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Full Paper

Synthesis and Analgesic Activity of Annelated Xanthine Derivatives in Experimental Models in Rodents

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A series of annelated derivatives of xanthine were synthesized and assayed as potential analgesic agents. All synthesized xanthine derivatives were tested in the writhing test and hot-plate test. The pharmacological assays demonstrated that all the compounds prepared, without exception, displayed a significant activity in the mouse writhing assay. The analgesic action of the most active compounds, expressed as ED_{50} was found to be 1.4–4.3 times more potent than that of acetylsalicylic acid used as the reference compound. However, only some of the compounds demonstrated analgesic activity in the hot-plate test. The analgesic effect of some compounds is probably related to their agonistic, antagonistic, or partial agonistic activity at the adenosine receptors.

Keywords: Adenosine receptors / Analgesic activity / Pyrimido[2,1-*f*]purinediones / Xanthines

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Introduction

Analgesic pharmacotherapy utilizes not only the classical drugs (opioids and NSAIDs) but also agents belonging to other pharmacological groups (analgesic adjuvants) used as an add-on therapy in the treatment of intractable pain, especially neuropathic pain syndromes [1].

Currently, the search for new drugs that interfere with other mechanisms of the pain response is in progress. Adenosine is one of the factors involved in the pain response [2]. The role of adenosine receptors in nociception is complex and may involve different mechanisms in the central nervous system and in peripheral tissues [3]. A_1AR agonists show antinociceptive effects in multiple preclinical models of acute and chronic pain [4]. Spinal administration of adenosine receptor agonists induces 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 [3]. Adenosine can produce analgesic or pronociceptive effects through the activation of peripheral A_1 and A_2 receptors, respectively [3].

Literature data on the effects of agonists and antagonists of the A_{2A}R in pain control are conflicting. For example, studies with A_{2A}R agonists have shown their antinociceptive effects in the writhing test, a visceral pain model, but the hypoalgesia observed in A_{2A}R knockout mice and the antinociceptive effects of a specific A_{2A}R antagonist in inflammatory models suggest that this is the absence or blockade of the A_{2A}R which has therapeutic potential in pain states [5]. In contrast, A_{2B} receptor blockade may result in analgesic effect [3]. Adenosine A_3 receptor activation produces pain behaviors due to the release of histamine and 5-hydroxytryptamine from mast cells and their subsequent actions on the sensory nerve endings.

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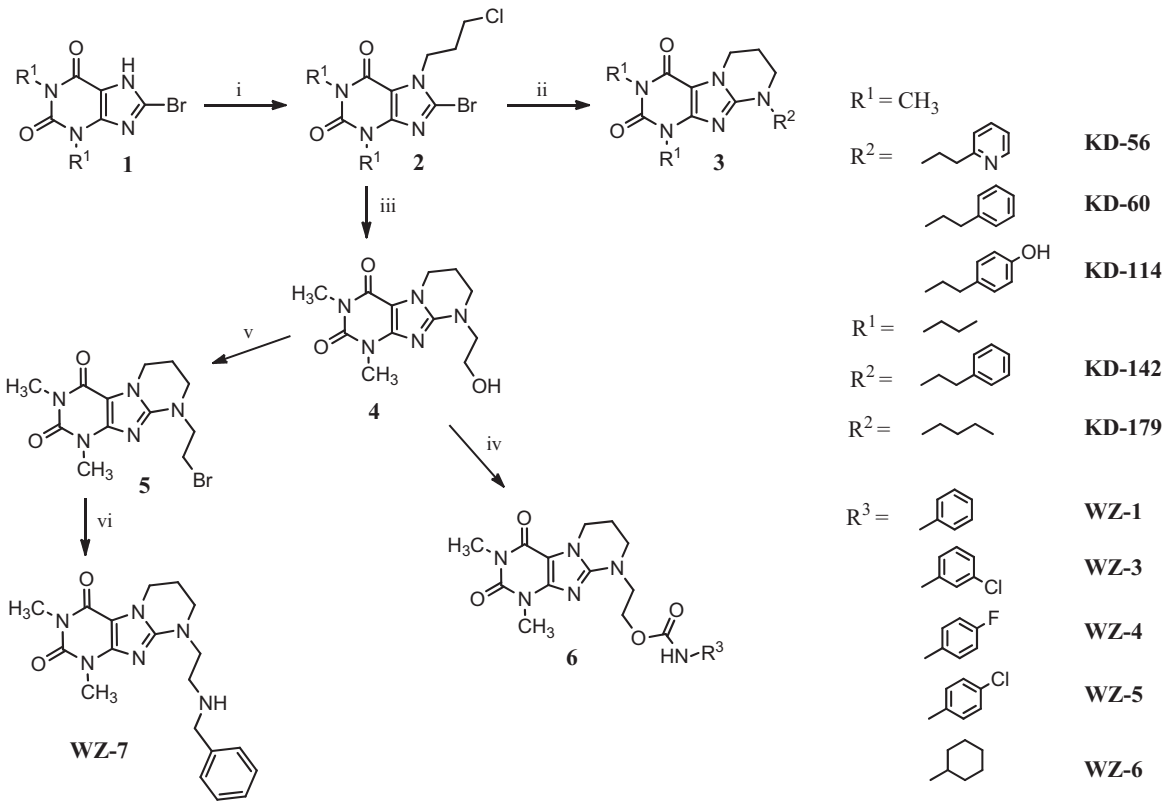
The endogenous adenosine systems contribute to antinociceptive properties of caffeine, opioids, noradrenaline, 5-hydroxytryptamine, tricyclic antidepressants, and transcutaneous electrical nerve stimulation [6]. There are many reports suggesting that some xanthine derivatives, for example, caffeine, possess analgesic properties in laboratory animals. Caffeine, an antagonist of adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors, is known as an adjuvant analgesic in combination with non-steroidal anti-inflammatory drugs (NSAIDs) and acetaminophen in humans [3]. Caffeine potentiated the analgesic effects of morphine and decreased the morphine-induced hyperactivity in mice [7]. In preclinical studies, caffeine produced intrinsic antinociceptive effects in several rodent models [6]. Though it is thought that caffeine analgesia is produced, at least in part, through adenosine receptor antagonism, it is unclear which receptor subtypes are involved [3].

This paper reports the synthesis and results of a preliminary pharmacological screening of annelated xanthine analogs. The synthesized compounds were tested for analgesic activity as well as for adenosine receptor binding affinity. For selected compounds, antiproliferative effect and metabolic stability were checked.

Results and discussion

Chemistry

The tetrahydropyrimido[2,1-*f*]purinediones were synthesized as depicted in Scheme 1. As starting materials, 7-(3-chloropropyl)-8-bromotheophylline (**2A**) and 7-(3-chloropropyl)-8-bromo-1,3-dipropylxanthine (**2B**) were used, which could be obtained as previously described by us [8–10]. They were cyclized with arylalkylamines (**KD-56**, **KD-60**, **KD-114**, and **KD-142**) and alkylamines **4** and **KD-179** under various reaction conditions regarding the amount of amine, the reaction medium, and the reaction time [11]. 4-Hydroxyphenylethylamine (tyramine) was obtained from its hydrochloride by alkalization to pH 9 of water solution of its hydrochloride by 10% aqueous sodium carbonate. The amine **WZ-7** was obtained as product of reaction of **5** (resulting from the reaction of bromination of **4** with PBr_3) with benzylamine



(i) $Cl(CH_2)_3Br$ (ii) R^2NH_2 (iii) $NH_2(CH_2)_2OH$ (iv) R^3NCO (v) PBr_3 (vi) $PhCH_2NH_2$

Scheme 1. Chemical structures of the investigated compounds.

performed upon microwave irradiation under solvent free conditions. Compound **WZ-7** was isolated as hydrochloride. The reaction of **4** and (un)substituted phenyl or cycloalkyl isocyanates carried out in acetonitrile yielded the carbamates **WZ-1**, **WZ-3**, **WZ-4**, **WZ-5**, and **WZ-6**.

Pharmacology

Radioligand receptor binding assays for adenosine receptors

The affinity data for different adenosine receptor subtypes are summarized in Table 1. The compound **KD-179** ($K_i = 0.28$ μM) showed the strongest affinity for A_1 receptors, the compounds **KD-56** ($K_i = 0.32$ μM), **KD-114** ($K_i = 0.23$ μM), and **KD-179** ($K_i = 0.30$ μM) for A_{2A} receptors, being not selective; the compound **KD-142** ($K_i = 0.59$ μM) for A_{2B} receptors, and **KD-179** ($K_i = 0.66$ μM) for A_3 receptors—both compounds were not selective. Compounds **WZ-1**–**WZ-7** with the exception of **WZ-7** (showing weak affinity for A_1 and A_{2A}Rs) were deprived of adenosine receptors affinity.

The hot-plate test

All annelated xanthine derivatives were examined in the hot plate test, but only some of them showed analgesic activity (Tables 2 and 3). The paws of mice are very sensitive to heat at temperatures which are not damaging to the skin. The reaction time is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics (COX-inhibitors) generally do not affect the responses [12].

The hot-plate test as a method of evaluating analgesic activity of substances showed that five derivatives of xanthine (**WZ-1**, **WZ-4, WZ-6, WZ-7**, and **KD-60**) in a dose-dependent manner prolonged the nociceptive reaction time in mice. The results were statistically significant. The highest analgesic activity was observed for the compound **WZ-7**. The calculated ED_{50} value for **WZ-7** is 31.4 mg/kg, for **WZ-1** is 120.7 mg/kg, whereas for **WZ-6** is 150.7 mg/kg. The compounds **WZ-4** and **KD-60** tested only at a dose of 100 mg/kg statistically significantly prolonged the nociceptive reaction time in mice. In comparison to the reference compound which was morphine, none of the xanthine derivatives had a stronger analgesic activity than the reference compound (Tables 2 and 3).

The writhing test

The analgesic activity of the new compounds was also evaluated in the writhing test. This test consists in the intraperitoneal injection of a chemical irritant followed by subsequent counting of "writhes" that is characteristic contractions of abdominal muscles, accompanied by a hind limb extensor motion [12]. Based on the available literature data, this assay reveals significant activity of compounds possessing anti-inflammatory properties, such as NSAIDs, which would abolish the reflex stimulated by substances like phenylbenzoquinone [12].

On the basis of the calculated ED_{50} values, it can be established that the compound **KD-142** ($ED_{50} = 9.0$ mg/kg) showed the strongest analgesic activity, and then in the decreasing order of analgesic potency: **WZ-5** ($ED_{50} = 10.2$ mg/kg), **KD-60** ($ED_{50} = 11.3$ mg/kg), **WZ-4** ($ED_{50} = 12.1$ mg/kg), **WZ-3** ($ED_{50} = 12.5$ mg/kg), **WZ-1** ($ED_{50} = 13.5$ mg/kg), **KD-179** ($ED_{50} = 16.8$ mg/kg), **KD-56** ($ED_{50} = 21.4$ mg/kg), **KD-114** ($ED_{50} = 27.2$ mg/kg), **WZ-6** ($ED_{50} = 42.1$ mg/kg), **WZ-7** ($ED_{50} = 80.9$ mg/kg). In comparison to the reference compound in this assay which was acetylsalicylic acid, except for **WZ-7**, the other compounds showed a stronger or similar (compound **WZ-6**) analgesic effect (Tables 4 and 5).

The results obtained from the analgesic tests showed that new compounds significantly increased the pain threshold on the hot plate and inhibited the writhing response induced by

Table 1. Adenosine receptor affinities of the investigated annelated xanthine derivatives.

Compound	Rat A_1 K_i [μM] ± SEM (% inhibition at indicated conc.)	Rat A_{2A} K_i [μM] ± SEM (% inhibition at indicated conc.)	Human A_{2B} K_i [μM] ± SEM (% inhibition at indicated conc.)	Human A_3 K_i [μM] ± SEM (% inhibition at indicated conc.)
WZ-1	>10 (3%)	>10 (35%)	>10 (6%)	>1 (0%)
WZ-3	>10 (10%)	>10 (33%)	>10 (4%)	>10 (3%)
WZ-4	>10 (12%)	≥10 (42%)	>10 (15%)	>10 (0%)
WZ-5	>10 (24%)	>10 (32%)	>10 (6%)	>10 (13%)
WZ-6	>10 (12)	≥10 (48%)	>10 (19%)	>10 (16%)
WZ-7[a]	7.55 ± 2.35	20.0 ± 2.0	>10 (8%)	>1 (0%)
KD-56	>25 (32%)	0.321 ± 0.028	ca. 10 (57%)	>10 (20%)
KD-60	>25 (22%)	>25 (37%)	13.4 ± 4.7	>10 (0%)
KD-114	≥25 (46%)	0.230 ± 0.080	7.20 ± 0.60	>10 (9%)
KD-142	0.620 ± 0.220	0.860 ± 0.090	0.590 ± 0.060	3.66 ± 0.77
KD-179[b]	0.28 ± 0.02	0.30 ± 0.09	1.32 ± 0.23	0.66 ± 0.02
Caffeine[c]	41.0	43.0	10.4	13.3

[a] Ref. [8].
[b] Ref. [9].
[c] Ref. [3].

Table 2. The influence of the test compounds on the pain reaction in the hot-plate test in mice.

Compound	Dose (mg/kg)	Time of reaction to pain stimulus (s) ± SEM	% Prolongation	ED_{50} (mg/kg)
Control	–	15.3 ± 1.8	–	–
WZ-1	50	20.3 ± 4.3	32.6	120.7
	100	21.7 ± 3.6	41.8	
	200	24.0 ± 2.7[a)]	56.8	
WZ-3	50	16.5 ± 4.1	7.8	–
	100	12.0 ± 2.7	–	
WZ-4	50	13.4 ± 2.9	–	–
	100	29.3 ± 6.4[b)]	91.5	
WZ-5	50	12.3 ± 4.8	–	–
	100	14.9 ± 3.3	–	
	200	15.9 ± 2.7	–	
WZ-6	50	17.3 ± 4.3	13.0	150.7
	100	20.3 ± 5.1	32.6	
	200	24.8 ± 4.3[a)]	62.0	
WZ-7	25	21.1 ± 7.1	37.9	31.4
	50	26.5 ± 6.4[b)]	73.2	
	100	40.1 ± 5.2[c)]	162.0	

[a)] $P < 0.05$.
[b)] $P < 0.01$.
[c)] $P < 0.001$.

phenylbenzoquinone, which suggests that the new compounds possess both centrally and peripherally mediated analgesic properties [12]. The central analgesic action may be mediated via the inhibition of central pain receptors, while the peripheral analgesic effect may be mediated through the inhibition of cyclooxygenase and/or lipoxygenase and other mediators. This hypothesis is based on the previous reports indicating that the hot-plate test and acetic acid writhing method are useful techniques for the evaluation of centrally and peripherally acting analgesic drugs, respectively [12].

The role of adenosine receptors in nociception is complex and may involve different mechanisms in the central nervous

Table 3. The influence of the test compounds on the pain reaction in the hot-plate test in mice.

Compound	Dose (mg/kg)	Time of reaction to pain stimulus (s) ± SEM	% Prolongation	ED_{50} (mg/kg)
Control	–	15.3 ± 1.8	–	–
KD-56	50	17.3 ± 1.4	13.0	–
	100	8.2 ± 2.1	–	
KD-60	50	11.4 ± 2.6	–	–
	100	30.7 ± 3.5[a)]	100.6	
KD-114	50	13.2 ± 2.4	–	–
	100	8.8 ± 1.7	–	
KD-142	50	8.8 ± 0.7	–	–
	100	11.7 ± 1.7	–	
KD-179	50	11.3 ± 0.8	–	–
	100	10.3 ± 0.9	–	
Control	–	18.4 ± 1.0	–	–
Morphine	6	30.6 ± 3.9[b)]	66.3	3.3
	3	29.9 ± 6.0[c)]	62.5	
	1	19.4 ± 2.1	5.4	

[a)] $P < 0.001$.
[b)] $P < 0.01$.
[c)] $P < 0.05$.

Table 4. The influence of the test compounds on the pain reaction in the writhing test in mice.

Compound	Dose (mg/kg)	Mean number of writhes ± SEM	% Inhibition	ED_{50} (mg/kg)
Control	–	26.0 ± 1.8	–	–
WZ-1	12.5	14.2 ± 2.4	45.3	13.5 (7.7–23.9)
	25	4.0 ± 1.3[a)]	84.6	
	50	0.0 ± 0[a)]	100	
	100	0.0 ± 0[a)]	100	
WZ-3	12.5	12.3 ± 2.5[b)]	52.6	12.5 (6.0–25.9)
	25	9.7 ± 1.6[c)]	62.6	
	50	4.2 ± 1.8[a)]	83.8	
	100	2.2 ± 0.9[a)]	91.5	
WZ-4	12.5	13.1 ± 2.7	49.6	12.1 (7.1–20.5)
	25	6.0 ± 2.6[c)]	76.9	
	50	2.7 ± 0.9[a)]	89.6	
	100	1.7 ± 0.3[a)]	93.4	
WZ-5	12.5	12.6 ± 3.1	51.5	10.2 (3.7–27.5)
	25	7.5 ± 2.8[c)]	71.1	
	50	6.2 ± 1.6[c)]	76.1	
	100	2.2 ± 0.5[a)]	91.5	
WZ-6	25	18.7 ± 3.4	28.0	42.1 (28.9–61.3)
	50	12.5 ± 2.7[b)]	51.9	
	100	3.4 ± 0.8[a)]	86.9	
WZ-7	50	21.5 ± 3.9	17.3	80.9 (67.0–97.7)
	100	9.0 ± 2.8[b)]	65.3	
	200	4.8 ± 2.1[c)]	81.5	

[a)] $P < 0.001$.
[b)] $P < 0.05$.
[c)] $P < 0.01$.

system and in peripheral tissues [5]. In connection with literature data about an important role of adenosine receptors, especially A_{2B} in the process of perception of pain, we attempted to synthesize compounds with antagonistic action at these receptors [3]. The novel tricyclic derivatives of xanthine, which were investigated for analgesic activity in the present study, are an example of these compounds.

On the other hand, the xanthine derivatives (except for the **WZ-5**, **WZ-6**, and **WZ-7**) showed a marked affinity for adenosine receptors (Table 1). The compound **KD-142** exhibiting the strongest analgesic effect in the writhing test, showed an affinity for all types of adenosine A_1 receptors ($K_i = 0.62\ \mu M$), A_{2A} ($K_i = 0.86\ \mu M$), A_{2B} ($K_i = 0.59\ \mu M$), A_3 ($K_i = 3.66\ \mu M$). Therefore, the analgesic effect of the test compound may be due to effects on these receptors [3]. However, compound **KD-179**, which had a stronger analgesic effect than aspirin, showed a very strong affinity for A_1 receptors ($K_i = 0.28\ \mu M$) and A_{2A} receptors ($K_i = 0.30\ \mu M$). The compounds **KD-56** and **KD-114**, which were also stronger than aspirin, tightly bound to the A_{2A} receptors ($K_i = 0.32\ \mu M$, $K_i = 0.23\ \mu M$, respectively). These results support a key role for the adenosine A_{2A} receptor (perhaps also A_1) in peripheral nociceptive pathways. There is a possibility that the analgesic effect of the tested xanthine derivatives may result from A_1 receptors activation and blockade or stimulation of A_{2A} receptors.

However, elucidation of the mechanism of analgesic action of other compounds that either did not bind to adenosine receptors or did not have a marked affinity requires further investigation.

Locomotor activity test

The effect of tested compounds on locomotor activity was determined in order to exclude false positive results in the assessment of analgesic activity. Investigations of the effects of the compounds on the spontaneous motor activity in mice showed that in comparison with the control group, the compounds administered at the doses 12.5–100 mg/kg did not influence on their analgesic effect in the writhing test (Tables 6 and 7). The exceptions are the two compounds, **WZ-4** and **KD-60**, which at an analgesic dose of 100 mg/kg, statistically significantly decreased locomotor activity by 58.4 and by 55.4%, respectively. Taking this into account, it can be concluded that the analgesic effect in part may be due to reduced mobility. On the other hand, these compounds showed significant analgesic effect in this test, at the doses 25 and 50 mg/kg, which did not influence in a statistically significant way the spontaneous locomotor activity of mice. We found that a high dose of the analgesic compound **WZ-7**

Table 5. The influence of the test compounds on the pain reaction in the writhing test in mice.

Compound	Dose (mg/kg)	Mean number of writhes ± SEM	% Inhibition	ED_{50} (mg/kg)
Control	–	26.0 ± 1.8	–	–
KD-56	25	12.5 ± 3.2[a)]	51.9	21.4 (15.3–20.9)
	50	1.5 ± 0.6[b)]	94.2	
	100	0.5 ± 0.1[b)]	98.0	
KD-60	12.5	13.2 ± 4.6	49.2	11.3 (6.5–19.5)
	25	4.7 ± 1.9[c)]	81.9	
	50	3.0 ± 1.2[b)]	88.4	
	100	3.2 ± 0.8[b)]	87.6	
KD-114	25	15.5 ± 3.1	40.3	27.2 (18.6–39.9)
	50	3.9 ± 2.4[b)]	85.0	
	100	1.7 ± 0.5[b)]	93.4	
KD-142	12.5	11.7+4.5[a)]	55.0	9.0 (3.5–22.9)
	25	7.0 ± 2.6[c)]	73.0	
	50	5.2 ± 1.0[b)]	80.0	
	100	2.5 ± 0.6[b)]	90.3	
KD-179	12.5	18.5 ± 2.7	28.8	16.8 (12.5–22.7)
	25	5.6 ± 2.1[c)]	78.4	
	50	1.2 ± 0.7[b)]	95.3	
	100	0.5 ± 0.4[b)]	98.0	
Acetylsalicylic acid	30	11.2 ± 2.1[a)]	56.9	39.1 (29.1–48.4)
	50	8.5 ± 1.3[c)]	67.3	
	100	3.2 ± 1.2[b)]	87.6	
Caffeine	25	24.3 ± 1.9	6.5	63.5 (49.8–86.7)
	50	20.5 ± 2.5	23.9	
	100	1.5 ± 0.1[b)]	94.2	

[a)] $P < 0.05$.
[b)] $P < 0.001$.
[c)] $P < 0.01$.

(200 mg/kg) statistically significantly decreased the number of light-beam crossings, as compared to the methylcellulose-treated animals. Taking this into consideration, it seems likely that its analgesic effect at this dose in the writhing test, in part, may be due to the effect on the locomotor activity. We did not observe any significant locomotor effects with the antinociceptive compound **WZ-7** at an analgesic dose of 100 mg/kg.

The compounds which showed significant analgesic activity in the hot-plate test: **WZ-1** (200 mg/kg), **WZ-4** (100 mg/kg), **WZ-6** (200 mg/kg), **WZ-7** (50, 100 mg/kg), and **KD-60** (100 mg/kg) at the same doses, statistically significantly decreased locomotor activity. Taking this into account, probably the analgesic effect of these compounds in the hot-plate test may be wholly or partly due to the influence on locomotor activity. Only compound **WZ-7** did not alter locomotor activity at 100 mg/kg, although animals showed significantly decreased hot-plate response latencies.

In summary, no locomotor effects were observed with **WZ-3**, **WZ-5**, **KD-56**, **KD-114**, **KD-142**, and **KD-179**, where strong analgesia was observed. As for the other compounds, this effect only at the highest dose. Thus, the analgesia observed after administration of these compounds: **WZ-1** (200 mg/kg), **WZ-4** (100 mg/kg), **WZ-6** (200 mg/kg), **WZ-7** (200 mg/kg), and **KD-60** (100 mg/kg) cannot easily be accounted for their locomotor effects.

Toxicity

Antiproliferative assay

In vitro toxicity screening of discovery compounds is an important area of drug discovery and include hERG block assays, mutagenicity/genotoxicity, teratogenicity, cytotoxicity, reactivity screens, selectivity screens, and drug–drug interactions which may cause toxic effects. Nowadays, the *in vitro* evaluation of toxicity of the new compounds is conducted at early discovery phases in parallel with the study on their efficacy [13]. During this work, the potential toxicity effect of the selected compounds was preliminary.

The antiproliferative effects of the selected compounds (**KD-60**, **KD-114**, and **KD-179**; **WZ-1**, **WZ-4**, and **WZ-7**) as well as the reference drug doxorubicin (DX) were determined against HEK-293 cell line by using colorimetric EZ4U method [14]. As seen in Fig. 1, the incubation of cells in the presence of compounds **WZ-1**, **WZ-4**, and **WZ-7** for 48 h showed no antiproliferative effect, even in very high concentrations. No effects were also observed for **KD-60** and **KD-114** (Fig. 2). The

Table 6. The influence of the test compounds on the locomotor activity.

Compound	Dose (mg/kg)	Mean number of movements ± SEM	Locomotor activity decrease (%)
Control	–	432.0 ± 58.6	–
WZ-1	25	387.0 ± 35.3	10.4
	50	320.0 ± 39.4	25.9
	100	249.4 ± 48.3	42.2
	200	160.5 ± 49.0[a)]	62.8
WZ-3	12.5	430.1 ± 39.6	–
	25	385.7 ± 32.9	10.7
	50	317.6 ± 45.0	26.4
	100	238.2 ± 41.7	44.8
WZ-4	25	398.7 ± 46.6	7.7
	50	312.0 ± 54.1	27.7
	100	179.4 ± 33.2[b)]	58.4
WZ-5	25	407.5 ± 51.4	5.6
	50	328.7 ± 45.1	24.0
	100	231.2 ± 37.8	46.4
WZ-6	50	319.5 ± 27.5	26.0
	100	243.9 ± 19.5	43.5
	200	193.5 ± 22.6[b)]	55.2
WZ-7	50	309.5 ± 32.9	28.3
	100	256.0 ± 21.8	40.7
	200	187.5 ± 25.1[b)]	56.5

[a)] $P < 0.01$.
[b)] $P < 0.05$.

antiproliferative effect was observed only for compound **KD-179** with calculated IC_{50} value of 39.54 μM.

Drug–drug interactions

To predict the potential drug–drug interactions, influence of chosen compounds on CYP3A4 cytochrome activity was also examined. The effects of selected compounds (**KD-60**, **KD-114**, and **KD-179**; **WZ-1**, **WZ-4**, and **WZ-7**) on CYP3A4 activity were tested by using luminescent CYP3A4 P450-Glo™ assay [15]. The results were compared to the reference compound, strong CYP3A4 inhibitor, ketoconazole. The obtained data showed that all examined compounds modulate cytochrome activity. As seen in Fig. 3, the compounds possessed dose-dependent and strong (**KD-60** and **KD-179**) or weak (**KD-114**) CYP3A4 inhibitor activity. Moreover, from compounds **WZ-1**, **WZ-4**, and **WZ-7**, only **WZ-4** did not inhibit significantly the activity of CYP3A4 (Fig. 4). Relative to the calculated IC_{50} value for ketoconazole ($IC_{50} = 0.14$ μM), **KD-60** showed similar, very strong, and comparable inhibition activity with calculated $IC_{50} = 0.21$ μM. Additionally, **KD-179** and **WZ-7**, with calculated IC_{50} values 1.79 and 1.01 μM, respectively, may be considered as a strong CYP3A4 inhibitors.

Conclusions

Pain perception, the endpoint of the whole nociception process, occurs in the cortex and the limbic system. These structures are responsible for the cognitive role of pain, including its realization and affective reactions related to pain (including anxiety, irritation, and aggression). Pain treatment may involve both psychological and pharmacological approaches. In our search for new more efficacious analgesics, we identified several derivatives of xanthines that display antinociceptive activity in two basic animal models of acute pain, i.e., the hot-plate test and the writhing test.

The preliminary tests performed in the present study indicated that new compounds demonstrated analgesic effect first of all in the writhing test. Analgesic effect of some compounds is probably related to their agonism/or antagonism or partial agonist at adenosine receptors. Preliminary screening tests documented their potent central and/or peripheral analgesic activity and affinity for different adenosine receptor subtypes. Using the spontaneous locomotor activity test, we showed that the results presented here could not be falsely attributed to sedative influence of xanthines. Explanation of the mechanism of action of some derivatives which did not bind to adenosine receptors or did not have a marked affinity, requires further investigations.

Experimental

Chemistry

Melting points were measured in open capillaries on a Mel-Temp. II apparatus (LD Inc., USA) and were not corrected. The

Table 7. The influence of the test compounds on locomotor activity.

Compound	Dose (mg/kg)	Mean number of movements ± SEM	Locomotor activity decrease (%)
Control	–	432.0 ± 58.6	–
KD-56	25	389.1 ± 51.3	9.9
	50	357.5 ± 42.8	17.2
	100	271.8 ± 39.8	37.0
KD-60	25	364.5 ± 24.8	15.7
	50	294.2 ± 39.4	31.8
	100	192.4.2 ± 36.2[a)]	55.4
KD-114	50	345.9 ± 43.5	19.9
	100	264.7 ± 51.8	38.7
KD-142	12.5	435.2 ± 41.9	–
	25	375.0 ± 39.7	13.1
	50	308.2 ± 47.1	28.6
	100	254.5 ± 42.0	41.0
KD-179	25	396.6 ± 31.0	8.1
	50	337.2 ± 42.9	21.9
	100	248.5 ± 37.9	42.4

[a)] $P < 0.01$.

purity of the synthesized compounds was confirmed by TLC performed on Merck silica gel 60 GF_{254} aluminum sheets (Merck, Darmstadt, Germany), using the following developing system: $CHCl_3$/AcOEt (1:1, v/v). Spots were detected by their absorption under UV light ($\lambda = 254$ nm). Column chromatography was performed on Merck silica gel 60 (70–230 mesh) using CH_2Cl_2/AcOEt 1:1, v/v as an eluent. Infrared spectra were measured with an FT IR 410 spectrometer (Jasco) in KBr pellets and are reported in cm^{-1}. ^1H NMR spectra were obtained on a Varian Mercury spectrometer (Varian Inc., Palo Alto, CA, USA) in DMSO-d_6, operating at 300 MHz. Chemical shifts are reported in δ values (ppm) relative to undeuterated solvent signals. The *J* values are expressed in Hertz (Hz). Signal multiplicities are represented by the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), cycl (cyclohexyl). ^{13}H NMR spectra were acquired on a Varian Mercury spectrometer (Varian Inc.) in DMSO-d_6, operating at 75 MHz. Mass spectrometry analyses: samples were prepared in a mixture of acetonitrile/water (50:50, v/v). The LC/MS were carried out on a system consisting of a Waters Acquity UPLC, coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Retention times (t_R) are given in minutes. The UPLC/MS purity of all final compounds was determined (%). Elemental analyses for C, H, and N were carried out by a micro method using the elemental Vario-EL III Elemental Analyser (Hanau, Germany) and were found within ±0.4% of the theoretical values. All tested compounds possessed a purity of not less than 95%.

*2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4,7,8-hexahydropyrimido[1,2-a]purin-9(6H)-yl)ethyl phenylcarbamate (**WZ-1**)*

White powder, m.p: 234–236°C (ethanol); TLC $R_f = 0.63$; yield 77.5%.; $C_{19}H_{22}N_6O_4$ (MW 398.38). ^1H NMR (300 MHz, DMSO d_6) δ: 2.05 (q, $J = 5.56$ Hz, 2H, CH_2–C<u>H_2</u>–CH_2), 3.10 (s, 3H, N_1–CH_3), 3.17 (s, 3H, N_3–CH_3), 3.44 (t, $J = 5.50$ Hz, 2H, 8–CH_2), 3.74 (t, $J = 5.23$ Hz, 2H, N_9–CH_2), 4.03 (t, $J = 5.91$ Hz, 2H, 6–CH_2), 4.29 (t, $J = 5.23$ Hz, 2H, CH_2–O), 6.93 (t, $J = 7{,}43$ Hz,1H, Ar4′–H),

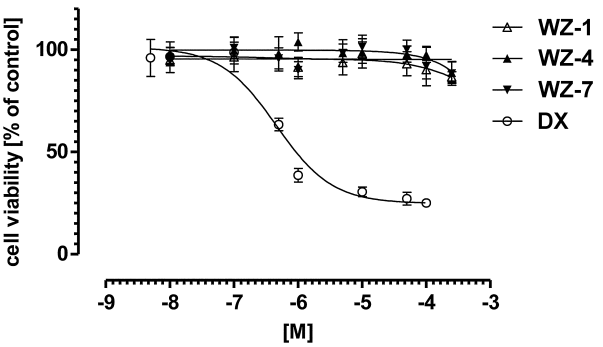


Figure 1. The antiproliferative effect of **WZ-1**, **WZ-4**, **WZ-7**, and DX (reference) against HEK-293 cell line.

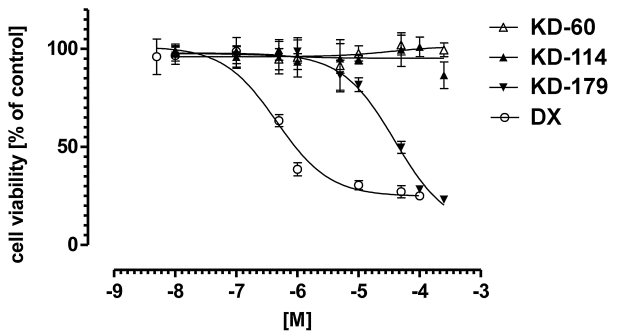


Figure 2. The antiproliferative effect of **KD-60**, **KD-114**, **KD-179**, and DX (reference) against HEK-293 cell line.

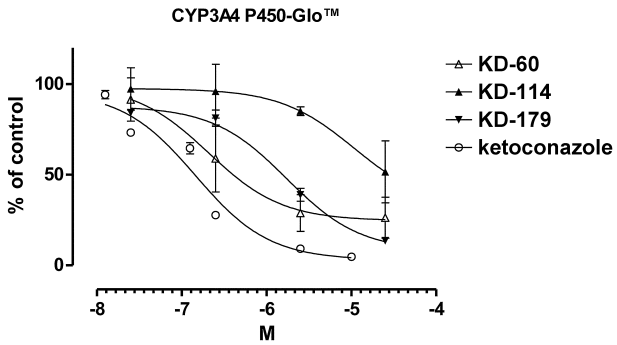


Figure 3. The effect of **KD-60**, **KD-114**, **KD-179**, and ketoconazole on CYP3A4 activity.

7.20 (t, $J = 7.98$ Hz, 2H, Ar3′–H + Ar5′–H), 7.37 (d, $J = 7.43$ Hz, 2H, Ar2′–H + Ar6′-H), 9.54 (s, 1H, NH); ^{13}C NMR (75 MHz; DMSO d_6) δ: 21.21 (CH_2); 27.57 (N_3CH_3); 29.58 (N_1CH_3); 41.81 (N_9CH_2); 44.93 (N_9CH_2); 48.63 (N_5CH_2); 61.79 (OCH_2); 102.29 ($C_{10'}$) (ArCH); 118.46 (ArCH); 122.75 (ArCH); 129.06 (ArCH); 139.47 (ArCH); 148.54 ($C_{9'}$); 151.34 (C=O); 151.85(C=O); 153.01 ($C_{4'}$); 153.75 (OC=O); IR ν: 3289 (N–H), 3076 (C–H), 2949 (–CH_2–), 1727 (C=O), 1698 (C=O), 1222, 1068, 849 (C–H), 759 (–CH_2–); UV (λ_{max}) = 300 nm (logε = 5.2); LC/MS$^{\pm}$: purity 97.48%, t_R = 4.43; (ESI) *m/z* $[M+H]^+$: 399.36. Anal. calcd. for $C_{19}H_{22}N_6O_4$: C, 57.27; H, 5.57; N, 21.10; Found: C, 57.17; H, 5.59; N, 21.00.

*2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4,7,8-hexahydropyrimido[1,2-a]purin-9(6H)-yl)ethyl 3-chlorophenylcarbamate (**WZ-3**)*
White solid, m.p: 244–252°C (ethanol); TLC: $R_f = 0.64$; yield 69.6%; $C_{19}H_{21}N_6O_4Cl$ (MW 432.87). ^{1}H NMR (ppm) δ: 2.03–2.07 (m, 2H, CH_2–C<u>H</u>$_2$–CH_2), 3.11 (s, 3H, N_1–CH_3), 3.17 (s, 6H, N_3–CH_3), 3.43 (t, $J = 5.36$ Hz, 2H, 8–CH_2), 3.75 (t, $J = 4.96$ Hz, 2H, N_9–CH_2), 4.03 (t, $J = 5.78$ Hz, 2H, 6–CH_2), 4.31 (t, $J = 5.01$ Hz, 2H, CH_2–O), 6.97–6.99 (m, 1H, Ar5′–H), 7.20–7.25 (m, 2H, Ar4′–H + Ar6′–H), 7.49 (s, 1H, Ar2′–H), 9.74 (s, 1H, NH). ^{13}C

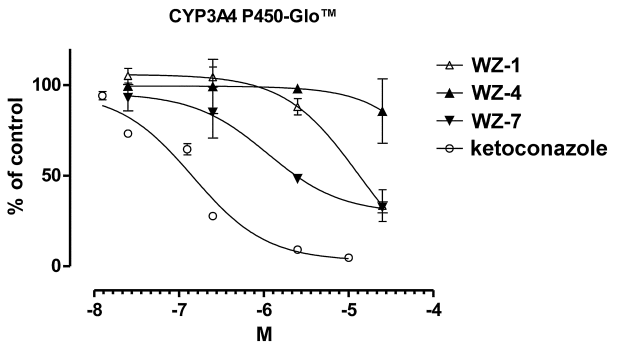


Figure 4. The effect of **WZ-1**, **WZ-4**, **WZ-7**, and ketoconazole on CYP3A4 activity.

NMR (75 MHz; DMSO d_6): 21.22 (CH_2); 27.57 (N_3CH_3); 29.55 (N_1CH_3); 41.82 (N_9CH_2); 44.82 (N_9CH_2); 48.59 (N_5CH_2); 62.03 (OCH_2); 102.29 ($C_{10'}$); 105.00 (ArCH); 116.83 (ArCH); 117.78 (ArCH); 122.42 (ArCH); 130.75 (ArCH); 133.55 (ArCH); 141.09 (ArCH); 148.51($C_{9'}$); 151.33 (C=O); 151.91 (C=O); 153.01 ($C_{4'}$); 153.66 (OC=O). IR ν: 3289 (N–H), 3083 (C–H), 2943 (–CH_2–), 1733 (C=O), 1696 (C=O), 1224, 1064, 778 (C–H), 748 (–CH_2–); UV (λ_{max}) = 300 nm (logε = 4.6); LC/MS$^{\pm}$: purity 96.47%, t_R = 5.12; (ESI) *m/z* $[M+H]^+$: 433.33; Anal. calcd. for $C_{19}H_{21}N_6O_4Cl$: C, 52.72; H, 4.89; N, 19.42. Found: C, 52.73; H, 4.77; N, 19.40.

*2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4,7,8-hexahydropyrimido[1,2-a]purin-9(6H)-yl)ethyl 4-fluorophenylcarbamate (**WZ-4**)*
White crystals, m.p: 262–266°C (ethanol); TLC: $R_f = 0.60$; yield 66.9%. $C_{19}H_{21}N_6O_4F$ (MW = 416.41). ^{1}H NMR (ppm) δ: 2.03–2.06 (m, 2H, CH_2–C<u>H</u>$_2$–CH_2), 3.11 (s, 3H, N_1–CH_3), 3.18 (s, 3H, N_3–CH_3), 3.43 (t, $J = 5.37$ Hz, 2H, 8–CH_2), 3.73 (t, $J = 5.01$ Hz, 2H, N_9–CH_2), 4.03 (t, $J = 5.78$ Hz, 2H, 6–CH_2), 4.28 (t, $J = 5.09$ Hz, 2H, CH_2–O), 7.05 (t, $J = 8.80$ Hz, 2H, Ar2′-H + Ar6′–H), 7.37 (br.s, 2H, Ar3′-H + Ar5′–H), 9.58 (s, 1H, NH). ^{13}C NMR: 21.21 (CH_2); 27.55 (N_3CH_3); 29.58 (N_1CH_3); 41.82 (N_9CH_2); 44.88 (N_9CH_2); 48.65 (N_5CH_2); 61.84 (OCH_2); 102.29 ($C_{10'}$); 115.46 (ArCH); 115.76 (ArCH); 119.95 (ArCH); 135.82 (ArCH); 148.53 ($C_{9'}$); 151.34 (C=O); 151.88 (C=O); 153.01 ($C_{4'}$); 153.84 (–OC=O); 156.41; 159.56. IR ν: 3300 (N–H), 3071 (C–H), 2939 (–CH_2–), 1731 (C=O), 1695 (C=O), 1646, 1218, 1074, 841 (C=H), 749 (–CH_2–); UV (λ_{max}) = 300 nm (logε = 4.8); LC/MS$^{\pm}$: purity 97.21%. t_R = 4.61; (ESI) *m/z* $[M+H]^+$: 417.31; Anal. calcd. for $C_{19}H_{21}N_6O_4F$: C, 54.80; H, 5.08; N, 20.18. Found: C, 54.74; H, 4.96; N, 20.18.

*2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4,7,8-hexahydropyrimido[1,2-a]purin-9(6H)-yl)ethyl 4-chlorophenylcarbamate (**WZ-5**)*
White crystals, m.p: 262–266°C; (ethanol); TLC: $R_f = 0.62$; yield 66.9%. $C_{19}H_{21}N_6O_4Cl$ MW = 432.87. ^{1}H NMR (ppm) δ: 2.03–2.07 (m, 2H, CH_2–C<u>H</u>$_2$–CH_2), 3.11 (s, 3H, N_1–CH_3), 3.17 (s, 3H, N_3–CH_3), 3.42–3.45 (m, 2H, 8–CH_2), 3.74 (t, $J = 4.98$ Hz, 2H, N_9–CH_2), 4.03 (t, $J = 5.78$ Hz, 2H, 6–CH_2), 4.29 (t, $J = 4.95$ Hz, 2H, CH_2–O), 7.25 (t, $J = 8.80$ Hz, 2H, Ar3′–H + Ar5′–H), 7.38 (d, $J = 7.43$ Hz, 2H, Ar2′–H + Ar6′–H), 9.68 (s, 1H, NH). ^{13}C NMR: 21.21 (CH_2); 27.56 (N_3CH_3); 29.58 (N_1CH_3); 41.82 (N_9CH_2); 44.83 (N_9CH_2); 48.61 (N_5CH_2); 61.92 (OCH_2); 102.29 ($C_{10'}$); 119.84 (ArCH); 126.36 (ArCH); 128.94 (ArCH); 138.49 (ArCH); 148.51 ($C_{9'}$); 151.33 (C=O); 151.90 (C=O); 153.01 ($C_{4'}$); 153.72 (–OC=O). IR ν: 3299 (N–H), 2939 (–CH_2–), 1735 (C=O), 1698 (C=O), 1638, 1219, 1088, 750 (–CH_2–); UV (λ_{max}) = 300 nm (logε = 4.6); LC/MS$^{\pm}$: purity 100.0%; t_R = 5.16; (ESI) *m/z* $[M+H]^+$: 433.33; Anal. calcd. for $C_{19}H_{21}N_6O_4Cl$: C, 52.72; H, 4.89; N, 19.42. Found: C, 52.71; H, 4.75; N, 19.38.

*2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4,7,8-hexahydropyrimido[1,2-a]purin-9(6H)-yl)ethyl cyclohexylcarbamate (**WZ-6**)*
White crystals, m.p: 262–266°C; (ethanol); TLC: $R_f = 0.62$; yield 12.36%. $C_{19}H_{28}N_6O_4$: MW = 404.46. ^{1}H NMR (ppm) δ: 0.96–

1.24 (m, 5H, cyclohexyl), 1.48–1.99 (m, 6H, cyclohexyl), 2.00–2.06 (m, 2H, CH_2–C<u>H</u>$_2$–CH_2), 3.13 (s, 3H, N_1–CH_3), 3.31 (s, 3H, N_3–CH_3), 3.39 (t, $J = 5.39$ Hz, 2H, 8–CH_2), 3.63 (t, $J = 5.23$ Hz, 2H, N_9–CH_2), 4.02 (t, $J = 5.91$ Hz, 2H, 6–CH_2), 4.12 (t, $J = 5.28$ Hz, 2H, CH_2–O), 7.03 (d, $J = 7.70$ Hz, 1H, NH). ^{13}C NMR: 21.18 (CH_2); 25.03 (cyclC); 25.58 (cyclC); 27.60 (N_3CH_3); 29.76 (N_1CH_3); 33.03 (cyclC); 33.80 (cyclC); 41.79 (N_9CH_2); 45.15 (N_9CH_2); 48.80 (N_5CH_2); 49.86 (cyclC); 61.17 (OCH_2); 102.27 ($C_{10'}$); 148.64 ($C_{9'}$); 151.43 (C=O); 151.72 (–C=O); 153.01 ($C_{4'}$); 155.59 (–OC=O). IR ν: 3313 (N–H), 2933 (–CH_2–), 1708 (C=O), 1689 (C=O), 1652, 1225, 1044, 749 (–CH_2–); UV (λ_{max}) = 300 nm (logε = 4.6); LC/MS$^{\pm}$: purity 98.62%; $t_R = 4.86$; (ESI) *m/z* $[M+H]^+$: 405.41; Anal. calcd. for $C_{19}H_{28}N_6O_4$: C, 56.42; H, 6.98; N, 20.78. Found: C, 57.35; H, 7.09; N, 20.53.

*9-(2-Benzylamino)ethyl)-1,3-dimethyl-6,7,8,9-tetrahydropyrimido[1,2-a]purine-2,4(1H,3H)-dione hydrochloride (**WZ-7**)*

White crystals, m.p: 280–285°C; (ethanol); TLC: $R_f = 0.30$; yield 22.45%; $C_{19}H_{24}N_6O_2$: MW = 368.43. ^{1}H NMR (ppm) δ: 2.06–2.10 (m, 2H, CH_2–C<u>H</u>$_2$–CH_2), 3.14 (s, 3H, N_1–CH_3), 3.29 (s, 3H, N_3–CH_3), 3.14–3.21 (m, 2H, C<u>H</u>$_2$–NH–), 3.34 (t, 2H, 8–CH_2), 3.73 (t, $J = 6.05$ Hz, 2H, N_9–CH_2), 4.03 (t, $J = 5.91$ Hz, 2H, –NH–C<u>H</u>$_2$), 4.19 (t, $J = 5.64$ Hz, 2H, 8–CH_2), 7.38–7.43 (m, 3H, Ar3′–H + Ar4′–H + Ar5′–H), 7.54–7.56 (m, 2H, Ar2′–H + Ar6′–H), 9.48 (s, 2H, NH_2^+). ^{13}C NMR: 21.02 (CH_2); 27.67 (N_3CH_3); 29.80 (N_1CH_3); 41.72 (N_9CH_2); 44.41 (N_9CH_2); 45.05 (NCH_2); 45.80 (NCH_2); 50.17 (N_5CH_2); 102.46 ($C_{10'}$); 129.07 (ArCH); 129.31 (ArCH); 130.40 (ArCH); 132.52 (ArCH); 148.34 ($C_{9'}$); 151.39 (C=O); 151.76 (C=O); 153.13 ($C_{4'}$). IR ν: 2950 (–CH_2–), 1699 (C=O), 1654, 881 (C–H), 748 (–CH_2–); UV (λ_{max}) = 300 nm (logε = 4.7); LC/MS$^{\pm}$: purity 98.03%. $t_R = 2.89$; (ESI) *m/z* $[M+H]^+$: 369.31; Anal. calcd. for $C_{19}H_{24}N_6O_2 \times HCl \times 0.5\ H_2O$ (MW 413.91): C, 55.13; H, 6.33; N, 20.30. Found: C, 55.12; H, 6.17; N, 20.37.

Pharmacology

Animals

The experiment was carried out on male Albino Swiss mice weighing 18–30 g. The animals were housed in constant temperature facilities exposed to 12:12 light-dark cycle and maintained on a standard pellet diet; tap water was given *ad libitum*. Control and experimental groups consisted of six animals each. The investigated compounds were administered intraperitoneally (*ip*) in the form of a suspension in 0.5% methylcellulose. Control animals received the equivalent volume of solvent.

All procedures were conducted according to guidelines of ICLAS (International Council on Laboratory Animals Science) and were approved by The Local Ethics Committee for Animal Experimentation.

Statistical analysis

The data are expressed as the mean ± SEM (standard error of the mean). Student's *t*-test was used to compare the results between two different groups of animals (the group given the investigated compound vs. the control group) from the writhing and hot-plate tests. The difference between the means was statistically significant if $P < 0.05$.

Adenosine radioligand binding assay

The affinity of the examined compounds for adenosine receptors was determined in cooperation with the University of Bonn.

The hot-plate test

In the hot-plate test, mice are treated *ip* either with the test compound or the vehicle 30 min before placing the animal on a hot-plate apparatus (Hot Plate 2A Type Omega) with the temperature set at 55–56°C. The time until the animal licks its hind paws or jumps is recorded by means of a stopwatch [16].

The writhing syndrome test

Mice are treated with 0.25 mL of 0.02% phenylbenzoquinone solution 30 min after *ip* administration of the investigated compound or the vehicle. Then the mice are placed individually in glass beakers and 5 min are allowed to elapse. After that period of time, a 10-min observation is conducted for each animal and the number of characteristic writhes is counted. The analgesic effect of the tested substances was determined by a decrease in the number of the observed writhes [17]. The analgesic effect of individual doses was expressed in percent. The ED_{50} values and their confidence limits were estimated by the method of Litchfield and Wilcoxon [18].

Locomotor activity test

Tested compounds and the vehicle (methylcellulose) were administered *ip* 30 min before the assay. The effects of various doses of the investigated compounds administered by the *ip* route were recorded in the photoresistor actimeters (Multi-Serv, Lublin, Poland, 30 cm in diameter, illuminated by two light beams) connected to the counter for the recording of light-beam interruptions. The mice were placed individually in the actimeters and the number of light-beam crossings was counted during the 30-min session. Each experimental group consisted of six mice. The data obtained in the 30th minute of the observation period were used for the statistical analysis.

Antiproliferative assay

Cell line: Human embryonic kidney HEK-293 cell line was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum, 100 mg/mL streptomycin, and 100 U/mL penicillin. Cells were cultured at 37°C in an atmosphere 5% CO_2. HEK-293 cell line (ATCC CRL1573) was kindly donated by Prof. Dr. Christa Müller (Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn).

In vitro antiproliferative assay: EZ4U non-radioactive cell proliferation and cytotoxicity assay (Biomedica) was used to define the antiproliferative activity of examined compounds.

First, the cells were seeded in 96-well plate at a concentration of 1.5×10^4 cells/well in 200 μL culture medium and cultured for 24 h to reach 60% of confluence. Next, the stock solutions of examined compounds in DMSO were diluted into fresh growth medium (the maximal DMSO concentration did not exceed 1%) and added into the microplates at the final concentrations from 0.01 to 250 μM. After 48 h of incubation, 20 μL of EZ4U labeling mixture was added to each well and the cells were incubated under the same conditions for 5 h. The absorbance of the samples was measured using a microplate reader (PerkinElmer) at 492 nm. The activity of the standard drug doxorubicin (Ebewe) was estimated as we described previously by using EZ4U at the concentrations from 0.005 to 100 μM [14]. GraphPad Prism 5.01 software was used to calculate the IC_{50} values.

Drug–drug interactions

The luminescent CYP3A4 P450-Glo™ assay was purchased from Promega. The CYP3A4 inhibitor ketoconazole was purchased from Sigma–Aldrich. The enzymatic reactions were performed in white polystyrene, flat-bottom Nunc™ MicroWell™ 96-well microplates (Thermo Scientific). The CYP3A4 P450-Glo™ assays were performed according to the manufacturer's procedure. The final concentrations of all examined compounds were 0.025–25 μM. The effect of ketoconazole was estimated as we described previously [19]. The luminescence signal was measured with a microplate reader in luminescence mode (PerkinElmer). For calculation, the total luminescence, the average luminescence of the control reaction containing inactive membranes was subtracted from the luminescence of CYP3A4 containing reactions. The luminescence of the reactions containing luciferin-free water instead of the tested compound indicated the total (100%) CYP3A4 activity [15].

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The authors have declared no conflicts of interest.

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