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THE EFFECT OF SEROTONIN 5-HT$_{1A}$, 5-HT$_2$ RECEPTOR LIGANDS, KETOPROFEN AND THEIR COMBINATION IN MODELS OF INDUCED PAIN IN MICE

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Anstract: The present study was carried out to investigate the effects of the 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione (compounds **1**-**4**) in two animal models of induced pain and to compare their effects with ketoprofen and with their combination. All experiments were performed on albino mice. Mice were evaluated for their responsiveness to noxious stimuli using: the hot-plate test and the phenylbenzoquinone- induced writhing test. All compounds showed analgesic activity only in the writhing test. The analgesic activities of compounds **3** and **4** were similar to ketoprofen. The compounds slightly increased the analgesic effect of ketoprofen when used in combination in the visceral type of pain. The possible mechanisms of the antinociceptive effect of these compounds are thought to involve the activation of analgesic effect mediated by the serotonergic pathways or combination of this mechanism with other important mediators playing a role in pain modulation.

Keywords: analgesic activity, serotonergic receptors, writhing test, xanthine derivatives

Chronic pain represents one of the most important problems. This is a common unpleasant sensory and emotional experience associated with actual or potential tissue damage. For instance, it has been estimated that 10-13% of the general population in Europe, are being disabled by persistent pain. Conventionally, analgesic treatment is accomplished by three principal drug groups: nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and local anesthetics, administered through different routes and modalities (1). Pain relief can be achieved and adverse effects minimized by a combination of analgesics (2). Analgesics are complemented with co-analgesic (adjuvant) drugs, specially in pain of inflammatory and neuropathic type (1, 3, 4). In the case of neuropathic pain, treatment with opioids may be of limited efficacy and combination with co-analgesics is necessary (4–8).

Co-analgesic drugs are defined as drugs that have a primary indications other than pain but are analgesic in some painful conditions or are capable of decreasing the side effects of analgesic drugs (4). Co-analgesics can also be defined as drugs that have weak or non-existent analgesic action when administered alone, but can enhance analgesic actions when co-administered with known analgesic agents (4). Such combinations increase analgesia without increasing the dose of analgesics, and therefore, can reduce the incidence of adverse effects (4). The combined treatment with the two types of drugs at doses much lower than therapeutic doses may be of great value in pain therapy (9).

Co-analgesic drugs include antidepressants, antipsychotics, anticonvulsants, antiarrhythmics, corticosteroids and others (4, 8, 10). Efficacy of these drugs depends on the type of pain, its pathophysiology, clinical status and adequacy of pain intervention. There has been an increase in the number of these drugs and they now play an important role in the management of chronic pain (10).

In chronic pain, antidepressants are an essential part of the therapeutic strategy in addition to classical analgesics (3, 8, 10). These drugs belong to the groups of tetracyclic antidepressants (TeCAs) (amoxapine, maprotiline), tricyclic antidepressants (TCAs) (amitriptyline, doxepin, imipramine), selec-

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tive serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs) (duloxetine, venlafaxine, milnacipran), and atypical antidepressants (bupropion, trazodone, mirtazapine, nefazodone), (3, 8). Antidepressants effective in chronic pain act *via* different mechanisms as can be seen in Table 1, (3, 10). However, other mechanisms have also been suggested..

Antidepressants are commonly used to treat the following chronic pain conditions: arthritis, central pain syndrome, fibromyalgia, low back pain, migraines, nerve damage from diabetes (diabetic neuropathy) (8, 11). Their effectiveness is best documented for painful diabetic neuropathy. On the other hand, some clinical data suggest that headache can occur after prolonged use of amitriptyline in patients with depression but without pain symptoms (3). The explanation of this phenomenon requires further research.

The analgesic potency of antidepressant drugs has been suggested to result from the inhibition of monoamine reuptake in the central nervous system, which consequentially leads to an increased activity of the antinociceptive descending pathways (3). The antidepressants have an analgesic effect that may be, at least partly, independent of their effect on depression (3). The dose necessary to achieve optimal analgesia is usually lower than that required for antidepressant therapy, which may suggest separation of analgesic and antidepressant effect (3).

The present study is, therefore, intended to investigate the analgesic potential of 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione, used alone or in combination with ketoprofen in two models of induced pain in mice, representing different types of pain stimuli. Our previous study demonstrated a potent antidepressant-like activity of several derivatives of 8-alkoxypurine-2,6-dione in the forced swimming test in mice and showed their strong affinity for the 5-HT_2 and 5-HT_{1A} receptors (12).

EXPERIMENTAL

Chemistry

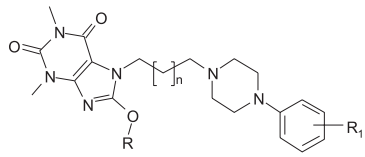
The structures of the investigated compounds **1**–**4** are presented in Table 2. Compounds **1**–**4** were synthesized by nucleophilic substitution of previously obtained 7-chloroalkyl-8-alkoxy-1,3-dimethyl-3,7-dihydropurine-2,6-diones with the appropriate phenylpiperazines in the presence of K_2CO_3. The synthesis and physicochemical data of compounds **1**–**4** were described elsewhere (13). The investigated compounds were pharmacologically tested as hydrochloride salts.

Table 1. The mechanism of action of antidepressants effective for chronic pain (3, 11).

Group	Drugs	**Mechanisms**
TCA	Amitriptyline Doxepin Imipramine	5-HT > NE reuptake inhibition
TeCA	Amoxapine	NE and 5-HT reuptake inhibition
SSRI	Citalopram Fluoxetine Paroxetine Sertraline Fluvoxamine	5-HT > NE reuptake inhibition
SNRI	Duloxetine Venlafaxine Milnacipran	5-HT NE > DA reuptake inhibition
Atypical antidepressant	Bupropion Trazodone Mirtazapine Nefazodone	DA NE reuptake inhibition 5-HT_2 receptor blockade 5-HT reuptake inhibition a_2-NE and 5-HT_2 presynaptic agonist; 5-$HT_{2/3}$ receptor blockade 5-HT_2 receptor blockade 5-HT reuptake inhibition

TCA - tricyclic antidepressants, TeCA - tetracyclic antidepressants, SSRI - selective serotonin reuptake inhibitors, SNRI - serotonin and noradrenaline reuptake inhibitors; NE - noradrenaline, DA - dopamine, HT - hydroxytryptamine.

Table 2. Structure and binding affinity data for serotonin 5-HT_{1A} and 5-HT_2 receptors of the investigated compounds.



Compound	R	n	R_1	K_i (nM) ± SEM	
				5-HT_{1A}	5-HT_2
1	C_3H_7	2	m-Cl	15 ± 1[a]	28 ± 2[a]
2	C_3H_5	2	m-Cl	12 ± 2[a]	15 ± 1[a]
3	C_2H_5	1	m-Cl	190 ± 28[a]	23 ± 2[a]
4	C_3H_7	1	m-Cl	288 ± 18[a]	25 ± 2[a]

[a] Data taken from ref. (12).

Table 3. The influence of the investigated compounds and ketoprofen on the pain reaction in the "writhing syndrome" test in mice.

Compound	Dose mg/kg	Mean number of writhings ± SEM
Control		30.5 ± 0.7
1	5	8.7 ± 0.8**
2	5	9.3 ± 0.2**
3	5	4.7 ± 0.6***
4	5	3.9 ± 0.4***
Ketoprofen	5	1.9 ± 0.1***

Data are presented as the means ± SEM of 6-8 mice per group. The results were analyzed by Student's *t*-test. ** $p < 0.01$,*** $p < 0.001$ *vs.* control.

Animals

The experiments were carried out on male albino-Swiss mice (body weight 20-26 g). The animals were housed in constant temperature facilities exposed to 12 : 12 h light-dark cycle and maintained on a standard pellet diet, tap water was *ad libitum*. Control and experimental groups consisted of six to eight animals each. The investigated compounds were administered intraperitoneally (*i.p.*) as a suspension in 0.9% NaCl.

Statistical analysis

The statistical significance was calculated using Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

Methods

The writhing syndrome test

Mice were treated with 0.25 mL of 0.02% phenylbenzoquinone solution 30 min after *i.p.* administration of the investigated compound or the vehicle. Then, the mice were placed individually in glass beakers and 5 min were allowed to elapse. After that period, each animal was observed for 10 min and the number of characteristic writhes was counted. The control group was given *i.p.* 0.9% NaCl. The analgesic effect of the tested substances was determined by a decrease in the number of writhes observed (14). The ED_{50} values and their confidence limits were estimated by the method of Litchfield and Wilcoxon (15).

The hot plate test

In the hot plate test, mice were treated *i.p.* 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 controlled at 55-56^{o}C. The time elapsing until the animal licks its hind paws or jumps is recorded using a stop-watch (16). The ED_{50} values and their

confidence limits were estimated by the method of Litchfield and Wilcoxon (15).

RESULTS

Analgesic activity in the writhing syndrome test

All xanthine derivatives were tested in the writhing test. In this test, pain was induced by injection of an irritant (such as phenylbenzoquinone) into the peritoneal cavity of mice. In this test, the investigated compounds (**1**–**4**) administered at a dose 5 mg/kg, showed significant analgesic properties (Table 3). Ketoprofen was a drug of reference in this assay. The mean number of writhing responses in the vehicle-treated mice was 30.5. The strongest analgesic effect was observed for compounds **3** and **4**. Compound **3** injected *i.p.* at a dose 5 mg/kg body weight, reduced the number of writhes in response to an irritating stimulus by 84.6%. Administration of compound **4** at a same dose, produced a strong analgesic effect (inhibition by 87.2%). Compound **1** reduced the number of abdominal writhings by 71.4% with respect to the control group, while compound **2** statistically significantly diminished the number of abdominal constrictions by 69.5%, as compared to the 0.9% NaCl-pretreated animals.

In this model of pain, each drug was used alone to test its analgesic effect, and in combination with ketoprofen (at the dose 5 mg/kg) to look for a possible potentiation of its analgesic effect.

The combination of the investigated compound **3** and ketoprofen produced a slightly higher effect in the writhing test, compared to each of them used alone (reduction of the number of writhes by 96.8%), (Table 4). The combination of the investigated compound **4** and ketoprofen also produced a slightly higher effect in the writhing test, compared to each of them used alone (reduction by 97.6%). As regards compound **2** and ketoprofen, this combination also increased the analgesic activity in the writhing test (95.5% increase *versus* 69.5 and 93.7%, respectively), (Table 4). Compound **1** and ketoprofen reduced the number of writhes in response to an irritating stimulus by 95.2%. This combination produced higher effect in the writhing test, compared to compound **1** (71.4%) and ketoprofen (93.7) used alone.

Hot plate test

As demonstrated in Table 5, in the hot plate test the investigated compounds **1**-**4** did not exerted an analgesic activity at the dose of 5 mg/kg body weight. Morphine, which was a drug of reference in this assay, showed a high antinociceptive potency.

DISCUSSION AND CONCLUSION

Serotonin (5-hydroxytryptamine, 5-HT), like noradrenaline, is believed to be one of important modulators of painful stimulus transmission while spinal serotonergic receptors play an important role in the inhibition of nociceptive reaction connected with the release of stimulatory amino acids and substance P (17). Many types and subtypes of 5-HT receptors were identified in the CNS, however, only four of them - $5HT_{1-4}$ were found in the spinal cord (17). When administered intrathecally, 5-HT had an antinociceptive effect in acute pain models. In the periphery, 5-HT was shown to produce analgesic response in pain accompanying inflammatory process.

Animal studies demonstrated antinociceptive action of serotonin *via* spinal 5-HT receptors in the writhing test. Analgesic action of different substances in that test could result from their effect on the serotonergic system (18) probably mediated by $5\text{-}HT_2$ and $5\text{-}HT_3$ receptors. It was abolished by antagonists of these receptors (ketanserin, cyprohep-

Table 4. The influence of the combination of the investigated compounds and ketoprofen on pain reaction in the "writhing syndrome" test in mice.

Compound	Dose mg/kg	Mean number of writhings ± SEM
Control		31.5 ± 2.6***
1 + Ketoprofen	5	1.5 ± 0.8***
2 + Ketoprofen	5	1,4 ± 0.2***
3 + Ketoprofen	5	1.0 ± 0.1***
4 + Ketoprofen	5	0.8 ± 0.1***

Data are presented as the means ± SEM of 6–8 mice per group. The results were analyzed by Student's *t*-test. *** $p < 0.001$ *vs.* control.

Table 5. The influence of the investigated compounds and ketoprofen on the pain reaction in the hot plate test in mice.

Compound	Dose mg/kg	Time of reaction to ain stimulus (s) ± SEM
Control		30.0 ± 2.4
1	5	27.2 ± 1.3
2	5	29.2 ± 3.0
3	5	31.6 ± 2.2
4	5	28.3 ± 1.6
Morphine	5	50.2 ± 0.7 **
Ketoprofen	5	19.2 ± 3.1

Data are presented as the means ± SEM of 6–8 mice per group. The results were analyzed by Student's *t*-test. ** $p < 0.01$ *vs*. control.

tadine, ondansetron). The $5HT_1$ receptors seemed to play a less important role in this model of pain and their antagonists did not abolish nociceptive action of agonists. The role of $5HT_{1A}$ receptors in thermal pain models is unclear (17). It appears that $5HT_{1A}$ receptors do not play a role in the hot plate test and antinociceptive action in this test can result from the stimulation of serotonin type 3 and probably 2 receptors.

Summarizing, the problem of implication of specific serotonin receptor subtypes in different animal models of pain is very complex and deserves further clarification (19, 20). It should be remembered that the role of serotonin in the CNS and its receptor-mediated actions, including analgesic effect, are closely connected with other neurotransmitter systems (19, 20).

Animal studies revealed analgesic potential of antidepressant drugs which is partly mediated by serotonin receptors but can also stem from complex interactions between serotonergic, adrenergic and opioid systems (21). Due to their antinociceptive actions, some antidepressant drugs can be used as co-analgesics (3, 10, 11). Trazodone which presented a weak activity of a serotonin reuptake inhibitor and $5\text{-}HT_{2A}$ receptor antagonist, also showed analgesic activity in animal studies (22) but the clinical trials did not confirm its beneficial effects in chronic pain (3).

In our previous studies, all investigated compounds were demonstrated to be highly active $5\text{-}HT_{2A}$ receptor ligands. Compounds **3** and **4** showed 8.3- and 11.5-fold stronger affinity for $5\text{-}HT_2$ receptor than for $5\text{-}HT_{1A}$ whereas compounds **1** and **2** were found to be highly active $5\text{-}HT_{1A}$ receptor ligands. On the other hand, several derivatives of 8-alkoxypurine-2,6-dione revealed a potent antidepressant-like activity in the forced swimming test in mice (12). In our study, the most potent effect in the forced swimming test was produced by compounds **3** and **4** (12). The investigated compounds exhibited a similar mechanism of action as trazodone (23). Perhaps it was because of the combination of the weak activity of a serotonin reuptake inhibitor and a $5\text{-}HT_{2A}$ receptor antagonist.

Pain is often associated with inflammatory conditions. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used analgesic agents. NSAIDs are clinically effective because they alleviate pain and inflammation (24-26) Thus, the NSAID (ketoprofen) was selected in the present study as a standard drug to be co-administered with the tested compounds (**1**–**4**), 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione.

The aim of the first stage of our studies was to determine analgesic activity of the test compounds and ketoprofen in the writhing test and the hot plate test.

7-(3-Chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione were administered at a dose of 5 mg/kg, because, as previous studies have shown, it was the lowest used dose that did not reduce the motility of animals (12). Higher doses of 10-30 mg/kg significantly decreased mobility. Therefore, this dose was used in order to exclude false-positive results in the tests assessing analgesic activity.

The analgesic activities of the 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione **1**–**4** were measured using the phenylbenzoquinone-induced writhing syn-

drome test. This test consists in intraperitoneal injection of the chemical irritant followed by subsequent counting of "writhes", i.e., characteristic contractions of abdominal muscles accompanied by a hind limb extensor motion. This test detects peripheral analgesic activity; however, some psychoactive agents (including clonidine and haloperidol) also show activity in this test. Compounds with anti-inflammatory properties, such as NSAIDs show a significant activity in this assay, viz. they abolish the reflex stimulated by the administration of an irritating substance, like phenylbenzoquinone

All tested compounds **1**–**4** demonstrated a statistically significant analgesic activity in this test with compounds **3** and **4** revealing the highest (similar to ketoprofen) potency. However, the observed effect was not stronger than that of ketoprofen alone at the same dose. It is probable that analgesic activity of the test compounds is mediated by 5-HT_2 receptors because they bound several times more strongly to 5-HT_2 than to 5-HT_{1A} receptors. It was additionally confirmed by the fact that analgesic activity of different substances in the writhing test could result from the stimulation of 5-HT_2 receptors while 5-HT_2 played a less important role (18, 27).

Peripheral 5-HT_2 and presynaptic 5-HT_{1A} receptors have been clearly shown to be involved in 5-HT-induced hyperalgesia (27). On the other hand, it is not excluded that 5-HT_{1A} receptors also played a role in this effect. In order to clarify the mechanism of action, we planned to determine analgesic activity of the test compounds, given together with antagonists of serotonin receptors. In addition, the involvement of serotonin receptors in the analgesic effect was supported by the fact that 5-HT when applied peripherally, was a potent proinflammatory and noxious agent, which caused hyperalgesia both in humans and rodents (27). 5-HT, released from platelets, mast cells, and basophils in injured or inflamed tissues, may play a role in inflammatory chemical milieu. Hyperalgesia, which is the major symptom of inflammation and tissue injury, probably caused by the phenylbenzoquinone, was the result of sensitization of nociceptors by a variety of inflammatory mediators. 5-HT is one of these mediators; indeed it has been shown be able to sensitize peripheral nerve fibers to other inflammatory mediators, such as bradykinin (27).

Next, we determined analgesic activity of the test compounds in another model of pain, namely the hot plate test. None of the tested xanthine derivatives prolonged the reaction time to a thermal stimulus. The proven activity in the writhing test with the concomitant lack of activity in the hot plate test indicates rather peripheral than central mechanism of analgesic action of the test compounds.

The next stage of research aimed to determine whether the test compounds, shown previously to possess antidepressant activity (12), can be used as co-analgetics. To answer this question, the compounds were tested for analgesic activity in the writhing test when administered in combination with ketoprofen.

The compounds slightly enhanced the analgesic effect of ketoprofen in the writhing test when given together.

In summary, compounds **1**-**4** were found to have analgesic effect when given alone, however, their effects differed in dependence on the pain model used. The analgesic activities were observed only in the writhing test. The strongest effect (similar to ketoprofen) was shown by compounds **3** and **4**. The possible mechanism of the antinociceptive effect of these compounds is thought to involve the activation of an analgesia mediated by serotonergic pathways or a combination of this mechanism with other important mediators playing a role in pain modulation. The compounds slightly enhanced the analgesic effect of ketoprofen in the writhing test when given in combination.

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