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Research report

# A novel dihydro-pyrazolo(3,4d)(1,2,4)triazolo(1,5a)pyrimidin-4-one (AJ23) is an antagonist at adenosine $A_1$ receptors and enhances consolidation of step-down avoidance

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## HIGHLIGHTS

- AJ23 is a non-xanthine compound with micromolar affinity at adenosine $A_1$ receptors.
- AJ23 has little affinity at non-purine receptors for other neuroactive agents.
- Adenosine depression of fEPSPs in the hippocampus is blocked by AJ23.
- Injected into the hippocampus AJ23 improves consolidation.

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## ABSTRACT

Adenosine $A_1$ receptor antagonists are of potential value in the treatment of cognitive dysfunction. We have developed compound AJ23 (7-methyl-1-phenyl-1,8-dihydro-pyrazolo-(3,4d)(1,2,4)-triazolo(1,5a)-pyrimidin-4-one) as a novel, non-xanthine based antagonist at $A_1$ receptors. It has micromolar affinity at human $A_1$ receptors with a 45-fold selectivity for $A_1$ over $A_{2A}$ receptors and little affinity for many other receptors and transporters tested in a screening panel. AJ23 blocks $A_1$ receptors in the rat hippocampus, increasing the baseline size of excitatory post-synaptic potentials and blocking the inhibitory effects of adenosine. When administered directly into the rodent hippocampus this compound improves consolidation in a step-down avoidance learning task. The results suggest that AJ23 or derivatives may represent possible leads for further chemical development towards a chemically novel group of antagonists at $A_1$ receptors with potential value as cognitive enhancers.



## 1. Introduction

Glutamate and acetylcholine are among those neurotransmitters in the central nervous system (CNS) with the most well-established roles in various forms of learning and memory [1]. Glutamate mediates transmission between the major pathways intrinsic to the hippocampus, with important roles in the modulation of synaptic plasticity as well as learning and memory processes during behavioural tasks [2]. Cholinergic neurons are also involved prominently in cognitive functions [3].

A common feature of glutamatergic and cholinergic terminals in the CNS is their possession of adenosine $A_1$ receptors which suppress the release of the primary transmitters [4,5] and may also, therefore, have crucial roles in cognition [6–8]. Activation of $A_1$ receptors can impair several forms of learning [9–13]. Conversely,

*Abbreviations:* AJ23, 7-methyl-1-phenyl-1,8-dihydropyrazolo-(3,4d)(1,2,4)triazolo(1,5a)pyrimidin-4-one; ACSF, artificial cerebrospinal fluid; CHO, Chinese Hamster Ovary; DMSO, $d^6$ – dimethylsulphoxide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; HEK, Human Embryonic Kidney; 5-HT, 5-hydroxytryptamine; IBRO, International Brain Research Organization; IR, infrared; $-K_D$, dissociation constant; LTP, long-term potentiation; LTD, long-term depression; NMR, nuclear magnetic resonance; PEI, polyethyleneimine; THF, tetrahydrofuran; ZM-241385, (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3a][1,3,5]triazin-5-ylamino]ethyl)phenol.

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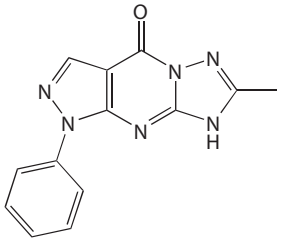

**Fig. 1.** Structure of 7-methyl-1-phenyl-1,8-dihydro-pyrazolo(3,4d)(1,2,4)triazolo (1,5a)pyrimidin-4-one (AJ23).

the blockade of $A_1$ receptors facilitates learning [13,14] such that selective antagonists represent suitable targets for the development of potential value in the treatment of cognitive dysfunction associated with ageing, stroke-induced brain damage, or neurodegenerative disorders [15].

We have now designed and synthesised a novel compound, 7-methyl-1-phenyl-1,8-dihydro-pyrazolo-(3,4d)(1,2,4)- triazolo-(1,5a)-pyrimidin-4-one (AJ23) (Fig. 1) which represents a new chemical structure acting as a selective $A_1$ receptor antagonist. The structure of AJ23 is not based around the xanthine nucleus, which has been responsible for terminating the development of several previous adenosine antagonists [16–20]. This difficulty, together with the understanding that xanthines were not simple, classically competitive antagonists, but their binding to receptors showed a complex relationship to the binding of adenosine itself [21,22], led to previous attempts to synthesise non-xanthine compounds [23–26] showing better potency, selectivity or toxicity profiles than xanthine-based compounds.

## 2. Methods

### 2.1. Synthesis

AJ23 was synthesised by adapting the route of El-Sherbeny et al. [27] to related compounds (Fig. 2). Pyrazole 1 was prepared by the literature method [28,29] and then converted into cyclic diamine 2 in modest yield over three steps. Reaction with triethyl orthoacetate then gave AJ23.

#### 2.1.1. Synthetic procedures

Reagents were obtained from commercial sources and used as received. Tetrahydrofuran (THF) was distilled from sodium and benzophenone. Where dry dichloromethane (DCM) is specified, it was dried by distillation from $CaH_2$.

#### 2.1.2. Preparation of 5,6-diamino-1-phenyl-1,5-dihydropyrazolo(3,4d)pyrimidin-4-one (2)

Thiophosgene (9.0 ml) was added to a stirred and cooled (0 °C) mixture of dry DCM (DCM, 300 ml), and calcium carbonate (100 g). A solution of ester 1 (21.9 g) dissolved in 37% HCl (125 ml) was added slowly dropwise over 2 h. After being stirred for an additional 12 h, the reaction mixture was filtered and the solid material was washed with DCM. The layers of the filtrate were separated and the aqueous solution extracted with DCM (4 times). The combined organics were washed twice with aqueous HCl and the solvent was removed under reduced pressure. The resulting crude isothiocyanate was dissolved in THF (60 ml) and added dropwise to stirred liquid ammonia (−78 °C) (20 ml). The reaction mixture was stirred at −78 °C for 3 h and then allowed to warm to room temperature and stirred for a further 12 h. The resulting brown sludge was dissolved in THF (20 ml) then the solvent removed reduced pressure to give the crude thiourea. This was taken up in minimal ethanol (50 ml), hydrazine monohydrate (28.0 ml) was added dropwise and the reaction mixture stirred at reflux for 12 h. After cooling the solid was obtained by filtration and dried under vacuum to give the diamine 2 (7.95 g, 35%). M.p. 294–295 °C. IR ($\upsilon_{max}$): 3320 (NH), 3232 (NH), 1681 $cm^{-1}$ (C=O). $^1$H NMR (400 MHz, DMSO-$d_6$): 8.15 (2H, d, *J* 7.7 Hz, ArH), 8.08 (1H, s, H-3), 7.56–7.51 (2H, m, ArH), 7.35 (1H, t, *J* 7.4 Hz, ArH). $^{13}$C NMR (100 MHz, DMSO-$d_6$): 101.4 (C), 121.1 (CH), 126.2 (CH), 129.2 (CH), 136.5 (CH), 139.3 (C), 155.1 (C), 157.1 (C), 157.4 (C). LRMS (FAB): 243 [(M+H)$^+$, 50%], 157 (60), 79 (100).

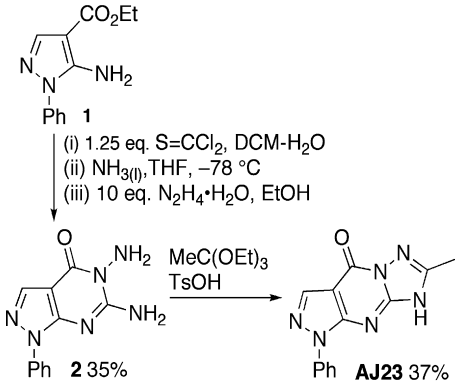


**Fig. 2.** Scheme for the synthesis of AJ23 as detailed in Section 2.

#### 2.1.3. Preparation of 7-methyl-1-phenyl-1,8-dihydro-pyrazolo(3,4d)-(1,2,4)triazolo(1,5a)pyrimidin-4-one (AJ23)

A suspension of diamine 2 (1.00 g) and p-toluene sulphonic acid monohydrate (1.00 g) in triethyl orthoacetate (20 ml) was heated under reflux for 20 h. After cooling, the solid was collected and recrystallised from ethanol to give the target compound AJ23 (405 mg, 37%) as an amorphous solid. M.p. > 300 °C. IR ($\upsilon_{max}$): 3070 (NH), 1716 $cm^{-1}$ (C=O). $^1$H NMR (400 MHz, DMSO-$d_6$): 13.85 (1H, broad s), 8.31 (1H, s, CH), 8.12 (2H, d, *J* = 8.4 Hz, ArH), 7.58 (2H, apparent t, *J* = 8.0 Hz, ArH), 7.38 (1H, t, *J* = 7.4 Hz, ArH), 2.78 (3H, s, $CH_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$): 14.9 ($CH_3$), 101.6 (C), 121.7 (CH), 127.2 (CH), 130.2 (CH), 137.9 (CH), 139.7 (C), 144.3 (C), 152.7 (C), 153.9 (C), 155.4 (C). LRMS (EI+): 266 ($M^+$, 7%), 78 (90), 63 (100).

### 2.2. Radioligand binding

Displacement binding studies were performed with cloned human $A_1$ and $A_{2A}$ receptors. The human recombinant adenosine $A_1$ receptor (ES-010-M; Euroscreen) was stably expressed in CHO-K1 cells and the membrane suspensions (received as frozen aliquots in 7.5 mM Tris–HCl, pH 7.5, containing 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose) were diluted in assay buffer after thawing. The binding ligand used was tritiated 1,3-dipropyl-8-cyclopentylxanthine ([$^3$H]-DPCPX) (Tocris Cookson; specific activity 3.811 TBq/mmol) and was used in assays at 20 nM.

The human recombinant $A_{2A}$ receptor (RBHA2AM; PerkinElmer) was stably expressed in HEK-293 cells and the membrane suspensions (received as frozen aliquots in 50 mM Tris–HCL, pH 7.4, containing 10% sucrose) were diluted in assay buffer on thawing. The $A_{2A}$ receptor ligand used was 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3a][1,3,5]triazin-5-ylamino]ethyl)phenol ([$^3$H]ZM-241385) (Tocris Cookson; specific activity 0.777 TBq/mmol) and was used in assays at 20 nM.

The assay method was based on a protocol described previously [30,31]. Filter plates (Millipore Multiscreen MHAF B3H60) were presoaked in 0.3% polyethyleneimine (PEI). 10 μg of membrane protein were used in a final assay volume of 0.2 ml. For $A_{2A}$ binding assays, a Tris–HCl buffer (50 mM Tris–HCl, 0.5 mM EDTA and 10 mM $MgCl_2$, pH 7.4) supplemented with 1 U/ml adenosine deaminase was used, whilst for $A_1$ receptor binding a HEPES buffer (20 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, pH 7.4) was used. Stock solutions of AJ23 were prepared in dimethylsulphoxide (DMSO; final concentration ≤ 1%). Non-specific binding was measured in the presence of 100 μM known non-radioactive ligands and accounted for less than 5% of total binding.

After incubation for 1 h at 25 °C, assays were terminated by rapid filtration using a Millipore manifold at a pressure of 700 mbar. Filters were washed with 3 × 200 μl of the relevant assay buffer before counting in a scintillation counter (Wallac Microbeta). Data were analysed using GraphPad Prism (GraphPad, San Diego, CA). For nonlinear regression analysis, the Cheng–Prusoff equation and $K_D$ values of 1.6 nM (human $A_1$) for [$^3$H]DPCPX and 1 nM (human $A_{2A}$) for [$^3$H]ZM-241385 were used to calculate $K_i$ values from $IC_{50}$ values.

A commercial screen (Cerep Ltd., France) was also conducted of AJ23 displacement of ligand binding at 25 neurotransmitter receptors and 3 transporters using an automated, robotic system. Most of the receptor panel were recombinant human receptors (h) stably expressed in Chinese Hamster Ovary (CHO) or Human Embryonic Kidney (HEK) cells. Since human receptor clones were not available for some ligands, receptors for noradrenaline, GABA, acetylcholine (muscarinic), neuropeptide Y, opiate, phencyclidine, 5-HT and sigma ligands were obtained from homogenates of rat cerebral cortex. The results are expressed as the percentage inhibition by AJ23 (10 μM) of ligand specific binding, using an appropriate high-affinity selective radiolabelled ligand for each receptor or site examined (Table 1).

### 2.3. Hippocampal electrophysiology using rat brain slices

All procedures were in accordance with the regulations and recommendations of the Animals (Scientific Procedures) Act, 1986 of the United Kingdom Home Office. Hippocampal slices were prepared as described previously [32,33]. Briefly, male Wistar rats weighing 100–150 g were killed by administering an overdose of urethane (10 ml/kg body weight i.p., as a 25% solution in water) followed by cervical dislocation. The brain was rapidly removed into ice-cold artificial cerebrospinal fluid (aCSF) of composition: (in mM) NaCl 115; $KH_2PO_4$ 2.2; KCl 2; $MgSO_4$ 1.2;

**Table 1**
AJ23 binding assays for target specificity (h = human).

| Target site | Test compound | AJ23 (10 μM) inhibition (%) |
|---|---|---|
| Adrenoceptor (α1) | Prazosin | 10 |
| Adrenoceptor (α2) | Yohimbine | 7 |
| Adrenoceptor (β, h) | Atenolol | 6 |
| Angiotensin AT 1(h) | Saralasin | 3 |
| Benzodiazepine (central) | Diazepam | 5 |
| Benzodiazepine 2 (h) | NPC567 | 11 |
| Cholecystokinin ($CCK_A$ (h); CCK1) | CCK-8 | −7 |
| Dopamine D1 (h) | SCH23390 | 5 |
| Dopamine D2S (h) | (+)Butaclamol | −6 |
| Endothelin $ET_A$ (h) | Endothelin-1 | 7 |
| GABA | GABA | −4 |
| NMDA | CGS19755 | −5 |
| Histamine H1 (h) | Pyrilamine | −1 |
| Melanocortin MC4 (h) | NDP-αMSH | 3 |
| Muscarinic | Atropine | 0 |
| Neurokinin NK1 (h) | [$Sar^9$, $Met(O_2)^{11}$]-SP | −2 |
| Neuropeptide Y | NPY | −7 |
| Nicotinic (neuronal, αBGTX-insensitive) | Nicotine | 4 |
| Opiate (non-selective) | Naloxone | −5 |
| Nociceptin ORL1 (h)(NOP) | Nociceptin | −1 |
| Phencyclidine (PCP) | Dizocilpine (MK-801) | −3 |
| 5-HT (non-selective) | 5-HT | 21 |
| sigma (non-selective) | Haloperidol | 13 |
| Glucocorticoid (h)(GR) | Dexamethasone | 1 |
| Vasopressin V1a (h) | [$d(CH_2)_5{}^1$, $Tyr(Me)_2$]AVP | 5 |
| Transporters | | |
| Norepinephrine (NET) (h) | Protriptyline | −12 |
| Dopamine (DAT) (h) | BTCP | −5 |
| Serotonin (5-HTT) (h) | Imipramine | −5 |

$NaHCO_3$ 25; $CaCl_2$ 2.5; D-glucose 10, gassed with 5% $CO_2$ in oxygen. The hippocampi were chopped into 450 μm transverse slices using a McIlwain tissue chopper. The slices were preincubated at room temperature for at least 1 h in a water-saturated atmosphere of 5% $CO_2$ in $O_2$ before individual slices were transferred to a 1 ml capacity superfusion chamber where submerged slices were superfused with aCSF at 28–30 °C and a flow rate of 3–4 ml/min. The Schaffer collaterals and commissural afferents to the CA1 region were stimulated using a concentric bipolar electrode (Harvard Apparatus, Edenbridge, UK) positioned in the stratum radiatum (0.1 Hz stimulation using a pulse duration of 50–300 μs). The stimulating electrode tip was located immediately internal to the stratum pyramidale at the border between the CA1 and CA2 regions. Recording electrodes were constructed from fibre-containing borosilicate glass capillary tubing (Harvard Apparatus, Edenbridge, Kent, UK), with the tips broken back under microscopic control to 2–4 μm, DC resistance approximately 5 MΩ when filled with a solution of 1 M NaCl. Population synaptic potentials (fEPSPs) in the CA1 stratum pyramidale were amplified and captured on a micro1401 interface (CED, Cambridge Electronic Design, Cambridge, UK) for storage on computer and subsequent analysis using Signal software (CED, Cambridge, UK). The slope of the fEPSP was measured along the most linear part of the potential, usually between cursors set at approximately 20% and 60% of the peak amplitude.

### 2.4. Step-down inhibitory avoidance

Effects on learning and memory were tested using a step-down inhibitory avoidance model of learning in rats treated with scopolamine as described previously [34]. All experiments were performed in accordance with the Review Committee of the Veterinary School (CICUAL), University of Buenos Aires and the International Brain Research Organization (IBRO), and are in compliance with the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985) and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Adult male Wistar rats (200–300 g) bred in-house were maintained in groups of 4–6 under a 12 h light/dark inverted cycle with water and food available ad libitum. They were anesthetized with ketamine/xylazine (i.p.; 75 and 10 mg/kg, respectively), and were bilaterally implanted with 27-gauge guide cannulae directed at a point 1.00 mm above the CA1 region of the dorsal hippocampus (coordinates *A*: −4.3 mm, *L*: 4.0 mm, *V*: 2.2 mm, modified from [35] for the rats from our colony). The location of the cannula tips was confirmed by post mortem examination at the end of the experiment.

Four to five days after the implantation of the cannula into the hippocampus (see above), each rat received an i.p. injection of 0.5 mg/kg (−)scopolamine hydrochloride (Sigma Chemicals), a dose which is known to have an amnesic effect for this task, 30 min before the training session. AJ23 was injected via a 30-gauge needle inserted through the guide cannula and protruding 1 mm beyond its tip, into the CA1 pyramidal cell layer of the dorsal hippocampus. Animals were randomly divided into groups; in one group the rats received a bilateral infusion of AJ23 (10 nmol), and in the other, the control group, the rats received a bilateral infusion of vehicle (5% DMSO in water) in a final volume of 1 μl, before (pre-training experiment) or after (post-training experiment) the training session.

Rats were initially trained in the step-down inhibitory avoidance task. A rat was placed on an elevated isolated platform (25 cm × 7 cm × 2.5 cm high) at the left side of an acrylic box (50 cm × 25 cm × 25 cm), with the floor made of parallel bronze bars (0.5 cm calibre, 0.5 cm apart). The latency was measured for the animal to step-down from the platform, placing all four paws on an electrifiable grid (training latency), at which point the animal was given a 2.0 s, 0.5 mA scrambled foot-shock, causing it to return to the platform or remain on the grid from which it was immediately removed and returned to its home-box.

After 24 h, a retention test was performed in which the step-down latency (test latency) was recorded up to a maximum of 120 s, but no shock was delivered. The difference between test and training latencies was taken as an indication of retention: a better memory should result in a higher test latency and a greater difference from the training latency.

In a pre-training paradigm, animals received a second foot-shock in the test session and were re-tested in an additional session 24 h after the first. This sequence was performed to evaluate if those animals were able to learn the task.

### 2.5. Data analysis

Data from hippocampal slices are presented as mean ± standard error. Baseline values were obtained from a stable 10 min period of evoked potential size prior to the addition of any drugs, with the first of those potentials being defined as 100%. This allowed the 10 min pre-drug period to provide an indication of baseline variance. Repeated measures ANOVA was used for statistical comparisons followed by the Bonferroni post hoc test for individual comparisons.

For the behavioural tasks, non-parametric statistics were used because an upper time limit (120 s) was specified for stepping-down from the platform. Results are presented as medians with percentile ranges ($P_{25}/P_{75}$). Statistical differences between test latencies were evaluated using a Kruskal–Wallis ANOVA. When significant differences were found, training and test latencies in each group were compared by the Wilcoxon test. Latencies for both training and test sessions, and differences between them (test minus training latencies) were compared between groups (AJ23 versus vehicle) using the Mann–Whitney '*U*' test, to evaluate effects of drug treatment. Analysis was limited to those animals in which the hippocampal cannulae were found on autopsy to be within 1 mm of the target coordinates.

## 3. Results

### 3.1. Binding experiments

In the binding studies, AJ23 displaced radiolabelled DPCPX from human $A_1$ receptors with a $K_i$ of 2.2 μM (Fig. 3). It displaced radiolabelled ZM241385 from human $A_{2A}$ receptors with a $K_i$ of >100 μM, and at a concentration of 100 μM, it displaced ZM241385 to 55% of its control value. AJ23 thus has a selectivity of approximately 45-fold for human $A_1$ versus $A_{2A}$ receptors.

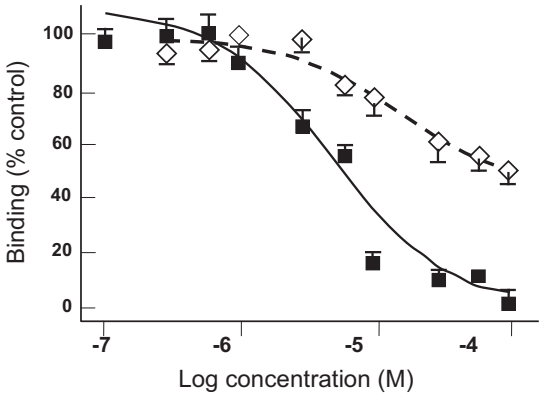


**Fig. 3.** Displacement by AJ23 of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) from human $A_1$ receptors (solid line, solid squares) and of ZM241385 from human $A_{2A}$ receptors (broken line, open diamonds). The $K_i$ at $A_1$ receptors was ~2.2 μM.

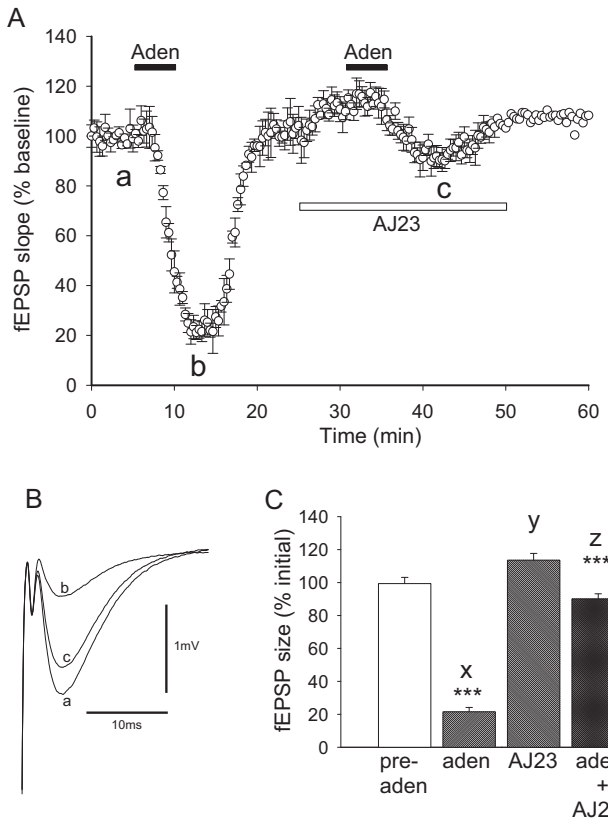


**Fig. 4.** Effects of AJ23 on the depression of evoked fEPSPs by adenosine.
A: A graph showing the time course of these experiments with symbols indicating the mean ± s.e. mean ($n = 4$) of the fEPSP slope. Adenosine at 30 μM (5 min) depressed potential size which recovered to the original baseline level. The addition of AJ23 (10 μM) for 5 min before a second application of adenosine increased the resting potential size and reduced the purine's inhibitory effect.ĝB: Sample fEPSPs recorded at points a, b and c in panel A: (a) during the initial baseline period (b) near the peak of the first response to adenosine and (c) near the peak response to the second application of adenosine in the presence of AJ23.
C: Bar chart summarising, respectively, the potential size in the initial baseline period, at the maximum point of inhibition by 30 μM adenosine alone, immediately before the second application of adenosine (showing the increased size produced by AJ23) and during the combined presence of adenosine and AJ23.
x*** $p < 0.001$, ($n = 4$), relative to the initial baseline.
y ($n = 4$), not significant relative to initial baseline.
z*** $p < 0.001$, ($n = 4$), relative to inhibition by adenosine alone.

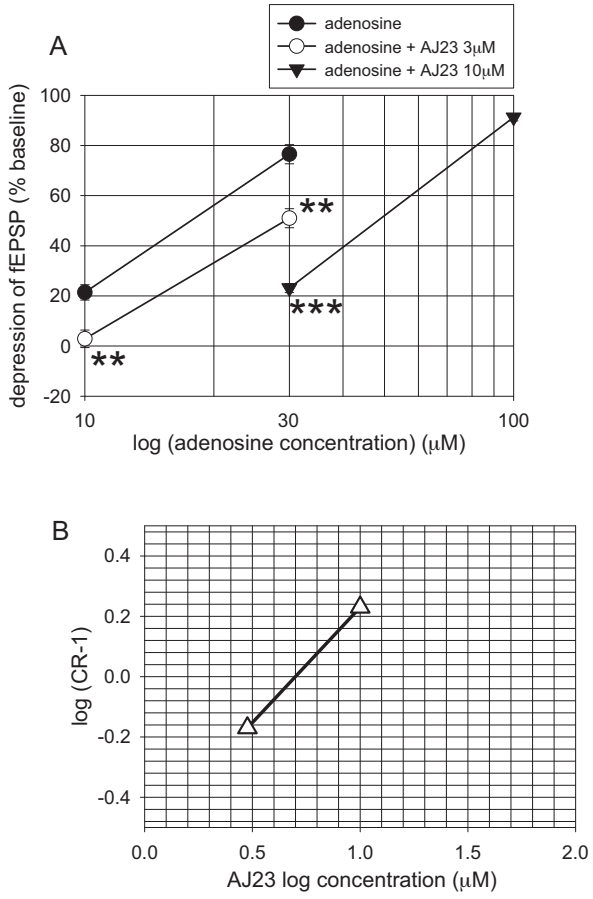


**Fig. 5.** A: Concentration–response plot for the effect of adenosine on the depression of fEPSPs in hippocampal slices using adenosine alone (filled circles), in the presence of AJ23 3 μM (open circles) and AJ23 10 μM (filled triangles).
B: Schild plot using the concentration ratios derived from panel (A).

The general screen of AJ23 against the binding to receptors or transporters of a range of neurotransmitters and related ligands indicated little displacement of any transmitter ligands including those for amino acid, monoamine and some peptide ligands. The maximum displacement recorded using 10 μM AJ23 was a 21% displacement of 5-HT (Table 1).

### 3.2. Effects on hippocampal slices

As an in vitro functional assay of glutamatergic neurotransmission in the brain, hippocampal slices were tested using the adenosine-induced depression of evoked population potentials as the target response, since this effect is known to be mediated by $A_1$ receptors. Adenosine at concentrations of 10 μM, 30 μM or 100 μM was superfused for 10 min, depressing fEPSP amplitude by 21% and 77% and 100% respectively. The potentials were allowed to recover to baseline levels and results were discarded if the original level was not regained ±10%. Preliminary experiments indicated that AJ23 was active in the low micromolar range and, in view of the limited quantity of compound available which made it difficult to perform full concentration–response curves, it was examined in detail at concentrations of 3 μM and 10 μM. The compound was added to the superfusion medium for 5 min before a second application of adenosine (Fig. 4), and was maintained for a further 15 min, throughout the response to adenosine. The inclusion of AJ23 at 10 μM increased the size of the baseline fEPSPs to 113% of the initial level and inhibited the response to 30 μM adenosine such that fEPSP size was now reduced by only 23% (a reduction of 70% in the response to adenosine alone at this concentration, $p < 0.001$, $n = 4$) (Fig. 4). In the presence of AJ23, adenosine at 10 μM inhibited fEPSPs by only 11% (different from adenosine alone, $p = 0.02$, $n = 6$). At the lower concentration of 3 μM, AJ23 produced a smaller but still statistically very significant antagonism of adenosine, with no significant increase in the baseline fEPSP size (Fig. 5A). Using the Gaddum equation, these changes would suggest a dissociation constant, $K_D$, for AJ23 of approximately 5 μM, a value consistent with that of 2.2 μM determined from the analysis of radioligand binding. In addition, a Schild plot from these data indicates an apparent p$K_B$ value of 1.0 μM (Fig. 5B), again consistent with the previous conclusion. The slope of the Schild plot is 0.75, which is low for a fully competitive interaction. This may indicate that there is an element of non-competitive behaviour in the antagonist action of AJ23. However, as yet there have been no studies on the effects of AJ23 on the metabolism of adenosine in brain tissue, and it may be that an inhibition of adenosine uptake or metabolism, both of which are effective removal processes in brain, could account for this value.

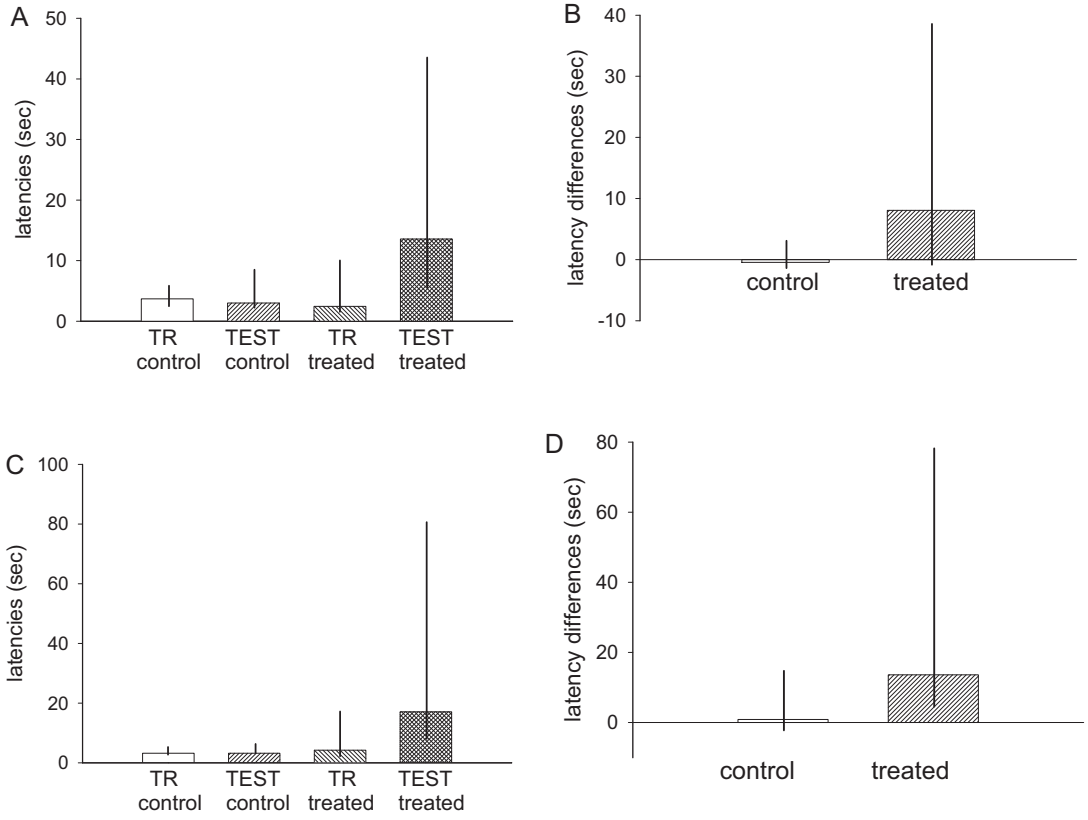


**Fig. 6.** Effect of pre- and post-training intrahippocampal infusion of AJ23 on scopolamine-induced amnesia of a step-down inhibitory avoidance task.
A: Bar diagram showing latencies for training (TR) and test (TEST) session in an inhibitory avoidance task for scopolamine-treated rats, receiving either AJ23 (treated) or saline (control) solution into the dorsal hippocampus before training.
*Significant differences compared to control test latencies (Mann–Whitney test, $p = 0.0155$).
B: Bar diagram showing differences ([test latency – training latency] for each animal).
*Significant differences compared to control differences (Mann–Whitney test, $p = 0.0363$).
Median with interquartile ranges corresponding to percentiles $P_{25}$ and $P_{75}$. Control $n = 13$, treated $n = 12$.
C: Bar diagram showing latencies for training (TR) and test (TEST) sessions of an inhibitory avoidance task, for scopolamine-treated rats receiving either AJ23 (treated) or saline (control) solution into the dorsal hippocampus immediately after training.
*Significant differences compared to control test latencies (Mann–Whitney test, $p = 0.0052$);
#Significant differences compared to treated training latencies (Wilcoxon test, $p = 0.0003$).
D: Bar diagram showing differences ([test latency–training latency] for each animal).
*Significant differences compared to control differences (Mann–Whitney test, $p = 0.0201$). Median with interquartile ranges corresponding to percentiles $P_{25}$ and $P_{75}$. Control: $n = 18$, treated: $n = 20$).

### 3.3. Step-down avoidance

Naive animals had training latencies of 4.09 [3.76/7.14] s (median [percentiles $P_{25}/P_{75}$]), and test latencies were 50.02 [12.17/120.00] s, with differences between test and training latencies of 46.26 [8.35/114.00] s. There were highly significant differences between test and training latencies (Wilcoxon test, $p = 0.0005$), and latency differences were significantly different from 0 (Wilcoxon signed rank test, $p = 0.0005$; $n = 13$).

#### 3.3.1. Pre-training administration of AJ23

In the first step-down inhibitory avoidance experiment (Fig. 6A), 10 nmol of AJ23 were infused into the hippocampus through each cannula, in a final volume of 1 μl per hippocampus, just before training.

For animals injected with scopolamine (30 min before training, i.p.), whether receiving AJ23 or not, there were no significant differences between training latencies ($p = 0.93$; Fig. 6A). In the test session, the scopolamine-injected group spent less time on the platform than the AJ23-injected animals previously injected with scopolamine (Mann–Whitney, $p = 0.015$; Fig. 6A). Hence, there were significant differences between test latencies of scopolamine-alone rats compared to scopolamine-AJ23-treated rats (Mann–Whitney, $p = 0.036$; Fig. 6B).

On the second day of testing (first test session), animals received a shock when they stepped onto the grid, but were re-tested 24 h later without any drug treatment. A significant increase in test latencies confirmed that the animals were able to achieve the criteria for the task; test latency medians were for control: 48.61 [$P_{25} = 6.03$; $P_{75} = 120.00$] s and for treated: 77.31 [14.06/120.00] s. These results support the concept that intrahippocampal injection of AJ23 exerted an effect either during training (acquisition) and/or immediately after training (consolidation), antagonising the amnesic effect caused by scopolamine.

#### 3.3.2. Post-training administration of AJ23

The post-training paradigm indicates whether the facilitatory effect of AJ23 on performance was produced on memory acquisition or consolidation, or both. Animals injected with scopolamine i.p. (as in the pre-training experiment) were then trained on the task, but AJ23 was infused into the hippocampus immediately after training.

Fig. 6C summarises the median latencies in the training and test sessions for pre-training (30 min, i.p.) scopolamine-injected rats injected with AJ23 (treated) or saline (control) into the

hippocampus immediately after training (i.e. during memory consolidation). Rats injected only with scopolamine did not reach the criterion for the task since the test latencies were not significantly different from training latencies, confirming the amnesic effect of scopolamine. The test latencies of AJ23-treated animals were significantly higher than their respective training latencies, showing acquisition and long-term memory formation of the task, and therefore confirming the ability of AJ23 to reverse the amnesic effect of scopolamine (Mann–Whitney, $p = 0.005$; Fig. 6C). There were significant differences between test latencies of AJ23-treated compared to non-treated rats, since the treated group spent longer on the platform. When the latency differences were compared, it was clear that the AJ23-treated rats were able to retain the learned behaviour (Mann–Whitney, $p = 0.02$; Fig. 6D).

## 4. Discussion

Glutamate release from neurones is inhibited by activation of adenosine $A_1$ receptors in many regions of the CNS including neocortex [36,37] and hippocampus [38]. Similarly acetylcholine release can be suppressed by $A_1$ receptor activation in cortex [39–41] and hippocampus [42,43]. The blockade of adenosine $A_1$ receptors, therefore, represents a valid target for developing compounds able to improve cognitive function, since an increased release should be produced of major excitatory neurotransmitters – especially glutamate [44] and acetylcholine [45] which are known to have major roles in learning and memory processing. The blockade of adenosine $A_1$ receptors is sufficient to improve cognition in standard paradigms such as scopolamine-induced deficits in mammals [46–48] including humans [49].

The present data indicate that AJ23, representing a novel chemical group of compounds acting at $A_1$ receptors, has relevant, desired characteristics for a novel lead compound. It has micromolar potency in displacing the high affinity $A_1$ receptor ligand DPCPX from human receptors, whilst being around 45-fold less potent at human $A_{2A}$ receptors. This selectivity at adenosine receptors is strengthened by a lack of displacement at a wide range of binding sites for neuroactive substances acting at receptors or transporter sites, suggesting that AJ23 should lack major unwanted effects on most aminergic and peptidergic neuronal systems. Some uncertainty remains about the mechanism of antagonism produced by AJ23. The early xanthine-based antagonists at adenosine receptors, such as theophylline were competitive antagonists [50]. However, one characteristic of competitive antagonists is the production of a Schild plot slope of unity, whereas AJ23 generates a slope of 0.75. A clarification of this slight anomaly will require measurements of the concentrations of adenosine and AJ23 under the experimental conditions used for assessing the Schild parameters. There are several, widely recognised reasons which may underlie disparities such as that found with AJ23. For example, if the antagonist is overall competitive but binds more effectively to the receptor than the agonist, this component of irreversibility will lower the value of the Schild slope. Equally, if the time course of binding is relatively slow, so that the Schild parameters are not assessed at a truly equilibrium point, a similar disparity will be seen. Finally, if the agonist is depleted in the test system, so that its effective concentration in the medium is underestimated, a slope less than unity may be measured. In the case of adenosine, for which both high affinity uptake processes and enzymic destruction are active in brain slices, it is highly likely that these factors contribute to the Schild slope disparity.

The functional activity of AJ23 is clear from its ability to raise the resting firing rate of neurones in the hippocampus, and to block the inhibitory activity of adenosine produced by the $A_1$ receptor-mediated depression of glutamate release from terminals of the Schaffer collateral and commissural axons. It has been a consistent finding in similar previous studies that adenosine is responsible for limiting the release of glutamate at these synapses and that $A_1$ receptor blockade raises neuronal excitability by removing tonic inhibition.

The step-down avoidance paradigm was performed to assess whether the selectivity and potency of AJ23 were sufficient to produce behavioural effects, and whether any effects would be consistent with an ability to enhance cognitive function. The results show that AJ23 can reverse the amnesic effect of scopolamine. In addition, the cognitive enhancement is most likely mediated within the hippocampus and its associated structures, since injection was made directly into that region. At least as importantly, the results are entirely consistent with the view that the hippocampal actions of scopolamine on associative memory of inhibitory avoidance and, by implication, of any compound that overcomes the amnesic effect of scopolamine in the hippocampus, are mediated primarily by effects on consolidation of the memory record. Thus, AJ23 was able to prevent the effects of scopolamine even after the acquisition of the avoidance response, an interaction that reflects the ability of caffeine to produce a specific effect [48]. The amnesic effect of scopolamine was mainly retrograde since AJ23 was able to rescue the memory trace even when injected after training. It is referred to as retrograde amnesia because a memory trace could only be rescued during consolidation if it was previously acquired and the memory trace converted to long-term memory. The results therefore indicate a significant effect of AJ23 on the memory consolidation process.

In assessing the behavioural effects of any compound it is essential to eliminate major disruptive factors that could result from non-specific effects on sensory or motor systems in the CNS, or toxic actions on organs that would influence behaviour. Hence, preliminary work on AJ23 toxicity was performed under a commercial contract (Porsolt Ltd., France) [51]. The possible toxic effects of AJ23 (0.125, 0.5, 2 and 8 mg/kg) were examined after oral administration, using the Irwin Test series in the rat [52] a panel of 36 observations covering behavioural modifications, physiological and neurotoxicity symptoms, rectal temperature and pupil diameter, with observations at intervals between 15 min and 24 h after injection. Significant effects were seen at 2 mg/kg, with increased reactivity to touch in all mice after 30 min. Only at 8 mg/kg was there a reduction of overall activity, suggesting a slight degree of sedation after 60 and 120 min. In addition we used an open-field test of locomotion and exploration using the same dose and intrahippocampal route of administration employed in the learning tasks. This test showed no significant differences ($p > 0.3$) between rats injected in the hippocampus with AJ23 or vehicle, implying that nonspecific changes of locomotor and exploratory activity could not have interfered with activity in the learning tasks.

There is some evidence for activity of pyrazolotriazolopyrimidine compounds at adenosine $A_{2A}$ and, since $A_{2A}$ receptors are the second most abundant adenosine receptors in the brain, we included this subtype in our initial screening, but found a much lower affinity of AJ23. Whilst not at the level of discrimination required for clinical trials, the data do indicate the ability of compounds related to the chemical series represented by AJ23 to produce selectivity at $A_1$ and $A_{2A}$ receptors that would be important in clinical practice. During ischaemic episodes, for example, as occurs during a stroke, large amounts of adenosine are release from neurons and glia to the extent that both these receptor subtypes may be activated [53,54]. Since the two subtypes of receptor can have opposite actions on the release of glutamate, with A1 receptors producing inhibition and A2A receptors producing enhancement [4,5], an ideal derivative might require to have a greater degree of selectivity.

A number of previous studies with pyrazolotriazolopyrimidine derivatives have shown activity at adenosine $A_3$ receptors but those

compounds had substitutions that were larger and more complex than those present in AJ23, and the nature of those substituents had a major bearing on activity of the compounds [55]. In addition, most of the pyrazolotriazolopyrimidine compounds active at $A_3$ receptors are antagonists, and there is little evidence that such an action would have pro-cognitive effects. Indeed, $A_3$ receptor antagonists or knockout animals lacking $A_3$ receptors show reduced cognitive function [56], making it very unlikely that the effects of AJ23 on behaviour were caused by $A_3$ receptor antagonism.

It should be emphasised that learning and more complex aspects of cognition are likely to be dependent on factors other than simply changes in neurotransmitter release, and for which adenosine receptors may not be relevant. Thus, reduced levels of the vesicular glutamate transporter Vglut1 is accompanied by lower levels of LTP together with behavioural deficits of spatial reversal learning, and recognition memory [57,58]. Equally, adenosine $A_1$ receptors may regulate other elements of neuronal physiology and pathology, such as the processing of β-amyloid and tau phosphorylation [59].

The overall picture that arises from this work, therefore, is that AJ23 is an antagonist at $A_1$ receptors in the CNS, with the ability to reverse the learning and memory deficits induced by scopolamine in a widely used avoidance model of cognitive dysfunction. Taken together with the absence of any general signs of toxicity, abnormal behaviour or alterations of motor and exploratory behaviour using the same dose and route of administration that resulted in a clear and significant reversal of scopolamine-induced amnesia, the results imply that this compound could represent the first in a new chemical class of non-xanthine compounds that might have beneficial effects in cognitive dysfunctional conditions such as Alzheimer's disease by blocking $A_1$ receptors. These properties make it a promising, chemically novel lead compound for further chemical development and for testing in cognitive tasks of learning and memory.

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