Design, synthesis and evaluation of amino-3,5-dicyanopyridines and thieno[2,3-b]pyridines as ligands of adenosine A₁ receptors for the potential treatment of epilepsy

Gaofenngwe Nkomba¹ · Gisella Terre'Blanche¹ ² · Helena D. Janse van Rensburg ¹ · Lesetja J. Legoabe¹

Received: 29 March 2022 / Accepted: 7 May 2022 / Published online: 24 May 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

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 A₁ agonists that could be used in pharmaco-resistant epilepsy (PRE). Compound 7c (thieno[2,3-b]pyridine derivative), displayed good binding affinity to the rA₁ 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 rA₁/rA₂A ARs with cyclisation was revealed. Amino-3,5-dicyanopyridines exhibited greater affinity towards rA₁ ARs ($K_i < 10$ nM) than rA₂A. Compound 6c had the best rA₁ affinity ($K_i = 0.076$ nM). Novel compounds (6d, 6k, 6l, 6m, 6n, 6o, 6p) were highly selective towards rA₁ ARs ($K_i$ between 0.179 and 21.0 nM). Based on their high selectivity for A₁ 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 rA₁ 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).

Keywords Amino-3,5-dicyanopyridines · Thieno[2,3-b]pyridines · Intramolecular cyclisation · Adenosine A₁/A₂A receptors · Epilepsy

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00044-022-02908-9.

Helena D. Janse van Rensburg heleenvanrens@gmail.com

1 Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa
2 Pharmaceutical Chemistry, School of Pharmacy, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa

Abbreviations

| Abbreviation | Comment |
|--------------|---------|
| $[^3]$H      | 1,3-$[^3]$H-dipropyl-8-cyclopentylxanthine |
| DPCPX        | |
| $[^3]$H      | $[^3]$H-5′-N-ethylcarboxamidoadenosine |
| NECA         | carbon-13 |
| $^{13}$C     | hydrogen-1 / protium |
| A₁ AR        | adenosine A₁ receptor subtype |
| A₂A AR       | adenosine A₂A receptor subtype |
| A₂B AR       | adenosine A₂B receptor subtype |
| A₃ AR        | adenosine A₃ receptor subtype |
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 A1, A2A, A2B and A3 [2] which have been linked to both inhibition (A1 and A3) and activation (A2A and A2B) 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 pathophysiological 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 ischaemia, seizures, or trauma to prevent neuronal injury [10]. The neuroprotective effects of adenosine may be due to stimulation of A1 receptors and blockade of A2A receptors [11]. Therefore, ARs are potential therapeutic targets for treatment 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]. Currently 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. suppression of seizures) [16].

Approximately one-third of epileptic patients on treatment remain poorly controlled [17]. Pharmacoresistance 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 seizures 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 A1 ARs [3]. Firstly, the released adenosine binds to presynaptic A1 receptors, which blocks the influx of Ca^{2+} through voltage-dependent calcium channels leading to inhibition of glutamate release, and hence, decreased excitation of postsynaptic glutamate receptors [11, 21]. Secondly, postsynaptic activation of A1 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 A1 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 A1 AR agonists that may be useful as antiepileptic agents. Initially the approach for discovering AR agonists as antiepileptics has been restricted to modification 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 A1 AR selective 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 cardiovascular 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 A1 AR agonists for the potential management 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 compounds 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 addition to treatment of CNS disorders [42].

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

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](image1)

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

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-dicyanopyridine 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 A1 AR agonists for the potential treatment of neurological conditions, such as epilepsy. Modifications at R2 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 A1 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 (R2) 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 A1 and A2A 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 (Et3N) 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-4H-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 alkylation 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 carbonyl condensation reactions. The target thieno[2,3-b]pyridines were synthesised was based on intermediates with A1 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 (-CONH2) 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 66q–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 (1H) and carbon-13 (13C) nuclear magnetic resonance (NMR) spectra, mass spectroscopy and HPLC (see supplementary material). It should be noted that, protons on the NH2-group (e.g., 6l) and the OH-group (e.g., 6k) are not always visible on a 1H 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 13C 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 13C 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) A1 and A2A ARs were determined by radioligand binding assays and are expressed as inhibition constant (Ki, nM) values (Table 1). All test compounds displayed specific binding values <20% at a maximum tested concentration of 100 μM (rA1 screening), and therefore, all underwent full biological assay for determination of Ki values (nM). Compounds 6a–j and 7a–d displayed specific binding values <20% at a maximum tested concentration of 100 μM (rA2A screening) and hence qualified for full rA2A radioligand binding assay, unlike compounds 6k–s with specific binding values >20%. The radioligand binding assays were validated with N6-cyclopentyladenosine (CPA) (A1 agonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (A1 antagonist), istradefylline (A2A antagonist) and caffeine (A1/A2A 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 R2 and aryl positions of the test compounds to assess how different substituents can influence both rA1 and rA2A ARs binding affinity as well as selectivity. As shown in Table 1, all test compounds displayed greater affinity toward the rA1 than rA2A AR. Compound 6c had the best rA1 AR affinity (Ki = 0.076 nM) of the present series. The latter compound together with 6b displayed better rA2A AR affinity than the other test compounds with Kj values of 48.3 nM (6c) and 48.0 nM (6b), respectively, but remain selective for the rA1 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 rA1 and rA2A AR affinity from the open ring structures to the closed ring structures. The only thieno[2,3-b]pyridine derivative that showed moderately good rA1 AR affinity is compound 7c (rA1/Ki = 61.9 nM). The general poor activity of thieno[2,3-b]pyridines relative to amino-3,5-dicyanopyridines 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](attachment:Scheme_3.pdf)
Table 1 $K_i$ values of test compounds and reference compounds at rat A₁ and A₂A ARs

| # | R²  | Aryl | $K_i \pm$ SEM (nM)$^a$ (Specific binding (%))$^b$ | $rA_1^c$ vs 1 nM [³H]DPCPX | $rA_2^d$ vs 4 nM [³H]NECA | $rA_1^c + 0.1$ mM GTP vs 1 nM [³H]DPCPX | GTP shift$^e$ | Sf$^f$ |
|---|-----|------|-----------------|------------------|------------------|------------------|----------|-----|
| Amino-3,5-dicyanopyridines | | | | | | | | |
| 6a | o | | 139 ± 18.8$^a$ | 1473 ± 256$^a$ | – | – | 11 |
| 6b | o | | 0.213 ± 0.019$^a$ (2.9)$^f$ | 48.0 ± 11.1$^a$ (35)$^g$ | – | – | 255 |
| 6c | o | | 0.076 ± 0.002$^a$ (0.49)$^f$ (0.21)$^h$ | 48.3 ± 10.1$^a$ (71)$^g$ (52)$^h$ | 0.069 ± 0.006$^a$ | 1 | 636 |
| 6d | o | | 10.3 ± 0.643$^a$ | 1205 ± 367$^a$ | 11.3 ± 0.663$^a$ | 1 | 117 |
| 6e | o | | 60.4 ± 3.83$^a$ | 338 ± 79.1$^a$ | – | – | |
| 6f | o | | 48.0 ± 4.36$^a$ | 751 ± 12.0$^a$ | – | – | 16 |
| 6g | o | | 26.6 ± 6.75$^a$ | 429 ± 55.0$^a$ | – | – | 16 |
| 6h | o | | 7.54 ± 0.768$^a$ (4.12)$^i$ | (581)$^j$ | – | – | |

Note: $^a$ SEM, $^b$ Specific binding, $^c$ A₁ receptor, $^d$ A₂A receptor, $^e$ GTP shift, $^f$ Sf

3,5-Dicyanopyridine derivatives (6a-6s) and Thieno[2,3-b]pyridine derivatives (7a-7d)
### Table 1 (continued)

| #  | R²    | Aryl          | 3,5-Dicyanopyridine derivatives (6a-6s) | Thieno[2,3-b]pyridine derivatives (7a-7d) |
|----|-------|---------------|----------------------------------------|------------------------------------------|
|    |       |               | Kᵢ ± SEM (nM) ± SEM | (Specific binding (%))                      |                                         |
|    |       |               | rA₁ vs 1 nM [³H]DPCPX | rA₂Δ vs 4 nM [³H]NECA | rA₁Δ + 0.1 mM GTP vs 1 nM [³H]DPCPX | GTP shift | St⁰ |
| 6i |       |               | 4.57 ± 0.284 ± 0.284 | 634 ± 94.3 ± 94.3 | – | – | 139 |
| 6j |       |               | Not determined (3.5) | 20.6 ± 6.56 ± 6.56 (15) | – | – | – |
| 6k |       |               | 8.82 ± 0.760 ± 0.760 | (22) | – | – | – |
| 6l |       |               | 21.0 ± 5.56 ± 5.56 | (27) | – | – | – |
| 6m |       |               | 0.179 ± 0.013 ± 0.013 | (80) | – | – | – |
| 6n |       |               | 0.831 ± 0.076 ± 0.076 | (35) | 1.94 ± 0.509 ± 0.509 | 2 | – |
| 6o |       |               | 1.64 ± 0.228 ± 0.228 | (25) | 2.25 ± 0.159 ± 0.159 | 1 | – |
| 6p |       |               | 0.430 ± 0.012 ± 0.012 | (30) | – | – | – |
Table 1 (continued)

| #  | R² | aryl                        | 3,5-Dicyanopyridine derivatives | Kᵢ ± SEM (nM)ᵃ (Specific binding (%))ᵇ | Thieno[2,3-b]pyridine derivatives | (7a-7d) |
|----|----|-----------------------------|---------------------------------|----------------------------------------|-----------------------------------|---------|
|    |    |                             |                                 | rₐ₁⁻ vs 1 nM [³H]DPCPX               | rₐ₂₇⁻ vs 4 nM [³H]NECA            | rₐ₁⁻ + 0.1 mM GTP vs 1 nM [³H]DPCPX | GTP shift | St⁹ |
| 6q |    |                             |                                 | 0.383 ± 0.069ᵃ (1.4)ᵇ                | (44)b                              | 1.82 ± 0.582ᵃ                         | 5       | –   |
| 6r |    |                             |                                 | 1.36 ± 0.040ᵇ (1.5)ᵇ                | (44)b                              | –                                   | –       | –   |
| 6s |    |                             |                                 | 4.06 ± 0.759ᵇ (5.0)ᵇ                | (27)b                              | –                                   | –       | –   |

**Thieno[2,3-b]pyridines**

| 7a |    |                             | 1008 ± 58.3ᵃ                     | 308 ± 93.6ᵇ                        |                                   |         |     |
| 7b |    |                             | 556 ± 28.1ᵃ                      | 561 ± 12.1ᵃ                       |                                   |         |     |
| 7c |    |                             | 61.9 ± 2.11ᵃ                     | 1062 ± 126ᵃ                      | 145 ± 28.8ᵃ                      | 2       |     |
| 7d |    |                             | 305 ± 15.3ᵃ                      | 162 ± 24.4ᵇ                      |                                   | –       | –   |
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: $rA1K_i = 4.06$ nM), benzoic acid substituent (6l: $rA1K_i = 21.0$ nM) and a carbonyl containing substituent (6a: $rA1K_i = 139$ nM) which displayed the lowest affinity for rA1 AR. In terms of selectivity, 6b also showed affinity toward the rA2A AR ($K_i = 4.06$ 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 ($6d: rA1K_i = 10.3$ nM vs 6a: $rA1K_i = 139$ nM) but had no effect on rA2A AR affinity. In general, the presence of the 3-methoxyphenyl substituent resulted in increased

### Table 1 (continued)

| # | R² | aryl | $rA1^c$ vs 1 nM [³H]DPCPX | $rA2A^d$ vs 4 nM [³H]NECA | $rA1^c + 0.1$ mM GTP vs 1 nM [³H]DPCPX | GTP shift$^e$ | SI$^f$ |
|---|---|---|---|---|---|---|---|
| Reference compounds | | | | | | | |
| CPA (A1 agonist) | | | 6.5 ± 0.4$^a$ (15.3)$^b$ (7.9)$^c$ | – | 36.5 ± 2.28$^a$ | 6 | – |
| DPCPX (A1 antagonist) | | | 0.5 ± 0.1$^a$ (0.6)$^b$ (0.3)$^c$ | – | 0.4 ± 0.032$^a$ | 1 | – |
| Istradefylline (A2A antagonist) | | | – | 3 ± 0.9$^a$ (13; 2.2)$^b$ (11.1)$^c$ | – | – | – |
| Caffeine (A1/A2A antagonist) | | | 52 800 ± 7 400$^a$ (44 000$^a$ (41 000$^a$ (26 000$^a$ | 27 800 ± 5 100$^a$ (43 000$^a$ (32 000$^a$ (22 000$^a$ (33 000$^a$ | – | 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 $rA2A K_i$ (nM) by the $rA1 K_i$ (nM)

$^a$Literature value: human adenosine A1 receptor and [³H]DPCPX; human adenosine A2A receptor and [³H]ZM241385 [56]
$^b$Literature value: rat adenosine A1 receptor and [³H]DPCPX; rat adenosine A2A receptor and [³H]ZM241385 [56]
$^c$Literature value: human adenosine A1 receptor and [³H]DPCPX; human adenosine A2A receptor and [³H]ZM241385 [79]
$^d$Literature value: human adenosine A1 receptor and [³H]DPCPX [57]

$^a$Literature value: rat adenosine A1 receptor and [³H]DPCPX [26]
$^b$Literature value: rat adenosine A1 receptor and [³H]DPCPX [75]
$^c$Literature value: rat adenosine A1 receptor and [³H]DPCPX [52]
$^d$Literature value: rat adenosine A1 receptor and [³H]DPCPX [80]
$^e$Literature value: rat adenosine A1 receptor and [³H]DPCPX [60]
$^f$Literature value: rat adenosine A1 receptor and [³H]DPCPX [62]
$^g$Literature value: rat adenosine A1 receptor and [³H]DPCPX [63]
$^h$Literature value: rat adenosine A1 receptor and [³H]DPCPX [81]
affinity for rA1 ARs but had no influence on rA2A 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 rA1 AR binding affinity.

For compounds 6e, 6m, 6n and 6q, 4-hydroxyphenyl was introduced at the R² position while maintaining almost all the same aryl groups mentioned earlier. Generally, these compounds displayed rA1 AR affinity of 1 nM or smaller (except 6e, aryl = CONH₂), with 6m (aryl = -methylpyridine) being the best with rA1/Kᵱ = 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, rA1 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 rA1 AR affinity.

Replacing 4-hydroxyphenyl with 3-hydroxyphenyl at position R² was also studied (6e vs 6f and 6m vs 6j), although a definite trend could not be observed within the limited data at hand. Comparison of 6f (3-hydroxyphenyl) to its 4-methoxyphenyl substituted counterpart 6d showed a four-fold decrease in rA1 AR affinity, although rA1 selectivity was maintained.

Comparison of 6a, 6d, 6e and 6f showed that the meta position is preferred to the para position whether OCH₃ or OH-group substitution is incorporated, and furthermore, it seems that a OCH₃-group is preferred to an OH-group.

Comparing 6l and 6k with 4-OCH₃ and 4-SCH₃ revealed that introducing a sulphur component increased binding affinity for compound 6k (Kᵱ = 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 rA1 AR affinity but had no mentionable affinity for rA2A ARs. Compound 6q (R = 4-OCH₂-CH₂OH) had the best affinity of these compounds with Kᵱ = 0.383 nM. SARs of amino-3,5-dicyanopyridines against rA1 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 rA1 ARs compared to their corresponding intermediate amino-3,5-dicyanopyridines (Fig. 4). These compounds all had a -CONH₂-group at the aryl position. The results indicate that ring closure from the intermediate open ring to fused ring structures decreased activity towards both rA1 and rA2A ARs. This corresponds with a study by [7] in which intramolecular cyclisation of the 6-amino-3,5-dicyanopyridines, specifically BAY606583 (a potent A₂B 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 rA1 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 rA1 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 rAKᵱ values of compounds reported in the presence of GTP by the rARKᵱ 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-OCH₃ group at position R²), 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 physicochemical 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 Å²). 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<sub>1</sub> AR
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 TPSA < 140Å² (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, 6d, 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 (logP) 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 rA1 ARs compared to thieno[2,3-b]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 R2 substitution, it was observed that 3- and 4-methoxyphenyl groups favoured rA1 AR binding compared to their 3- and 4-hydroxyphenyl counterparts. Looking at the aryl substitution, the methylpyridine substituent displayed the overall best rA1 AR affinity. Novel compounds (6d, 6k, 6l, 6m, 6n, 6o and 6p) proved to be highly selective with low nanomolar rA1 AR affinity (Ki values between 0.179 nM and 21.0 nM). The only thieno[2,3-b]pyridine derivative that displayed moderately good rA1 AR activity (Ki = 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 A1 ARs; however, compounds 6c, 6d and 6o (notably all containing a 3-OCH3 group at position R2) behaved as rA1 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 rA1 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 13C 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(CH3)4) as reference. Chemical shifts were reported in parts per million (ppm) in relation to the solvent peak (DMSO-d6: residual CH3 at 2.50 ppm for 1H NMR and 39.52 ppm for 13C 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 H2S 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-dihydropyrididine-2-thiolate (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 H2O, EtOH and hexane, dried (30 °C) and recrystallised from MeOH to yield the title compound 6a as whiteish 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); 13C NMR (151 MHz, DMSO) δ 168.8 (C-CON), 166.1 (C-6), 160.8 (C-2), 159.6 (C-4), 158.0 (C-14), 130.1 (C-C1), 125.7 (CH, C-2”, C-6”), 115.3 (C, C-3’, C-5’), 115.3 (C, C-5), 114.1 (C, CN), 93.3 (C, C-5), 85.9 (C, C-3), 55.3 (CH3, OCH3), 33.3 (CH2, S-CH2–); APICI-HRMS m/z: calculated for C16H17N3OS [M + H]+ = 290.12; found 290.1277; Purity (HPLC, λ = 254): 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); 13C NMR (151 MHz, DMSO) δ 166.1 (C-6), 160.8 (C-2), 159.6 (C-4), 158.1 (C, C-2”), 157.7 (C, C-2’), 155.7 (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 (CH3, OCH3), 35.3 (CH2, -SCH2–), 23.9 (CH2, -methylpyridine); APICI- HRMS m/z: calculated for C21H19N4S [M + H]+ = 340.0863, found 340.0871; Purity (HPLC, λ = 280): 100% C-4), 157.7 (C, C-2”), 155.7 (C, C-6”), 137.0 (C, C-4”), 129.9 (CH, C-5”), 121.8 (CH, C-3”), 120.4 (CH, C-6”), 115.1 (CH, C-4”), 115.1 (CH, C-2”), 115.0 (C, CN), 93.3 (C, C-5), 86.0 (C, C-5), 55.3 (CH3, OCH3), 33.3 (CH2, -SCH2–); APICI- HRMS m/z: calculated for C16H14N2O2S [M + H]+ = 280.08, found 280.0844; Purity (HPLC, λ = 254): 100% C-4), 157.7 (C, C-2”), 155.7 (C, C-6”), 137.0 (C, C-4”), 129.9 (CH, C-5”), 121.8 (CH, C-3”), 120.4 (CH, C-6”), 115.1 (CH, C-4”), 115.1 (CH, C-2”), 115.0 (C, CN), 93.3 (C, C-5), 86.0 (C, C-5), 55.3 (CH3, OCH3), 33.3 (CH2, -SCH2–); APICI- HRMS m/z: calculated for C16H14N2O2S [M + H]+ = 280.08, found 280.0844; Purity (HPLC, λ = 254): 100% C-4), 157.7 (C, C-2”), 155.7 (C, C-6”), 137.0 (C, C-4”), 129.9 (CH, C-5”), 121.8 (CH, C-3”), 120.4 (CH, C-6”), 115.1 (CH, C-4”), 115.1 (CH, C-2”), 115.0 (C, CN), 93.3 (C, C-5), 86.0 (C, C-5), 55.3 (CH3, OCH3), 33.3 (CH2, -SCH2–); APICI- HRMS m/z: calculated for C16H14N2O2S [M + H]+ = 280.08, found 280.0844; 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-bromoacetic acid (0.690 g, 5 mmol) to yield 6g which was recrystallised from MeOH as light orange solid (0.822 g, 38.6%); RF: 0.40 (DCM/MeOH: 10:1); mp: 244.5–245.1 °C; 1H 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); 13C NMR (151 MHz, DMSO) δ 168.8 (C, COOH), 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 (C, C-2'), 115.2 (C, CH-5'), 108.8, (C, CN) 108.5 (C, CN), 101.7 (CH2, C at dioxol), 93.4 (C, C-5), 86.1 (C, C-3), 33.3 (CH2, -SCH2); APCI-HRMS m/z: calculated for C16H12N5O3S [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-carboxaldehyde (0.414 g, 5 mmol) and 3-(bromomethyl)benzolic 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; 1H 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); 13C 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 (CH2, -SCH2); APCI-HRMS m/z: calculated for C19H13N4O3S [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; 1H 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 (dd, J = 8.2, 2.4, 0.7 Hz, 1H), 6.91–6.83 (m, 2H), 4.56 (s, 2H), 2.46 (s, 3H); 13C 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'), 157.0 (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 (CH2, -SCH2), 23.9 (CH3, Methylpyridine); APCI-HRMS m/z: calculated for C20H17N5O2S [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)benzolic 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; 1H 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); 13C NMR (151 MHz, DMSO) δ 166.01 (C, COOH), 136.8 (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 (CH2, -SCH2), 14.0 (CH3, SCH3); APCI-HRMS m/z: calculated for C24H20N4O2S2 [M + H]+ + 3433.0787, found 3433.0784; 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)benzolic 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; 1H 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
2-amino-4-(4-hydroxyphenyl)-6-(((6-methylpyridin-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (6m) 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 (DCM/PE/EtOAC 1:1); mp: 190.8–191.7 °C; 1H 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); 13C 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 (CN, CN), 93.1 (C, C-5), 85.8 (C, C-3), 35.4 (CH2, -SCH2-), 24.0 (CH3 Methylpyridine); APCI-HRMS m/z: calculated for C20H16Na2O2S [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; 1H 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); 13C 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 (CH2, -SCH2-), 20.9 (CH3 Methylbenzyl); APCI-HRMS m/z: calculated for C21H15F2N3S [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; 1H 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); 13C 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-4), 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 (CH3, OCH3), 32.9 (CH3, -SCH2-); APCI-HRMS m/z: calculated for C22H16F6N3S [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 6p 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; 1H 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); 13C 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 (CH3, Methylpyridine); APCI-HRMS m/z: calculated for C20H15F2N3S [M + H]+ = 376.1027, found 376.1039; Purity (HPLC, λ = 254): 100% General procedure for the synthesis of 6q–6s and 7a:

2-amino-4-((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 H₂O, 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/ EtOAc 10:1:1); mp: 162.1–163.5 °C; ¹H 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); ¹³C 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′″, C-5″″), 127.7 (CH, C-2″, C-6″″), 118.7 (CH, C-5″″), 115.3 (CH, C-3′, C-5′), 115.3 (C, CN), 114.5 (C, C-5), 85.9 (C, C-3), 69.7(CH₂, OCH₂CH₂OH), 59.4 (CH₂, OCH₂CH₂OH), 29.2 (CH₂-SCH₂); APCI-HRMS m/z: calculated for C₂₅H₁₉ClN₅O₂S₂ [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/ EtOAc 10:1:1); mp: 234.6 °C; ¹H NMR (600 MHz, DMSO) δ 7.92 (m, 2H), 7.89 (s, 1H), 7.53–7.52 (m, 2H), 7.39–7.33 (m, 2H), 6.92–6.87 (m, 2H), 4.63 (s, 2H); ¹³C NMR (151 MHz, DMSO) δ 166.9 (C, C-3″), 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′), 125.5 (C, C-2″), 121.9 (C, C-3′, C-5′), 115.6 (CH, C-5″), 115.5 (C, CN), 115.4 (C, CN), 93.3 (C, C-5), 85.9 (C, C-3), 29.3 (CH₂-SCH₂); APCI-HRMS m/z: calculated for C₂₅H₁₄ClN₅O₂S₂ [M+H] + = 528.0663, found 528.0652; Purity (HPLC, λ = 254): 100% General procedure for synthesis of 7b and 7c

2-amino-6-(((2-(4-chlorophenyl)thiazol-4-yl)methyl)thio)-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (6s) 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, 31.8%): RF: 0.72 (DCM/PE/ EtOAc 10:1:1); mp: 263.9–264.9 °C; ¹H 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); ¹³C 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 (CH₃, OCH₃); APCI-HRMS m/z: calculated for C₁₅H₁₂FN₃O₂S [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 (7a) Prepared as for 6q from 4-fluorobenzaldehyde (0.536 ml, 5 mmol) and 2-bromoacetonamide (0.693 g, 5 mmol) yield compound 7a which was recrystallised from MeOH as yellow to greenish solid (0.687 g, 42.0%): RF: 0.77 (DCM/PE/EtOAc 10:1:1); mp: 257.5–260.7 °C; ¹H 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); ¹³C 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 C₁₅H₁₁FN₃O₂S [M+H] + = 328.0663, found 328.0652; Purity (HPLC, λ = 254): 100%
(0.034 g, 19.3%); Rf: 0.56 (DCM/PE/EtOAc 10:1:1); mp: 296.82 °C; 1H 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); 13C 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-2'), 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 (CH2, at dioxol), 90.5 (C, C-5); APCI-HRMS m/z: calculated for C16H12N5O3S [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 H2O, 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; 1H 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); 13C 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 C16H12N5O3S [M + H]+ 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 [3H]DPCPX (120Ci/mmol) and [3H]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, istрадифylline, caffeine and anhydrous magnesium chloride (MgCl2) 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 A1 and A2A 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 A1 and A2A AR ligand 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 re-centrifuged 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 A1 and A2A receptor radioligand binding assays The degree of binding affinity the test compounds showed toward A1 and A2A ARs were determined through radioligand binding assays, as previously described in literature [53, 74–76]. The A1 AR radioligand binding assay used rat whole brain membranes (expressing A1 ARs) and 0.1 nM 1,3-[3H]-dipropyl-8-cyclopentylxanthine ([3H] DPCPX) as radioligand while the A2A AR radioligand binding assay used rat striatal membranes (expressing A2A ARs) and 4 nM [3H]-5'-N-ethylcarboxamidoadenosine ([3H] NECA) as radioligand. In A2A AR radioligand binding assays, 50 nM CPA was also added to reduce the binding of [3H]NECA to adenosine A1 receptors and 10 mM MgCl2 was also included to increase radioligand binding and decrease non-specific binding. Adenosine deaminase was included in both A1 and A2A binding assay to inactivate any remaining endogenous adenosine. The incubations were carried out in 4 mL polystyrene 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 A1 assay consisted of: (i) test compound (10 µL), (ii) 0.1 nM [3H]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 A2A assay consisted of: (i) 120 μg rat striatal membranes, 0.2 units/mL adenosine deaminase. 10 mM MgCl₂ (membrane suspension, 790 μL), (ii) test compound (10 μL), (iii) 50 nM CPA (100 μL) and (iv) 4 nM [³H]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 [³H]DPCPX and [³H]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 A₁ 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 A₁ 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 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 [³H]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 absence 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 IC₅₀ (half maximal inhibitory concentration) values were calculated, were obtained by plotting the specific binding against the logarithm of the test compounds’ concentrations. Subsequently, the IC₅₀ values were used to calculate the Ki values for the competitive inhibition of [³H]DPCPX (Kᵢ = 0.36 nM) against rat whole brain membranes and [³H]NECA (Kᵢ = 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 Ki 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.

**Acknowledgements** This study was funded by the North-West University (NWU). The authors wish to thank Dr. D. Otto for NMR analyses and Dr. J. Jordaan for MS analyses both from Chemical Research Beneficiation at NWU, as well as Dr R. Lemmer. for DSC analyses, Prof F. Van der Kooy for HPLC analyses and Ms S. Lowe for assistance with biological assays from the Centre of Excellence for Pharmaceutical Sciences (Pharmacen), NWU.

**Compliance with ethical standards**

**Conflict of interest** The authors declare no competing interests.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**References**

1. Sheth S, Brito R, Mukherjea D, Rybak LP, Ramkumar V. Adenosine receptors: expression, function and regulation. Int J Mol Sci. 2014;15:2024–52.
2. Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov. 2006;5:247–64.
3. Abbraccchio MP, Cattabeni F. Brain adenosine receptors as targets for therapeutic intervention in neurodegenerative diseases. Ann N Y Acad Sci. 1999;890:79–92.
4. Chen JF, Eltzschig HK, Fredholm BB. Adenosine receptors as drug targets–what are the challenges? Nat Rev Drug Discov. 2013;12:265–86.
5. Effendi WI, Nagano T, Kobyashi K, Nishimura Y. Focusing on adenosine receptor ligands. Florence: University of Florence Y Acad Sci. 1999;890:79–92.
6. Boison D. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. Neuroscientist. 2005;11:25–36.
7. Betti M. Design, Synthesis and Pharmacological evaluation of new adenosine receptor ligands. Florence: University of Florence (Thesis -PhD); 2016.
8. Klotz KN. Adenosine receptors and their ligands. Naunyn Schmiedebergs Arch Pharm. 2000;362:382–91.
9. Cunha RA. How does adenosine control neuronal dysfunction and neurodegeneration? J Neurochem. 2016;139:1019–55.
10. Benarroch EE. Adenosine and its receptors: multiple modulatory functions and potential therapeutic targets for neurologic disease. Neurology. 2008;70:231–6.
11. Wardas J. Neuroprotective role of adenosine in the CNS. Pol J Pharmacol. 2002;54:313–26.
12. Stone TW, Ceruti S, Abbracchio MP. Adenosine receptors and neurological disease: neuroprotection and neurodegeneration. Handb Exp Pharmacol. 2009;193:535–87.
13. Ribeiro J, Sebastiao A, De Mendonça A. Adenosine receptors in the nervous system: pathophysiological implications. Prog Neurobiol. 2002;68:377–92.
14. Masino SA, Kawamura MJ, Ruskin DN. Adenosine receptors and epilepsy: current evidence and future potential. Int Rev Neurobiol. 2014;119:233–55.
15. Epilepsy: a public health imperative. Summary. Geneva: World Health Organization; 2019 (WHO/MSD/MER/19.2). Licence: CC BY-NC-SA 3.0 IGO.
16. Tomé ÂR, Silva H, Cunha RA. Role of the purinergic neuromodulation system in epilepsy. Open Neurosci J. 2010;4:64–83.
17. Gouder N, Fritschi JM, Boisson D. Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. Epilepsia. 2003;44:87–85.
18. Müller CE. A1 adenosine receptors and their ligands: overview and recent developments. IL Farm. 2001;56:77–80.
19. Dal Ben D, Lambertucci C, Buccioni M, Marti Navia A, Marucci G, Spinaci A, et al. Non-Nucleoside Agonists of the Adenosine Receptors: An Overview. Pharmaceuticals. 2019;12:1–22.
20. Gao ZG, Jacobson KA. Emerging adenosine receptor agonists: an update. Expert Opin Emerg Drugs. 2011;16:597–602.
21. Jacobson KA, Van Galen PJ, Williams M. Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. J Med Chem. 1992;35:407–22.
22. Knutsen LJS, Lau J, Sheardown MJ, Eskesen K, Thomsen C, Weis JU, et al. Anticonvulsant Actions of Novel and Reference Adenosine Agonists. In: Belardinelli L, Pellegr A, editors. Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology. Norwell, MA USA: Kluwer Academic Publishers; 1995. p. 479–87.
23. Jacobson KA, Tosh DK, Jain S, Gao ZG. Historical and Current Adenosine Receptor Agonists in Preclinical and Clinical Development. Front Cell Neurosci. 2019;13:1–17.
24. Alinaghizadeh F, Zahedifar M, Seifi M, Sheibani H. Cascade synthesis of thiou [2, 3-b] pyridines by using intramolecular cyclization reactions of 3-cyano-2-(organymethylthio) pyridines. J Braz Chem Soc. 2016;27:663–9.
25. Litvinov VP, Dotsenko VV, Krivokolyosko SG. Thiopropylpyridines: synthesis, properties, and biological activity. Russ Chem Bull. 2005;54:864–904.
26. Al-Trawneh SA, El-Abadleh MM, Zahra JA, Al-Taweel SA, Zani F, Incerti M, et al. Synthesis and biological evaluation of tetracyclic thiopyridopyrimidines as antibacterial and antitumor agents. Bioorg Med Chem. 2011;19:2541–8.
27. Amorim R, de Meneses MDF, Borges JC, da Silva Pinheiro LC, Caldas LA, Cunha JF, et al. Thiou [2, 3-b] pyridine derivatives: a new class of antiviral drugs against Mayaro virus. Arch Virol. 2017;162:1577–87.
28. Bernardino AM, Pinheiro LC, Ferreira VF, Azevedo AR, Carneiro J, Souza TM, et al. Synthesis and antiviral activity of new 4-(phenylamino) thiou [2, 3-b] pyridine derivatives. Heterocycl Commun. 2004;10:407–10.
29. Adachi I, Yamasori T, Hiramatsu Y, Sakail K, Mihara S-I, Kawakami M, et al. Studies on dihydroxypropyridines. III. Synthesis of 4, 7-dihydrothiobenzotriazole-2-[2,3-b]pyridines with vasodilator and anti-hypertensive activities. Chem Pharm Bull. 1988;36:4389–402.
30. Bhakker RH, Jain MR, Gadad RA, Prajapati VM, Patel DN, Gupta AA, et al. Synthesis and antidiabetic activity of 2, 5-disubstituted-3-imidazol-2-yl-pyrido [2, 3-b] pyridines and thiou [2, 3-b] pyridines. Bioorg Med Chem. 2007;15:6782–95.
31. Liao H, Li Y, Wang X-Y, Wang B, He H-Y, Liu J-Y, et al. Synthesis, preliminary structure–activity relationships, and in vitro biological evaluation of 6-aryl-3-amino-thiou [2, 3-b] pyridine derivatives as potential anti-inflammatory agents. Bioorg Med Chem Lett. 2013;23:2349–52.
32. Ouf SA, Gaber HM. New fused pyridines: Synthesis of certain pyridothienopyrimidine derivatives as anti-dermatophytic agents. Afrinadif: Rev de quimica teorica y aplicada. 2005:62:337–45.
33. Masch A, Nasereddin A, Alder A, Bird MJ, Schweda SI, Preu L, et al. Structure-activity relationships in a series of antipsodalidal thiou [2,3-b]pyridines. Malar J. 2019;18:1–10.
34. Hassan AY, Surt MG, Said MM, El-Sebae S. Utility of thiou [2,3-b] pyridine derivatives in the synthesis of some condensed heterocyclic compounds with expected biological activity. Univers Org Chem. 2013;1:1–15.
35. Saku O, Saki M, Kurokawa M, Ikeda K, Takizawa T, Usaka N. Synthetic studies on selective adenosine A2A receptor antagonists: Synthesis and structure–activity relationships of novel benzofuran derivatives. Bioorg Med Chem Lett. 2010;20:1090–3.
36. Dyachenko IV, Dyachenko VD, Dorovatovskii PV, Khrutsev VN, Nenadjenko VG. Multicomponent Synthesis of 4-Alkyl(Aryl, Hetaryl)‐2‐alkoxycarbonyl(aroyl, carbamoyl)‐ 3,6‐diamino‐5‐cyanopyridothienopyrimidine derivatives as anti-dermatophytic agents. Bioorg Med Chem Lett. 1998;8:345–49.
37. Artemov V, Ivanov V, Koshkarov A, Shestopalov A, Litvinov V. Synthesis of pyrido [3'2',4']pyrimidines: Synthesis and characterization of several new fused pyridine heterocycles. Heterocycl Chem. 2005;18:405–13.
38. Liu H, Zaplishnyy V, Mikhaylichenko L. Facilitating Students’ Review of the Chemistry of Nitrogen-Containing Heterocyclic Compounds and Their Characterization through Multistep Synthesis of Thiou [2, 3-b] Pyridine Derivatives. J Chem Educ. 2016;93:1785–7.
