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Synthesis and evaluation of new N^6-substituted adenosine-5′-*N*-methylcarboxamides as A_3 adenosine receptor agonists

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ABSTRACT

A number of N^6-substituted adenosine-5′-*N*-methylcarboxamides were synthesised and their pharmacology, in terms of their receptor affinity, selectivity and cardioprotective effects, were explored. The first series of compounds, **4a**–**4f** and **5a**–**5f**, showed modest receptor affinity for the A_3AR with K_i values in the low to mid μM range. However, the incorporation of a 4-(2-aminoethyl)-2,6-di-*tert*-butylphenol group in the N^6-position (in compounds **4g** and **5g**) significantly improved the affinity with K_i values of 30 and 9 nM, respectively. Improvements in affinity, as well as selectivity were seen when a functionalised linker was introduced. The N^6-phenyl series, compounds **7a**–**7d**, demonstrated low to mid nanomolar receptor affinities (K_i = 2.3–45.0 nM), with **7b** displaying 109-fold selectivity for the A_3AR (vs A_1). The N^6-benzyl series **9a**–**9c** also proved to be potent and selective A_3AR agonists and the longer chain length linker **13** was tolerated at the A_3AR without abrogation of affinity or selectivity. Cardioprotection was demonstrated by a simulated ischaemia cell culture assay, whereby **7b**, **7c**, **9a**, **9b** and **9c** all showed cardioprotective effects at 100 nM comparable or better than the benchmark A_3AR agonist IB-MECA, but which were indistinguishable by statistical analysis. For example, compound **9c** reduced cell death by 68.0 ± 3.6%.

1. Introduction

Adenosine, a ubiquitous endogenous nucleoside, has four distinct receptor subtypes, namely A_1, A_{2A}, A_{2B} and A_3 and is a member of the GPCR family. It helps to regulate a wide array of physiological processes and the A_3 subtype has been shown to be involved in inflammation,[1] cancer[2] and cardioprotection.[3,4] In particular, a cardioprotective response is elicited by the phenomenon of ischaemic preconditioning (IPC), whereby a brief period of ischaemia prior to an extended coronary occlusion protects the heart and results in a marked reduction in infarct size. Several different adenosine receptors subtypes have been implicated in preconditioning. The A_3 receptor has been implicated since the partially selective A_3 agonist IB-MECA emulates IPC.[5] Recently a new highly selective A_3 agonist, CP-532,903, was also found to protect the mouse heart from reperfusion injury by activating the sarcolemmal ATP-sensitive potassium channel.[6] Apart from adenosine, opiates and bradykinin have also demonstrated an ability to trigger IPC.[7] Other important A_3 receptor mediated factors include the role of nitric oxide, angiogenesis and aspects of reperfusion injury.[8–10] Protein kinase C (PKC) is activated by the A_3 receptor causing an increased opening of K_{ATP} channels and favours formation of phosphatidylethanolamine via phospholipase D through Rho A.[11–15] Opening of mitochondrial and sarcolemmal potassium channels is well known to protect cardiomyocytes from cell death. The AMISTAD I and II trials utilised adenosine as an adjunct for thrombolytic therapy and reperfusion to treat acute myocardial infarction, but the results were initially ambiguous.[16,17] However, normalisation of infarct size to the area at risk resulted in significant infarct size reduction in patients treated with adenosine within 4 h of infarction.[18]

A number of pharmacologically important agonists displaying A_3AR selectivity have been synthesised and are shown in Figure 1. IB-MECA is currently in Phase II trials for the treatment of colorectal cancer and the structurally similar Cl-IB-MECA is planned to be used in conjunction with chemotherapy.[19] MRS3558 is in preclinical development for the treatment of arthritis, whereas CP-532,903 recently demonstrated protection against myocardial ischaemia/reperfusion injury via sarcolemmal K_{ATP} channels, as well as anti-inflammatory effects.[6,19,20] These compounds exhibit a number of structural similarities. They are

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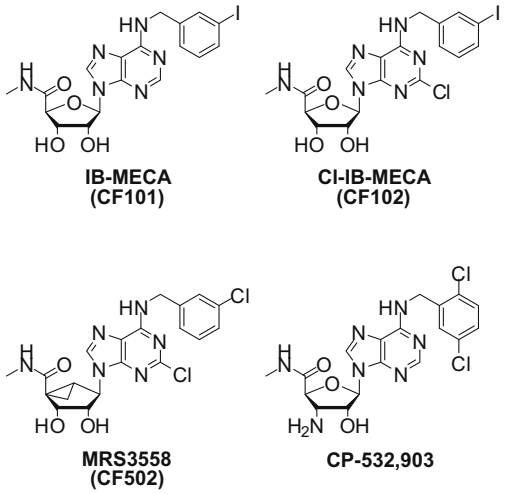


Figure 1. Pharmacologically important A_3AR agonists.

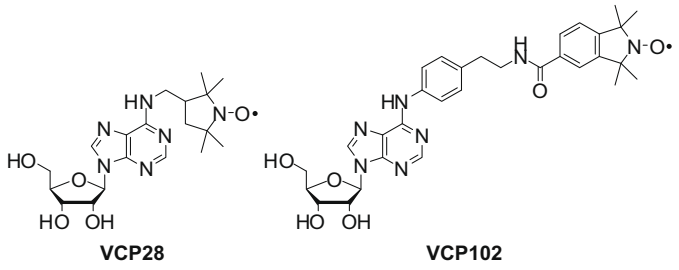


Figure 2. A_1AR agonists with attached nitroxide moieties.

all nucleosides possessing a 5′-*N*-methylcarboxamido group, N^6-benzyl group and a *meta*-substituted halogen on that benzyl group. All of these modifications typically confer A_3AR selectivity.

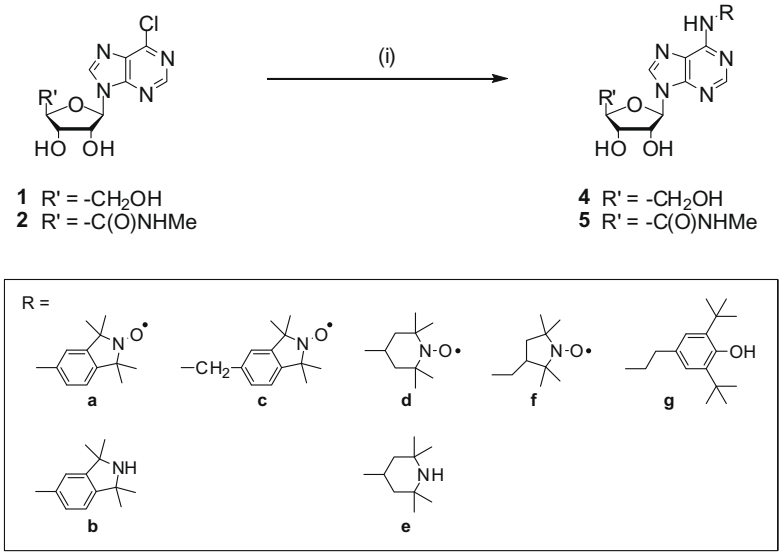
Recently, we reported a series of novel A_1 adenosine receptor agonists, which were both potent and selective via receptor binding assays.[21] Among the compounds synthesised were **VCP28** (Fig. 2), which had good affinity (K_i = 50 nM) and selectivity (over 400-fold selectivity for the A_1 over the A_3AR) and **VCP102**, with higher affinity still (K_i = 7 nM), but lower selectivity (~3-fold A_1AR selectivity). These compounds also contained functionality known to possess antioxidant activity (i.e., nitroxides and, in other cases, hindered phenols). Cardioprotective effects can also occur by the action of antioxidants via direct scavenging by superoxide, a key radical, reducing oxidative damage and protecting the heart from ischaemia and reperfusion injury. The well known antioxidant resveratrol has been implicated in cardioprotection.[22] Clinically, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, as well as anti-hyperlipidemic agents, like the statins have been developed as anti-oxidative therapeutics.[23,24] In addition to providing a potential second mode of cardioprotective activity, the antioxidant functionality and associated linkers were found to make an important contribution to adenosine receptor affinity and selectivity.

We now report the design, synthesis and preliminary pharmacological evaluation of a series of related A_3 adenosine receptor agonists. This current study builds on our previous series of A_1AR agonists by incorporating features known to impart selectivity for the A_3 adenosine receptor.

2. Results and discussion

A key feature present in many potent and selective A_3AR agonists (such as those depicted in Fig. 1) is a 5′-*N*-methyl carboxamido moiety. In our first series of compounds, a 5′-*N*-methyl carboxamide group was incorporated into the structures of N^6-substituted adenosines previously found to be moderately potent, but non-selective A_1/A_3AR agonists.[21] These compounds were prepared from the reaction of the appropriate amine with the 5′-modified 6-chloropurine riboside **2** in the presence of the base *N*,*N*-diisopropylethylamine (DIPEA). This provided the N^6-substituted compounds **5a**–**g** in yields of 46–100% (Scheme 1). Two corresponding adenosines (compounds **4c** and **4g**) were prepared for comparative purposes from the reaction of the appropriate amine with 6-chloropurine riboside (**1**).

A second series of compounds was prepared which featured a functionalised N^6-phenyl or benzyl group. Both of these substituents are known to interact favourably with a hydrophobic binding domain, with the latter typically conferring greater selectivity for the A_3AR. The necessary aniline precursors **6a**–**d** were prepared by coupling 4-(2-aminoethyl)aniline with the appropriate benzoic



Scheme 1. Reagents and conditions: R–NH_2 (**3**), N(*i*-Pr)$_2$Et, *t*-BuOH, 83 °C.

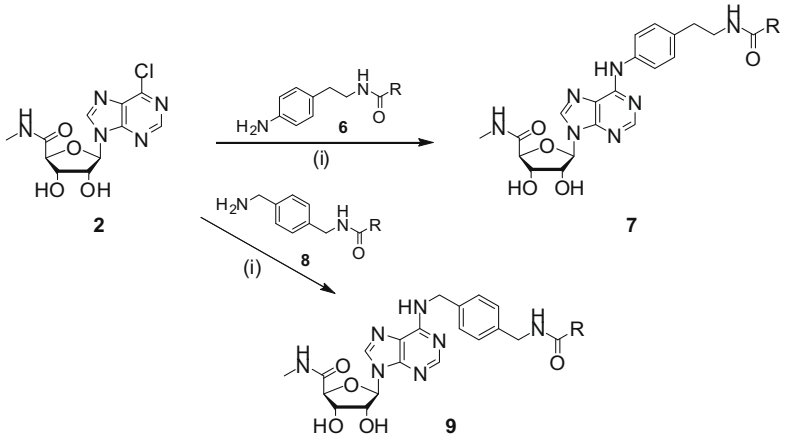
acid. In an analogous fashion, the substituted benzyl amines **8a–c** were prepared from the reaction of *p*-xylylenediamine with various benzoic acids. Compound **2** was then reacted with **6** and **8** to give the targeted N^6-substituted adenosine-5′-*N*-methylcarboxamides, **7** and **9**, respectively (Scheme 2).

A longer chain length linker was also employed to determine if activity at the A_3AR would be abrogated by the increased distance of the hindered phenol moiety from the nucleoside. To this end, 4-aminobenzonitrile was reacted with succinic anhydride in chloroform to generate the amide **10**, which was then coupled to 4-(2-aminoethyl)-2,6-di-*tert*-butylphenol (**3g**) using EDC, HOBt and DIPEA in DMF. Reduction of the nitrile group subsequently gave the benzyl amine derivative **12**. The reaction of this amine (**12**) with the 5′-*N*-methylcarboxamide derivative of 6-chloropurine riboside **2** afforded the desired N^6-substituted adenosine-5′-*N*-methylcarboxamide **13** (Scheme 3).

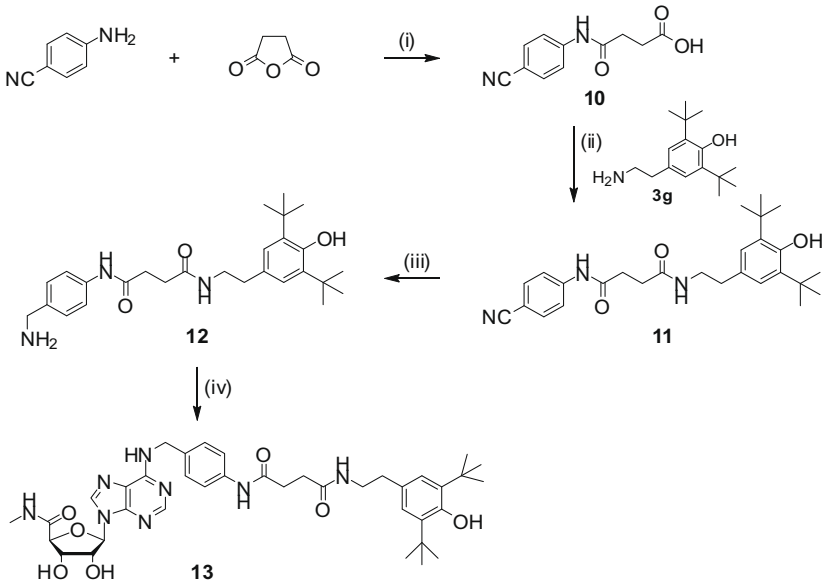
The receptor binding affinity of the first series of N^6-substituted adenosine-5′-*N*-methylcarboxamides is reported in Table 1. All compounds showed low to sub-micromolar K_i values to the high affinity agonist binding conformation of the A_3AR with good selectivity over A_{2A}AR. However, selectivity versus the A_1AR was generally only modest.

The well established A_3AR affinity induced by the 5′-*N*-methyl carboxamido group is evident through a direct comparison to the results from our previously published series of substituted nucleosides.[21] Nucleosides **5a–g** were the direct 5′-*N*-methyl carboxamido analogues of **4a–4g**, possessing low- to sub-micromolar affinities at the A_3AR. All had greater A_3AR selectivity, except **5c** which had a similar selectivity profile to **4c** (i.e., both ~4-fold A_3 selective). In the case of **5g**, selectivity increased to 69-fold (A_3 vs A_1AR) with a highly potent binding affinity (K_i value of 9 nM), as well as a 3.3-fold increase in potency. This illustrates that the presence of the 5′-*N*-methyl carboxamido moiety does not detract from the A_3AR potency or selectivity, but instead enhances it and considerably so in some cases.

In the next stage of this study, a functionalised linker was utilised to incorporate improvements in A_3AR affinity (Table 2). The N^6-phenyl series (compounds **7a–d**) were highly potent with K_i values for A_3AR ranging from 2.25 to 45 nM. Compounds **7a**, **7c** and **7d** were mildly selective for the A_3AR, whereas **7b** was

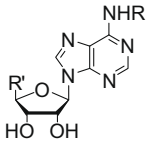


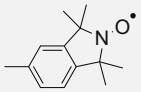
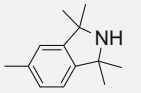
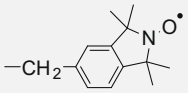
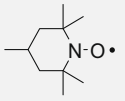
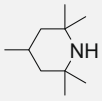
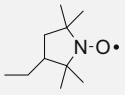
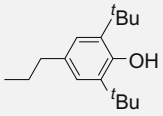
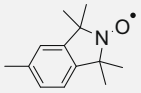
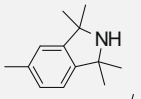
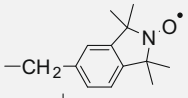
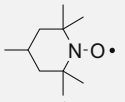
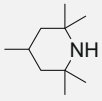
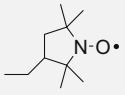
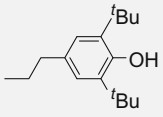
Scheme 2. Reagents and conditions: (i) R–NH_2, N(*i*-Pr)$_2$Et, *t*-BuOH, 83 °C.



Scheme 3. Reagents and conditions: (i) $CHCl_3$, 25 °C; (ii) **3g**, EDCI, HOBt, N(*i*-Pr)$_2$Et, DMF, 25 °C; (iii) H_2, Pd/C, MeOH, 25 °C; (iv) **2**, N(*i*-Pr)$_2$Et, *t*-BuOH, 83 °C.

Table 1
Receptor affinity of the target compounds

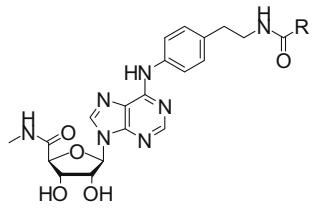


Entry	No.	R	R′	K_i A_1AR (μM)	K_i A_{2A}AR (μM)	K_i A_3AR (μM)	A_1/A_3
1	**4a**[21]		–CH_2OH	0.10 ± 0.03 (4)	>10 μM	0.79 ± 0.56 (3)	0.13
2	**4b**[21]		–CH_2OH	0.29 ± 0.03 (4)	>10 μM	0.28 ± 0.17 (2)	1.0
3	**4c**		–CH_2OH	1.14 ± 0.10 (4)	>10 μM	0.28 ± 0.16 (2)	4.1
4	**4d**[21]		–CH_2OH	0.05 ± 0.01 (4)	>10 μM	8.56 ± 3.20 (2)	0.0058
5	**4e**[21]		–CH_2OH	1.58 ± 0.45 (4)	>10 μM	25.0 ± 6.40 (3)	0.063
6	**4f**[21]		–CH_2OH	0.05 ± 0.01 (4)	>10 μM	21.2 ± 8.30 (2)	0.0024
7	**4g**		–CH_2OH	0.15 ± 0.03 (4)	20% at 10 μM (3)	0.03 ± 0.008 (5)	5.0
8	**5a**		–C(O)NHMe	0.80 ± 0.22 (4)	>10 μM	0.53 ± 0.25 (3)	1.5
9	**5b**		–C(O)NHMe	1.17 ± 0.17 (4)	>10 μM	0.07 ± 0.03 (2)	16
10	**5c**		–C(O)NHMe	1.29 ± 0.23 (4)	>10 μM	0.32 ± 0.14 (3)	4.0
11	**5d**		–C(O)NHMe	1.89 ± 0.43 (4)	>10 μM	4.16 ± 1.90 (2)	0.45
12	**5e**		–C(O)NHMe	0.96 ± 0.22 (4)	>10 μM	2.48 ± 1.28 (2)	0.39
13	**5f**		–C(O)NHMe	0.57 ± 0.19 (4)	>10 μM	1.02 ± 0.2 (2)	0.56
14	**5g**		–C(O)NHMe	0.62 ± 0.13 (4)	>10 μM	0.009 ± 0.003 (4)	69

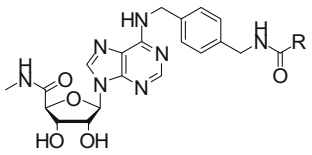
Another common way to augment A_3AR selectivity is to incorporate a methylene spacer between the N^6-position and the substituent. Nitroxide **4c**, with only the methylene spacer added as compared to previously reported compound **4a**,[21] enhanced A_3AR selectivity fourfold, coupled with a K_i of 280 nM. This is further demonstrated by comparison of **5c** versus **5a** (4 vs 1.5-fold for A_3AR) with increased potency.

>100-fold selective versus A_1AR. Building on these findings, four N^6-benzyl adenosine-5′-*N*-methylcarboxamides (compounds **9a**–**c** and **13**) were prepared and evaluated. All of these compounds showed an elevated A_3AR selectivity (14–19-fold), and retained low nanomolar affinities. It is noteworthy to add that the hindered phenol component of **13** was well tolerated even at a considerable

Table 2
Receptor affinity of the target compounds



7



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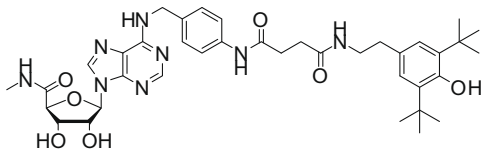
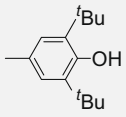
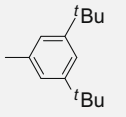
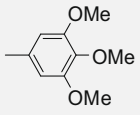
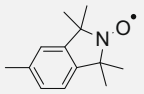
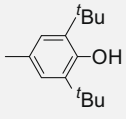
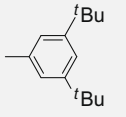
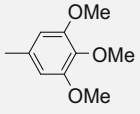


13

Entry	No.	R	K_i A_1AR (nM)	K_i A_{2A}AR (nM)	K_i A_3AR (nM)	A_1/A_3
1	**7a**		243 ± 53 (4)	>10,000	45 ± 12 (2)	5.4
2	**7b**		245 ± 77 (5)	>10,000	2.25 ± 0.59 (5)	109
3	**7c**		26.2 ± 9.5 (5)	27% at 10 μM	4.26 ± 1.01 (5)	6.2
4	**7d**		53.1 ± 11.3 (4)	>10,000	25.0 ± 2.0 (2)	2.1
5	**9a**		105 ± 14 (3)	>10,000	5.63 ± 0.90 (3)	19
6	**9b**		128 ± 22 (3)	>10,000	9.02 ± 1.96 (3)	14
7	**9c**		76.8 ± 6.7 (2)	>10,000	5.04 ± 0.19 (3)	15
8	**13**		42.1 ± 22.8 (4)	>10,000	2.22 ± 0.91 (5)	19

distance from the nucleoside core, exhibiting a K_i value of 2.22 nM. Further studies are under way to engender even better A_3AR selectivity and affinity.

2.1. Cardioprotective effects

Cultured H9C2 embryonic rat atrial cardiomyocytes were exposed to conditions used previously to mimic in vivo ischaemia.[21,25] This type of ischaemic model results in membrane dysfunction and allows entry of propidium iodide. Under simulated ischaemia conditions, 10–50% of all cells were stained positively for propidium iodide. When cells were incubated in the simulated ischaemia conditions in the presence of the prototype A_3 agonist IB-MECA, the number of PI-positive cells was reduced by 54.1 ± 6.7% ($n = 4$, $P < 0.05$). The series of adenosine receptor agonists tested in this assay all demonstrated cardioprotective properties at the same concentration (100 nM, Fig. 3). All five novel A_3 agonists tested showed equal or greater protective activity than IB-MECA, with compound **9b** reducing cell death in this model by 68.0 ± 3.6% ($n = 4$, $P < 0.05$). There were no significant differences between the level of protection conferred by the different analogues tested ($n = 4$, $P < 0.05$). The protective effects of IB-MECA and analogues **7b**, **7c**, **9a**, **9b** and **9c** were all significantly reduced in the presence of the A_3 antagonist 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(+)-dihydropyridine-3,5-dicarboxylate (MRS-1191) at 100 nM agonist concentration ($n = 4$, $P < 0.05$, Fig. 3). These data indicate that the adenosine receptor analogues evaluated have significant A_3-mediated cardioprotective effects at nanomolar concentrations.

Interestingly, compounds **9a** and **9b** have similar structures (differing only by the presence of a phenolic –OH in **9a**) and also similar receptor affinities, selectivity profile and cardioprotective effects. This suggests that the antioxidant hindered phenol moiety present in compound **9a** does not make a significant contribution to cardioprotective effects observed at the test concentration.

3. Conclusions

A number of N^6-substituted adenosine-5′-*N*-methylcarboxamides were synthesised and their pharmacology, in terms of their receptor affinity, selectivity and cardioprotective effects, were explored. The first series of compounds, **4a**–**4f** and **5a**–**5f**, showed

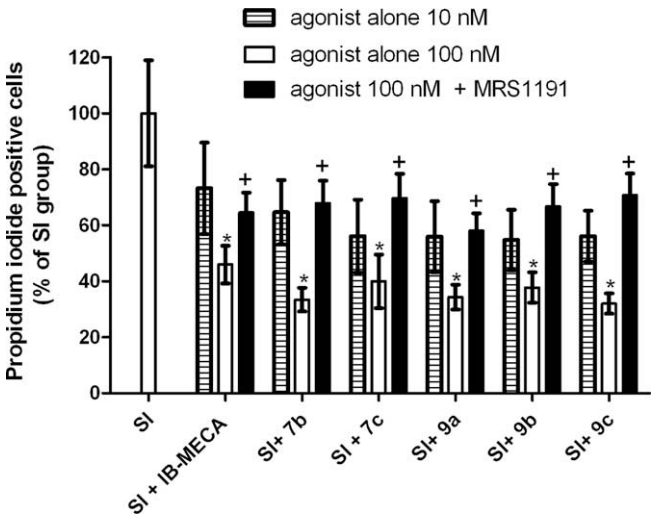


Figure 3. Cardioprotection exerted by novel A_3 agonists in a cell culture ischaemia model. Cells were incubated in hypoxic simulated ischaemia (SI) medium for 12 h. Propidium iodide exclusion was then used to determine the number of viable cells, and cell death calculated for each treatment, with the simulated ischaemia treatment normalised to 100%. * indicates a significant difference to the SI group, + indicates a significant difference to the relevant agonist treatment without antagonist.

low to mid micromolar receptor affinity for the A_3AR. However, the incorporation of a 4-(2-aminoethyl)-2,6-di-*tert*-butylphenol group in the N^6-position, for example, **4g** and **5g** significantly improved the affinity with K_i values of 30 and 9 nM, respectively. Improvements in affinity, as well as selectivity, were seen when a functionalised linker was introduced. The N^6-phenyl series, **7a**–**7d**, demonstrated low to mid nanomolar receptor affinities (2.3–45.0 nM), with **7b** displaying 109-fold selectivity for the A_3AR (vs A_1). The N^6-benzyl series **9a**–**9c** also proved to be potent and selective A_3AR agonists and the longer chain length linker **13**, was tolerated at the A_3AR without abrogation of affinity or selectivity. Cardioprotection was demonstrated by a simulated ischaemia cell culture assay whereby, **7b**, **7c**, **9a**, **9b** and **9c** all showed cardioprotective effects at 100 nM that are comparable or better than the benchmark A_3AR agonist, IB-MECA, but which were indistinguishable by statistical analysis. For example, compound **9c** reduced cell death by 68.0 ± 3.6%. No significant protective effect was observed at the lower concentration of 10 nM. These effects were reversed by the A_3AR antagonist MRS-1191, suggesting an A_3 receptor dominated mechanism of action. Further research is currently underway to determine the exact nature of the cardioprotection mechanism of these promising lead compounds.

4. Experimental

4.1. General experimental

Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. All microwave reactions took place in a Biotage Initiator Microwave Synthesiser. All NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer and ^{1}H and ^{13}C NMR spectra were recorded at 300.13 MHz and 75.4 MHz, respectively. Thin layer chromatography was conducted on 0.2 mm plates using Merck Silica Gel 60 F_{254}. Column chromatography was achieved using Merck Silica Gel 60 (particle size 0.063–0.200 μm, 70–230 mesh). 5-Amino-1,1,3,3-tetramethylisoindolin-2-yloxyl (**3a**),[26] 5-aminoisoindoline (**3b**),[27] 2,3-*O*-isopropylidene-1-(6-chloropurin-9-yl)-1-deoxy-*N*-methyl-β-D-ribofuranuronamide,[28] *N*-[2-(4-aminophenyl)ethyl]-3,5-di-*tert*-butyl-4-hydroxybenzamide (**6a**)[21] and 1,1,3,3-tetramethylisoindol-2-yloxyl-5-carboxylic acid *N*-[2-(4-aminophenyl)ethyl]amide (**6d**)[21] were prepared as previously reported. 4-Amino-TEMPO (**3d**), 4-amino-2,2,6,6-tetramethylpiperidine (**3e**), 3-(aminomethyl)PROXYL (**3f**), 4-cyanomethyl-2,6-di-*tert*-butylphenol and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid were purchased from Sigma–Aldrich. 6-Chloropurine riboside (**1**) was purchased from Advanced Molecular Technologies.

4.1.1. 1-(6-Chloropurin-9-yl)-1-deoxy-*N*-methyl-β-D-ribofuranuronamide (2)

Dowex-50W (H^+) resin (1.12 g) was added to 2,3-*O*-isopropylidene-1-(6-chloropurin-9-yl)-1-deoxy-*N*-methyl-β-D-ribofuranuronamide (462 mg, 1.31 mmol) in de-ionised water (12.5 mL) and the suspension stirred at 25 °C for 16 h. The reaction mixture was filtered through sintered glass, washed with water and the filtrate evaporated to give the title compound (**2**) as a white solid (338 mg, 82%). Mp 136 °C dec. ^{1}H NMR (CD_3OD): δ 2.83 (s, 3H), 4.45 (dd, J = 4.8, 2.4 Hz, 1H), 4.50 (d, J = 2.4 Hz, 1H), 4.84 (dd, J = 6.9, 4.8 Hz, 1H), 6.20 (d, J = 6.9 Hz, 1H), 8.79 (s, 1H), 8.84 (s, 1H). ^{13}C NMR (CD_3OD): δ 26.2, 74.3, 74.9, 86.3, 90.9, 133.6, 148.0, 152.0, 153.0, 153.2, 172.5. ESMS calcd for $C_{11}H_{13}ClN_5O_4^+$ (M+H) 314.1 and 316.1, found 314.1 and 316.1.

4.1.2. 5-(Aminomethyl)-1,1,3,3-tetramethylisoindol-2-yloxyl (3c)

5-Bromo-1,1,3,3-tetramethylisoindolin-2-yloxyl[29] (2.274 g, 8.45 mmol) was dissolved in dry DMF (12 mL) before zinc cyanide (1.191 g, 1.21 equiv) and tetrakis(triphenylphosphine)palladium (4.88 g, 0.47 equiv) were added. The reaction mixture was stirred under an atmosphere of N_2 at 138 °C for 25 h. The reaction was quenched by pouring onto aqueous ammonia (10% solution, 50 mL) and extracted with $CHCl_3$ (3 × 80 mL). The combined organic extracts were washed with aqueous ammonia (10% solution) followed by brine. Drying ($MgSO_4$) and evaporation afforded a crude black oil (8 g), which solidified upon storage below −10 °C. The reaction product was purified using silica gel column chromatography (100% chloroform), giving 5-cyano-1,1,3,3-tetramethylisoindolin-2-yloxyl as a yellow powder (1.23 g, 68% yield). This powder was recrystallised from $CHCl_3$. Mp 125–126 °C. IR (cm^{-1}): 2228.

5-Cyano-1,1,3,3-tetramethylisoindolin-2-yloxyl (480 mg, 2.2 mmol) was dissolved in dry toluene (5 mL). $LiAlH_4$ (1 M solution in diethyl ether, 4.47 mL, 2 equiv) was added and the reaction was refluxed under a nitrogen atmosphere for 18 h. A further 2.5 mL of $LiAlH_4$ solution was added at this time, and reflux continued for another 6 h. The reaction was cautiously quenched by the addition of water before it was made acidic (pH 1) through the addition of 2 M HCl and then washed with $CHCl_3$. The aqueous layer was basified (pH 14) with 5 M sodium hydroxide and repeatedly extracted with $CHCl_3$. The organic extracts which contained product were combined, washed with brine, dried ($MgSO_4$) and the solvent evaporated to give crude 5-(aminomethyl)-2-hydroxy-1,1,3,3-tetramethylisoindoline as a clear, colourless oil. This product oxidised on exposure to air for 24 h to give 5-(aminomethyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl as a yellow oil. The reaction product was further purified using column chromatography (ethyl acetate). The desired product was isolated as a yellow foam (306 mg, 48% yield) and was crystallised as dark yellow plates from acetonitrile. Mp 83–85 °C. ESMS ($M+H^+$): calcd m/z = 220.3, found m/z = 220.1.

4.1.3. 4-(2-Aminoethyl)-2,6-di-*tert*-butylphenol (3g)[30]

To a suspension of $LiAlH_4$ (320 mg, 8.56 mmol) in anhydrous Et_2O (25 mL) was added 4-cyanomethyl-2,6-di-*tert*-butylphenol (1.00 g, 4.07 mmol) in anhydrous Et_2O (25 mL) dropwise at 0 °C

under N_2. The dark mixture was gently refluxed for 4 h, then cooled to 0 °C and water and 1 M NaOH was added until the $LiAlH_4$ had decomposed. The white solid was filtered and the filtrate extracted with Et_2O (3 × 50 mL), washed with brine (2 × 50 mL), dried ($MgSO_4$), and evaporated under reduced pressure to give (**3g**) (0.89 g, 88%) as a yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.50 (s, 18H), 2.72 (t, J = 7.0 Hz, 2H), 3.00 (t, J = 7.0 Hz, 2H), 5.11 (br s, 1H), 7.05 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 30.4, 34.3, 40.1, 43.9, 125.3, 130.4, 136.0, 152.2. ESMS calcd for $C_{16}H_{28}NO^+$ (M+H) 250.2, found 250.2.

4.1.4. N^6-(1,1,3,3-Tetramethylisoindolin-2-yloxyl-5-ylmethyl)adenosine (4c)

5-(Aminomethyl)-1,1,3,3-tetramethylisoindol-2-yloxyl (**3c**) (72 mg, 0.33 mmol) and 6-chloropurine riboside (**1**) (116 mg, 1.23 equiv) were dissolved in *t*-BuOH (2 mL). DIPEA (120 μL, 2 equiv) was added to the solution and the reaction was refluxed under a nitrogen atmosphere for 94 h. The solvent was evaporated from the reaction mixture under vacuum and the resultant pale yellow foam product was purified further using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). N^6-(1,1,3,3-Tetramethylisoindolin-2-yloxyl-5-ylmethyl)adenosine (**4c**) was isolated as a yellow foam in quantitative yield. HR-MS (ESI) calcd for $C_{23}H_{30}N_6O_5^+$ (M+H) 470.2272, found 470.2270.

4.1.5. N^6-(4-(2-Aminoethyl)-2,6-di-tert-butylphenol)adenosine (4g)

To a mixture of 6-chloropurine riboside (**1**) (100 mg, 0.35 mmol) in *t*-BuOH (5 mL) was added DIPEA (183 μL, 1.05 mmol) and amine **3g** (109 mg, 044 mmol) and the mixture was heated at reflux for 24 h. The solution was evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give the desired riboside **4g** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.23) (163 mg, 93%). ^{1}H NMR (CD_3OD): δ 1.38 (s, 18H), 2.90 (t, J = 7.1 Hz, 2H), 3.44 (t, J = 7.1 Hz, 2H), 3.77 (d, J = 12.6 Hz, 1H), 3.92 (d, J = 12.6 Hz, 1H), 4.19 (d, J = 2.4 Hz, 1H), 4.35 (dd, J = 5.7, 2.4 Hz, 1H), 4.75 (dd, J = 6.3, 5.7 Hz, 1H), 5.97 (d, J = 6.3 Hz, 1H), 7.02 (s, 2H), 8.21 (s, 1H), 8.25 (s, 1H). ^{13}C NMR (CD_3OD): δ 29.5, 34.1, 35.3, 42.1, 62.1, 71.3, 74.2, 86.8, 90.0, 120.0, 124.9, 129.7, 137.8, 140.0, 147.6, 152.1, 152.3, 154.9. HR-MS (ESI) calcd for $C_{26}H_{38}N_5O_5^+$ (M+H) 500.2867, found 500.2878.

4.1.6. N^6-(1,1,3,3-Tetramethylisoindolin-2-yloxyl-5-yl)adenosine-5′-*N*-methylcarboxamide (5a)

A mixture of **2** (46 mg, 0.145 mmol) and 5-amino-1,1,3,3-tetramethylisoindolin-2-yloxyl (32 mg, 1.07 equiv) was dissolved in anhydrous *t*-BuOH (2.5 mL). DIPEA (50 μL, 2 equiv) was added to the solution, and the reaction immersed in an oil bath and refluxed under a nitrogen atmosphere for 68 h. The reaction solvent was evaporated under vacuum to give a yellow foam which was purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). The title compound (**5a**) was isolated as a yellow oil (52 mg, 74% yield). HR-MS (ESI) calcd for $C_{23}H_{29}N_7O_5^+$ (M+H) 483.2225, found 483.2215.

4.1.7. N^6-(1,1,3,3-Tetramethylisoindolin-5-yl)adenosine-5′-*N*-methylcarboxamide (5b)

5-Aminoisoindoline (**3b**) (48 mg, 0.252 mmol) and **2** (83 mg, 1.04 equiv) were combined in a reaction flask and dissolved in anhydrous *t*-BuOH (2.5 mL). DIPEA (93 μL, 2.1 equiv) was added to the solution before the reaction was refluxed under a nitrogen atmosphere for 20 h. The reaction solvent was then diluted with methanol and evaporated to give an off-white foam. This crude reaction product was purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1) to give the title compound (**5b**) as an off-white foam (117 mg, 100% yield). ^{1}H NMR (CD_3OD): δ 1.55 (s, 6H), 1.57 (s, 6H), 2.89 (s, 3H), 4.35 (dd, J = 4.8, 1.5 Hz, 1H), 4.50 (d, J = 1.5 Hz, 1H), 4.78 (dd, J = 7.5, 4.8 Hz, 1H), 6.07 (d, J = 7.5 Hz, 1H), 7.21 (d, J = 8.1 Hz, 1H), 7.69 (dd, J = 8.1, 2.1 Hz, 1H), 7.74 (d, J = 2.1 Hz, 1H), 8.37 (s, 1H), 8.47 (s, 1H). ^{13}C NMR (CD_3OD): δ 26.2, 31.6, 31.7, 64.3, 64.5, 73.6, 75.0, 86.6, 90.7, 115.3, 121.9, 122.1, 122.8, 139.8, 142.9, 144.8, 150.0, 150.1, 153.6, 153.9, 172.7. HR-MS (LSIMS) calcd for $C_{23}H_{30}N_7O_4^+$ (M+H) 468.2354, found 468.2353.

4.1.8. N^6-(1,1,3,3-Tetramethylisoindolin-2-yloxyl-5-ylmethyl) adenosine-5′-*N*-methylcarboxamide (5c)

Compound **2** (42 mg, 0.134 mmol) and 5-(aminomethyl)-1,1,3,3-tetramethylisoindol-2-yloxyl (**3c**) (25 mg, 1.03 equiv) were dissolved in anhydrous *t*-BuOH (4 mL). DIPEA (40 μL, 2 equiv) was added to the solution and the reaction was refluxed under a nitrogen atmosphere for 112 h. After this time, the reaction solvent was evaporated to give a yellow foam which was then purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). The title compound (**5c**) was isolated as a pale yellow oil (46.4 mg, 81% yield). HR-MS (ESI) calcd for $C_{24}H_{31}N_7O_5^+$ (M+H) 497.2381, found 497.2372.

4.1.9. N^6-(2,2,6,6-Tetramethylpiperidin-1-yloxyl-4-yl) adenosine-5′-*N*-methylcarboxamide (5d)

Compound **2** (55 mg, 0.175 mmol) and 4-amino TEMPO (37 mg, 1.24 equiv) were combined in a reaction flask and dissolved in anhydrous *t*-BuOH (2.5 mL). DIPEA (65 μL, 2.1 equiv) was added to the solution, and the reaction was refluxed under a nitrogen atmosphere for 30 h. After this time the solvent was evaporated under vacuum to give an orange foam. This crude reaction product was purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). The title compound (**5d**) was isolated as an orange foam (47 mg, 60% yield). HR-MS (ESI) calcd for $C_{20}H_{31}N_7O_5^+$ (M+H) 449.2381, found 449.2375.

4.1.10. N^6-(2,2,6,6-Tetramethylpiperidin-4-yl)adenosine-5′-*N*-methylcarboxamide (5e)

Compound **2** (82 mg, 0.261 mmol) and 4-amino-2,2,6,6-tetramethylpiperidine (52 μL, 1.16 equiv) were dissolved in anhydrous *t*-BuOH (2.5 mL). DIPEA (100 μL, 2.2 equiv) was added to the solution and the reaction was refluxed under a nitrogen atmosphere for 24 h. The reaction solvent was evaporated under vacuum to give a yellow foam, which was purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). The title compound (**5e**) was isolated as an off-white oil/foam (75 mg, 66% yield). ^{1}H NMR (CD_3OD): δ 1.19 (s, 6H), 1.22 (br s, 1H), 1.26 (br d, J = 12.6 Hz, 2H), 1.34 (s, 6H), 2.00 (dd, J = 12.6, 3.3 Hz, 2H), 2.87 (s, 3H), 4.32 (dd, J = 4.8, 1.2 Hz, 1H), 4.48 (d, J = 1.2 Hz, 1H), 4.74 (dd, J = 7.5, 4.8 Hz, 1H), 6.01 (d, J = 7.5 Hz, 1H), 8.23 (s, 1H), 8.32 (s, 1H). ^{13}C NMR (CD_3OD): δ 26.2, 28.1, 34.4, 44.6, 45.7, 53.0, 73.6, 75.1, 86.7, 90.7, 121.6, 142.1, 149.6, 154.2, 155.9, 173.0. HR-MS (ESI) calcd for $C_{20}H_{32}N_7O_4^+$ (M+H) 434.2510, found 434.2505.

4.1.11. N^6-(2,2,5,5-Tetramethylpyrrolidin-1-yloxyl-3-ylmethyl) adenosine-5′-*N*-methylcarboxamide (5f)

Compound **2** (62 mg, 1.36 equiv) and 3-(aminomethyl)PROXYL (**3f**) (25 mg, 0.145 mmol) were dissolved in anhydrous *t*-BuOH (2.5 mL). DIPEA (65 μL, 2.56 equiv) was added to the solution and the reaction was refluxed under a nitrogen atmosphere for 29 h. At this time the reaction solvent was evaporated to give a yellow foam, which was then purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). The title compound (**5f**) was isolated as a pale yellow oil which solidified over time (30 mg, 46% yield). HR-MS (ESI) calcd for $C_{20}H_{31}N_7O_5^+$ (M+H) 449.2381, found 449.2375.

4.1.12. N^6-(3,5-Di-*tert*-butyl-4-hydroxyphenethyl)adenosine-5′-*N*-methylcarboxamide (5g)

To a solution of compound **2** (100 mg, 0.32 mmol) in *t*-BuOH (5 mL) was added DIPEA (167 μL, 0.96 mmol) and amine **3g** (95 mg, 0.38 mmol) and the mixture was heated at reflux for 24 h. The solution was then evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give desired riboside **5g** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.25) (130 mg, 77%). ^{1}H NMR (CD_3OD): δ 1.37 (s, 18H), 2.86–2.91 (m, 5H), 3.82 (br s, 2H), 4.36 (d, J = 4.8 Hz, 1H), 4.51 (s, 1H), 4.76 (d, J = 7.8, 4.8 Hz, 1H), 6.02 (d, J = 7.8 Hz, 1H), 7.03 (s, 2H), 8.20 (s, 1H), 8.27 (s, 1H). ^{13}C NMR (CD_3OD): δ 24.7, 29.5, 34.1, 35.3, 42.0, 72.1, 73.6, 85.1, 89.2, 120.2, 124.9, 129.7, 137.8, 140.5, 147.7, 152.3, 152.4, 155.0, 171.4. HR-MS (ESI) calcd for $C_{27}H_{39}N_6O_5^+$ (M+H) 527.2976, found 527.2985.

4.1.13. *N*-[2-(4-Aminophenyl)ethyl]-3,5-di-*tert*-butyl-4-hydroxybenzamide (6b)

To a solution of 3,5-di-*tert*-butylbenzoic acid (216 mg, 0.92 mmol), EDCI (212 mg, 1.10 mmol) and HOBt (149 mg, 1.10 mmol) in DMF (5 mL) was added DIPEA (481 μL, 2.76 mmol) and *p*-aminoethylaniline (145 μL, 1.10 mmol) and the mixture was stirred at 25 °C for 16 h. The solution was then poured into water, forming a light brown precipitate, which was filtered and washed with water to give the amide **6b** (301 mg, 93%) as a brownish solid. ^{1}H NMR ($CDCl_3$): δ 1.39 (s, 18H, 6 × CH_3), 2.89 (t, J = 6.5 Hz, 2H), 3.66 (br s, 2H), 3.71 (q, J = 6.5 Hz, 2H), 6.15 (br s, 1H), 6.72 (d, J = 8.2 Hz, 2H), 7.11 (d, J = 8.2 Hz, 2H), 7.55 (s, 2H), 7.59 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 31.4, 34.8, 34.9, 41.3, 115.4, 121.0, 125.4, 128.9, 129.7, 134.5, 145.0, 151.1, 168.1. ESMS calcd for $C_{23}H_{33}N_2O^+$ (M+H) 353.5, found 353.2.

4.1.14. *N*-(4-Aminophenethyl)-3,4,5-trimethoxybenzamide (6c)

To a solution of 3,4,5-trimethoxybenzoic acid (743 mg, 3.50 mmol), EDCI (805 mg, 4.20 mmol) and HOBt (567 mg, 4.20 mmol) in DMF (10 mL) was added DIPEA (1.83 mL, 10.5 mmol) and *p*-aminoethylaniline (553 μL, 4.20 mmol) and the reaction mixture was stirred at 25 °C for 16 h. The reaction was poured into water, forming a light brown precipitate, which was filtered and washed with water to give the amide **6c** (901 mg, 78%) as an off-white solid. ^{1}H NMR ($CDCl_3$): δ 2.86 (t, J = 6.5 Hz, 2H), 3.67–3.70 (m, 4H,), 3.92 (s, 9H), 6.18 (br s, 1H), 6.70 (d, J = 8.1 Hz, 2H), 6.96 (s, 2H), 7.08 (d, J = 8.1 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 34.7, 41.4, 56.2, 60.8, 104.1, 115.3, 128.6, 129.6, 130.2, 140.7, 145.0, 153.1, 167.1. ESMS calcd for $C_{18}H_{23}N_2O_4^+$ (M+H) 331.2, found 331.1.

4.1.15. N^6-[4-[2-[3,5-Di-*tert*-butyl-4-hydroxybenzamido]ethyl]phenyl]adenosine-5′-*N*-methylcarboxamide (7a)

Compound **2** (60 mg, 1.16 equiv) and the substituted aniline **6a** (61 mg, 0.164 mmol) were dissolved in anhydrous *t*-BuOH (4 mL). DIPEA (75 μL, 2.62 equiv) was added to the solution and the reaction was refluxed under a nitrogen atmosphere for 43 h. At this time, the reaction solvent was evaporated to give a yellow foam which was then purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). The title compound (**7a**) was isolated as an off-white powder (81 mg, 78% yield). Mp 170 °C. ^{1}H NMR (CD_3OD) : δ 1.44 (s, 18H), 2.88 (s, 3H), 2.91 (t, J = 7.2 Hz, 2H), 3.60 (t, J = 7.2 Hz, 2H), 4.34 (dd, J = 4.8, 1.2 Hz, 1H), 4.50 (d, J = 1.2 Hz, 1H), 4.76 (dd, J = 7.5, 4.8 Hz, 1H), 6.05 (d, J = 7.5 Hz, 1H), 7.28 (d, J = 8.7 Hz, 2H), 7.61 (s, 2H), 8.33 (s, 1H), 8.41 (s, 1H). ^{13}C NMR (CD_3OD) : δ 26.2, 30.6, 35.3, 36.0, 41.3, 73.1, 74.4, 86.2, 90.5, 121.9, 122.3, 125.2, 126.2, 130.3, 136.3, 137.7, 137.8, 141.8, 149.4, 153.1, 153.6, 158.1, 170.8, 172.2. HR-MS (LSIMS) calcd for $C_{34}H_{44}N_7O_6$ (M+H) 646.3348, found 646.3351.

4.1.16. N^6-[4-[2-[3,5-di-*tert*-butylbenzamido]ethyl]phenyl]adenosine-5′-*N*-methylcarboxamide (7b)

To a solution of **2** (100 mg, 0.32 mmol) in *t*-BuOH (5 mL) was added DIPEA (167 μL, 0.96 mmol) and amine **6b** (225 mg, 0.65 mmol) and the mixture was heated at reflux for 48 h. The solution was then evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give the desired riboside **7b** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.23) (123 mg, 61%). ^{1}H NMR (CD_3OD): δ 1.36 (s, 18H), 2.88 (s, 3H), 2.94 (t, J = 7.2 Hz, 2H), 3.63 (t, J = 7.2 Hz, 2H), 3.81 (s, 3H), 3.87 (s, 6H), 4.36 (d, J = 4.8 Hz, 1H), 4.51 (s, 1H), 4.77 (dd, J = 7.5, 4.8 Hz, 1H), 6.06 (d, J = 7.5 Hz, 1H), 7.28 (d, J = 8.4 Hz, 2H), 7.62 (m, 3H), 7.75 (d, J = 8.4 Hz, 2H), 8.31 (s, 1H), 8.39 (s, 1H). ^{13}C NMR (CD_3OD): δ 24.7, 30.4, 34.5, 34.7, 41.3, 72.1, 73.5, 85.1, 89.2, 120.2, 120.9, 121.2, 125.3, 129.0, 134.0, 134.9, 137.1, 141.4, 148.6, 151.0, 152.1, 152.5, 169.9, 171.3. HR-MS (ESI) calcd for $C_{34}H_{44}N_7O_5^+$ (M+H) 630.3398, found 630.3413.

4.1.17. N^6-[4-[2-[3,4,5-Trimethoxybenzamido]ethyl]phenyl]adenosine-5′-*N*-methylcarboxamide (7c)

To a solution of **2** (100 mg, 0.32 mmol) in *t*-BuOH (5 mL) was added DIPEA (167 μL, 0.96 mmol) and amine **6c** (216 mg, 0.65 mmol) and the mixture was heated at reflux for 48 h. The solution was then evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give the desired riboside **7c** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.14) (128 mg, 66%). ^{1}H NMR (CD_3OD): δ 2.88 (s, 3H), 2.92 (t, J = 7.2 Hz, 2H), 3.63 (t, J = 7.2 Hz, 2H), 3.81 (s, 3H), 3.87 (s, 6H), 4.35 (d, J = 4.8 Hz, 1H), 4.50 (s, 1H), 4.76 (dd, J = 7.5, 4.8 Hz, 1H), 6.05 (d, J = 7.5 Hz, 1H), 7.13 (s, 2H), 7.26 (d, J = 8.4 Hz, 2H), 7.73 (d, J = 8.4 Hz, 2H), 8.30 (s, 1H), 8.38 (s, 1H). ^{13}C NMR (CD_3OD): δ 24.7, 34.6, 41.3, 55.4, 59.8, 72.1, 73.5, 85.1, 89.2, 104.6, 120.7, 120.8, 128.9, 129.8, 134.8, 137.1, 140.7, 141.3, 148.5, 152.1, 152.5, 153.0, 171.3, 173.7. HR-MS (ESI) calcd for $C_{29}H_{34}N_7O_8^+$ (M+H) 608.2463, found 608.2469.

4.1.18. N^6-[4-[2-[1,1,3,3-Tetramethylisoindolin-2-yloxyl-5-amido]ethyl]phenyl]adenosine-5′-*N*-methylcarboxamide (7d)

Compound **2** (64 mg, 1.10 equiv) and 1,1,3,3-tetramethylisoindol-2-yloxyl-5-carboxylic acid *N*-[2-(4-aminophenyl)ethyl]amide (**6d**) (66 mg, 0.186 mmol) were taken up in anhydrous *t*-BuOH (4 mL). DIPEA (75 μL, 2.31 equiv) was added to the solution and the reaction was refluxed under a nitrogen atmosphere for 43 h. The reaction solvent was evaporated under vacuum to give an off-white foam which was purified using column chromatography ($CHCl_3$/MeOH/NH_3, 89:10:1). The title compound (**7d**) was isolated as an off-white powder (87 mg, 74% yield). Mp 170 °C. HR-MS (ESI) calcd for $C_{32}H_{38}N_8O_6^+$ (M+H) 630.2909, found 630.2911.

4.1.19. *N*-(4-(Aminomethyl)benzyl)-3,5-di-*tert*-butyl-4-hydroxybenzamide (8a)

To a stirred solution of *p*-xylylenediamine (544 mg, 3.99 mmol) in DMF (10 mL) at 0 °C was added dropwise, a solution of 3,5-di-*tert*-butyl-4-hydroxy-benzoic acid (200 mg, 0.80 mmol), EDCI (169 mg, 0.88 mmol), HOBt (119 mg, 0.88 mmol) and DIPEA (417 μL, 2.39 mmol) in DMF (5 mL). The resultant mixture was stirred for 16 h at 25 °C, at which time it was poured onto ice/water (100 mL) and stirred for 1 h. The small amount of precipitate that formed was filtered, washed with water and the filtrate extracted with $CHCl_3$ (3 × 30 mL), washed with brine, dried over $MgSO_4$, filtered and the filtrate evaporated to give the amide **8a** (109 mg, 37%) as a yellow oil. ^{1}H NMR (DMSO): δ 1.38 (s, 18H), 4.43 (d,

J = 6.0 Hz, 2H), 5.20 (br s, 1H), 7.15–7.22 (m, 4H), 7.64 (s, 2H), 8.97 (t, J = 6.0 Hz, 1H).

4.1.20. *N*-(4-(Aminomethyl)benzyl)-3,5-di-*tert*-butylbenzamide (8b)

To a stirred solution of *p*-xylylenediamine (654 mg, 4.80 mmol) in DMF (10 mL) at 0 °C was added dropwise, a solution of 3,5-di-*tert*-butylbenzoic acid (225 mg, 0.96 mmol), EDCI (202 mg, 1.06 mmol), HOBt (143 mg, 1.06 mmol) and DIPEA (500 μL, 2.88 mmol) in DMF (5 mL). The mixture that formed was stirred for 16 h at 25 °C, at which time it was poured onto ice/water (100 mL) and the resultant solution stirred for 1 h. The precipitate that formed was filtered, washed with water to give the amide **8b** (219 mg, 65%). ^{1}H NMR DMSO: δ 1.32 (s, 18H), 4.47 (d, J = 6.0 Hz, 1H), 7.24–7.32 (m, 4H), 7.56 (s, 1H), 7.73–7.75 (m, 2H), 8.98 (t, J = 6.0 Hz, 1H).

4.1.21. *N*-(4-(Aminomethyl)benzyl)-3,4,5-trimethoxybenzamide (8c)

To a stirred solution of *p*-xylylenediamine (321 mg, 2.35 mmol) in DMF (10 mL) at 0 °C was added dropwise, a solution of 3,4,5-trimethoxybenzoic acid (100 mg, 0.47 mmol), EDCI (109 mg, 0.57 mmol), HOBt (77 mg, 0.57 mmol) and DIPEA (100 μL, 0.57 mmol) in DMF (5 mL). The mixture that formed was stirred for 16 h at 25 °C, at which time it was poured onto ice/water (100 mL) and the resultant solution stirred for 1 h. The small amount of precipitate that formed was filtered (to give dimer, 12%), washed with water and the filtrate extracted with $CHCl_3$ (3 × 30 mL), washed with brine, dried over $MgSO_4$, filtered and the filtrate evaporated to give the amide **8c** (89 mg, 57%). ^{1}H NMR (DMSO): δ 3.71 (s, 3H), 3.83 (s, 6H), 4.46 (d, J = 6.0 Hz, 2H), 7.20–7.30 (m, 6H), 8.97 (t, J = 6.0 Hz, 1H).

4.1.22. N^6-[4-[2-[3,5-Di-*tert*-butyl-4-hydroxybenzamido]ethyl] benzyl]adenosine-5′-*N*-methylcarboxamide (9a)

To a solution of compound **2** (70 mg, 0.22 mmol) in *t*-BuOH (5 mL) was added DIPEA (116 μL, 0.67 mmol) and amine **8a** (165 mg, 0.44 mmol) and the mixture was heated at reflux for 24 h. The reaction mixture was then evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give the desired product **9a** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.18) (104 mg, 72%). ^{1}H NMR (CD_3OD): δ 1.45 (s, 18H), 2.87 (s, 3H), 4.34 (d, J = 4.8 Hz, 1H), 4.50 (s, 1H), 4.54 (s, 2H), 4.75 (dd, J = 7.5, 4.8 Hz, 1H), 4.82, (br s, 2H), 6.02 (d, J = 7.5 Hz, 1H), 7.29–7.34 (m, 4H), 7.71 (s, 2H), 8.21 (s, 1H), 8.30 (s, 1H). ^{13}C NMR (CD_3OD): δ 24.7, 29.2, 34.2, 42.9, 43.5, 72.0, 73.5, 85.1, 89.2, 120.2, 124.2, 125.0, 127.3, 128.3, 137.3, 137.6, 138.3, 140.7, 152.0, 154.8, 157.3, 169.7, 171.4. HR-MS (ESI) calcd for $C_{34}H_{44}N_7O_6^+$ (M+H) 646.3348, found 646.3350.

4.1.23. N^6-[4-[2-[3,5-Di-*tert*-butylbenzamido]ethyl]benzyl] adenosine-5′-*N*-methylcarboxamide (9b)

To a solution of compound **2** (73 mg, 0.23 mmol) in *t*-BuOH (5 mL) was added DIPEA (125 μL, 0.72 mmol) and amine **8b** (164 mg, 0.47 mmol) and the mixture was heated at reflux for 24 h. The reaction mixture was then evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give the desired product **9b** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.25) (110 mg, 75%). ^{1}H NMR (CD_3OD): δ 1.35 (s, 18H), 2.87 (s, 3H), 4.34 (d, J = 4.8 Hz, 1H), 4.50 (s, 1H), 4.56 (s, 2H), 4.76 (dd, J = 7.8, 4.8 Hz, 1H), 4.82, (br s, 2H), 6.02 (d, J = 7.8 Hz, 1H), 7.31–7.38 (m, 4H), 7.64 (s, 1H), 7.74 (s, 2H), 8.22 (s, 1H), 8.31 (s, 1H). ^{13}C NMR (CD_3OD): δ 24.6, 30.4, 34.5, 42.9, 43.5, 72.0, 73.5, 85.1, 89.2, 120.2, 121.3, 125.4, 127.3, 127.4, 133.6, 137.7, 138.0, 140.7, 151.0, 152.5, 154.9, 169.5, 171.4. HR-MS (ESI) calcd for $C_{34}H_{44}N_7O_5^+$ (M+H) 630.3398, found 630.3406.

4.1.24. N^6-[4-[2-[3,4,5-Trimethoxybenzamido]ethyl]benzyl] adenosine-5′-*N*-methylcarboxamide (9c)

To a solution of compound **2** (65 mg, 0.21 mmol) in *t*-BuOH (5 mL) was added DIPEA (108 μL, 0.62 mmol) and amine **8c** (137 mg, 0.42 mmol) and the reaction mixture was heated at reflux for 24 h. The solution was then evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give the desired riboside **9c** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.15) (101 mg, 80%). ^{1}H NMR (CD_3OD): δ 2.89 (s, 3H), 3.83 (s, 3H), 3.90 (s, 6H), 4.34 (d, J = 4.5 Hz, 1H), 4.50 (s, 1H), 4.58 (s, 2H), 4.77 (dd, J = 7.8, 4.8 Hz, 1H), 4.85 (br s, 2H), 6.04 (d, J = 7.5 Hz, 1H), 7.22 (s, 2H), 7.35 (d, J = 7.8 Hz, 2H), 7.40 (d, J = 7.8 Hz, 2H), 8.26 (s, 1H), 8.34 (s, 1H). ^{13}C NMR (CD_3OD): δ 24.6, 43.0, 43.5, 55.4, 59.7, 72.0, 73.5, 85.1, 89.2, 104.7, 120.2, 126.4, 127.3, 127.4, 129.5, 137.9, 140.8, 152.5, 153.1, 154.9, 168.1, 171.4. HR-MS (ESI) calcd for $C_{29}H_{34}N_7O_8^+$ (M+H) 608.2463, found 608.2469.

4.1.25. 4-(4-Cyanophenylamino)-4-oxobutanoinc acid (10)

A solution of *p*-aminobenzonitrile (1.00 g, 8.47 mmol) and succinic anhydride (0.89 g, 8.90 mmol) in $CHCl_3$ (50 mL) was stirred for 16 h at 25 °C. The white precipitate that formed was filtered and washed with $CHCl_3$ to give (**10**) (1.40 g, 76%) as a white solid. ^{1}H NMR (DMSO): δ 2.54 (t, J = 6.0 Hz, 2H, CH_2), 2.62 (t, J = 6.0 Hz, 2H, CH_2), 7.77 (s, 4H, ArH), 10.39 (br s, 1H, NH), 12.14 (br s, 1H, COOH). ESMS calcd for $C_{11}H_9N_2O_3^-$ (M−H) 217.1, found 217.1.

4.1.26. *N*-(4-Cyanophenyl)-N^4-(3,5-di-*tert*-butyl-4-hydroxyphenethyl)succinamide (11)

To a solution of the acid **10** (438 mg, 2.00 mmol), EDC (462 mg, 2.41 mmol) and HOBt (326 mg, 2.41 mmol) in DMF (10 mL) was added DIPEA (770 μL, 4.42 mmol) and amine **3g** (500 mg, 2.00 mmol) and the mixture was stirred for 16 h at 25 °C. Water (150 mL) was then added and the mixture was extracted with EtOAc (3 × 50 mL), washed with water (5 × 30 mL), dried with $MgSO_4$ and evaporated at reduced pressure. The resultant red oil was purified by SiO_2 column chromatography (hexane/EtOAc, 1:1) and gave the amide **11** as a colourless foam. ^{1}H NMR ($CDCl_3$): δ 1.49 (s, 18H), 2.62–2.82 (m, 6H), 3.58 (q, J = 6.0 Hz, 2H), 5.17 (br s, 1H), 5.74 (t, J = 6.0 Hz, 1H), 7.01 (s, 2H), 7.60 (d, J = 8.7 Hz, 2H), 7.73 (d, J = 8.7 Hz, 2H), 9.47 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 30.4, 31.1, 32.6, 34.3, 35.6, 41.5, 106.3, 119.0, 119.5, 125.1, 129.1, 133.0, 136.4, 142.9, 152.5, 171.4, 172.5. ESMS calcd for $C_{27}H_{36}N_3O_3^+$ (M+H) 450.3, found 450.4.

4.1.27. N^1-(4-(Aminomethyl)phenyl)-N^4-(3,5-di-*tert*-butyl-4-hydroxyphenethyl)succinamide (12)

To a solution of **11** (220 mg, 0.49 mmol) in MeOH (10 mL) and 4 M HCl (1 mL) was added 10% Pd on carbon (50 mg) and the mixture was stirred vigorously under H_2 for 4 h, then filtered through Celite and washed with diethyl ether. The filtrate was evaporated to give the hydrochloride of **12** (170 mg, 71%), which was used directly in the next step.

4.1.28. N^6-[4-(4-(3,5-Di-*tert*-butyl-4-hydroxyphenethylamino)-4-oxobutanamido)benzyl] adenosine-5′-*N*-methylcarboxamide (13)

To a solution of **2** (65 mg, 0.21 mmol) in *t*-BuOH (5 mL) was added DIPEA (147 μL, 0.83 mmol) and amine **12** (160 mg, 0.35 mmol) and the mixture was heated at reflux for 24 h. The solution was then evaporated at reduced pressure onto SiO_2 and purified by column chromatography to give the desired product **13** ($CHCl_3$/MeOH/NH_3, 89:10:1, R_f = 0.17) (85 mg, 56%). ^{1}H NMR (CD_3OD): δ 1.43 (s, 18H), 2.54 (t, J = 6.6 Hz, 2H), 2.64–2.72 (m, 4H), 2.89 (s, 3H), 3.34–3.39 (m, 2H), 4.35 (d, J = 4.8 Hz, 1H), 4.50 (s, 1H), 4.76 (dd, J = 7.8, 4.8 Hz, 1H), 6.04 (d, J = 7.8 Hz, 1H), 7.00 (s, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 8.26 (s,

1H), 8.35 (s, 1H). ^{13}C NMR (CD_3OD): δ 24.6, 29.5, 30.7, 31.7, 34.1, 35.2, 41.2, 43.3, 72.0, 73.6, 85.1, 89.2, 119.9, 120.1, 124.6, 127.7, 129.9, 134.5, 137.6, 137.9, 140.7, 152.0, 152.5, 154.9, 155.0, 171.4, 171.5, 173.1. HR-MS (ESI) calcd for $C_{38}H_{51}N_8O_7^+$ (M+H) 731.3875, found 731.3882.

4.2. Receptor binding assays

Competitive binding assays were performed using recombinant human adenosine receptors with the radioligands [^{125}I]-ABA (A_1 receptor agonist), [^{3}H]-ZM241385 (A_{2A} receptor antagonist), [^{125}I]-ABOPX (A_{2B} receptor antagonist) and [^{125}I]-ABA (A_3 receptor agonist).[31] Radioligand binding assays were conducted at 21 °C for 2–3 h in 100 μL of buffer containing 10 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 5–20 μg membrane protein and 1 U/mL adenosine deaminase. The use of agonist radioligands for A_1 and A_3 receptors and the inclusion of $MgCl_2$ creates conditions that stabilise the high affinity state of receptors complexed with G proteins. Non-specific binding was measured in the presence of 100 μM NECA. Competition binding experiments were carried out with 0.5–0.6 nM radioligand. To detect bound radioligand, membranes were filtered over Whatman GF/C filters using a Brandel cell harvester (Gaithersburg, MD) and washed three times over 15–20 s with ice-cold buffer (10 mM Tris, 1 mM $MgCl_2$, pH 7.4). B_{max} and K_D values were calculated by nonlinear least-squares interpolation to a single-site binding model.

In competition binding assays, IC_{50} values for competing compounds were derived by fitting the data to a four-parameter logistic equation: $B = \text{specific}/(1 + (IC_{50})^n) + \text{nonspecific}$, where B = specific binding and n = the Hill slope. K_i was derived from IC_{50} as described by Linden.[32]

The K_i values determined for human A_1 and A_3 receptors represent binding to the G-protein coupled high affinity conformational state that is detected by the use of an agonist radioligand.[32]

4.3. Propidium iodide assay

4.3.1. Simulated ischaemic (SI) buffer

Simulated ischaemic buffer components (in mM); 137 NaCl, 3.5 KCl, 0.88 $CaCl_2{\cdot}2H_2O$, 0.51 $MgSO_4{\cdot}7H_2O$, 5.55 D-glucose, 4 HEPES, 2% FBS (foetal bovine serum) with 10 mM 2-deoxy-D-glucose and 20 mM DL-lactic acid.[33]

4.3.2. Method for simulated ischaemia

H9C2 (2-1) embryonic rat cardiomyocyte cell lines (ATCC, USA) were used for this assay. The cell line was grown in Dulbecco's modified Eagle's medium contain 4 mM L-glutamate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 100 U/mL penicillin and 100 mg/mL streptomycin supplemented with 10% foetal bovine serum in 5% CO_2 incubator. Cells were used at 60–70% confluence and plated in the density of 5×10^4 cells/mL in a 96-well plate 1 day prior to assay. For simulated ischaemia, media from each well was replaced with 100 μL/well SI buffer and the plate was placed in the nitrogen incubator for 12 h at 37 °C. The control group was kept in the normal oxygen incubator for the same period of time. In the control group HEPES buffer without 2-deoxy-D-glucose and DL-lactic acid was used.

At the end of the simulated ischemic protocol 5 μM propidium iodide was added to each well and after 5 min images were taken from an inverted microscope (Nikon Eclipse TE2000U; Nikon Instruments, Tokyo, Japan) with 535 nM excitation and 617 nM emission wavelength. Duplicate wells were used for each experiment, and each experiment was repeated three times. From each well four images were taken, and propidium iodide positive cells were quantified using 'Scion Image' software (Scion Image Alpha version 4.0.3.2) then normalised to SI group considered as a 100% cell death.

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