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RESEARCH PAPER

Anti-nociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A_1 adenosine receptors

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Background and purpose: Allopurinol is a potent inhibitor of the enzyme xanthine oxidase, used primarily in the treatment of hyperuricemia and gout. It is well known that purines exert multiple effects on pain transmission. We hypothesized that the inhibition of xanthine oxidase by allopurinol, thereby reducing purine degradation, could be a valid strategy to enhance purinergic activity. The aim of this study was to investigate the anti-nociceptive profile of allopurinol on chemical and thermal pain models in mice.
Experimental approach: Mice received an intraperitoneal (i.p.) injection of vehicle (Tween 10%) or allopurinol (10–400 mg kg^{-1}). Anti-nociceptive effects were measured with intraplantar capsaicin, intraplantar glutamate, tail-flick or hot-plate tests.
Key results: Allopurinol presented dose-dependent anti-nociceptive effects in all models. The opioid antagonist naloxone did not affect these anti-nociceptive effects. The non-selective adenosine-receptor antagonist caffeine and the selective A_1 adenosine-receptor antagonist, DPCPX, but not the selective A_{2A} adenosine-receptor antagonist, SCH58261, completely prevented allopurinol-induced anti-nociception. No obvious motor deficits were produced by allopurinol, at doses up to 200 mg kg^{-1}. Allopurinol also caused an increase in cerebrospinal fluid levels of purines, including the nucleosides adenosine and guanosine, and decreased cerebrospinal fluid concentration of uric acid.
Conclusions and implications: Allopurinol-induced anti-nociception may be related to adenosine accumulation. Allopurinol is an old and extensively used compound and seems to be well tolerated with no obvious central nervous system toxic effects at high doses. This drug may be useful to treat pain syndromes in humans.
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Keywords: allopurinol; purines; pain; adenosine; guanosine; xanthine oxidase; anti-nociception

Abbreviations: P_1, purinergic receptor type 1; P_2, purinergic receptor type 2; A_1, adenosine receptor type 1; A_{2A}, adenosine receptor type 2A; A_{2B}, adenosine receptor type 2B; A_3, adenosine receptor type 3; DMSO, dimethyl sulphoxide; TFL, tail-flick latency; MPE, maximum possible effect; CSF, cerebrospinal fluid; HPLC, high-performance liquid chromatography; ADA, adenosine deaminase; ROS, reactive oxygen species; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; SCH58261, 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo-[4,3-e]-1,2,4-triazolo [1,5c]pyrimidine

Introduction

Allopurinol [1,5-dihydro-4*H*-pyrazolo(3,4-*d*)pyrimidin-4-one] is a structural analogue of hypoxanthine and a potent inhibitor of the enzyme xanthine oxidase that catalyses the transformation of hypoxanthine to xanthine and uric acid, reducing both uric acid formation and purine degradation (Pacher *et al.*, 2006; Day *et al.*, 2007). Allopurinol is used primarily in the treatment of hyperuricemia and gout (Rundles, 1982). Besides its hypouricemic effects, allopurinol has been studied for several other indications, including treatment of seizures, psychiatric disorders, ischaemia-reperfusion injury, protozoal diseases and as a measure of liver impairment (Das *et al.*, 1987; Van Waeg *et al.*, 1988; Garcia Garcia *et al.*, 1990; Day *et al.*, 1994; Akhondzadeh *et al.*, 2005).

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Both healthy and hyperuricemic patients exhibit a reduction of uric acid levels after allopurinol, probably leading to accumulation of purines, including the neuromodulator adenosine, which may explain its beneficial anticonvulsant and antipsychotic effects (Tada *et al*., 1991; Wada *et al*., 1992; Zagnoni *et al*., 1994; Lara *et al*., 2000; 2003; Machado-Vieira *et al*., 2001). Of note, positive effects of allopurinol in refractory epilepsy (Tada *et al*., 1991), aggressive behaviour (Lara *et al*., 2000; 2003), mania (Machado-Vieira *et al*., 2001) and schizophrenia (Lara *et al*., 2001a; Brunstein *et al*., 2005) have been suggested to be secondary to its inhibitory effect on purine degradation and thus enhancing activities of adenosine, despite the lack of direct data to support this hypothesis.

The purinergic system involves adenosine and adenosine triphosphate (ATP) as major endogenous effectors, acting on P_1 and P_2 receptors respectively (Ralevic and Burnstock, 1998). Adenosine is mainly an inhibitory neuromodulator, regulating synaptic activity and release of several neurotransmitters, such as noradrenaline, dopamine, serotonin, acetylcholine and glutamate (Brundege and Dunwiddie, 1997; Ralevic and Burnstock, 1998). It is well known that adenosine and its analogues exert multiple effects on pain transmission at peripheral and central sites (Sawynok, 1998; Sawynok and Liu, 2003). Anti-nociceptive effects of adenosine may be related to the inhibition of intrinsic neurons by an increase in K^+ conductance and pre-synaptic inhibition of sensory nerve terminals, decreasing the release of substance P and glutamate (Sollevi, 1997); attenuation by NMDA-induced production of nitric oxide also may be involved (Bhardwaj *et al*., 1995). Adenosine has been shown to mediate opioid analgesia (Bennett, 2000).

Caffeine and theophylline are the classic P_1 adenosine antagonists currently used in humans, but adenosine agonists for human use are still lacking. We hypothesized that the inhibition of xanthine oxidase by allopurinol, thereby reducing purine degradation, could be a valid strategy to enhance purinergic activity, which is in line with the anticonvulsant and neuropsychiatric effects observed with allopurinol treatment (Tada *et al*., 1991; Wada *et al*., 1992; Zagnoni *et al*., 1994; Lara *et al*., 2000; 2001a; 2003; Machado-Vieira *et al*., 2001; Brunstein *et al*., 2005). Based on the considerations above, the aims of the present study were: (i) to investigate the anti-nociceptive activity induced by allopurinol in chemical and thermal pain models in mice; (ii) to identify, by means of pharmacological as well as neurochemical approaches, possible mechanisms by which allopurinol causes anti-nociception in mice; and (iii) to evaluate the acute toxicity induced by allopurinol using behavioural paradigms.

Methods

Animals

All animal procedures and studies followed the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) and our institutional protocols for experiments with animals, designed to avoid suffering and limit the number of animals used. The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. Male adult Swiss albino mice (2–3 months of age, 30–40 g) were kept on a 12 h light/dark cycle (light on at 7:00 a.m.) at temperature of 22 ± 1°C, housed in plastic cages (five per cage) with tap water and commercial food pellets *ad libitum*. All behavioural procedures were conducted between 8:00 and 10:00 a.m. In all experiments of nociceptive behavioural, the animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments.

Drug administration

Experiments were performed according to the method described by Schmidt *et al*. (2000): 20 min before the experiment, animals were placed individually in acrylic boxes, which also served as observation chambers. After this adaptation period, treatments were performed. Animals were given an intraperitoneal (i.p.) injection (10 mL kg^{-1}) of vehicle (saline or 10% Tween) or allopurinol (10–400 mg kg^{-1}). In order to investigate the mechanism of action of allopurinol, some animals were also pre-treated (15 min in advance) with an i.p. injection of the non-selective (A_1 and A_{2A}) adenosine receptor antagonist caffeine (30 mg kg^{-1}), the selective A_1 adenosine receptor antagonist DPCPX (0.1 mg kg^{-1}), the selective A_{2A} adenosine receptor antagonist SCH58261 (0.5 mg kg^{-1}) or the non-selective opioid receptor antagonist naloxone (1 mg kg^{-1}). Adenosine (100 mg kg^{-1}) and morphine sulphate (6 mg kg^{-1}) were used as positive controls for those experiments. Caffeine, adenosine, DPCPX and SCH58261 doses were based on earlier work (Lara *et al*., 2001b; Peana *et al*., 2006; Dall'Igna *et al*., 2007).

Capsaicin-induced nociception

The method used for capsaicin-induced licking was similar to that described by Sakurada *et al*. (1993). Thirty minutes after i.p. treatments, 20 µL of capsaicin (1.6 µg per paw) was injected intraplantarly (i.pl.), under the plantar skin of the right hind paw (Hamilton microsyringe with a 26-gauge needle). Animals were observed individually for 5 min after capsaicin administration for the time spent licking the injected paw, which was recorded and considered a measure of nociception.

Glutamate-induced nociception

The procedure used was similar to that described previously (Beirith *et al*., 2002). Thirty minutes after i.p. treatments, a volume of 20 µL of glutamate solution (10 µmol per paw prepared in saline) was injected i.pl., as described above. Mice were observed individually for 15 min following glutamate injection and the amount of time spent licking the injected paw was recorded and considered as indicative of nociception.

Tail-flick test

Nociception was assessed with a tail-flick apparatus (Albrasch Electronic Equipments, Brazil), as described in detail else-

where (D'Amour and Smith, 1941). A source of light was positioned below the tail, focused on a point 2.3 cm rostral to the tip of the tail and the time that the mouse took to withdraw its tail from the noxious stimulus was recorded. Deflection of the tail activates a photocell and automatically terminates the trial. A cut-off time of 10 s was employed in order to prevent tissue damage (a mouse that did not flick its tail by 10 s was considered as fully analgesic). On day one, animals were first habituated with the tail-flick apparatus through three separate measures (data not shown). On day two, baseline tail-flick latency (TFL) was measured for each mouse prior to the treatments. Animals displaying at least two TFL of 10 s on the baseline were excluded from the study. Immediately after the third TFL measurement, animals received i.p. treatments and 30 min thereafter were submitted to the tail-flick apparatus. Data for tail-flick are expressed as mean per cent of maximum possible effect (% MPE) ± SEM, according to the following formula (Calcagnetti *et al.*, 1990): % MPE: 100 × (post-drug latency – baseline latency) × (cut-off time – baseline latency)$^{-1}$.

Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described by Eddy and Leimbach (1953), with minor modifications. In these experiments, the hot-plate apparatus (Ugo Basile, model-DS 37, Italy) was maintained at 55 ± 0.5°C. Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placing of the animal on the hot-plate and the occurrence of licking of hind paws or jumping off the surface was recorded as response latency. On day one, the animals were first habituated to the apparatus. On day two, mice were tested and animals displaying baseline latencies of more than 15 s were excluded from the study. An automatic 20 s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Thirty minutes after i.p. treatments, animals were placed on the heated surface and response latency recorded as described above. Data for hot-plate are expressed as mean % MPE ± SEM.

Hole-board test

The hole-board apparatus (Ugo Basile, Italy) consisted of grey Perspex panels (40 cm × 40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor. Photocells below the surface of the holes measured the number of head-dips. The board was positioned 15 cm above the table and divided into nine squares of 10 cm × 10 cm with a water-resistant marker. Thirty minutes after i.p. treatments, each animal was placed singly in the centre of the board facing away from the observer and its behaviour recorded for 5 min. The number of head-dips, crossings (number of squares crossed with all four paws), rearings, groomings and defecations was recorded, as well as the latency to start locomotion (Vinadé *et al.*, 2003).

Measurement of motor performance

In order to evaluate non-specific muscle relaxant or neurotoxic effects, we evaluated the effects of allopurinol in the rotarod test and in spontaneous locomotor activity test. The rotarod apparatus (Ugo Basile, Italy) consists of a rotating (18 r.p.m.) bar (2.5 cm diameter), subdivided by disks into six compartments. As previously described (Leal *et al.*, 2000), mice were initially trained to remain on the rotarod apparatus for 120 s. Those not remaining on the bar for at least two out of three consecutive trials were discarded. On the day after training, the latency to fall from the rotarod (one trial with a maximum of 60 s) was determined 30 min after i.p. treatments. The method to evaluate spontaneous locomotor activity was adapted from Creese *et al.* (1976). Activity cages (45 cm × 25 cm × 20 cm, Albarsch Electronic Equipment, Brazil), equipped with three parallel photocells, automatically recorded the number of crossings. Animals were individually habituated to the activity cage for 10 min before receiving the i.p. treatments. Animals were placed again in the activity cages 30 min after treatments, and the crossings were recorded for 15 min.

Potentiation of barbiturate sleeping time in mice

In order to investigate sedative properties of allopurinol, mice pre-treated with allopurinol (50, 100 or 200 mg kg^{-1}) or vehicle (30 min in advance) received an i.p. injection of sodium pentobarbital (30 mg kg^{-1}). After the barbiturate injection, the sleeping time (time elapsed between loss and recuperation of righting reflex) was recorded. Criterion for recuperation of righting reflex is that animals have to regain their normal posture for three consecutive times when challenged to remain on their backs (Yamamoto *et al.*, 1987).

Rectal temperature

This was measured by using a flexible probe before and 30 min after allopurinol (50, 100 or 200 mg kg^{-1}) or vehicle treatment.

Cerebrospinal fluid sampling

Groups of mice were treated similarly with i.p. administration of allopurinol (200 mg kg^{-1}) or vehicle. After 30 min, mice were anaesthetized with sodium thiopental (60 mg kg^{-1}, 10 mL kg^{-1}, i.p.) and placed in a stereotaxic apparatus, where the cerebrospinal fluid (CSF) was drawn (10–20 μL per mouse) by direct puncture of the cisterna magna, with an insulin syringe (27 gauge × 1/2 in length), with the help of a magnifying glass. All samples were centrifuged at 10 000× *g* in an Eppendorf centrifuge for 5 min to obtain cell-free supernatants and stored in separate tubes in –70°C until analysis.

High-performance liquid chromatography procedure

High-performance liquid chromatography was performed with aliquots obtained from the CSF cell-free supernatants. The following purines were measured according to Domanski *et al.* (2006): ATP, adenosine diphosphate, adenosine monophosphate, adenosine, guanosine triphosphate, guanosine diphosphate, guanosine monophosphate, guanosine, inosine monophosphate (IMP), inosine, hypoxanthine, xanthine and uric acid. Analyses were performed with Shimadzu

Class-VP chromatography system consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto-injector valve with 50 μL loop, and an UV detector. Separations were achieved on a Supelco C18 250 mm × 4.6 mm, 5 μm particle size column. The mobile phase flow rate was 1.2 mL min^{-1} and column temperature was 24°C. Buffer composition remained unchanged (A: 150 mmol L^{-1} phosphate buffer, pH 6.0, containing 150 mmol L^{-1} potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, 100% at 12.30 min and 0% at 12.40 min. Samples of 10 μL were injected every 18 min into the injection valve loop. Absorbance was read at 254 nm.

Statistical analysis

Data are expressed as mean ± SEM, except the ID_{50} values (i.e. the dose of allopurinol necessary to reduce the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). CSF concentrations of purines are expressed as mean ± SEM in μmol·L^{-1}. Data were submitted to Kolmogorov–Smirnov, Levene and Bartlett tests for normality evaluation. Statistical analysis between groups was performed using one-way ANOVA plus the *post hoc* Student–Newman–Keuls multiple comparisons test when necessary. All results with $P < 0.05$ were considered statistically significant.

Drugs

Allopurinol, capsaicin, adenosine, caffeine, naloxone and glutamate were purchased from Sigma Chemicals (St Louis, MO, USA). DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was purchased from Tocris (Northpoint, UK). SCH58261 [5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo-(4,3-e)-1,2,4-triazolo(1,5c)pyrimidine] was provided by S. Weiss (Vernalis, UK). Sodium thiopental and morphine sulphate were obtained from Cristália (SP, Brazil). Allopurinol was dissolved in a 10% Tween solution. The dose of Tween (10%) did not cause any detectable effect. Capsaicin was diluted in 5% DMSO (dimethyl sulphoxide); DPCPX and SCH58261 were diluted in 10% DMSO. All other drugs were dissolved in saline (NaCl 0.9%) and buffered with 0.1 N NaOH or 0.1 N HCl to pH 7.4 when necessary. All other chemicals were purchased from local suppliers. Drug and molecular target nomenclature used in this manuscript conforms to the Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Figures 1–4 (panel A) show that i.p. administration of allopurinol produced anti-nociception in the tail-flick, hot-plate, i.pl. glutamate and i.pl. capsaicin tests in mice. Mean ID_{50} values (and their respective 95% confidence limits) in the glutamate and capsaicin tests were 102.5 (61.9–169.8) and 119.1 (54.0–262.7 mg kg^{-1}) respectively, and maximal inhibitions were 63 ± 12% and 58 ± 8% respectively. Vehicle (10% Tween) did not affect nociception as compared with control (sham) animals (data not shown). Morphine (6 mg kg^{-1} – positive control) produced anti-nociception in all models.

Figure 4B shows that the non-selective opioid-receptor antagonist naloxone completely prevented morphine-induced anti-nociception, without affecting anti-nociception induced by allopurinol. As shown in Figure 4C, i.p. adenosine (100 mg kg^{-1}), as well as allopurinol, produced anti-nociceptive effects against capsaicin-induced pain, an effect

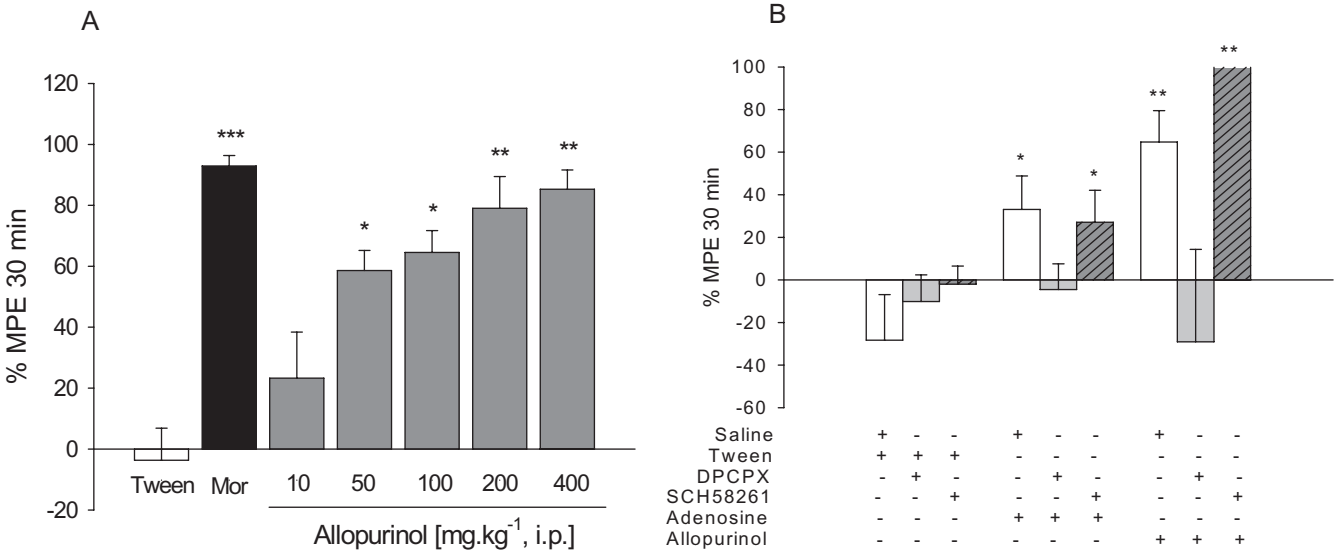

Figure 1 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) or morphine (6 mg kg^{-1}; Mor) on tail-flick test; mean baseline latencies (s) were: Tween – 6.5 ± 0.4; morphine – 5.4 ± 0.3; allopurinol 10 to 400 mg kg^{-1} – 7.4 ± 0.4, 6.4 ± 0.4, 6.8 ± 0.4, 6.9 ± 0.5 and 6.9 ± 0.5 s respectively. B. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on tail-flick test. The columns represent mean values of % of maximum possible effect (% MPE) and vertical bars represent SEM. n = 8–12 animals per group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

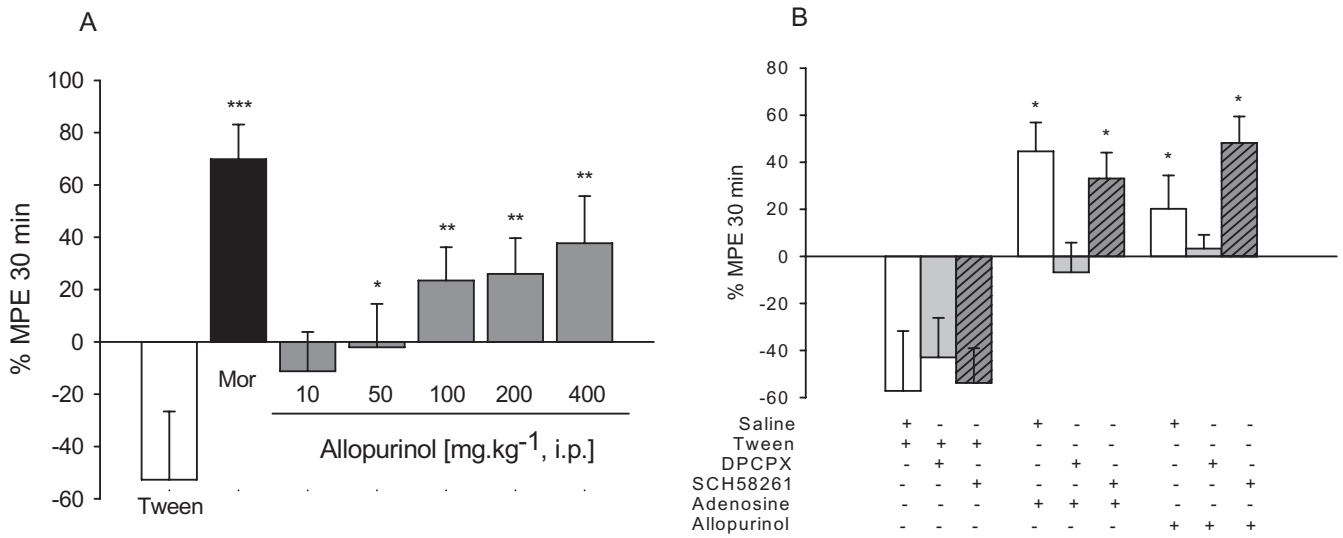

Figure 2 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) or morphine (6 mg kg^{-1}; Mor) on the hot-plate test; mean baseline latencies (s) were: Tween – 10.4 ± 0.7; morphine – 7.4 ± 0.4; allopurinol 10 to 400 mg kg^{-1} – 8.5 ± 0.4, 9.0 ± 0.7, 10.2 ± 0.6, 8.8 ± 0.7 and 10.2 ± 0.9 s respectively. B. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on hot-plate test. The columns represent mean values of % of maximum possible effect (% MPE) and vertical bars represent SEM. n = 8–12 animals per group. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

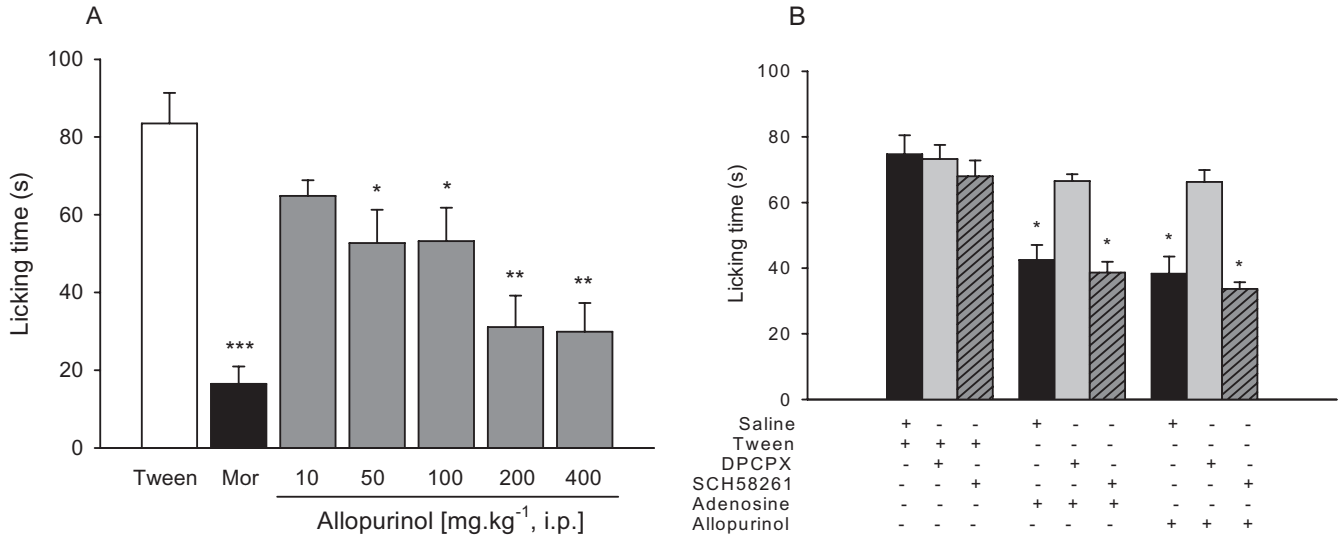

Figure 3 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) or morphine (6 mg kg^{-1}; Mor) on glutamate-induced pain. B. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on glutamate-induced pain. The columns represent mean time spent licking the injected hind paw and vertical bars represent SEM. n = 8–12 animals per group. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

prevented by pre-treatment with the non-selective adenosine receptor antagonist caffeine (30 mg kg^{-1}). The results depicted in Figures 1–3 (panel B) and Figure 4D show that the selective A_1 adenosine receptor antagonist DPCPX (0.1 mg kg^{-1}), but not the selective A_{2A} adenosine receptor antagonist SCH58261 (0.5 mg kg^{-1}), had no effect *per se*, but prevented anti-nociception induced by allopurinol and adenosine in the tail-flick, hot-plate, i.pl. glutamate and i.pl. capsaicin pain tests.

In the hole-board model (Table 1), neither i.p. allopurinol (10–200 mg kg^{-1}) nor vehicle affected latency to first head-dip, number of head-dips, groomings and defecations. However, allopurinol, at the highest dose (400 mg kg^{-1}), induced a significant decrease in the number of crossings and rearings, compared with vehicle.

Allopurinol (10–200 mg kg^{-1}) did not induce motor deficits, ataxia or affected spontaneous locomotor activity, as evaluated by the performance in the rotarod test and in the activity cages. However, allopurinol (400 mg kg^{-1}) decreased latency to fall in the rotarod test and spontaneous locomotor activity measured by activity cages as shown in Table 1. Allopurinol had no effect on pentobarbital-induced sleeping time (n = 8

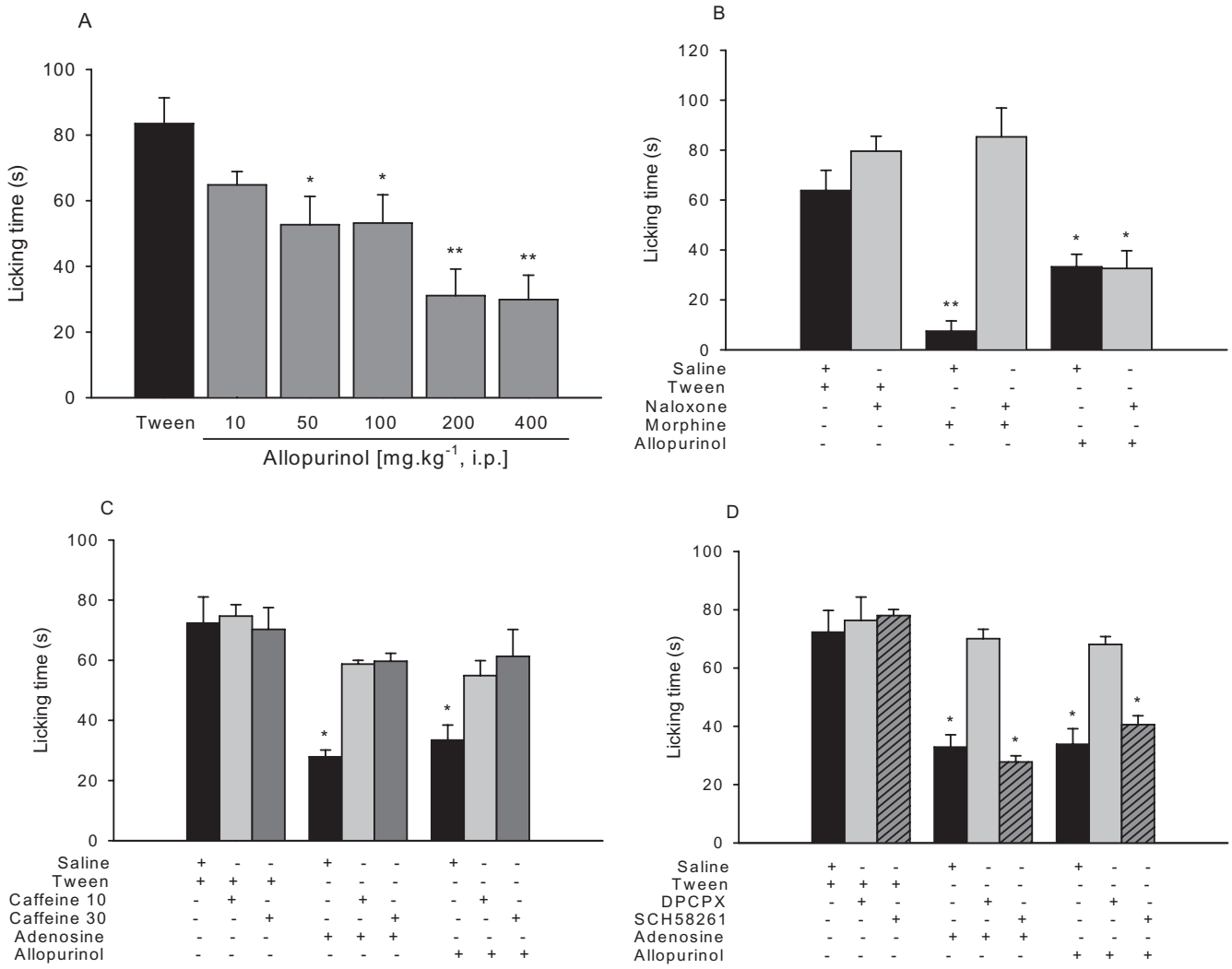

Figure 4 A. Anti-nociceptive effects of allopurinol (10–400 mg kg^{-1}, i.p.) on capsaicin-induced pain. B. Effects of naloxone (1 mg kg^{-1}, i.p.) on the anti-nociceptive effects of morphine (6 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on capsaicin-induced pain. C. Effects of caffeine (10 or 30 mg kg^{-1}, i.p.) on adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) anti-nociception on capsaicin-induced pain. D. Effects of DPCPX (0.1 mg kg^{-1}, i.p.) or SCH58261 (0.5 mg kg^{-1}, i.p.) on anti-nociceptive effects of adenosine (100 mg kg^{-1}, i.p.) or allopurinol (200 mg kg^{-1}, i.p.) on capsaicin-induced pain. The columns represent mean time spent licking the injected hind paw and vertical bars represent SEM. $n = 8$–12 animals per group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with control (10% Tween or saline + Tween), one-way ANOVA followed by Student–Newman–Keuls test.

Table 1 Effects of allopurinol on the hole-board, rotarod and spontaneous locomotor activity tests, used in mice

Treatment	*Allopurinol (mg kg^{-1})*					
	Tween 10%	*10*	*50*	*100*	*200*	*400*
Latency to head-dip (s)	6.0 (0.9)	6.4 (0.8)	5.5 (0.9)	7.5 (0.2)	5.3 (0.7)	8.5 (0.7)
Head-dips (*n*)	50.1 (5.7)	56.0 (2.2)	49.7 (8.6)	43.5 (6.2)	47.0 (7.5)	35.0 (6.3)
Squares crossed (*n*)	42.1 (5.4)	40.7 (5.9)	44.0 (7.2)	47.7 (5.9)	46.0 (9.4)	21.7 (9.4)*
Rearings (*n*)	2.0 (0.8)	2.3 (0.8)	2.6 (0.9)	1.8 (1.0)	1.7 (0.8)	0.7 (0.3)*
Groomings (*n*)	1.6 (0.3)	1.5 (0.3)	0.8 (0.4)	1.2 (0.4)	1.6 (0.6)	1.3 (0.4)
Fecal boli (*n*)	0.8 (0.4)	1.2 (0.4)	1.2 (0.5)	1.0 (0.3)	1.5 (0.5)	0.8 (0.5)
Latency to fall (s)	56.4 (1.6)	50.6 (7.0)	58.0 (1.5)	50.8 (6.8)	56.2 (2.5)	34.5 (5.8)*
Crossings (*n*)	234 (28)	250 (37)	222 (10)	213 (15)	212 (19)	140 (14)*

Vehicle (10% Tween) or allopurinol was given i.p., 30 min prior to the behaviour measurements: latency to the first head-dip; head-dips; squares crossed; rearings; groomings; fecal boli; latency to fall (rotarod); number of crossings (spontaneous locomotor activity). $n = 8$ animals per group. *$P < 0.05$ compared with control (10% Tween), one-way ANOVA followed by Student–Newman–Keuls test.

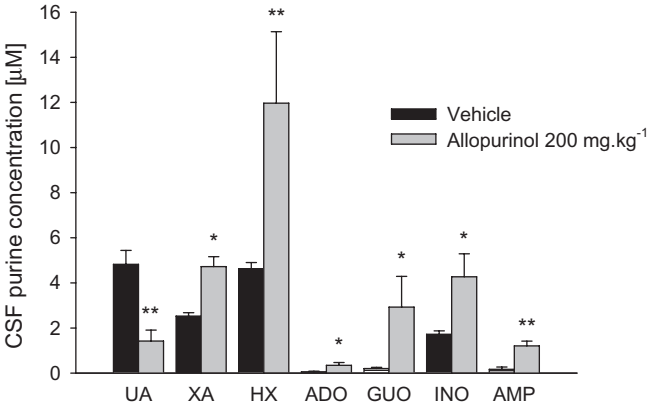

Figure 5 Effects of allopurinol (200 mg kg^{-1}, i.p.) on cerebrospinal fluid (CSF) concentration of purines. The columns represent mean (µmol·L^{-1}) and vertical bars represent SEM. Vehicle was 10% Tween. Vehicle or allopurinol was given i.p. 30 min prior to the CSF sampling. UA, uric acid; XA, xanthine; HX, hypoxanthine; ADO, adenosine; GUO, guanosine; INO, inosine; AMP, adenosine monophosphate. $n = 8$ animals per group. *$P < 0.05$ and **$P < 0.01$ compared with vehicle (control), one-way ANOVA followed by Student–Newman–Keuls test.

for all groups; Tween – 33.5 ± 5.8 min; allopurinol 50 mg kg^{-1} – 33.9 ± 10.4 min; allopurinol 100 mg kg^{-1} – 13.4 ± 8.7 min; and allopurinol 200 mg kg^{-1} – 21.2 ± 8.1 min; $P = 0.36$) and rectal temperature ($n = 8$ for all groups; Tween – 35.8 ± 0.2°C; allopurinol 50 mg kg^{-1} – 36.1 ± 0.3°C; allopurinol 100 mg kg^{-1} – 35.5 ± 0.3°C; and allopurinol 200 mg kg^{-1} – 36.1 ± 0.4°C; $P = 0.72$).

Figure 5 shows that the CSF concentration of uric acid was significantly reduced 30 min after treatment with allopurinol (200 mg kg^{-1}). Conversely, the CSF concentrations of xanthine, hypoxanthine, guanosine, adenosine, inosine and adenosine monophosphate were significantly increased in mice treated with allopurinol compared with mice receiving vehicle. The most significant changes were observed for adenosine (sevenfold increase) and guanosine (14-fold increase). Intraperitoneal administration of allopurinol did not affect CSF levels of ATP, adenosine diphosphate, guanosine triphosphate, guanosine diphosphate, guanosine monophosphate and IMP, compared with vehicle (data not shown). CSF allopurinol concentration was estimated to be 57.7 ± 5.9 µmol·L^{-1}, 30 min after a single i.p. dose of allopurinol (200 mg kg^{-1}). Allopurinol was not detected in the CSF of control animals.

Discussion and conclusions

In this study, i.p. administration of the xanthine oxidase inhibitor, allopurinol, produced dose-dependent anti-nociceptive effects in the i.pl. capsaicin, i.pl. glutamate, tail-flick and hot-plate pain models in mice. The opioid antagonist naloxone did not alter the anti-nociceptive effects of allopurinol. However, the non-selective adenosine receptor antagonist caffeine and the selective A_1 adenosine receptor antagonist DPCPX, but not the selective A_{2A} adenosine receptor antagonist SCH58261, prevented allopurinol-induced anti-nociception. No obvious motor deficits were produced by allopurinol at doses up to 200 mg kg^{-1}. This study also demonstrated that i.p. administration of allopurinol significantly increased CSF levels of purines, including the nucleosides adenosine and guanosine, and decreased CSF concentration of uric acid.

Although allopurinol has been traditionally used in the treatment of gout and its related symptoms (including pain), only anecdotal reports investigating the effects of allopurinol *per se* on pain are found in the literature (Pinelli *et al.*, 1991; Daskalopoulou *et al.*, 2005; Hacimuftuoglu *et al.*, 2006; Inkster *et al.*, 2007). Interestingly, the present study demonstrated that allopurinol produced anti-nociception in four different animal pain models. Although these animal models are essentially based on acute, short-lasting noxious stimuli, some differences between tests can be found. Tail-flick and hot-plate tests are thermal models of pain but the tail-flick refers predominantly to a spinal reflex with modest control by supraspinal structures, while the hot-plate test is a more complex pain model, producing two behavioural components (i.e. paw licking and jumping) considered to be supraspinally integrated responses (Le Bars *et al.*, 2001). Intraplantar injection of algogenic chemical agents (capsaicin or glutamate) usually produces similar nociceptive responses and represents a longer-lasting stimulus (tonic pain). However, i.pl. administration of glutamate produces a nociceptive response and paw oedema that are mainly mediated by non-NMDA receptors (Beirith *et al.*, 2002), while the capsaicin test involves a more complex mechanism, predominantly mediated by tachykinin and NMDA receptors (Sakurada *et al.*, 1993).

The rationale to administer allopurinol for pain is derived from evidence in basic and clinical research on the purinergic system. Purines and their analogues have been considered important targets for the development of new drugs for pain management, as the nucleoside adenosine and its analogues present anti-nociceptive effects at spinal, supraspinal and peripheral sites (Sawynok, 1998; Sawynok and Liu, 2003), and P_1 and P_2 receptors are closely involved in the mechanisms of pain transmission (Sawynok, 1998; Sawynok and Liu, 2003). Adenosine can alter pain transmission by acting on both nociceptive afferent and transmission neurons, and these actions are mediated primarily by adenosine A_1 receptors (Sawynok, 1998). Additional effects on inflammatory cells at peripheral sites (Fredholm, 1997) and on glia in the central nervous system (CNS) (Gebicke-Haerter *et al.*, 1996; Ogata and Schubert, 1996) mediated by adenosine A_{2A}, A_{2B} and A_3 receptors also occur, and these potentially can produce indirect effects on pain transmission. Endogenous adenosine can be released in the CNS and peripheral tissues, and the regulation of its levels by various pharmacological agents can alter pain processing through activation of adenosine A_1 receptors on neurons, and perhaps other receptors on adjacent structures (Sawynok and Liu, 2003). Anti-nociception induced by adenine-derived purines seems to be related to adenosine receptors, probably A_1 receptors, as adenosine antagonists such as caffeine and theophylline block their effect and adenosine uptake blockers and adenosine deaminase inhibitors enhance anti-nociception (McGaraughty *et al.*, 2001; Donnelly-Roberts

et al., 2008). Therefore, allopurinol, by inhibiting xanthine oxidase and production of uric acid, may produce accumulation of other purines (for example adenosine), which may account for its anti-nociceptive properties.

With regard to the mechanism of action of allopurinol, our findings demonstrated that the activation of a analoxone-sensitive opioid pathway is unlikely to be involved in the anti-nociception caused by allopurinol, as naloxone, under conditions where it fully reversed morphine-induced anti-nociception, had no effect against anti-nociception after allopurinol. However, caffeine and DPCPX, but not SCH58261, prevented allopurinol-induced anti-nociception; these results indicate that A_1 adenosine receptors and adenosine are involved in these effects. Importantly, there is no evidence that allopurinol presents any direct agonist or antagonist effect on adenosine receptors (Day *et al.*, 2007).

The basic mechanism of action of allopurinol and its metabolite oxypurinol is inhibition of xanthine oxidase (they bind strongly to the reduced form of xanthine oxidase and inhibit the enzyme). This leads to a decrease in the systemic concentration of uric acid and an increase in the concentration of the precursors, hypoxanthine and xanthine (Day *et al.*, 2007). In addition, hypoxanthine can be converted to inosine, IMP and consequently, to adenosine and guanosine (Day *et al.*, 2007). Thus, the primary effect of both allopurinol and oxypurinol is inhibition of uric acid production, and the overall result is the inhibition of the metabolism of xanthine and hypoxanthine leading to greater salvage of these purines by their conversion to inosine, adenosine and guanosine. These findings, both in CNS and periphery, have been extensively demonstrated after systemic administration of allopurinol in several studies in animals and humans (Kim *et al.*, 1987a,b; Ceballos *et al.*, 1994; Marro *et al.*, 2006). In fact, a significant concentration of allopurinol has been demonstrated in CSF after its systemic administration and a remarkable suppression of CSF uric acid levels has been observed (Kim *et al.*, 1987a; Enrico *et al.*, 1997; Akdemir *et al.*, 2001). Accordingly, in this study, we demonstrated a marked increase in the CSF concentrations of allopurinol (approximately 58 $\mu mol \cdot L^{-1}$) and of the nucleosides adenosine and guanosine and their metabolites, 30 min after an i.p. administration of allopurinol (200 mg kg^{-1}). Therefore, allopurinol-induced CSF adenosine accumulation may play a role in the anti-nociceptive action of allopurinol.

Although our findings indicate a role for adenosine in allopurinol-induced anti-nociception, we cannot rule out the influence of other purines. This study also demonstrated a significant increase in the CSF concentration of the nucleoside guanosine. Our group and others (Dobolyi *et al.*, 2000; Oses *et al.*, 2004; 2007; Cunha, 2005) have demonstrated that the nucleosides guanosine and adenosine closely interact in the CNS. More recently, we have proposed a specific guanine-based purinergic system with relevant physiological and pathological implications to the CNS, in addition to the well-characterized adenine-based purinergic system (Schmidt *et al.*, 2007). Of note, we have demonstrated that guanosine, as well as adenosine, may modulate pain transmission (Schmidt *et al.*, 2008). Therefore, it is not possible to exclude that the nucleoside guanosine may also influence allopurinol-induced anti-nociception. Unfortunately, a limitation of our study is that guanosine receptor antagonists are not available, which would more directly assess the role of guanosine in the anti-nociceptive effects of allopurinol.

Allopurinol was developed and has been extensively used as an inhibitor of the enzyme xanthine oxidase (Day *et al.*, 2007). Xanthine oxidase is a highly versatile flavoprotein enzyme that catalyses the oxidative hydroxylation of purine substrates and generates reactive oxygen species (ROS) (Borges *et al.*, 2002). ROS have been proposed to contribute to and/or maintain conditions of chronic pain (Kim *et al.*, 2006). More recently, some data indicated that ROS may also mediate acute pain transmission (Hacimuftuoglu *et al.*, 2006). Notably, there is overwhelming acceptance that xanthine oxidase activity is significantly increased in various pathological states, including some pain states (Khalil and Khodr, 2001). Therefore, the inhibition of this enzymatic pathway may be beneficial for treating pain (Lee *et al.*, 2007).

The administration of allopurinol has been shown to decrease tissue injury following ischaemia/reperfusion in a variety of *in vitro* and *in vivo* models (Garcia Garcia *et al.*, 1990; Reilly *et al.*, 1991). Recently, Inkster *et al.* (2007) showed that allopurinol treatment (50 and 250 mg kg^{-1}) had marked beneficial effects on nerve and vascular function in diabetic rats. That same study also demonstrated that allopurinol (150 mg kg^{-1}) attenuated diabetes-induced tactile allodynia, thermal and mechanical hyperalgesia. These effects may be related to a reduction on xanthine oxidase activity and consequently on aspects of ROS-mediated nerve dysfunction via adverse vascular effects (Inkster *et al.*, 2007).

Central and systemic administration of the nucleoside adenosine has been traditionally related to significant side effects, such as hypotension, sedation and impaired motor function (Sawynok and Liu, 2003). Therefore, if allopurinol induces anti-nociception by increasing levels of adenosine, the reduction in pain scores could be related to these alterations. However, our results show that allopurinol up to 200 mg kg^{-1} produced no obvious behavioural disturbances (hole-board) and did not alter motor coordination (rotarod) and spontaneous ambulation (activity cages). However, at a higher dose (400 mg kg^{-1}), some CNS side effects were observed (decreased locomotor activity as shown in the hole-board test and activity cages and impaired motor coordination as observed in the rotarod test).

In summary, to the best of our knowledge, this is the first study reporting the anti-nociceptive profile of allopurinol in well-established pain models. Allopurinol-induced anti-nociception may be related to an accumulation of adenosine, and perhaps guanosine, in the CNS. Although it is too early to propose the use of adenine- or guanine-based purines for clinical research, an interesting approach to investigate their role clinically is the investigation of purine derivatives already used in humans, such as allopurinol. Moreover, because allopurinol is a well-known and extensively used compound and seems to be well tolerated, with no obvious CNS toxic effects, except at high doses, this drug may be useful to treat pain syndromes in humans.

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Conflicts of interest

None.

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