Evaluation of chemical constituents of Rooibos (Aspalathus linearis) and Honeybush (Cyclopia intermedia) as adenosine A1/A2A receptor ligands

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Title

Evaluation of chemical constituents of Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*) as adenosine A_1/A_{2A} receptor ligands

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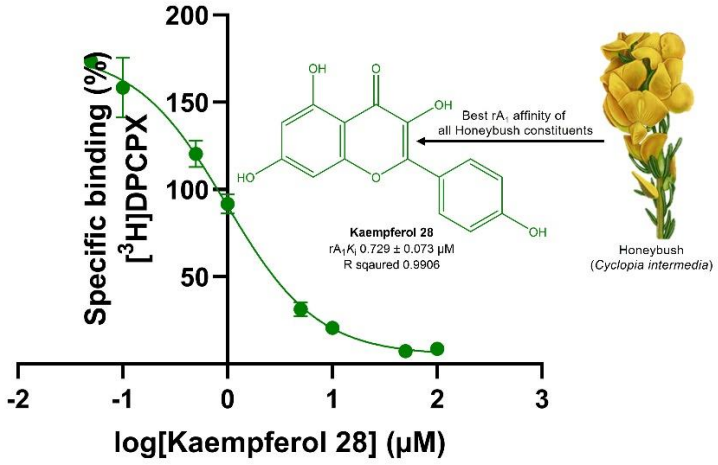
Abstract

Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*) are popular tisanes in South Africa and are of growing interest due to the wide variety of flavonoids and other phytochemicals they contain. Despite their history as herbal teas and traditional medicines, the chemical constituents of these tisanes have yet to be studied for their effects on adenosine receptors. Flavonoids have previously shown promising affinity toward the adenosine receptors. A series of 30 commercially available constituents of Rooibos and Honeybush were investigated via radioligand binding studies to determine their adenosine A_1 and A_{2A} receptor affinity at both rat and human subtypes in order to establish structure-activity relationships and identify novel adenosine receptor ligands. In addition, *in silico* evaluations of the 30 test compounds were also performed to investigate their physiochemical and pharmacokinetic properties. The most promising constituent was kaempferol (**28**) which showed sub-micromolar affinity towards the rat A_1 subtype (rA_1K_i = 0.7287 μM; hA_1K_i = 9.88 μM) and acted as an antagonist toward adenosine rA_1 receptors. Additionally, quercetin (**2**), chrysoeriol (**8**), luteolin (**9**), eriodyctiol (**12**), and naringenin (**27**) also showed adenosine A_1 and/or A_{2A} receptor affinity. It was observed that a flavonol scaffold is preferred to flavone and flavanone scaffolds, and within the flavonols, C4'-OH substitution on ring B is preferred to C3',4'-diOH substitution. These phytochemicals, specifically kaempferol (**28**), may be considered lead-like and valuable in designing novel ligands, based on *in vitro* and *in silico* evaluation.

Graphical abstract



Keywords

Rooibos, Honeybush, adenosine A_1 receptor, adenosine A_{2A} receptor, structure-activity relationships

Introduction

Adenosine is involved in cellular activities, and thus, engaged in both physiologic and pathophysiologic processes. Four adenosine receptor (AR) subtypes, A_1, A_{2A}, A_{2B} and A_3 [1,2] are members of the G protein-coupled receptor (GPCR) superfamily, which is also a therapeutic target for several pathologies [3]. The A_1 and A_{2A} ARs are distributed throughout the brain [4,5]. A_1 and A_3 ARs mainly couple to inhibitory G-proteins, while A_{2A} and A_{2B} ARs couple to stimulatory G-proteins [6]. A_1 ARs are mostly found in the central nervous system (cerebral cortex, cerebellum, hippocampus, thalamus, brainstem and spinal cord) as well as some peripheral organs. A_{2A} ARs are concentrated in the striatum, nucleus accumbens, and olfactory tubercle [7,8,9].

AR antagonists have therapeutic potential in the treatment of diverse pathological conditions, such as neurodegenerative disorders (including Parkinson's and Alzheimer's disease [10,11,12]), depression [13], specific cancers [14], type-2 diabetes, myocardial infarction, and asthma [15,16]. These antagonists can also be renal protective [17], cognitive enhancing [18], and anti-inflammatory [19].

Naturally occurring AR antagonists have been identified and include the alkylxanthines, caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) [20,21,22]. Other natural products (NPs), like caffeine, have also provided various drug leads worth optimizing by synthetic means to increase their AR affinity. AR antagonists are thus widely classified as xanthines [23]. However, due to these structures' weak photostability and low water solubility [24] there is an on-going search for non-xanthine antagonists. Some of these non-xanthine scaffolds include the flavonoids, which are phytochemicals and also found in tisanes like Rooibos and Honeybush.

Drug discovery through natural products (NPs) has attracted the attention of various research groups over the years. Plant-derived NPs is an alternative approach to obtain potential drug leads. Several studies have proven that the use of natural products can be effective in treating numerous pathological conditions, including neurodegenerative disorders. Additionally, NPs provide the advantage of abundant clinical experiences, structural diversity and numerous biological activities [25].

A number of herbs have traditionally been used as herbal infusions or “teas” in South Africa. Two Cape fynbos plants, *Aspalathus linearis* (Brum.f) Dahlg. (family Fabaceae; tribe Crotalarieae), better known as Rooibos, and various species of *Cyclopia* Vent. (family Fabaceae; tribe Podalyrieae), commonly known as Honeybush, have enjoyed commercial

success as herbal teas [26]. These teas are popular tisanes enjoyed for their taste and aroma and medicinal purposes [26,27].

Aspalathus linearis, known as Rooibos, is a leguminous bush indigenous to the mountain areas of the Western Cape Province of South Africa. It is gaining popularity as an alternative beverage worldwide, mainly due to the absence of caffeine and its low tannin content [28]. Rooibos tea is widely known as a natural cognitive enhancer as it can maintain alertness and concentration in the short-term and may also be beneficial for long-term cognitive health [29,30]. Traditional medicinal uses of Rooibos in South Africa include alleviating infantile colic, allergies, asthma, and dermatological problems [31]. It is asserted that Rooibos has a low toxicity profile with several health benefits [28,32] which include antioxidant [33], antiaging [34], anticancer [35], anti-inflammatory [36], antimutagenic [37] and antidiabetic [38] properties.

Rooibos tea (aqueous extract) can either be prepared by infusion of oxidised (fermented) or “green” (unfermented) plant leaves [39]. Various compounds have been isolated from such aqueous extracts [40], as well as from methanolic extracts [40, 41].

The short, woody shrub Honeybush (*Cyclopia intermedia*) is grown in the mountain slopes of the Langkloof district between the Eastern and Western Cape regions of South Africa. Honeybush tea is usually prepared by infusion of fermented leaves, stems, and flowers of the plant. During the fermentation process, the plant material changes colour from green to dark brown as the phenolic compounds are oxidized [27].

Aqueous extracts of both fermented and unfermented Honeybush have shown antimutagenic [42], antioxidant, immunomodulating [43,44], lipolytic [45], bone resorption [46] and antidiabetic [47] activities. Honeybush is traditionally used as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis [26].

In this pilot study, the chemical constituents of Rooibos and Honeybush (structures found in Appendix A, Table A1) will be investigated for their potential A_1 and A_{2A} AR affinity and the possibility of identifying novel AR ligands. The results will give insight into structure-activity relationships toward gaining AR affinity, and previously unknown AR drug leads and scaffolds may be identified.

Results and Discussion

In silico evaluation of physiochemical and pharmacokinetic properties

The physiochemical and pharmacokinetic properties of the test compounds were computed via SwissADME, and the results are summarised in Appendix A, Tables A2-4 and Figures A1-2.

For the purpose of this discussion, focus was placed on compounds **2**, **8**, **9**, **12**, **27** and **28** which showed promising rA_1 and/or rA_{2A} AR affinity. Table 1 summarises the results of the predicted physiochemical properties, whereas Table 2 gives the drug-likeness and medicinal chemistry friendliness of selected constituents found in Rooibos and/or Honeybush.

Table 1: Physiochemical properties of selected constituents found in Rooibos and/or Honeybush

Cmpd	Physiochemical properties								Lipophilicity	Water solubility[c]	
	Chemical formula	Molecular weight	Fraction Csp3	Num. rotatable bonds	Num. H-bond acceptors	Num. H-bond donors	Molar refractivity	TPSA (Å^2)[a]	$LogP_{o/w}$ (MLOGP)[b]	LogS (ESOL)[d]	LogS (Ali)[e]
2	C15H10O7	302.24	0.00	1	7	5	78.03	131.36	-0.56	-3.16	-3.91
8	C16H12O6	300.26	0.06	2	6	3	80.48	100.13	0.22	-4.06	-4.87
9	C15H10O6	286.24	0.00	1	6	4	76.01	111.13	-0.03	-3.71	-4.51
12	C15H12O6	288.25	0.13	1	6	4	73.59	107.22	0.16	-3.26	-3.90
27	C15H12O5	272.25	0.13	1	5	3	71.57	86.99	0.71	-3.49	-3.99
28	C15H10O6	286.24	0.00	1	6	4	76.01	111.13	-0.03	-3.31	-3.86

[a]Topological Polar Surface Area (TPSA, Å); [b]$LogP_{o/w}$ (MLOGP); [c]LogS scale: insoluble < -10 < poorly < -6 < moderately < -4 < soluble < -2 < very < 0 < highly; [d]LogS (ESOL); [e]LogS (Ali)

Table 2: Drug-likeness and medicinal chemistry friendliness of selected constituents found in Rooibos and/or Honeybush

Cmpd	*Drug-likeness*						*Medicinal chemistry friendliness*		
	Num. violations						Num. violations		
	Lipinski[a]	Ghose[b]	Veber[c]	Egan[d]	Muegge[e]	Bioavailability score[f]	PAINS[g]	Brenk[h]	Lead-likeness[i]
2	0	0	0	0	0	0.55	1 (catechol)	1 (catechol)	0
8	0	0	0	0	0	0.55	0	0	0
9	0	0	0	0	0	0.55	1 (catechol)	1 (catechol)	0
12	0	0	0	0	0	0.55	1 (catechol)	1 (catechol)	0
27	0	0	0	0	0	0.55	0	0	0
28	0	0	0	0	0	0.55	0	0	0

[a]Lipinski: MW < 500, MLOGP < 4.15, N or O < 10, NH or OH < 5; [b]Ghose: 160 < MW < 480, -04 < WLOGP < 5.6, 40 < MR < 130, 20 < atoms < 70; [c]Veber: Num. rotatable bonds < 10, TPSA < 140; [d]Egan: WLOGP < 5.88, TPSA < 131.6; [e]Muegge: 200 < MW < 600, -2 < XLOGP < 5, TPSA < 150, num. rings < 7, num. carbon > 4, num. heteroatoms > 1, num. rotatable bonds < 15, num. H-bond acceptors < 10, num. H-bond donors < 5; [f]The probability that a compound will have > 10% bioavailability in rat or measurable Caco-2 permeability; [g]PAINS: pan assay interference structures; [h]Brenk: structural alert; [i]Lead-likeness: 250 < MW < 350, XLOGP3 < 3.5, num. rotatable bonds < 7

Oral administration is considered the preferred route of drug delivery and Lipinski's "rule of five" outlines the physiochemical properties that support the likelihood of absorption upon oral administration [57]. Of these properties, the partition coefficient for n-octanol/water, or LogPo/w, (which determines the balance between lipophilicity and water solubility) is of great importance. A topological method was used to calculate MLogP [58,59], and should ideally be <4.15.

The drug-likeness of the compounds was assessed by rule-based filters from Lipinski [57], Ghose [60], Veber [61], Egan [62], and Muegge [63]. The rules violated can be observed in Appendix A, Table A4. None of the test compounds that show adenosine A_1 and/or A_{2A} receptor affinity (**2**, **8**, **9**, **12**, **27** & **28**) violated any of Lipinski's five rules. The bioavailability score (the likelihood that a compound will have >10% bioavailability in rat Caco-2 permeability) is 0.55 for all six test compounds (**2**, **8**, **9**, **12**, **27** & **28**). Thus, adhering to Lipinski's five rules [64].

The WLOGP-versus-TPSA referential, also known as the BOILED-Egg model (Appendix A, Figure A2), can be used to predict the passive human gastrointestinal absorption (HIA) and blood–brain-barrier (BBB) permeation [65]. Since A_1 and A_{2A} ARs are greatly expressed in the central nervous system, a drug that targets these receptors should be able to cross the BBB. Unfortunately, none of the test compounds that show promising affinity are predicted to cross the BBB but are expected to have high GI absorption. Yet, compounds **15**, **16**, **17**, **24** and **26** are expected to permeate the BBB. According to Daina & Zoete (2016) it is predicted that moderately polar (TPSA<79 $Å^2$) and relatively lipophilic (MLogP 0,4-6,0) molecules have a high probability of entering the CNS and, thus, cross the BBB.

It was also predicted that compounds **2**, **8**, **9**, **12**, **27** and **28** are soluble to moderately soluble in water. A soluble molecule makes drug development easier [66] and also influences GI absorption [67].

Two methods were employed to identify potentially problematic fragments in the present series. The first method identifies pan assay interference compounds (PAINS) and compounds having substructures that are likely to hinder biological assays and exhibit target promiscuity are identified [68]. The Brenk method identifies fragments that may be potentially toxic, chemically reactive, metabolically unstable, or have poor pharmacokinetics [69]. Only three out of the six test compounds with affinity toward rA_1 and/or A_{2A} subtypes produced alerts; quercetin (**2**), luteolin (**9**) and eriodyctiol (**12**), which all contain a catechol. It is seen as problematic since a catechol is rapidly oxidized to reactive quinones, which can damage biological macromolecules and interfere with membrane function. They can also produce toxic reactive oxygen species [70].

In vitro evaluation of adenosine A_1 and A_{2A} receptor affinity

Competitive binding

The degree of binding affinity that the test compounds (**1-30**) possess towards rat A_1 and A_{2A} ARs was determined via radioligand binding studies in either duplicate (specific binding (%)) or triplicate (inhibition constant (K_i, μM)) and expressed as mean ± standard error of the mean (SEM). Test compounds were initially assessed at a high maximum concentration of 100 μM using rat whole brain membranes (rA_1) or rat striatal membranes (rA_{2A}). Only compounds **2**, **8**, **9**, **12**, **27** and **28**, which displayed specific binding values of ≤20% at a maximum tested concentration of 100 μM, underwent full biological assays for determination of inhibition constant values (K_i, μM). These compounds were further investigated at human (h) A_1 and/or A_{2A} receptor subtypes. All other test compounds displayed specific binding values >20%; thus, no K_i values were determined. The A_1 and A_{2A} AR radioligand binding assays were validated with CPA (A_1 agonist) and DPCPX (A_1 antagonist) as reference compounds. Results are summarized in Table 3.

Table 3: Competition of test compounds for [^{3}H]DPCPX binding at adenosine A_1 receptors and [^{3}H]NECA binding at adenosine A_{2A} receptors

Cmpd	K_i value ± SEM (μM)[a] (Specific binding (%))[b]					Selectivity index				GTP shift
	rA_1 vs 0.1 nM [^{3}H]DPCPX[c]	**hA_1 vs 1 nM [^{3}H]DPCPX[d]**	**rA_{2A} vs 4 nM [^{3}H]NECA[e]**	**hA_{2A} vs 30 nM [^{3}H]NECA[f]**	***r*A_1 + 0.1 mM GTP vs 0.1 nM [^{3}H]DPCPX[c]**	**$rA_{2A}K_i$/ rA_1K_i**	**$hA_{2A}K_i$/ hA_1K_i**	**hA_1K_i/ rA_1K_i**	**$hA_{2A}K_i$/ $rA_{2A}K_i$**	**rA_1K_i + 0.1 mM GTP/rA_1K_i**
1	(100)[b]	-	61 [b]	-	-	-	-	-	-	-
2	2.47 ± 0.64[a]	3.38 ± 0.91 [a]	6.99 ± 0.89 [a]	-	1.49 ± 0.18 [a]	2.83	-	1.37	-	0.60
3	(88)[b]	-	(80)	-	-	-	-	-	-	-
4	(100)[b]	-	(98)[b]	-	-	-	-	-	-	-
5	(97)[b]	-	(78)[b]	-	-	-	-	-	-	-
6	(100)[b]	-	(100)[b]	-	-	-	-	-	-	-
7	(69)[b]	-	(79)[b]	-	-	-	-	-	-	-
8	1.39 ± 0.17[a]	2.41 ± 1.33 [a]	3.94 ± 1.76[a]	7.99 ± 0.63[a]	1.29 ± 0,019[a]	-	3.32	1.74	0.028	0.93
9	2.32 ± 0.10[a]	2.28 ± 0.52 [a]	2.50 ± 1.27[a]	13.1 ± 5.15[a]	1.59 ± 0.15[a]	-	5.74	0.98	5.25	0.69
10	(100)[b]	-	(100)[b]	-	-	-	-	-	-	-
11	(63)[b]	-	(73)[b]	-	-	-	-	-	-	-
12	55.3 ± 1.18 [a]	10.7 ± 3.91 [a]	17.0 ± 2.87[a]	10.4 ± 5.38[a]	26.4 ± 2.80 [a]	-	0.97	0.19	0.61	0.48
13	(42)[b]	-	(75)[b]	-	-	-	-	-	-	-
14	(87)[b]	-	(83)[b]	-	-	-	-	-	-	-
15	(88)[b]	-	(100)[b]	-	-	-	-	-	-	-
16	(92)[b]	-	(100)[b]	-	-	-	-	-	-	-
17	(73)[b]	-	(100)[b]	-	-	-	-	-	-	-
18	(100)[b]	-	(100)[b]	-	-	-	-	-	-	-
19	(98)[b]	-	(100)[b]	-	-	-	-	-	-	-
20	(96)[b]	-	(100)[b]	-	-	-	-	-	-	-
21	(89)[b]	-	(80)[b]	-	-	-	-	-	-	-
22	(100)[b]	-	(100)[b]	-	-	-	-	-	-	-
23	(52)[b]	-	(92)[b]	-	-	-	-	-	-	-
24	(98)[b]	-	(100)[b]	-	-	-	-	-	-	-
25	(100)[b]	-	(100)[b]	-	-	-	-	-	-	-
26	(90)[b]	-	(100)[b]	-	-	-	-	-	-	-
27	31.9 ± 1.70[a]	14.1 ± 2.71[a]	(8)[b]	-	20.7 ± 1.94[a]	-	-	0.44	-	0.65
28	0.729 ± 0.073[a]	9.88 ± 0.56[a]	(39)[b]	-	0.73 ± 0.054[a]	-	-	13.6	-	1.00
29	(41)[b]	-	(79)[b]	-	-	-	-	-	-	-
30	(69)[b]	-	(100)[b]	-	-	-	-	-	-	-
	Reference standards									
CPA	0.0065 ± 0.0004[a] 0.0059	-	-	-	0.037 ± 0.002[a] 0.035	-	-	-	-	5.62
DPCPX	0.0005 ± 0.0001[a] 0.0006	0.004 ± 0.0002[a] 0.003	0.149 ± 0.023[a] 0.157	0.053 ± 0.005[a] 0.129	0.0004 ± 0.00003[a] 0.0005	298	13.3	8	0.36	0.80
IST	0.125 ± 0.006[a]	0.741 ± 0.005[a]	0.003 ± 0.001[a]	0.042 ± 0.005[a]	-	0.024	0.057	5.93	14	-

	0.230	0.841	0.002	0.026

[a] Inhibition constant (K_i, µM) is presented as the mean ± standard error of the mean (SEM) of experiments performed in triplicate; [b] Specific binding (%) at maximum tested concentration of 100 µM is presented as the mean of experiments performed in duplicate; [c] Dissociation constant (K_d): 0.36 nM; [d] K_d: 3.86 nM; [e] K_d:15.3 nM; [f] K_d:20.1 nM; Values without SEM are taken from the literature; r: rat; h: human; [^{3}H]DPCPX: 8-cyclopentyl-1,3-dipropylxanthine, tritiated at dipropyl-2,3- positions, selective adenosine A_1 receptor antagonist; [^{3}H]NECA: 5'-N-ethylcarboxamidoadenosine, tritiated at adenine-2,8 position, non-selective adenosine A_1, A_{2A}, and A_3 receptor agonist; GTP: guanosine-5'-triphosphate; CPA: N^6-cyclopentyladenosine; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine; IST: istradefylline

Rooibos

Rooibos contains flavonoids – representing three flavonoid subclasses, namely, flavone, flavonol, and flavanone (Figure 1) [41]. The health benefits of Rooibos are probably due to these polyphenols and major flavonoids [71].

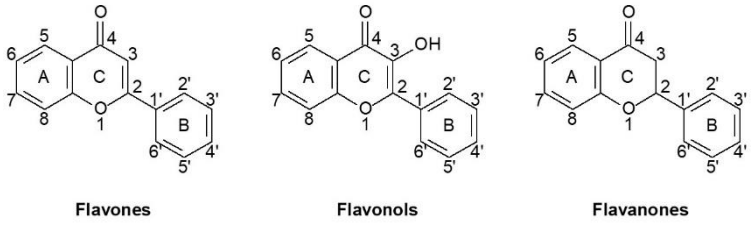


Figure 1. Basic chemical structures of flavonoid subclasses.

The aqueous Rooibos extracts mainly consist of two unique compounds namely the C-C-linked dihydrochalcone glucoside aspalathin (**1**) [72,73] and the cyclic dihydrochalcone aspalalinin [74]. Only aspalathin (**1**) was evaluated in the present study but was devoid of rA_1 and A_{2A} AR affinity [72,73].

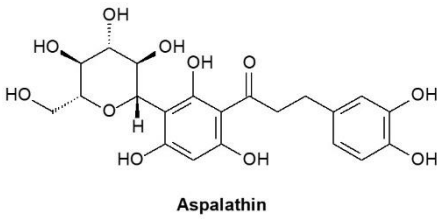


Figure 2. Chemical structure of aspalathin (**1**).

The flavonol quercetin (**2**) showed A_1 AR affinity (rA_1K_i = 2.47 μM & hA_1K_i = 3.38 μM). The flavonol glycoside derivatives, isoquercitrin (**3**) (quercetin 3-O-β-D-glucopyranoside), rutin (**4**) (quercetin-3-O-rutinoside) and its galactoside derivative hyperoside (**5**) (quercetin 3-O-β-D-galactopyranoside) showed no rA_1 or A_{2A} AR affinity. This may be due to the glycoside or galactoside groups present in these compounds. The flavonol quercetin (**2**) has previously been shown to possess affinity for the A_1 (inhibition constant (K_i) = 2.47 μM) and A_{2A} (K_i = 6.99 μM) ARs in the low micromolar range [75].

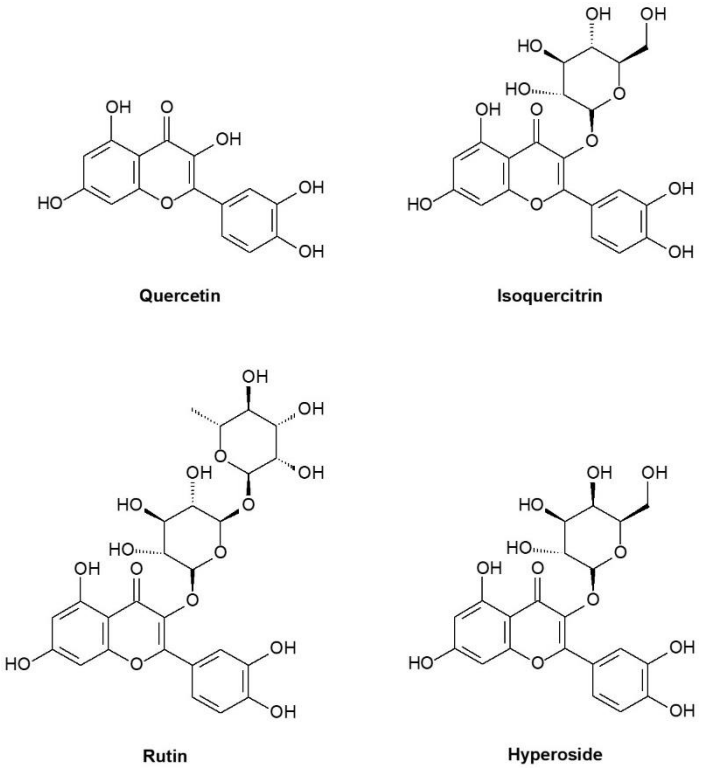


Figure 3. Chemical structures of quercetin (**2**), iso-quercetin (**3**), rutin (**4**) and hyperoside (**5**).

The flavones chrysoeriol (**8**) (rA_1K_i = 1.39 μM; $rA_{2A}K_i$ = 3.94 μM and hA_1K_i = 2.41 μM; $hA_{2A}K_i$ = 7.99 μM) and luteolin (**9**) (rA_1K_i = 2.32 μM; $rA_{2A}K_i$ = 2.50 and hA_1K_i = 2.24 μM; $hA_{2A}K_i$ = 13.1 μM) showed promising affinity for both A_1 and A_{2A} AR subtypes for both species. Luteolin (**9**) has previously showed affinity toward the rA_1 and rA_{2A} ARs (half maximal inhibitory concentration (rA_1IC_{50}) = 1.19 μM; $rA_{2A}IC_{50}$ = 0.84 μM)) [76]. Rooibos is also a source of nothofagin (3-dehydroxydihydrochalone glucoside) and its flavone analogues, vitexin (**6**) (apigenin-8- C-glucoside), isovitexin (**7**) (apigenin-6-C-glucoside), as well as other flavones orientin (**10**) (luteolin-8-C-glucoside) and iso-orientin (**11**) (luteolin-6-C-glucoside) showed no mentionable affinity. Again, the presence of a glycoside group may possibly hinder affinity.

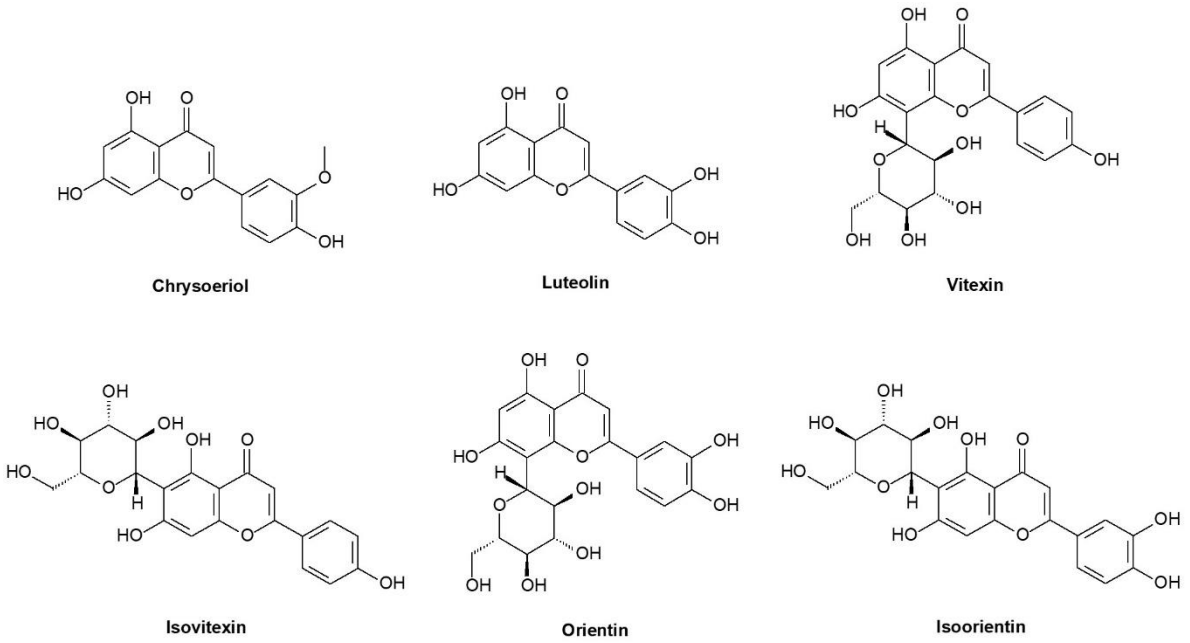


Figure 4. Chemical structures of chrysoeriol (**8**), luteolin (**9**), vitexin (**6**), isovitexin (**7**), orientin (**10**) and isoorientin (**11**).

The flavanone eriodyctiol (**12**) and the flavan-3-ol catechin (**13**) are two other phytochemicals that have also been detected in Rooibos [28]. Albeit weak, eriodyctiol (**12**) showed affinity towards A_1 and A_{2A} subtypes (rA_1K_i = 55,3 μM; $rA_{2A}K_i$ = 17.0 μM and hA_1K_i = 10.7 μM; $hA_{2A}K_i$ = 10.4 μM).

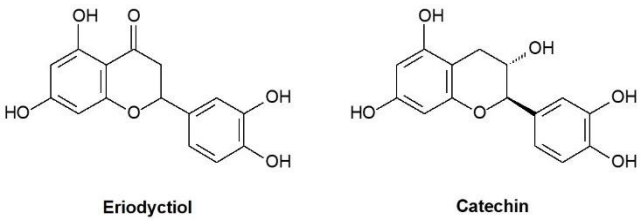


Figure 5. Chemical structures of eriodyctiol (**12**) and catechin (**13**).

Various phenolic acids are also present in Rooibos extracts and include caffeic acid (**14**), ferulic acid (**15**), p-coumaric acid (**16**), p-hydroxybenzoic acid (**17**), vanillic acid (**18**), protocatechuic acid (**19**) [73], and syringic acid (**20**) [77]. Although some of them are predicted to cross the BBB, none of these acids showed affinity towards rA_1 and rA_{2A} receptors.

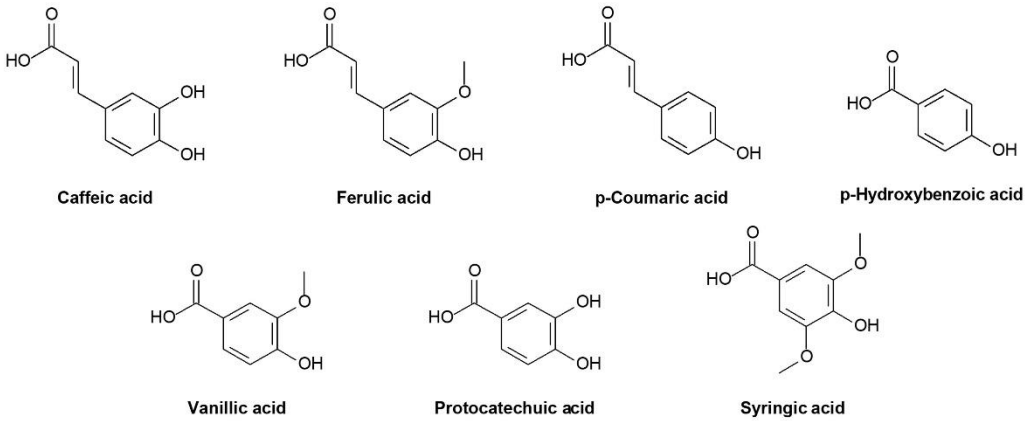


Figure 6. Chemical structures of caffeic acid (**14**), ferulic acid (**15**), p-coumaric acid (**16**), p-hydroxybenzoic acid (**17**), vanillic acid (**18**), protocatechuic acid (**19**), and syringic acid (**20**).

Honeybush

The two major phenolic constituents in the leaves of Honeybush are mangiferin (**21**) (a glycosyl xanthone) and the flavanone hesperitin (**23**) [27]. In the processed leaves and stems the xanthones isomangiferin (**22**), the isoflavone formononetin (**24**), the inositol (+)-pinitol (**25**) and the phenolic acid, tyrosol (**26**) were identified. None of these constituents showed affinity toward either the rA_1 or rA_{2A} ARs.

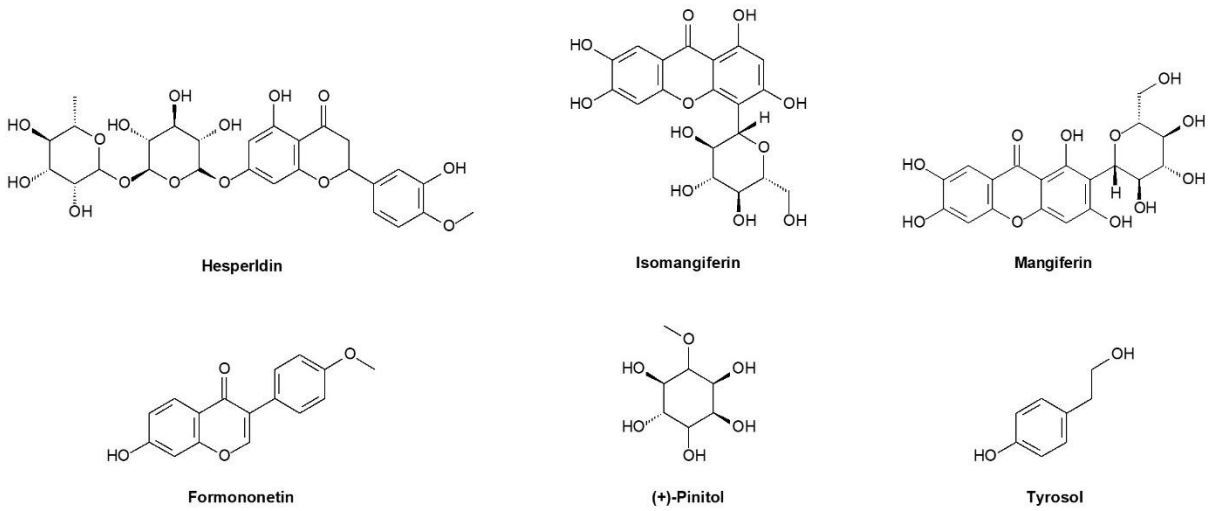


Figure 7. Chemical structures of mangiferin (**21**), isomangiferin (**22**), hesperitin (**23**), formononetin (**24**), (+)-pinitol (**25**) and tyrosol (**26**).

The flavanone, naringenin (**27**), found in Honeybush, showed affinity towards the rA_1 AR (rA_1K_i = 31.9 µM; hA_1K_i = 14.1 µM). The flavonol, kaempferol (**28**) - also found in Honeybush - showed the best affinity for the A_1 AR in the series (rA_1K_i = 0,729 µM; hA_1K_i = 9,88 µM).

Compounds structurally related to kaempferol, such as kaempferide (**29**) and rhamnocitrin (**30**) – although not present in Honeybush – was also tested; however, neither showed affinity toward rA_1 and A_{2A} ARs. Thus, it can be observed that the -OCH_3 group at position C4' (kaempferide) and position C7 (rhamnocitrin), respectively, decreased affinity.

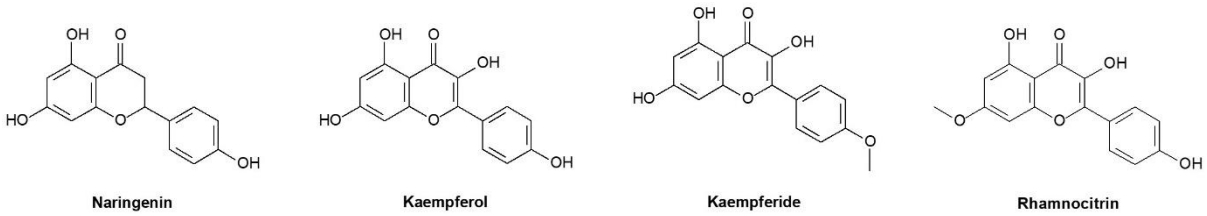


Figure 8. Chemical structures of naringenin (**27**), kaempferol (**28**), kaempferide (**29**) and rhamnocitrin (**30**).

Structure-activity relationships

Structure-activity relationships (SAR) based on K_i values (μM) of **2**, **8**, **9**, **12**, **27** and **28** showed a coherent relationship between the constituents and rA_1 and rA_{2A} AR affinity. The influence on AR affinity of different polar (-OH) and/or non-polar (-OCH_3) groups, the number of these groups and their position on rings A, B and C of the flavones, flavonols and/or flavanones was explored. For the flavones, a comparison of chrysoeriol (**8**: C3'-OCH_3) to luteolin (**9**: C3'-OH) showed that C3'-OH substitution decreased rA_1 affinity. Although both substitution patterns led to dual AR affinity, it may be said that a C3'-OCH_3-C4'-OH substitution pattern (**8**) is preferred for rA_1 affinity and a catechol group (**9**) for rA_{2A} affinity. For the flavonols, a mono-hydroxy substituted compound, such as kaempferol (**28**), exhibited higher affinity than one which was 3',4'-dihydroxy substituted (for example quercetin (**2**)). Indicating that a catechol group (and potentially increased polarity), led to decreased rA_1 affinity. However, when the flavanones were compared, naringenin (**27**: C3'-OH) showed lower activity than its di-hydroxy substituted counterpart eriodyctiol (**12**). From the limited data at hand, it seems that not only the number and position of the -OH or -OCH_3 group(s) influence affinity, but also the type of flavonoid. It was observed that the flavonols have the best affinity for the A_1/A_{2A} ARs, followed by the flavones and flavanones in decreasing order of affinity (flavonol>flavone>flavanones).

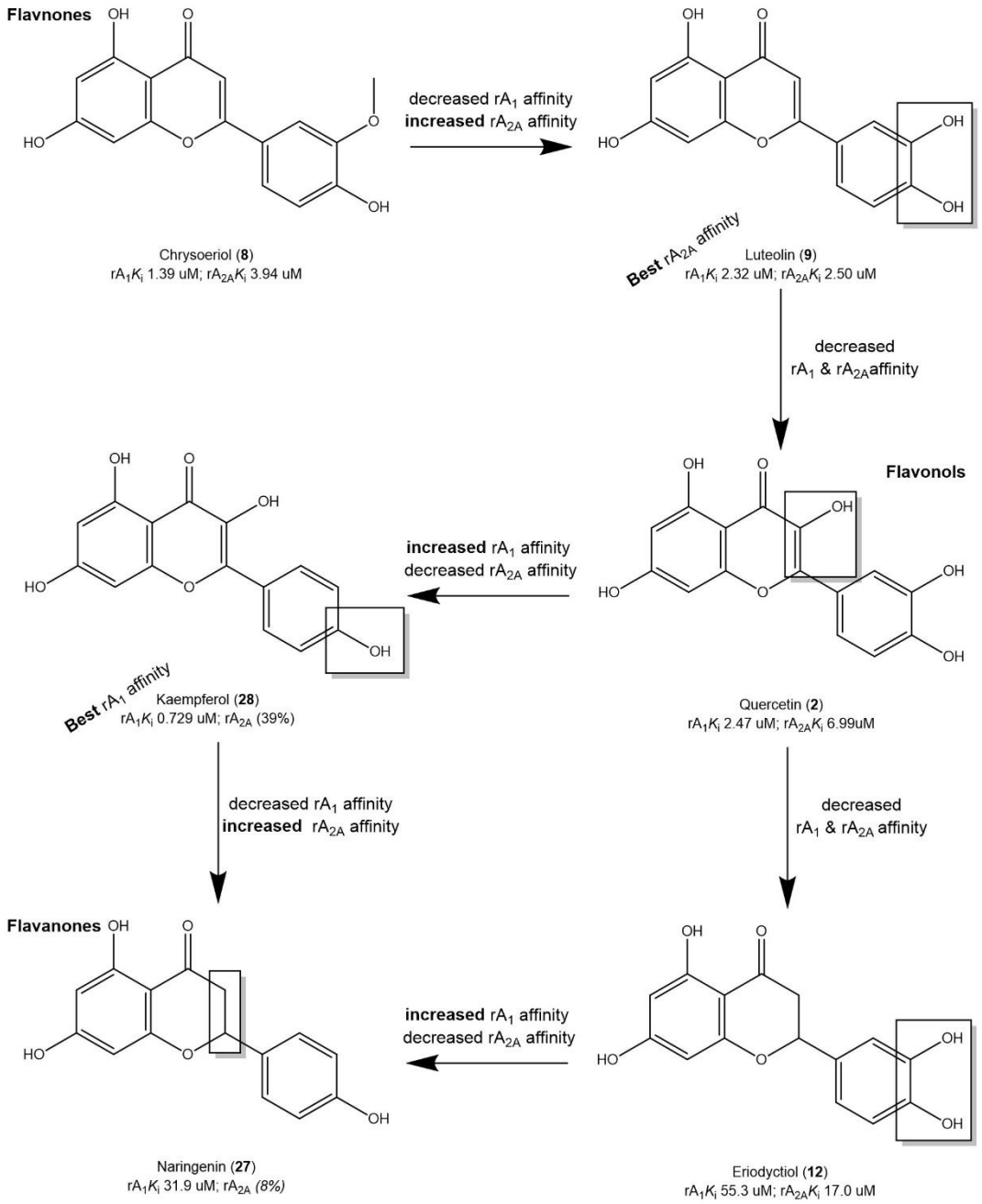


Figure 9. Summary of structure-activity relationships of constituents of Rooibos and Honeybush.

Functional binding

A GTP shift assay determined the type of binding affinity a test compound exhibits at the rat A_1 AR. The results are also summarized in Table 3. The six compounds with r A_1 affinity (2, 8, 9, 12, 27, and 28) were selected to determine functionality (agonism or antagonism). The results are also summarized in Table 3.

As seen in Table 1, of the 30 test compounds evaluated, compounds **2**, **8**, **9**, **12**, **27**, **28** possess A_1 AR affinity. Therefore, these compounds were selected to determine the type of activity they exert at rA_1 ARs. The most promising compound of the present test series was, however, compound **28,** with A_1 AR affinity in the sub-micromolar range (rA_1K_i = 0.729 μM).

GTP works by uncoupling the A_1 AR from its G-protein leading to a shift from a high to a low affinity state for agonists [78]. It is therefore possible to determine if a compound will act as an agonist, a partial agonist or an antagonist by comparing a test compound′s binding curves in the presence and absence of GTP [49]. With an antagonist, the binding curve is unaffected in the presence of GTP, and the antagonist test compound will have a GTP shift value of approximately 1 [79,80].

The results obtained suggested that compounds **2**, **8**, **9**, **12**, **27**, and **28** act as A_1 AR antagonists since there is no significant rightward shift of the binding curve in the presence of GTP and compounds have calculated GTP shifts of $\leq$1 (Fig. 10). Interestingly, GTP and endogenous adenosine binding interactions are controlled similarly by human and rat proteins in A_1 and A_{2A} AR binding studies [78]. As reference substances, CPA (an A_1 agonist) and DPCPX (an A_1 antagonist) were used to validate the GTP shift assays.

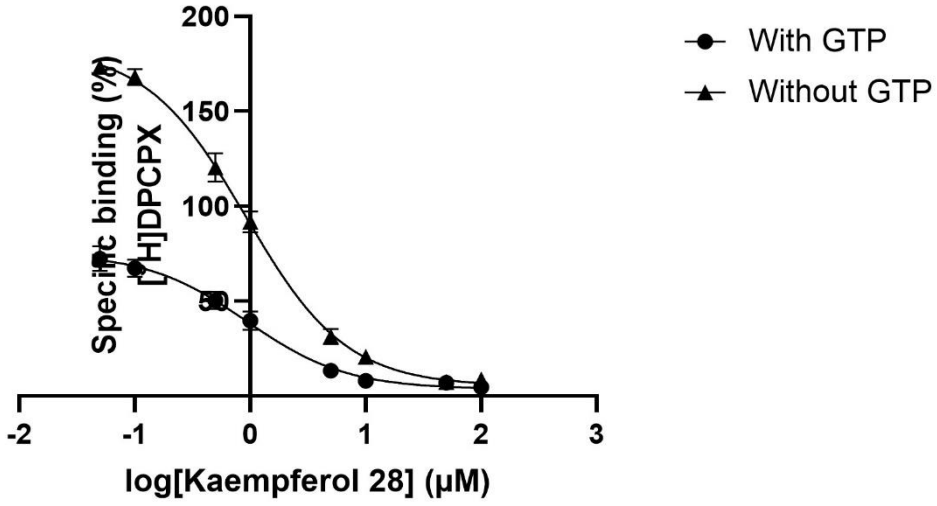


Figure 10. The binding curve of kaempferol (**28**) is an example of an A_1 AR antagonist which is determined via a GTP shift assay (with 100 μM GTP) in rat whole brain membranes expressing rA_1 ARs with [^{3}H]DPCPX as radioligand. **28** has a GTP shift of 1.

Correlation between species

The pK_i values of selected reference standards and test compounds (**2**, **8**, **9**, **12**, **27** and **28**) which showed affinity toward rat and human A_1 and/or A_{2A} receptor subtypes were compared, and thus, correlation coefficients were calculated. The R-squared value of the linear regression analysis is equal to the correlation coefficient (Appendix A, Figures A3-4).

Site-directed mutagenesis studies on several members of the G-protein coupled receptor superfamily exposed that a single amino acid can modulate ligand affinity, and thus, explain species differences [81,82]. Predictably, the rat and human A_1 receptor subtype (R squared 0.88) showed better correlation than the A_{2A} AR subtype (R squared 0.81); since a 95% sequence homology at the amino-acid level between rA_1 and hA_1 receptor subtypes exists, compared to only 82% between rA_{2A} and hA_{2A} [83]. Prototypical xanthine derivatives DPCPX (selective A_1 receptor antagonist) and IST (selective A_{2A} receptor antagonist) used as reference standards were markedly less potent at the human than rat receptors (Table 3). DPCPX and IST were, respectively, eight and fourteen times more selective toward the rat than human A_1 and A_{2A} receptor subtype; indeed, the affinity of xanthine-based antagonists is species-dependent. Therefore, it was suggested that different structural determinants may be responsible for binding of xanthine derivatives (such as DPCPX and IST) compared to other scaffolds.

Conclusions

Overall, the constituents of Rooibos and Honeybush did not show mentionable affinity toward either A_1 or A_{2A} receptor subtypes, except for compounds **2**, **8**, **9**, **12**, **27** and **28**. Of these compounds, the flavonol kaempferol (**28**) showed sub-micromolar AR affinity, making it the most promising test compound of the present evaluated NPs derived from either Rooibos or Honeybush. It was observed that a flavonol scaffold is preferred to flavone and flavanone scaffolds, and within the flavonol scaffold C4'-OH substitution on ring B is preferred to C3',4'-OH substitution. Based on the promising *in vitro* affinity and drug-like properties of kaempferol (**28**), it may be used as a lead compound to further synthesise a series of *para*-benzyl substituted flavonols to be evaluated as adenosine A_1 and A_{2A} receptor ligands.

Materials and methods

Chemistry

Test compounds **1-30** were obtained from commercial vendors such as AmBeed or Sigma-Aldrich and used without further purification.

In silico evaluation of physiochemical and pharmacokinetic properties

SwissADME (http://www.swissadme.ch), a free web tool, was used to predict physiochemical and pharmacokinetic properties of test compounds **1-30** from their molecular structures using the most relevant computational methods [48].

In vitro evaluation of adenosine A_1 and A_{2A} receptor affinity and activity

All reagents and solvents were commercially available. This includes [^{3}H]-8-cylcopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX; specific activity 120 Ci/mmol), 5′-N-[^{3}H]-ethylcarboxamideadenosine ([^{3}H]NECA; specific activity 27.1 Ci/mmol) and filter count (liquid scintillation cocktail) from PerkinElmer. Adenosine deaminase (5.9 mg protein/mL, 157 units/mg protein) from Sigma-Aldrich and Whatman GF/B 25 mm diameter filters from Merck. Residual radioactivity was measured with a Packard Tri-CARB 2810 TR liquid scintillation counter.

Membrane preparation

The North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCare) approved the collection of tissue samples from adult male Sprague-Dawley rats for the radioligand binding studies (application number NWU-00035-10-A5). Rat brain membranes were prepared (whole brain and striatal membranes were pooled separately) and stored as described in literature [49]. Chinese hamster ovary (CHO) cells expressing the human A_1 or A_{2A} AR were kindly donated by Prof. K.N. Klotz from the University of Würzburg, Germany. CHO cells were grown adherently and maintained in Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% foetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and Geneticin (G418, 0.2 mg/ml) at 37°C in 5% CO_2/95% air. Cells were split 2 or 3 times weekly at a ratio between 1:5 and 1:20, respectively. For radioligand binding studies, the culture medium was removed, cells were washed with phosphate-buffered saline (PBS) and frozen until preparation of membranes, upon which frozen cells in ice-cold hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4) was thawed again. The cell suspension was then homogenized on ice (Ultra-Turrax, 2 X× 15s at full speed) and the homogenate was spun for 10 min (4°C) at 1,000 g. The supernatant was then centrifuged for 30 min at 100,000 g. The membrane pellet was resuspended in 50 mM Tris/HCl buffer pH 7.4, frozen in liquid nitrogen and stored at –80°C. Protein content for rat and CHO cell membranes were determined using Bradford reagent and bovine serum albumin as reference standard [50].

Competitive binding studies

Rat (r): To determine rA_1 AR affinity, rat whole brain membranes (expressing A_1 ARs) and [^{3}H]DPCPX (selective A_1 antagonist) [51] were used, and for rA_{2A} affinity, rat striatal membranes (expressing A_{2A} ARs) and [^{3}H]NECA (non-selective A_1/A_{2A} antagonist) [52]. Each incubation of the rA_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) [51,49]. In turn, 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 N6-cyclopentyladenosine (CPA) (100 µL) and (iv) 4 nM [^{3}H]NECA (radioligand solution, 100 µL) [49,52]. The final volume of all incubations contained 1 mL of 50 mM Tris.HCl buffer (pH 7.7, 25°C) and 1% DMSO [49]. Non-specific binding of [^{3}H]DPCPX and [^{3}H]NECA was defined as binding in the presence of 100 µM CPA [49,51,52]. Specific binding was defined as the total binding minus the non-specific binding [49].

Human (h): To determine hA_1 AR affinity, CHO cell membranes expressing A_1 ARs and [^{3}H]DPCPX was used, and for hA_{2A} affinity, CHO cell membranes expressing A_{2A} ARs and [^{3}H]NECA [53]. Each incubation of the hA_1 or hA_{2A} assay consisted of: (i) test compound (2 µL), (ii) 0.1 nM [^{3}H]DPCPX or 30 nM [^{3}H]NECA (radioligand solution, 20 µL) and (iii) 20 µg CHO cell membranes expressing hA_1 AR or 65 µg CHO cell membranes expressing hA_{2A} AR and 0.2 units/mL adenosine deaminase (membrane suspension, 178 µL). Samples were incubated for 1h at 25°C, filtered and washed three times with 4 mL ice-cold binding and filtered individually as described for conventional radioligand binding studies. Non-specific binding of [^{3}H]DPCPX and [^{3}H]NECA was defined as binding in the presence of 100 µM CPA [49,51,52]. Specific binding was defined as the total binding minus the non-specific binding [49].

GTP shift assay: The guanosine triphosphate (GTP) shift assay resembles the [^{3}H]DPCPX radioligand binding assay; however, it requires the addition of 100 µM GTP. Non-specific binding was defined as binding in the presence of 10 µM DPCPX [54,55].

Data analysis

The competitive binding studies' data analysis was done using Microsoft Excel and GraphPad Prism Software. Sigmoidal dose-response curves, from which half maximal inhibitory concentration (IC_{50}) values were calculated, were obtained by plotting the specific binding against the logarithm of the test or reference compounds′ concentrations. Subsequently, the

IC_{50} values were used to calculate the inhibition constant (K_i) values for the competitive inhibition of [^{3}H]DPCPX (rK_d = 0.36 nM [51]; hK_d = 3.86 nM [53]) against A_1 ARs and [^{3}H]NECA (rK_d = 15.3 nM [52]; hK_d = 20.1 nM [53]) against A_{2A} ARs by the test compounds using the Cheng-Prusoff equation [56]. Descriptive statistics were used to represent K_i values (μM) as the mean ± standard error of the mean (SEM) of experiments performed in triplicate. Specific binding (%) values of the radioligand at a maximum tested concentration of 100 μM were represented as the mean of experiments performed in duplicate**.** Where applicable, selectivity index (SI) values were calculated for the A_1 isoform as a ratio of $A_{2A}K_i$ and A_1K_i values of test compounds. GTP shifts were calculated by dividing the K_i values of compounds in the presence of 100 μM GTP by the K_i values obtained in the absence of 100 μM GTP [55].

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Conflict of Interest

The authors declare that they have no conflict of interest.

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