilled H_2O, EtOH and hexane, dried (30 °C) and recrystallised from MeOH to yield **7b** as yellow powder (0.054 g, 31.8%): Rf: 0.75 (DCM/PE/EtAc 10:1:1); mp: 263.9–264.9 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.42 (d, $J = 8.4$ Hz, 2H), 7.30 (s, 2H), 7.14 (d, $J = 8.4$ Hz, 2H), 6.99 (s, 2H), 5.70 (s, 2H), 3.85 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 166.8 (C, CO), 163.4 (C, C-6), 160.2 (C, C-4'), 158.5 (C, between position 1 & 7 of thienopyridine), 152.3 (C, C-4), 146.3 (C, C-3), 129.7 (CH, C-2', C-6'), 125.5 (C, C-2), 116.0 (C, C-1'), 114.4 (C, between position 3 & 4 of thienopyridine), 114.3 (CH, C-3',C-5'), 92.9 (C, CN), 90.5 (C, C-5), 55.3 (CH$_3$, O$\underline{C}$H$_3$); APCI-HRMS *m/z*: calculated for $C_{16}H_{14}N_5O_2S$ [M + H] + 340.0863, found 340.0853; Purity (HPLC, λ = 254): 93.8%

3,6-diamino-4-(benzo[d][1,3]dioxol-5-yl)-5-cyanothieno[2,3-b]pyridine-2-carboxamide (7c)

Prepared as for **7b** from 2-((6-amino-4-(benzo[d][1,3]dioxol-5-yl)-3,5-dicyanopyridin-2-yl)thio)acetamide (**6g**) (0.134 g, 0.379 mmol) to yield **7c** which was washed with distilled H_2O and hexane and dried without further purification to yield an orange solid

(0.034 g, 19.3%): Rf: 0.56 (DCM/PE/EtOAc 10:1:1); mp: 296.82 °C; ^{1}H NMR (600 MHz, DMSO) δ 7.28 (s, 1H), 7.15–7.03 (m, 1H), 7.03–6.91 (m, 1H), 6.06 (dd, $J = 118.5$, 114.5 Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.8 (C, CO), 158.4 (C, C-6), 152.0 (C, between position 1 & 7 of thienopyridine), 148.5 (C, C-4), 147.5 (C, C3', C4'), 146.3 (C, C-3), 126.8 (C, C-1'), 122.0 (C, C-2), 115.9 (C, between position 3 & 4 of thienopyridine), 114.4 (CH, C-6'), 108.8 (CH, C-2'), 108.7 (C, CN), 101.7 (CH, C-5'), 92.8 (CH$_2$, at dioxol), 90.5 (C, C-5); APCI-HRMS m/z: calculated for $C_{16}H_{12}N_5O_3S$ [M + H] + 354.0655, found 354.0647; Purity (HPLC, λ = 254): 62.6%

3,6-diamino-5-cyano-4-(4-methoxyphenyl)thieno[2,3-b]pyridine-2-carboxamide (7d) Prepared by refluxing a solution of 2-((6-amino-3,5-dicyano-4-(3-hydroxyphenyl)pyridin-2-yl)thio)acetamide (**6f**) (0.201 g, 0.518 mmol) and KOH (0.080 g, 1.426 mmol) in EtOH (10 ml) for 6 h. After cooling, ice-cold water was added to the reaction mixture to precipitate the product. The resulting precipitate was filtered off, washed with distilled H$_2$O, EtOH and hexane, dried (30 °C) and recrystallised from MeOH to yield **7d** as greenish solid (0.163 g, 66.4%): Rf: 0.15 (DCM/PE/EtOAc 10:1:1); mp: 285.12 °C; ^{1}H NMR (600 MHz, DMSO) δ 9.97 (s, 1H), 7.39 (t, $J = 7.7$ Hz, 1H), 7.31 (s, 2H), 6.98 (s, 3H), 6.90–6.76 (m, 2H), 5.70 (s, 2H); ^{13}C NMR (151 MHz, DMSO) δ 166.7 (C,CO), 163.3 (C, C-6), 158.4 (C, C, between position 1 & 7 of thienopyridine), 157.6 (C, C-3'), 152.2 (C, C-4), 146.0 (C, C-1'), 134.8 (C, C-3), 130.4 (CH, C-5'), 118.3 (C, C-2), 116.9 (C, C between position 3 & 4 of thienopyridine), 115.8 (CH, C-6'), 114.7 (CH, C-4'), 113.9 (CH, C-2'), 93.0 (C, CN), 89.9 (C, C-5); APCI-HRMS m/z: calculated for $C_{15}H_{12}N_5O_2S$ [M + H] + 326.0706, found 326.0690; Purity (HPLC, λ = 254): 100%

Biology

In vitro evaluation

Materials and methods All reagents were commercially available and purchased from various manufacturers. Radioligands [^{3}H]DPCPX (120 Ci/mmol) and [^{3}H]NECA (27.1 Ci/mmol) were obtained from PerkinElmer. Adenosine deaminase from bovine spleen (157 units/mg, 5.9 mg/ml, or 130 units/mg, 6.8 mg/ml), CPA, DPCPX, istradefylline, caffeine and anhydrous magnesium chloride (MgCl$_2$) were all obtained from Sigma-Aldrich. Radioactivity was counted by a PerkinElmer Tri-CARB 2810 TR liquid scintillation analyser.

Ethics The collection of tissue samples for the A$_1$ and A$_{2A}$ AR radioligand binding assays were approved by the Health Sciences Ethics Office for Research, Training and Support,

North-West University (NWU-00418-21-A5) and were performed in accordance with the guidelines of the South African National Standard (SANS) document (The care and use of animals for scientific purposes).

Tissue samples Male Sprague–Dawley rats whole brain membranes (including striata and excluding cerebellum and brain stem) and rat striatal membranes were used for the A$_1$ and A$_{2A}$ AR radioligand binding assays, respectively, and prepared as described in literature [53]. Upon dissection, the tissue samples were snap frozen with liquid nitrogen and stored at −70 °C. The samples were later thawed on ice, weighed and disrupted for 90 s (whole brain) or 30 s (striata) with the aid of a Polytron homogeniser (model: Polytron PT 10-35 GT) in 10 volumes of ice-cold 50 mM Tris buffer (pH 7.7 at 25 °C). The resulting homogenate was centrifuged at 20,000 g for 10 min at 4 °C and the pellet was resuspended in 10 volumes of ice-cold Tris buffer, again with the aid of a Polytron homogeniser as above. The resulting suspension was recentrifuged and the pellet obtained was suspended in Tris buffer (pH 7.7 at 25 °C) to a volume of 5 mL/g original tissue weight. The whole brain and striatal membranes were aliquoted into microcentrifuge tubes and stored at −70 °C until needed. Protein concentration of the rat brain tissues was determined according to the Bradford protein assay, using bovine serum albumin as reference standard [74].

Adenosine A$_1$ and A$_{2A}$ receptor radioligand binding assays The degree of binding affinity the test compounds showed toward A$_1$ and A$_{2A}$ ARs were determined through radioligand binding assays, as previously described in literature [53, 74–76]. The A$_1$ AR radioligand binding assay used rat whole brain membranes (expressing A$_1$ ARs) and 0.1 nM 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine ([^{3}H] DPCPX) as radioligand while the A$_{2A}$ AR radioligand binding assay used rat striatal membranes (expressing A$_{2A}$ ARs) and 4 nM [^{3}H]-5′-N-ethylcarboxamidoadenosine ([^{3}H] NECA) as radioligand. In A$_{2A}$ AR radioligand binding assays, 50 nM CPA was also added to reduce the binding of [^{3}H]NECA to adenosine A$_1$ receptors and 10 mM MgCl$_2$ was also included to increase radioligand binding and decrease non-specific binding. Adenosine deaminase was included in both A$_1$ and A$_{2A}$ binding assay to inactivate any remaining endogenous adenosine. The incubations were carried out in 4 mL polypropylene tubes that were precoated with Sigmacote (Sigma–Aldrich). All incubations were prepared with 50 mM Tris buffer (pH 7.7 at 25 °C) to a volume of 1 mL. Each incubation of the A$_1$ assay consisted of: (i) test compound (10 μL), (ii) 0.1 nM [^{3}H]DPCPX (radioligand solution, 100 μL) and (iii) 120 μg rat whole brain membranes and 0.1 units/mL adenosine deaminase (membrane suspension, 890 μL). Whereas every incubation

of the A_{2A} assay consisted of: (i) 120 μg rat striatal membranes, 0.2 units/mL adenosine deaminase. 10 mM $MgCl_2$ (membrane suspension, 790 μL), (ii) test compound (10 μL), (iii) 50 nM CPA (100 μL) and (iv) 4 nM [^{3}H]NECA (radioligand solution, 100 μL). The final volume of all incubations contained 1 mL of 50 mM Tris.HCl buffer (pH 7.7, 25 °C) and 1% DMSO. (DMSO was used to prepare all stock solutions of the test compounds.) The incubations were vortexed and incubated for 60 min at 25 °C in a shaking waterbath. Half an hour after incubation was started, the incubations were vortexed again. The incubations were terminated via filtration through a prewetted 2.5 cm Whatman glass microfiber filter (grade GF/B) under reduced pressure using a Hoffeler vacuum system. The tubes were washed twice with 4 mL ice-cold Tris buffer and the filters were washed once more with 4 mL ice-cold Tris buffer. The damp filters were place in scintillation vials and 4 mL of scintillation fluid (Filter-Count) was added. The vials were shaken and incubated for 2 h before being counted (Packard Tri-CARB 2100 TR). Non-specific binding of [^{3}H]DPCPX and [^{3}H]NECA for the radioligand binding assays were defined as binding in the presence of 100 μM CPA or 10 μM DPCPX. Specific binding was defined as the total binding minus the non-specific binding.

GTP shift assays The type of binding affinity at the rat A_1 AR displayed by test compounds was determined via a GTP shift assay, as described in literature [52–54, 77]. The membrane preparation was performed under the same conditions as described above for the adenosine A1 receptor radioligand binding assay (see Tissue samples). A GTP shift assay follows similar method as A_1 AR radioligand binding assay, but additionally 100 μM GTP was added. GTP is thought to act by uncoupling the receptors from their G-proteins which causes agonists of the receptor to lose binding affinity [78]. The incubations were carried out in 4 mL polypropylene tubes that were precoated with Sigmacote (Sigma–Aldrich). All incubations were prepared with 50 mM Tris buffer (pH 7.7 at 25 °C) to a volume of 1 mL. Each incubation (i) test compound (10 μL), (ii) 0.1 nM [^{3}H]DPCPX (radioligand solution, 100 μL), (iii) 0.1 mM GTP and (iv) 120 μg rat whole brain membranes and 0.1 units/mL adenosine deaminase (membrane suspension, 790 μL). Non-specific binding was defined as binding in the presence of 10 μM DPCPX.

Statistical data analyses

All statistical data analyses were carried out with Microsoft Excel and GraphPad Prism Software. Sigmoidal dose response curves, from which IC_{50} (half maximal inhibitory concentration) values were calculated, were obtained by plotting the specific binding against the logarithm of the test compounds' concentrations. Subsequently, the IC_{50} values were used to calculate the Ki values for the competitive inhibition of [^{3}H]DPCPX ($K_d = 0.36$ nM) against rat whole brain membranes and [^{3}H]NECA ($K_d = 15.3$ nM) against rat striatal membranes by the test compounds by means of the Cheng–Prusoff equation. All incubations were carried out in triplicate and the K_i values are expressed as the mean ± standard error of mean (SEM). GTP shifts were calculated by dividing the Ki values of compounds reported in the presence of GTP by the Ki values obtained in the absence of GTP.

Compliance with ethical standards

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