

## Anticociceptive effects of novel A<sub>2B</sub> adenosine receptor antagonists

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**Running Title: Analgesic effects of A<sub>2B</sub> receptor antagonists**

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Number of text pages: 22

Number of tables: 3

Number of figures: 4

Number of references: 36

Number of words in the Abstract: 136

Number of words in the Introduction: 580

Number of words in the Discussion: 1463

Abbreviations

THC,  $\Delta^9$ -tetrahydrocannabinol; MC, methylcellulose; MPE, maximal possible effect; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; PSB-36, 1-butyl-8-(3-noradamantyl)-3-(3-hydroxypropyl)xanthine; DMPX, 3,7-dimethyl-1-propargylxanthine; MSX-3, phosphoric acid mono-(3-{8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl}-1,2,6,7-tetrahydropurin-3-yl)propyl)ester; PSB-50, 8-(p-bromophenyl)-1-propargylxanthine; PSB-53, 4-(1-butylxanthin-8-yl)benzoic acid; PSB-1115, 1-propyl-8-(p-sulfophenyl)xanthine; PSB-55, 8-{4-[2-(4-benzylpiperazin-1-yl)-2-oxo-ethoxy]phenyl}-1-butylxanthine; enprofylline,

3-propylxanthine; PSB-10, (*R*)-8-ethyl-4-methyl-2-(2,3,5-trichlorophenyl)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one; 8-SPT, 8-(*p*-sulfophenyl)theophylline; 8-SPC, 8-(*p*-sulfophenyl)caffeine).

Section assignment: Behavioral Pharmacology

## **ABSTRACT**

Caffeine, an adenosine A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptor antagonist, is frequently used as an adjuvant analgesic in combination with non-steroidal anti-inflammatory drugs or opioids. In this study we have examined the effects of novel specific adenosine receptor antagonists in an acute animal model of nociception. Several A<sub>2B</sub>-selective compounds showed antinociceptive effects in the hot-plate test. In contrast, A<sub>1</sub>- and A<sub>2A</sub>- selective compounds did not alter pain thresholds, and an A<sub>3</sub> adenosine receptor antagonist produced thermal hyperalgesia. Evaluation of psychostimulant effects of these compounds in the open field showed only small effects of some antagonists at high doses. Co-administration of low, sub-effective doses of A<sub>2B</sub>-selective antagonists with a low dose of morphine enhanced the efficacy of morphine. Our results indicate that analgesic effects of caffeine are mediated - at least in part - by A<sub>2B</sub> adenosine receptors.

## Introduction

Caffeine has intrinsic antinociceptive properties and is frequently used as an adjuvant analgesic drug (Malec and Michalska, 1988; Sawynok and Yaksh, 1993). Although it is thought that caffeine analgesia is produced, at least in part, through adenosine receptor antagonism it is unclear which receptor subtypes are involved. The adenosine receptor family comprises four subtypes,  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$  (Fredholm et al., 2001). They are widely distributed in the CNS and peripheral tissues: The  $A_1$  receptors are found in high density in the brain (cortex, cerebellum and hippocampus) and the dorsal horn of the spinal cord, and at lower levels in other brain regions and in peripheral tissues (Rivkees et al., 1995; Fredholm et al., 2001). The  $A_{2A}$  receptors show a more restricted expression pattern in the CNS with high levels in the striatum, nucleus accumbens and olfactory tubercle (Ongini and Fredholm, 1996). In the periphery,  $A_{2A}$  receptors are highly expressed in spleen, thymus, leukocytes and blood platelets (Ongini and Fredholm, 1996).  $A_{2B}$  and  $A_3$  receptors are widely distributed, but have a low density in the CNS (Dixon et al., 1996). In the periphery  $A_{2B}$  receptors are highly expressed in the large intestine, on mast cells and hematopoietic cells (Feoktistov and Biaggioni, 1995).  $A_3$  receptors show a species-dependent distribution: in rats, testis and mast cells express  $A_3$  receptors in high density (Salvatore et al., 1993), while humans exhibit high  $A_3$  receptor expression in the lung and in cells of the immune system (Salvatore et al., 1993).

$A_1$  receptors can couple to  $G_i$  thus inhibiting the formation of cAMP, while stimulation of  $A_2$  receptors, which bind to  $G_s$  leads to an increase in adenylate cyclase activity (Fredholm et al., 2001).  $A_1$  receptors also activate phospholipase C and phospholipase D (Fredholm et al., 2001).  $A_2$  receptors are further subdivided into subtypes  $A_{2A}$  and  $A_{2B}$ , based on the recognition that stimulation of the adenylate cyclase by adenosine (through  $G_s$  or in the striatum through  $G_{olf}$ ) in rat brain was mediated via distinct high affinity (localized in high density in striatal membranes) and low affinity binding sites (present in low density throughout the brain) (Daly et al., 1983).  $A_{2B}$  receptors can also stimulate phospholipase C via

$G_q$  activation (Feoktistov and Biaggioni, 1997).  $A_3$  adenosine receptors are coupled to  $G_i\alpha_2$ -,  $G_i\alpha_3$ -, and to a lesser extent to  $G_{q/11}$  protein (Palmer and Stiles, 1995).

The role of adenosine receptors in nociception is complex and may involve different mechanisms in the central nervous system and in peripheral tissues. For example, spinal administration of adenosine receptor agonists produces antinociception in a variety of animal models of pain, presumably through the activation of spinal  $A_1$  and to a lesser extent through  $A_2$  receptors (Holmgren et al., 1986; Sawynok, 1998). Adenosine can produce analgesic or pronociceptive effects (Doak and Sawynok, 1995) through the activation of peripheral  $A_1$  and  $A_2$  receptors, respectively (Taiwo and Levine, 1990; Doak and Sawynok, 1995). It has been suggested that antagonism of peripheral adenosine  $A_2$  receptors accounts, at least in part, for caffeine analgesia (Taiwo and Levine, 1990; Karlsten et al., 1992).

A number of studies have shown interactions between adenosine receptors and the opioid system. Caffeine potentiated the analgesic effects of morphine, decreased the morphine-induced hyperactivity in mice, and inhibited the development of tolerance to morphine in rats (Malec and Michalska, 1988).

In this study we examined the roles of specific adenosine receptor subtypes in analgesia and morphine synergism using several novel subtype-selective adenosine receptor antagonists. We demonstrate an analgesic effect of  $A_{2B}$  receptor-selective compounds.

## Experimental Procedures

**Chemicals.** The following compounds were tested in this study: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; PSB-36, 1-butyl-8-(3-noradamantyl)-3-(3-hydroxypropyl)xanthine; DMPX, 3,7-dimethyl-1-propargylxanthine; MSX-3, phosphoric acid mono-(3-{8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydro-purin-3-yl}propyl)ester; PSB-50, 8-(p-bromophenyl)-1-propargylxanthine; PSB-53, 4-(1-butylxanthin-8-yl)benzoic acid; PSB-1115, 1-propyl-8-(p-sulfophenyl)xanthine; PSB-55, 8-{4-[2-(4-benzylpiperazin-1-yl)-2-oxo-ethoxy]phenyl}-1-butylxanthine; enprofylline, 3-propylxanthine; PSB-10, (*R*)-8-ethyl-4-methyl-2-(2,3,5-trichlorophenyl)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one; 8-SPT, 8-(p-sulfophenyl)theophylline; 8-SPC, 8-(p-sulfophenylcaffeine); morphine-HCl; codeine sulfate; acetylsalicylic acid; caffeine. For structures and affinities of adenosine receptor antagonists and reference compounds see Table 1. Methylcellulose sodium salt, caffeine, morphine-HCl, codeine sulfate,  $\Delta^9$ -tetrahydrocannabinol (THC), acetylsalicylic acid sodium salt, DMPX, DPCPX and enprofylline were purchased from Sigma-Aldrich (Steinheim, Germany); carrageenan was from Carl Roth (Karlsruhe, Germany). PSB-36, MSX-3, PSB-50, PSB-53, PSB-1115, PSB-55, PSB-10, 8-SPT, and 8-SPC were synthesized in our laboratory. The adenosine receptor affinities of compounds used in this study, were determined experimentally by published standard procedures, or taken from the literature (see Table 1). For the selective  $A_{2B}$  antagonists PSB-1115 and PSB-53 effects on specific radioligand binding to 30 different receptors were determined by a company (Cerep, Poitiers, France) at a high concentration of 3  $\mu$ M.

**Animals.** Male NMRI mice (32 – 40 g, Charles River Deutschland GmbH, Sulzfeld) were housed in groups of 5 mice per cage at a temperature of 23 - 24°C and a 12 h light/dark cycle. Standard food pellets (Altromin 1324 R, Germany) and water were available *ad libitum*.

Experiments were approved by a local ethics committee. All animals were acclimatized for 2 weeks prior to the initiation of the experiments. Animals were used only once, and were euthanized after the test. The experiments were performed during the light phase between 07.00 and 16.00 h

**Hot-plate test.** Antinociceptive effects of the compounds were evaluated in the hot-plate test. Mice (10 per group) were injected intraperitoneally with drug, or the control animals with solvent (1% methylcellulose) in a volume of 10 ml/kg. At the times indicated, they were placed individually on a hot-plate apparatus (Columbus Instruments, Ohio, USA), a 25 x 25 cm metal surface maintained at  $52 \pm 0.1^\circ\text{C}$  and surrounded by a 40 cm high plexiglass wall. The latency of hindpaw licking was determined using a timer integrated into the hot-plate system. A cut-off time of 60 s was used to prevent tissue damage. The paw-surface temperature was determined 50 minutes after drug injection using a contact thermometer. The level of analgesia was expressed as % of the maximum possible effect (MPE):  $\text{MPE} = [(\text{latency}_{\text{drug-treated}} - \text{latency}_{\text{control}}) / (\text{cutoff time} - \text{latency}_{\text{control}})] \times 100$ . If possible, we calculated  $\text{ED}_{50}$  values for analgesic drug efficacy. We used only those compounds where data from at least five doses were available. All calculations were performed using logarithmic dose-values and sigmoid curve fit. The  $\text{ED}_{50}$  values were calculated by applying the Hill's equation using the Prism software (GraphPad Software Inc.).

**Open field test.** To determine possible psychostimulant drug effects, we determined the locomotor activity in the open field in mice that were treated under the same conditions as for the analgesia testing. Briefly, animals (6 - 8 per group) were injected ip with drug or MC (controls) and placed into the center of an open field apparatus (42 x 42 x 28 cm) after 30 min. Activity of the animals was tracked by an automatic monitoring system (ActiMot, TSE, Germany) for 10 minutes under normal lighting conditions. Horizontal motor activity was evaluated and expressed as distance travelled (in m).



**Statistical Analysis.** Data were analyzed by one-way ANOVA followed by Dunnett's test or in case of the interaction studies by Bonferroni test. The 0.05 level of probability was used as the criterion for significance. The group sizes are shown in the description of the methods above. All data were expressed as mean values  $\pm$  S.E.M..

## Results

**Adenosine receptor affinity and selectivity of compounds.** All non-commercially available compounds used in this study (shown in Table 1) were synthesized in our laboratory. The affinities and selectivities for adenosine receptor subtypes of the test compounds were described previously or determined by published standard procedures in radioligand binding assays at native ( $A_1$ ,  $A_{2A}$ ) or recombinant ( $A_{2B}$ ,  $A_3$ ) adenosine receptors.  $K_i$  values are summarized in Table 1. It should be noted that these binding data were mostly obtained with human and rat adenosine receptors. Mouse data were only available for some compounds at the  $A_{2B}$  receptor (Brackett and Daly, 1994), although the  $K_i$  values are likely to be similar in the highly homologous human, rat and mouse  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  receptors (Fredholm et al., 2001; Kim et al., 2002). Only the  $A_3$  adenosine receptor exhibits larger species differences, many antagonists exhibiting a considerably lower affinity for the rat than for the human  $A_3$  adenosine receptor subtype. Thus, it is likely that the  $A_3$  affinity at mouse  $A_3$  receptors may even be lower than at human  $A_3$  receptors for the compounds used in this study. The selectivity of the  $A_{2B}$  adenosine receptor antagonists PSB-53 and PSB-1115 was further investigated in radioligand binding assays at a series of 30 different receptors, including adrenergic, angiotensin, benzodiazepine, bradykinin, cholecystokinin, dopamine, endothelin, GABA, NMDA, histamine, muscarinic and nicotinic acetylcholine, neurokinin, opioid, serotonin, and vasopressin receptors, as well as neurotransmitter transporters for norepinephrine, dopamine and serotonin. At a high concentration of 3  $\mu$ M both compounds

showed only low to negligible binding to all investigated drug targets, except for the adenosine receptors (data not shown). Therefore the compounds appear to be truly selective  $A_{2B}$  adenosine receptor antagonists.

The compounds include (a) the potent  $A_1$ -selective antagonists DPCPX and PSB-36, both compounds exhibiting high selectivity over all other adenosine receptor subtypes, (b) the water-soluble prodrug MSX-3 of the potent  $A_{2A}$ -selective antagonist MSX-2, (c) the  $A_3$ -antagonist PSB-10, (d) four recently developed  $A_{2B}$ -selective adenosine receptor antagonists (PSB-1115, PSB-50, PSB-53, PSB-55), (e) the moderately potent standard antagonists caffeine, DMPX, and enprofylline, which has moderate selectivity for  $A_{2B}$  receptors (3-7-fold vs.  $A_1$ , 3-28-fold vs.  $A_{2A}$ , highly selective vs.  $A_3$ ), and (f) two sulfophenylxanthine derivatives, structurally related to the  $A_{2B}$ -selective antagonist PSB-1115, one of which is virtually inactive at adenosine receptors (8-SPC), the other one being a non-selective  $A_1/A_{2B}$  antagonist (see Table 1). The  $A_{2B}$ -selective antagonist PSB-1115 and the structurally related sulfophenyltheophylline (8-SPT) and sulfophenylcaffeine (8-SPC) would not penetrate into cells or into the brain (Daly, 2000), and can thus be used as pharmacological tools to distinguish extracellular from intracellular, and peripheral from central effects.

**Hot-plate test.** The antinociceptive activity of these compounds was evaluated in the hot-plate test in mice, an acute animal pain model. The results are summarized in Fig. 1, Table 2, and Table 3. A time course analysis of several adenosine receptor antagonists was performed. As shown in Table 2, PSB-1115, PSB-55 and PSB-10 showed their maximum effect after 30 minutes. This time point was chosen for all further studies with independent groups of animals. Caffeine, a virtually non-selective  $A_1/A_{2A}/A_{2B}$  adenosine receptor antagonist with a slight preference for the  $A_{2B}$  receptor produced a robust dose-dependent analgesia in this test. In contrast, the  $A_1$  selective antagonists DPCPX and PSB-36 were not effective in doses up to 100 mg/kg. The  $A_2$  non-selective antagonist DMPX, which shows a slight preference for the  $A_{2B}$  versus the  $A_{2A}$  adenosine receptor (Table 1), exhibited

antinociceptive activity at 30 mg/kg. When subtype-selective antagonists were used, we found no effect with the  $A_{2A}$ -selective compound MSX-3, while all  $A_{2B}$ -selective compounds (PSB-50, PSB-53, PSB-1115, PSB-55 and enprofylline) produced a dose-dependent effect in hot-plate analgesia. PSB-50 was effective at 75 mg/kg, but not at 100 mg/kg. However, we observed profound hypomotility/ataxia and sedation in animals treated with 100 mg/kg. Surprisingly, we found a dose-dependent decrease in hot-plate response latencies in mice treated with the  $A_3$  antagonist PSB-10. Thus, PSB-10 appeared to produce hyperalgesia. 8-SPT and 8-SPC, which are structurally similar to the  $A_{2B}$ -selective compound PSB-1115, but were either non-selective (8-SPT), or virtually inactive at adenosine receptors (8-SPC), did not show any antinociceptive efficacy. The antinociceptive effects of codeine sulfate, morphine hydrochloride, and acetylsalicylic acid were also tested and are shown for comparison (Table 3). To determine, if the compounds had any effects on the body temperature of the animals, which might interfere with the hot-plate result, we determined the paw-surface temperature using a contact thermometer 30 minutes after drug injection. The results are shown in Fig. 2. Neither PSB-1115, nor PSB-55, nor PSB-10, nor caffeine produced changes of the animals paw temperature. In contrast, injection of 4 mg/kg ethanol, which is known to produce hypothermia, led to a readily detectable, highly significant reduction in paw temperature.

**Open-field test.** To evaluate possible effects of active compounds on locomotor activity, we used the open field test (Fig. 3). High dose of enprofylline (100 mg/kg) reduced locomotor activity, but neither with an antinociceptive dose of 100 mg/kg of PSB-53, nor with enprofylline and PSB-50, at an analgesic dose of 75 mg/kg effects on the locomotor activity were seen. No significant locomotor effects were observed with the analgesic compounds DMPX and PSB-1115. The hyperalgesic compound PSB-10 also not reduced locomotor activity at a high dose of 100 mg/kg.

**Interaction with morphine.** Because caffeine is known to enhance the analgesic effect of morphine, we wanted to investigate possible interactions between  $A_{2B}$  antagonists and morphine. We therefore evaluated hot-plate response latencies of animals treated with a low (non-effective) dose of  $A_{2B}$  antagonists, a low dose of morphine, or a combination of both. As shown in Fig. 4, the efficacy of morphine was increased by each compound except PSB-50. We selected among the active compounds PSB-1115 (10 mg/kg) and tested whether it also affected the efficacy of  $\Delta^9$ -tetrahydrocannabinol (THC, 20 mg/kg, threshold-dose of THC). However, we did not find any significant enhancement of THC analgesia ( $p > 0.36$ ).

## Discussion

Adenosine has dual activity on nociception. It acts centrally within the spinal cord to suppress nociceptive signaling (Sawynok and Yaksh, 1993), presumably through the activation of A<sub>1</sub> and A<sub>2</sub> adenosine receptors (DeLander and Hopkins, 1986). In the periphery adenosine has algogenic activity, which is probably mediated by A<sub>2</sub> receptors (Taiwo and Levine, 1990; Karlsten et al., 1992; Sawynok and Yaksh, 1993). Caffeine, a virtually non-selective A<sub>1</sub>-, A<sub>2A</sub>-, and A<sub>3</sub>-adenosine receptor antagonist, exhibits antinociceptive effects and shows adjuvant analgesic properties in combination with opioid and non-opioid analgesics (Sawynok and Yaksh, 1993; Sawynok, 1998).

In this study we investigated the effects of systemic administration of adenosine receptor subtype-selective antagonists on pain sensation. Results obtained with subtype-selective antagonists may provide better insights into the (patho)physiological role of specific adenosine receptors in pain models than agonists, because agonists can target receptors that have no role in control by endogenous adenosine, while antagonists block physiological stimulation of the receptors.

A<sub>1</sub>-selective adenosine receptor antagonists were not hyperalgesic in the hot-plate test. Thus, although the activation of central A<sub>1</sub> receptors seems to play an important role in spinal antinociception (Reeve and Dickenson, 1995; Nakamura et al., 1997), the pharmacological blockade of this receptor has no effect on pain responses in the applied animal models. This cannot be due to lacking CNS penetration of DPCPX, since it has been shown that the compound does penetrate into the brain in concentrations sufficient to block A<sub>1</sub> receptors (Finlayson et al., 1997). In A<sub>1</sub> knockout mice the analgesic effect of intrathecal adenosine analogs was lost suggesting that this receptor subtype is responsible for the central analgesic effects of adenosine. These animals showed an increased pain sensation in the tail-flick test, but not in the von-Frey test (Johansson et al., 2001). It is likely that the role of A<sub>1</sub> receptors in the central processing of nociceptive signals may not have become evident in our models. In a

recent study, DPCPX had exhibited pronociceptive effects only at a low dose of 1 mg/kg, but not at 3 or 10 mg/kg (Bastia et al., 2002). This had been explained by the potentially low selectivity at higher concentrations. In fact, DPCPX is highly selective versus the other adenosine receptor subtypes in rodents. PSB-36, an even more selective A<sub>1</sub> antagonist, also did not show any pronociceptive effects in our hands.

The selective A<sub>2A</sub> antagonist MSX-3 was also ineffective, although A<sub>2A</sub> knockout mice displayed a hypoalgesic phenotype (Ledent et al., 1997). Disparate results from knockout and pharmacological studies are not uncommon. They may be due to pharmacokinetic effects, or to developmental effects of the gene knockout, or both. In this case, pharmacological blockade of the A<sub>2A</sub> receptors may not be sufficient to produce antinociception, or the central and peripheral effects of A<sub>2A</sub> receptor inhibition counterbalance each other. Recently, antinociceptive effects of the A<sub>2A</sub>-selective antagonist SCH-58261 were described (Bastia et al., 2002), which is structurally different from the styrylxanthine derivative MSX-3. However, the A<sub>2A</sub> antagonist was only effective in the hot plate test after intrathecal and not after systemic application.

Here we report for the first time that A<sub>2B</sub> receptor antagonists are potent analgesic agents. Because the virtually nonselective A<sub>2</sub> receptor antagonist DMPX (only slightly selective for A<sub>2B</sub> versus A<sub>2A</sub> receptors) was active in our models but not the A<sub>2A</sub>-selective antagonist MSX-3, we feel that the DMPX effects may be due to the inhibition of A<sub>2B</sub> receptors. A variable response was observed with compound PSB-55, which was effective only at the highest dose tested (100 mg/kg), although this compound has the highest affinity to the A<sub>2B</sub> receptors of all compounds tested (Hayallah et al., 2002). PSB-55 has a relatively high molecular weight and rather low water-solubility. We therefore feel that the probably unfavorable pharmacokinetic properties, of this compound may be responsible for the variable *in vivo* effects.

To determine possible locomotor effects of adenosine receptor antagonists, which may complicate the interpretation of hot-plate results, we studied locomotor activity after drug treatment in the open-field. We found that high dose of the analgesic compound enprofylline (100 mg/kg) reduced locomotor activity. However, no locomotor effects were observed with PSB-53 at 100 mg/kg where strong analgesia was observed, nor with enprofylline and PSB-50 at an analgesic dose of 75 mg/kg. Also, we did not observe any locomotor effects with the antinociceptive compounds DMPX and PSB-1115. In addition, PSB-10 also did not alter locomotor activity at 100 mg/kg, although animals showed significantly decreased hot-plate response latencies. Thus, the hot-plate analgesia observed after  $A_{2B}$  antagonist administration cannot easily be accounted for by their locomotor effects.

We found that the peripherally acting  $A_{2B}$  antagonist PSB-1115, which probably cannot penetrate the blood brain barrier due to its polar sulfonate group (Baumgold et al., 1992), is a potent analgesic compound. Therefore  $A_{2B}$  analgesia must be produced by a peripheral effect. Sulfonates such as PSB-1115 will also not penetrate cell membranes and therefore cannot inhibit intracellular enzymes (Daly, 2000). Thus, the observed effects clearly have to be due to an extracellular mechanism and are believed to be mediated by a blockade of peripheral  $A_{2B}$  adenosine receptors. This is confirmed by the lacking affinity of PSB-1115 and another  $A_{2B}$ -selective antagonist, PSB-53, for a series of 30 receptors, which are known drug targets.

The  $A_3$  adenosine receptor antagonist PSB-10 significantly increased pain sensation in the hot-plate test. It should be noted that PSB-10 is a very potent and selective  $A_3$  antagonist in humans (Ozola et al., 2003), but may be less potent at mouse  $A_3$  receptors. Highly potent and selective rodent  $A_3$  antagonists are currently not available (Müller, 2003).

Caffeine has a virtually non-selective  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  antagonist activity on adenosine receptors, with a slight preference for  $A_{2B}$  receptors. Caffeine analgesia in our models was similar to that described in the literature (Malec and Michalska, 1988), although the non-selective adenosine receptor blocker 8-SPT remained ineffective in our pain tests. The main

differences between caffeine and 8-SPT with regard to their actions at adenosine receptors are (i) the high polarity of 8-SPT, which cannot penetrate the blood-brain barrier in contrast to caffeine, and (ii) the (small) selectivity of caffeine for  $A_{2B}$  versus  $A_1$  adenosine receptors. Other activities of caffeine (alteration of catecholamine or acetylcholine release and turnover, inhibition of phosphodiesterases, influence on intracellular calcium concentrations, interaction with  $GABA_A$  receptors, or an as yet unknown mechanism) may contribute to its antinociceptive effects as well (Sawynok and Yaksh, 1993; Daly, 2000). Also, selectivity for  $A_{2B}$  versus  $A_1$  receptors may play a role since  $A_1$  antagonism may counteract the antinociceptive effect mediated by  $A_{2B}$  antagonists. *In vivo*, in the presence of high adenosine concentrations released by noxious stimuli, the  $A_{2B}$  selectivity of caffeine may even be higher than *in vitro*, since  $A_{2B}$  adenosine receptors exhibit low affinity for adenosine ( $EC_{50} \sim \mu M$ ) while  $A_1$  adenosine receptors are high affinity receptors ( $EC_{50}$  of adenosine  $\sim nM$ ) (Fredholm et al., 2001).

In summary, our results indicate an important role of adenosine  $A_{2B}$  and  $A_3$  receptors in pain signaling.  $A_{2B}$  receptors have previously been proposed to mediate pro-nociceptive effects at peripheral sites (Sawynok et al., 1997). Since the compounds in these studies are likely to produce their anti-nociceptive effects also peripherally, they support the role of  $A_{2B}$  receptors in peripheral pain signaling. However, our study revealed a pronociceptive effect of an  $A_3$  receptor antagonist, which was unexpected, because previous studies have indicated that those receptors may also be involved in peripheral pain signaling (Sawynok et al., 1997).

Caffeine is frequently used as an adjuvant analgesic in the medical practice for the treatment of various types of pain such as headache, postpartum pain, postoperative pain, and dental surgery pain (Sawynok and Yaksh, 1993) in combination with non-steroidal anti-inflammatory drugs (Cass and Frederik, 1962; Sawynok and Yaksh, 1993) or in combination with morphine (Malec and Michalska, 1988). There is a large body of evidence pointing to an interaction between the opioid and adenosine systems in the modulation of pain signaling.



Morphine enhances the release of adenosine from the spinal cord and cortex (Phillis et al., 1980; Cahill et al., 1996), presumably through a synergistic activation of mu and delta opioid receptors (Cahill et al., 1996). Pharmacological studies have indicated that the A<sub>1</sub> and A<sub>2A</sub> receptors may be downstream mediators of morphine analgesia (Keil and DeLander, 1994; Sawynok, 1998), although studies in A<sub>2A</sub> knockout mice failed to reveal any differences in mu-receptor dependent analgesia (Bailey et al., 2002). The results from this study indicate that the analgesic effects of centrally released adenosine may be counterbalanced by pronociceptive A<sub>2B</sub> receptor-mediated peripheral effects of adenosine. The lack of synergistic effects between A<sub>2B</sub> selective compounds and THC indicates that the synergism is specific for the opioid system and not due to a general increase in antinociceptive drug efficacy. Specific A<sub>2B</sub> receptor antagonists might therefore be valuable adjuvant drugs for opioid analgesia with minimal side effects.

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‡ Scholarship of the Egyptian government

O.M.A-S. was on leave from the Faculty of Pharmacy, Al-Azhar University with a scholarship of the Egyptian government

φ A.M.H. was on leave from the Faculty of Pharmacy, Assiut University with a scholarship of the Egyptian government

# Support from the Deutsche Forschungsgemeinschaft (FOR425), the State of North-

Rhine-Westfalia (Innovationsprogramm Forschung), and the BONFOR Program.

\* Supported by the Deutsche Forschungsgemeinschaft (FOR425)

Figure 1

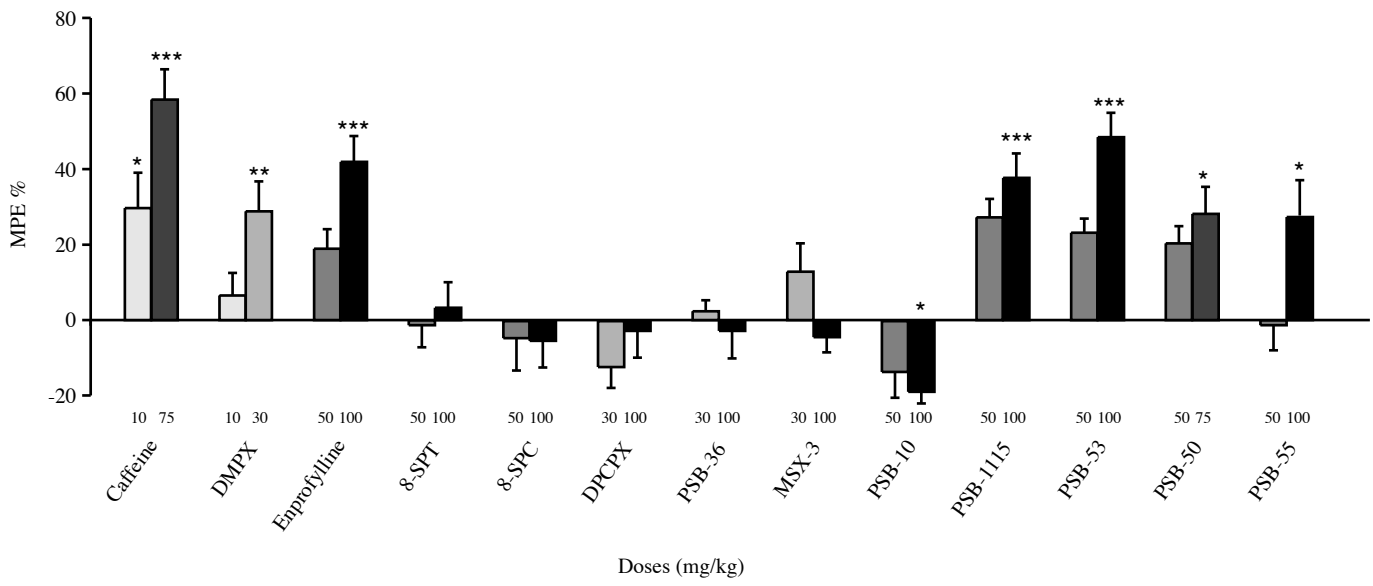


Figure 2

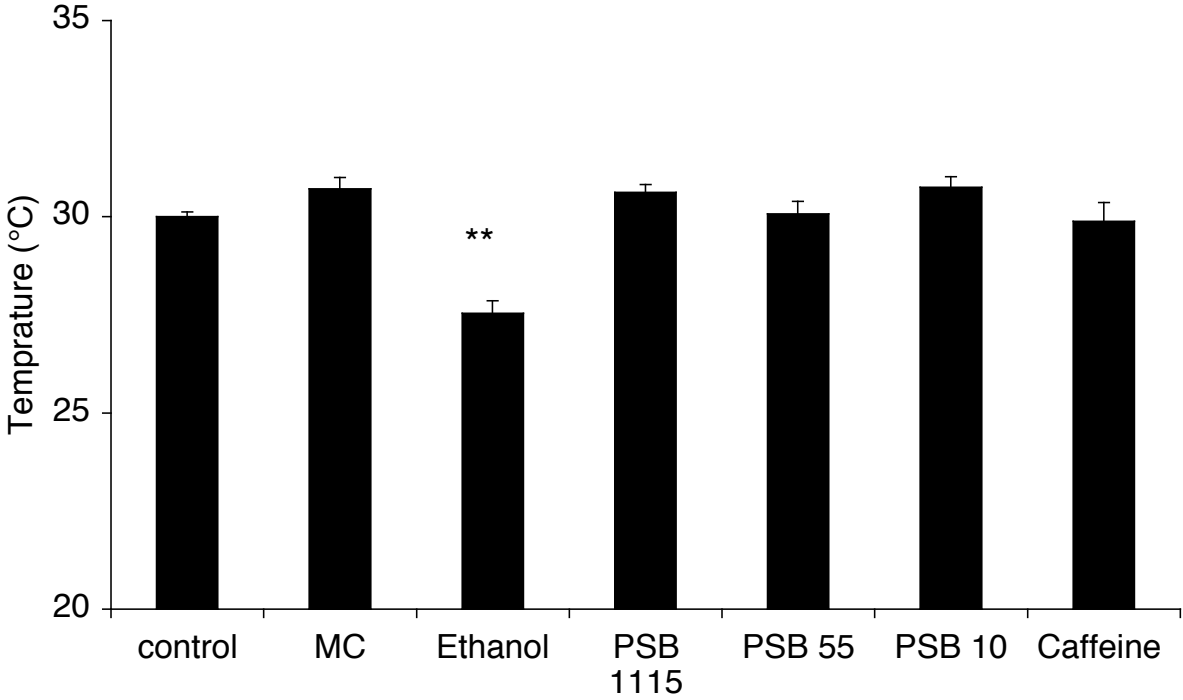




Figure 3

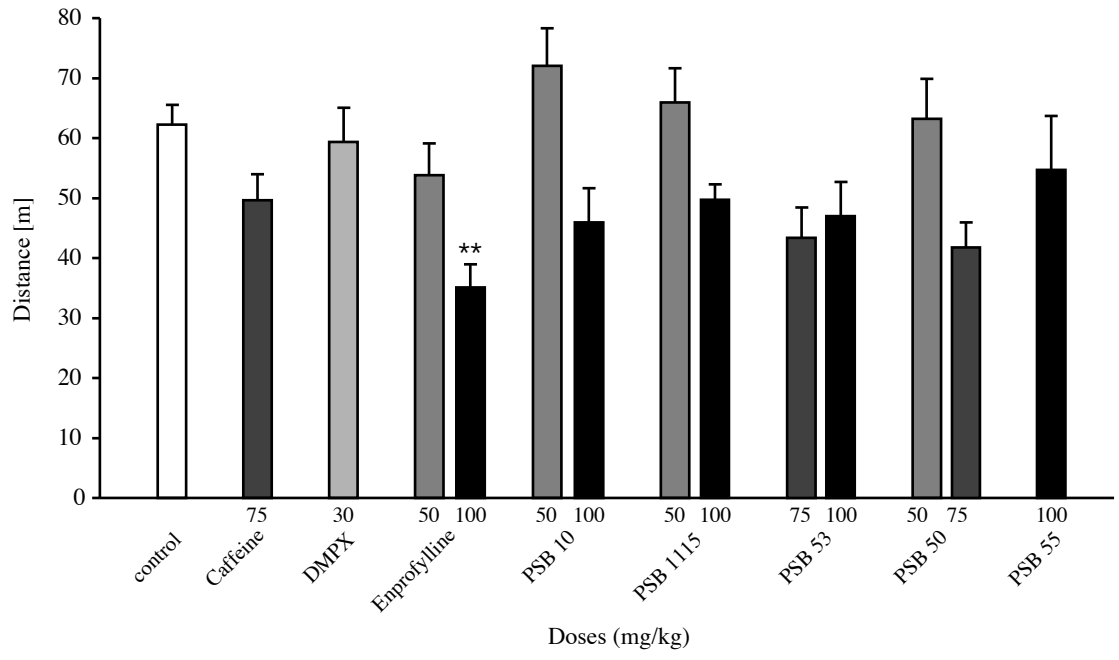
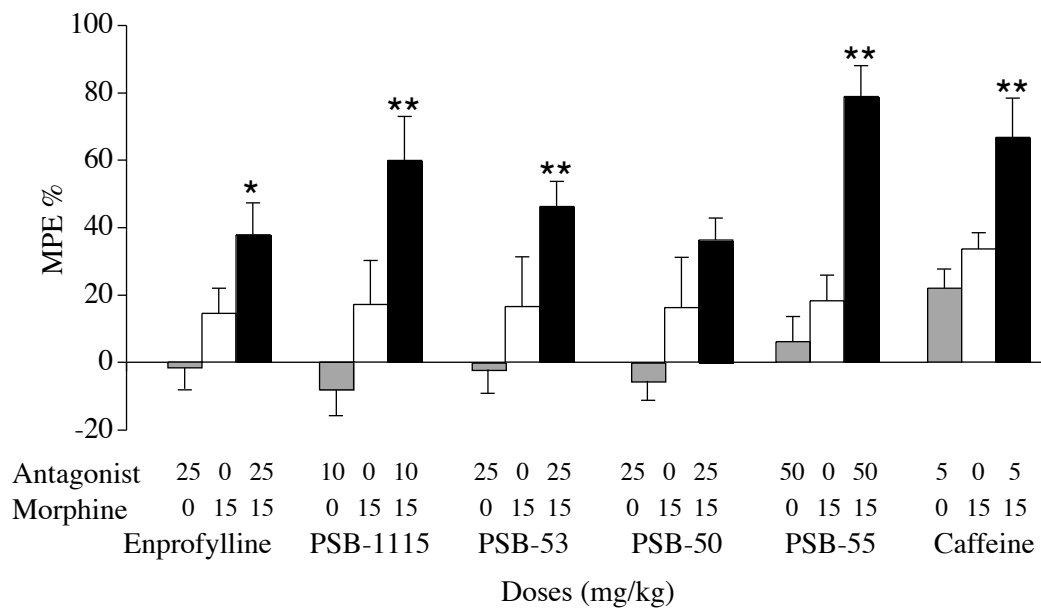
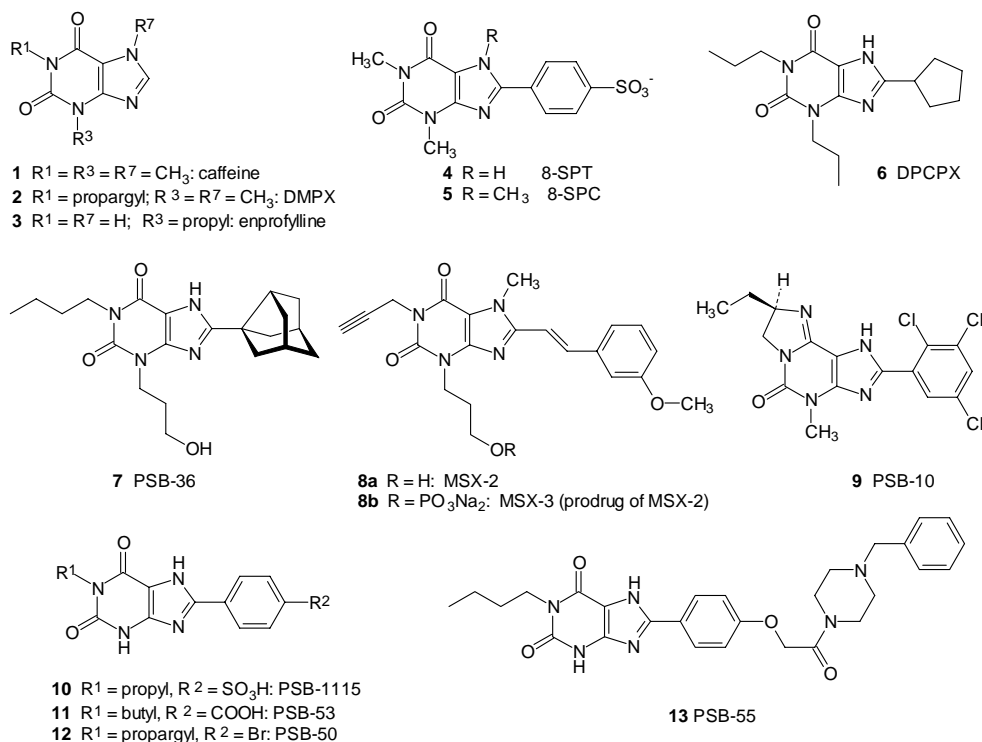


Figure 4



**Table 1** Structures of adenosine receptor antagonists and affinities at adenosine receptor subtypes



No.	Compound	K <sub>i</sub> [nM] (species) <sup>a</sup>			
		A <sub>2B</sub>	A <sub>1</sub>	A <sub>2A</sub>	A <sub>3</sub>
1	Caffeine	13,000 (m) <sup>d,1</sup>	41,000 (r) <sup>2</sup>	43,000 (r) <sup>2</sup>	>100,000 (r) <sup>3</sup>
		10,400 (h) <sup>4</sup>	44,900 (h) <sup>c</sup>	23,400 (h) <sup>c</sup>	13,300 (h) <sup>5</sup>
2	DMPX	2,500 (m) <sup>d,1</sup>	45,000 (r) <sup>6</sup>	16,000 (r) <sup>6</sup>	>10,000 (h) <sup>c</sup>
		4,130 (h) <sup>4</sup>			
3	Enprofylline	4,730 (h) <sup>4</sup>	32,000 (r) <sup>7</sup>	135,000 (r) <sup>7</sup>	158,000 (h) <sup>8</sup>
4	8-SPT	2,200 (r) <sup>9</sup>	3,200 (r) <sup>9</sup>	57,000 (r) <sup>9</sup>	>100,000 (r) <sup>9</sup> 5,890 (h) <sup>5</sup>
5	8-SPC	>>10,000 (h) <sup>c</sup>	> 100,000 (r) <sup>10</sup>	57,000 (r) <sup>10</sup>	>>10,000 (h) <sup>c</sup>
6	DPCPX	186 (r) <sup>4</sup>	1.0 (r) <sup>4</sup>	500 (r) <sup>4</sup>	>10,000 (r) <sup>3</sup>
		63.8 (h) <sup>4</sup>			795 (h) <sup>11</sup>
7	PSB-36 <sup>c</sup>	187 (h)	0.12 (r)	552 (r)	6,500 (r) 2,300 (h)
8b <sup>b</sup>	MSX-2 <sup>12</sup>	>10,000 (h)	900 (r)	8 (r)	>10,000 (h)

<b>9</b>	PSB-10	<b>30,000</b> (h) <sup>c</sup>	805 (r) <sup>13</sup>	6,040 (r) <sup>13</sup>	0.441 (h) <sup>13</sup>
<b>10</b>	PSB-1115	<b>53</b> (h) <sup>11</sup>	2,200 (r) <sup>11</sup>	24,000 (r) <sup>11</sup>	>10,000 (h) <sup>c</sup>
<b>11</b>	PSB-53 <sup>11</sup>	<b>24</b> (h)	481 (r)	3,800 (r)	4,622 (h)
<b>12</b>	PSB-50 <sup>11</sup>	<b>6.8</b> (h)	60 (r)	199 (r)	477 (h)
<b>13</b>	PSB-55 <sup>11</sup>	<b>1.3</b> (h)	37 (r)	550 (r)	475 (h)

<sup>a</sup> r = rat; m = mouse; h = human

<sup>b</sup> MSX-3 (**8a**) is a water-soluble phosphate prodrug of MSX-2 (**8b**)

<sup>c</sup> Results from our laboratory

<sup>d</sup> Data obtained from adenylate cyclase assays at NIH 3T3 intact cells

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**Table 2.** Time dependence of effects of different adenosine receptor antagonists on the hot-plate response of mice (hind-paw)

Comp.	Response latency (sec.)				
	15 min	30 min	60 min	90 min	120 min
MC	23.50 ± 2.45	25.88 ± 1.86	24.13 ± 2.13	22.63 ± 2.03	23.75 ± 2.43
PSB-1115	27.50 ± 2.20	33.38 <sup>(*)</sup> ± 2.66	28.13 ± 1.63	25.50 ± 2.37	26.13 ± 2.00
PSB-55	28.00 ± 1.45	34.0* ± 2.35	27.63 ± 2.64	23.50 ± 1.43	22.63 ± 2.12
PSB-36	23.75 ± 3.36	22.63 ± 1.15	18.88 ± 1.41	25.63 ± 2.56	22.63 ± 1.75
PSB-10	23.38 ± 1.29	18.88 ± 1.75	22.13 ± 1.37	21.13 ± 1.77	23.75 ± 1.95
MSX-3	23.50 ± 2.34	23.63 ± 2.26	24.88 ± 1.56	24.25 ± 1.88	24.25 ± 1.92
8-SPC	21.88 ± 1.44	24.88 ± 2.61	24.13 ± 2.81	23.50 ± 1.15	21.88 ± 3.08

Results are expressed as mean ± SEM, n = 8, (\*) 0.1 < p < 0.05; \* p < 0.05  
Dose used was 100 mg/kg for all compounds

**Table 3** Hot plate analgesia

No.	Compound	Dose (mg/kg)	Time of reaction on pain stimulus (s) <sup>a</sup>	ED <sub>50</sub>
<b>1</b>	Caffeine	0.00	27.10 ± 1.23	7.96
		1.00	26.80 ± 2.19	
		3.00	33.00 ± 2.36	
		5.00	34.30 ± 1.87	
		10.0	36.90 ± 3.13*	
		75.0	46.20 ± 2.74***	
<b>2</b>	DMPX	0.00	23.60 ± 1.97	> 50
		10.0	26.10 ± 2.15	
		30.0	34.20 ± 2.72**	
		50.0	lethal dose	
<b>3</b>	Enprofylline	0.00	24.60 ± 2.03	56.9
		10.0	22.30 ± 2.23	
		30.0	26.10 ± 2.38	
		50.0	31.30 ± 1.79	
		75.0	36.70 ± 2.55**	
		100	39.50 ± 2.36***	
<b>4</b>	8-SPT	0.00	24.50 ± 2.14	> 100
		30.0	26.50 ± 2.32	
		50.0	23.90 ± 2.11	
		100	25.80 ± 2.28	
<b>5</b>	8-SPC	0.00	27.30 ± 1.92	> 100
		30.0	24.10 ± 1.80	
		50.0	25.60 ± 2.77	
		100	25.40 ± 2.21	
<b>6</b>	DPCPX	0.00	27.10 ± 1.23	> 100
		10.0	24.10 ± 2.11	
		30.0	23.10 ± 1.93	
		100	26.10 ± 2.22	
<b>7</b>	PSB-36	0.00	24.40 ± 1.76	> 100
		30.0	25.10 ± 0.97	
		50.0	23.00 ± 1.96	
		100	23.40 ± 2.61	
<b>8</b>	MSX-3	0.00	25.90 ± 2.35	> 100
		30.0	30.20 ± 2.69	
		50.0	25.80 ± 2.39	
		100	24.40 ± 1.39	
<b>9</b>	PSB-10	0.00	24.70 ± 1.95	b
		30.0	24.20 ± 1.23	
		50.0	19.80 ± 2.28	
		100	18.00 ± 1.04*	

<b>10</b>	PSB-1115	0.00	23.50 ± 2.00	
		6.25	20.90 ± 1.61	
		12.5	30.30 ± 1.92	27.74
		25.0	30.40 ± 2.60	
		50.0	33.30 ± 1.63**	
		100	37.10 ± 2.34***	
<b>11</b>	PSB-53	0.00	27.20 ± 2.11	
		12.5	25.90 ± 2.29	
		25.0	31.00 ± 3.08	50
		50.0	34.80 ± 1.24	
		75.0	38.90 ± 2.19**	
		100	43.00 ± 1.86***	
<b>12</b>	PSB-50	0.00	27.20 ± 2.11	
		12.5	25.30 ± 1.78	
		25.0	25.60 ± 2.74	> 100
		50.0	33.80 ± 1.50*	
		75.0	36.50 ± 2.05	
		100	26.20 ± 2.34	
<b>13</b>	PSB-55	0.00	27.90 ± 1.75	
		30.0	27.50 ± 2.28	> 100
		50.0	28.40 ± 2.07	
		100	36.80 ± 3.01*	
<b>14</b>	Codeine sulfate	0.00	26.20 ± 1.99	
		5.00	23.88 ± 1.27	> 50
		20.0	25.98 ± 2.24	
		50.0	36.25 ± 3.90*	
<b>15</b>	Morphine-HCl	0.00	26.20 ± 1.99	
		3.00	29.30 ± 2.16	
		5.00	25.20 ± 2.56	
		10.0	31.50 ± 2.05	18.1
		15.0	33.60 ± 4.76	
		30.0	54.50 ± 2.82***	
		50.0	55.30 ± 3.18***	
<b>16</b>	Acetylsalicylic acid	0.00	27.10 ± 1.23	
		250	27.20 ± 1.10	>1000
		500	30.40 ± 1.28	
		1000	33.10 ± 2.44*	

<sup>a</sup> Results are expressed as mean ± SEM, n = 8 - 10, \*p < 0.05; \*\* p < 0.01; \*\*\*p < 0.001

<sup>b</sup> compound was pronociceptive

**Fig. 1.** Effects of adenosine receptor antagonists and reference compounds on hot-plate analgesia in mice. Columns show the analgesic effect of the drug in two doses as percentage of the maximal possible effect (MPE%). The degree of shading of the columns is proportional to the dose tested. The results obtained with additional doses are also shown in Table 3. Data are represented as mean  $\pm$  SEM, n = 10; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001 (control vs. drug treated group, one-way ANOVA followed by Dunnett's test).

**Fig. 2.** Effects of adenosine receptor antagonists on hind-paw temperature. Ethanol at a dose of 4 g/kg was used as a positive control. Adenosine receptor antagonists showed no significant reduction in the paw temperature at high doses of 100 or (in case of caffeine) 75 mg/kg. Data are represented as mean  $\pm$  SEM, n = 8; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001 (control vs. drug treated group, one-way ANOVA followed by Dunnett's test).

**Fig. 3.** Evaluation of effects of compounds on locomotor activity at analgesic doses. Data are represented as mean  $\pm$  SEM, n = 10; \*\* = p < 0.01 (control vs. drug treated group, one-way ANOVA followed by Dunnett's test).

**Fig. 4.** Influence of A<sub>2B</sub> adenosine receptor selective antagonists on the analgesic effect of morphine. Each column represents the analgesic effect of the drug treatment as percentage of the maximal possible effect (MPE%) (mean  $\pm$  SEM, n = 10); \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001 (morphine vs. drug + morphine treated group, one-way ANOVA followed by Bonferroni test).