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PHARMACOLOGY

ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF 8-METHOXY-1,3-DIMETHYL-2,6-DIOXO-PURIN-7-YL DERIVATIVES WITH TERMINAL CARBOXYLIC, ESTER OR AMIDE MOIETIES IN ANIMAL MODELS

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Abstract: The previous studies in a series of 8-methoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl derivatives revealed their analgesic properties. We extended the study with these compounds in aim to assess their impact on inflammatory process. For this purpose we used: the zymosan-induced peritonitis and the carrageenan-induced edema model. Furthermore, the antioxidant activity of the investigated compounds by the FRAP assay was determined. For the most active derivatives from evaluated series their influence on plasma TNF-α level was also tested *in vivo*. All investigated purine-2,6-dione derivatives **1**-**11** decreased neutrophils count and inhibited intensity of early vascular permeability. Furthermore, all evaluated compounds reduced the volume of edema caused by subcutaneous injection of carrageenan. Derivatives **1** (with ester moiety), **3** and **4** (with carboxylic group) showed the highest activity in the zymosan-induced peritonitis. In addition, a significant inhibition of plasma TNF-α level in rats with endotoxemia was observed following intraperitoneal administration of these compounds. In turn, compounds **6** and **8**-**11** containing amide moiety showed the greatest anti-inflammatory (antiedematous) effect in the carrageenan-induced paw edema model. All compounds did not show significant antioxidant properties. The present studies revealed that the presented purine-2,6-dione derivatives exhibit a significant anti-inflammatory activity and this effect may result from their ability to lower TNF-α level.

Keywords: anti-inflammatory activity, endotoxemia, purine-2,6-dione derivatives, TNF-α

Inflammation is a response of the immune system to physical and/or chemical and/or biological injury, defined as any process able to cause tissue or cell damage. Inflammatory processes are cause of a large number of diseases, such as atherosclerosis, cancer, asthma, arthritis, and many others (1).

Theophylline (Th) - 1,3-dimethyl-3,7-dihydropurine-2,6-dione has been widely used for the treatment of airway diseases for more than 80 years (2). More recently, it has been shown to have anti-inflammatory effects in asthma and chronic obstructive pulmonary disease (COPD) at lower concentrations. The molecular mechanism of the anti-inflammatory effect may be due to inhibition of PDE4 and histone deacetylase-2 activation, resulting in switching off activated inflammatory genes (3). Theophylline has a narrow therapeutic index; as a result, toxicity can be a significant problem with its chronic use.

This is the reason why new, more efficient and free of side effects anti-inflammatory medications are constantly searched for. The modification of a parent structure of theophylline by introduction of 4-arylpiperazinyl-alkyl substituent in the 7 position allowed to receive potent analgesic, antipyretic, and antiphlogistic agents (4). In this group, the most significant activity in several *in vivo* models (e.g., acetic acid writhing test, bradykinin-induced pain response, carrageenan-induced paw edema) was observed for compounds possessing 3 – 5 carbon alkyl chain and substituted phenyl ring with electron-withdrawing chloro/fluoro atom and methyl or trifluoromethyl group (4).

In the context of a research program that aims to contribute to the discovery of new anti-inflammatory and analgesic drug candidates, we described the synthesis and pharmacological evaluation of new 8-

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alkoxy-1,3-dimethyl-2,6-dioxopurin-7-yl derivatives with ester (**1**, **2**), carboxylic (**3**, **4**) and amide (**5, 6, 8–11**) terminal groups (5).

We also tested 8-oxo-purine-2,6-dione analogue (**7**) with an additional acid function in the form of an enol group (Fig. 1) (5).

The tested compounds **1–11** showed analgesic activity. The strongest analgesic and anti-inflammatory effects were observed for benzylamide (**6**) or 4-arylpiperazinamide (**8–11**) derivatives, which were more active than acetylic acid used as a reference drug (up to 23- and 36-fold increase in activity in writhing and formalin test, respectively). Several compounds more active than theophylline inhibited the phosphodiesterase activity in rat liver homogenates (5).

In the present paper, the results of further pharmacological studies in a group of 8-alkoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl derivatives (**1–11**) are described. They concern the evaluation of anti-inflammatory activity of these compounds in the zymosan-induced peritonitis model and the carrageenan-induced hind paw edema model in mice. We also assessed their activity in *in vitro* FRAP assay estimating the total ferric reducing antioxidant power. Finally, the effect of the most active theophylline derivatives on plasma TNF-α level in rat model of endotoxemia was investigated.

EXPERIMENTAL

Chemistry

The multistep syntheses of the investigated compounds **1-11** (Fig. 1) were previously reported (5). Firstly, in a reaction of 8-bromo-1,3-dimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione -7-alkylcarboxylates with sodium methanolate in a methanol medium the methyl 2-(8-methoxy-1,3-dimethyl-2,6-dioxo-2,3-dihydro-1*H*-purin-7(6*H*)-yl)acetate (**1**) and this higher butyrate homolog (**2**) were obtained. Corresponding acids **3** and **4** were prepared by alkaline hydrolysis of **1** and **2,** respectively, using acetone-water (1 : 2, v/v) solution of KOH and then by acidification with HCl. The methyl 2-(1,3-dimethyl-2,6,8-trioxo-2,3,8,9-tetrahydro-1*H*-purin-7(6*H*)-yl)acetate (**7**) was synthesized by acidic hydrolysis of **1** following by estrification with methanol. Benzylamides (**5**, **6**) and arylpiperazinamides (**8–11**) were prepared in a reaction of **3** and **4** with respective amine (benzylamine or 1-arylpiperazine derivatives), using 1,1'-carbonylimidazole (CDI) as carbonyl group activating agent in DMF medium (5). The chemical structure of compounds **1-11** were confirmed by spectral data (^{1}H-NMR, LC/MS) and elemental analyses and the purity were establish using LC/MS method. All the investigated compounds have purity over 98% (5).

Pharmacology

Animals

Experiments were carried out on male Wistar rats weighing 180-220 g and male albino Swiss mice (18–26 g). The animals were housed in constant temperature facilities exposed to 12 : 12 light-dark cycle and maintained on a standard pellet diet and tap water given *ad libitum*. Control and experimental groups consisted of 6-8 animals each. The investigated compounds were administered intraperitoneally (*i.p.*) in a form of suspension in 0.5% methylcellulose. Control animals received the equivalent volume of solvent.

Male Wistar rats weighing 200-250 g bred in-house from progenitors obtained from Charles River Laboratories (Sulzfed, Germany) were used to assess the influence of theophylline derivatives on plasma TNF-α levels in a model of endotoxemia. Animals were fasted overnight prior to drug administration but had free access to water.

All procedures were conducted according to guidelines of ICLAS (International Council on Laboratory Animals Science) and were approved by The Local Ethics Committee of the Jagiellonian University in Kraków (agreement nr 47/2014).

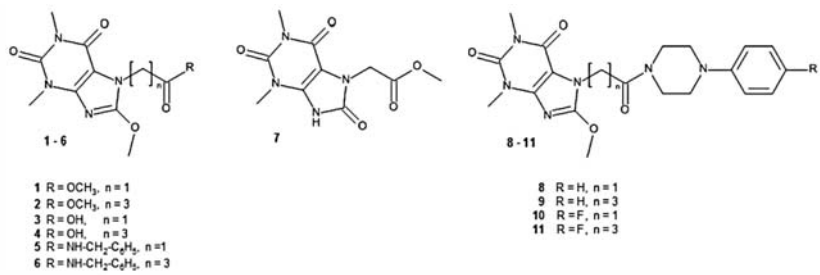

Figure 1. Chemical structures of the compounds **1–11** (5)

Drugs and chemicals

LPS (*Escherichia coli* 055:B5), methylcellulose, carrageenan, zymosan A, Evans blue, ketoprofen, indomethacin, acetate buffer, $FeCl_3 \cdot 6H_2O$, TPTZ (2,4,6-tripyridyl-s-triazine), theophylline, ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Carrageenan-induced edema model

Wistar rats weighing 180-220 g were used in the "hind paw edema" test. Animals were divided into four groups, one of them being the control. In order to produce inflammation, 0.1 mL of 1% carrageenan solution in water was injected into the hind paw subplantar tissue of rats, according to the modified method of C.A. Winter (6) and P. Lence (7). The development of paw edema was measured with a plethysmometer (Plethysmometer 7140, Ugo Basile). Prior to the administration of test substances, paw diameters were measured by dividers and recorded. The investigated compounds were administered at doses of 5, 10, 25, 50, 100 mg/kg, *i.p.* (as a suspension in methylcellulose), prior to carrageenan injection. Methylcellulose was administered by the same route, to the control group (methylcellulose had no effect on edema, data not shown). After these administrations, paw diameters were measured at 1, 2 and 3 h. The percent, of edema and edema inhibition were calculated according to the equations given below.

$$\text{Edema } \% = (N' \times 100) / N$$

$$\text{Edema inhibition } \% = (N - N' \times 100) / N$$

where N = paw diameters measured 1, 2 and 3 h after injection of carrageenan to the control group paw diameters at the beginning; N' = paw diameters measured 1, 2, and 3 h after injection of carrageenan to the test groups paw diameters at the beginning.

Zymosan-induced peritonitis

Peritoneal inflammation was induced as described previously (8). Zymosan A was freshly prepared (2 mg/mL) in sterile 0.9% NaCl. Thirty min after subcutaneous (*s.c.*) injection of the investigated compounds into the loose skin over the flank, zymosan A was injected *i.p.* in a volume of 0.25 mL. Four hours later, the animals were killed. The peritoneal cavity was lavaged with 1.5 mL of saline and after 30 s of gentle manual massage the exudates were retrieved. Cells were counted using an automatic cell counter (Countess, Invitrogen) following staining with Turk's solution. The investigated compounds suspended in 0.5% methylcellulose were injected *s.c.* at the dose of 50 mg/kg b.w. and pitched in an ultrasonic cleaner. The control group was given *s.c.* 0.5% methylcellulose 30 min prior to zymosan.

Vascular permeability

The compounds suspended in 0.5% methylcellulose were injected *s.c.* at the dose of 50 mg/kg b.w. Then, after 30 min, Evans blue was suspended in saline (10 mg/mL) and injected intravenously (*i.v.*) into the caudal vein (0.2 mL/mouse), which was immediately followed by *i.p.* injection of zymosan A. Thirty minutes later the animals were killed and their peritoneal cavities were lavaged with 1.5 mL of saline as described above. The lavage fluid was centrifuged and the absorbance of the supernatant was measured at 620 nm as described previously (9). The investigated compounds suspended in 0.5% methylcellulose were injected *s.c.* 30 min before Evans blue and zymosan. The control group was given *s.c.* 0.5% methylcellulose 30 min prior to zymosan. Indomethacin in a dose of 50 mg/kg b.w. was used as a reference compound.

Determination of the antioxidant activity by the FRAP assay

The FRAP assay was conducted according to Benzie and Strain (10) with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \times 3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution was prepared by mixing 10 parts of acetate buffer, 1 part of TPTZ solution, and 1 part of $FeCl_3 \times 6H_2O$ solution. Three hundred µL of the FRAP solution was mixed with 10 µL of the test compound solution and incubated at room temperature for 10 min in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm against ethanol. The results for the test compounds are expressed as an increase in absorbance of the test sample compared to a sample containing the solvent.

In the FRAP assay the antioxidant potential of the sample was determined from a standard curve plotted using $FeSO_4 \times 7H_2O$ in a concentration range between 37.5 and 1200 µM.

LPS-induced endotoxemia

Rats were cannulated in the jugular vein under ketamine/xylazine anesthesia three days before the experiment. All catheters were filled with heparinized saline and exteriorized *via* an incision on the back of the neck. In order to induce endotoxemia, an intravenous dose of 1 mg/kg lipopolysaccharide (LPS) from *E. coli* serotype 055:B5 was adminis-

tered prior to compound administration. Compounds **1**, **3**, **4**, **6**, **7**, **9** and theophylline (Th) as a reference drug suspended in 0.5% methylcellulose were given to rats (n = 4-5) at a dose of 50 mg/kg *i.p.* simultaneously with LPS. Control animals received LPS and a respective volume of vehicle by the same routes of administration as the treatment groups. Blood samples (300 μL) were collected into heparinized tubes at 0, 15, 30 min and 1, 1.5, 2, 3, and 4 h after LPS and compound administration. The animals were injected with an equal volume of 0.9% saline through the tubings after each blood collection. Blood was centrifuged at 4°C for 20 min (1500 × g). Plasma was stored at –80°C until assayed.

Determination of plasma TNF-α levels

Tumor necrosis factor α (TNF-α) concentrations in rat plasma were measured using ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Limit of quantification was 13 pg/mL.

Statistical analysis

The data are expressed as the mean ± SEM (standard error of the mean). To compare the results between two different groups of animals (the investigated compound group *vs.* the control group) Student's *t*-test was used. The difference of means was statistically significant if $p < 0.05$. The results of carrageenan-induced paw edema experiments are expressed as a percentage of change from control (pre-drug) values. The data were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's test. A probability value < 0.05 was considered statistically significant.

Table 1. Antiinflammatory effect of the compounds in the carrageenan-induced paw edema test.

Compound	Dose mg/kg	Change in edema volume [mL]		
		1 h	2 h	3 h
Control	-	0.9 ± 0.01	1.35 ± 0.01	1.52 ± 0.09
1	100	0.5 ± 0.09	0.25 ± 0.08**	0.030 ± 0.004***
	50	0.65 ± 0.07	0.35 ± 0.05*	0.25 ± 0.03**
2	100	0.25 ± 0.03*	0.23 ± 0.04**	0.18 ± 0.02**
	50	0.73 ± 0.08	0.35 ± 0.06*	0.25 ± 0.08*
3	100	0.6 ± 0.07	0.08 ± 0.006***	0.020 ± 0.001***
	50	0.75 ± 0.09	0.3 ± 0.07*	0.18 ± 0.02**
4	100	0.6 ± 0.07	0.38 ± 0.09*	0.050 ± 0.004***
	50	0.6 ± 0.04	0.325 ± 0.02*	0.25 ± 0.03**
5	100	0.5 ± 0.03	0.080 ± 0.007***	0.020 ± 0.001***
	50	0.6 ± 0.08	0.375 ± 0.09*	0.28 ± 0.02**
Control	-	0.9 ± 0.13	1.04 ± 0.1	1.22 ± 0.09
6	25	0.075 ± 0.005***	0.175 ± 0.01***	0.1 ± 0.07***
	10	0.475 ± 0.09*	0.175 ± 0.01***	0.1 ± 0.07***
7	100	0.52 ± 0.08	0.21 ± 0.05**	0.030 ± 0.005***
	50	0.61 ± 0.04	0.37 ± 0.05*	0.22 ± 0.03**
8	25	0.1 ± 0.02***	0.01 ± 0.002***	0.075 ± 0.005***
	10	0.175 ± 0.03**	0.125 ± 0.009***	0.15 ± 0.009***
9	25	0.2 ± 0.04***	0.075 ± 0.008***	0.125 ± 0.02***
	10	0.225 ± 0.01**	0.325 ± 0.02**	0.15 ± 0.009***
10	25	0.175 ± 0.03**	0.125 ± 0.04***	0.1 ± 0.006***
	10	0.65 ± 0.1	0.35 ± 0.07**	0.15 ± 0.03***
11	25	0.225 ± 0.05**	0.175 ± 0.01***	0.075 ± 0.003***
	10	0.2 ± 0.06**	0.125 ± 0.009***	0.15 ± 0.005***

Data are presented as the means ± SEM of 6–8 animals per group. The results were analyzed by one-way analysis of variance (ANOVA) * $p < 0.05$,** $p < 0.01$,*** $p < 0.001$ *vs.* control value at respective time points.

Comparisons between maximum TNF-α levels and the area under effect-time curve (AUEC) were performed using a one-way ANOVA with *post-hoc* Tukey HSD test (Statistica v. 10, StatSoft, USA). AUEC was calculated using Phoenix WinNonlin v. 6.3 (Pharsight Corporation, Mountain View, CA, USA).

RESULTS

Anti-inflammatory (antiedematous) effect in the carrageenan-induced edema model

All new 8-alkoxy-1,3-dimethyl-2,6-dioxopurin-7-yl derivatives were first administered at a dose of 10 mg/kg. In the case of the compounds that were not active at this dose, in the next step, they were administered at higher doses, while others at a lower dose. Therefore, the compounds **1**–**5** and **7** were administered at doses from 100 to 10 mg/kg body weight, whereas compounds **6** and **8**–**11** at doses from 25 to 5 mg/kg b.w. Ketoprofen was used as a reference compound. Ketoprofen administered *i.p.* at a dose of 100 mg/kg b.w. inhibited edema formation by 80.0%, 75.7%, and 80.6%, in three consecutive hours of the experiment, respectively. When administered *i.p.* at a dose of 20 mg/kg b.w. it inhibited edema formation by 20, 54 and 67% in three consecutive hours of the experiment, respectively.

All test compounds decreased the volume of edema induced by *s.c.* carrageenan injection into the hind paw of rats (Table 1).

A statistically significant antiedematous effect was observed after the administration of compound **1** at two doses. The doses of 100 mg/kg b.w. and 50 mg/kg b.w. reduced edema by 81.4-98.0% and 74.0-83.5% in the 2nd and 3rd hour of observation, respectively (Table 1). Compound **2** had a similar effect. Its administration at a dose of 100 mg/kg b.w. lowered edema by 72.2-88.1% throughout the whole experimental period. On the other hand, administration of this compound at a dose of 50 mg/kg b.w. decreased edema in the 2nd and 3rd hour of the experiment by 74.0 and 83.5%, respectively. Compound **3** also demonstrated a statistically significant effect in this experimental model. When administered *i.p.* at a dose of 100 mg/kg b.w., it inhibited edema formation by 94.0 and 98.6% in the 2nd and 3rd hour of the experiment, respectively, while its dose of 50 mg/kg b.w. reduced edema by 77.7 and 88.1%. Similarly, compound **4** when administered *i.p.* at a dose of 100 mg/kg b.w. inhibited edema development by 71.8 and 96.7% in the 2nd and 3rd hour of the experiment, respectively, and the differences reached statistical significance. Its administration at a dose of 50 mg/kg b.w. lowered edema by 75.9 and 83.5%. Anti-inflammatory effect of the lowest dose of 10 mg/kg b.w. was diminished.

Administration of compound **5** at a dose of 100 mg/kg b.w. *i.p.* caused edema inhibition by 94.0 and 98.6% in the 2nd and 3rd hour of the experiment, respectively, while given at a dose of 50 mg/kg b.w. lowered edema by 72.2 and 81.5% (Table 1).

Compound **6** also exhibited a statistically significant effect in this experimental model. When administered *i.p.* at a dose of 25 mg/kg b.w., it inhibited edema formation by 91.6, 83.2, and 91.8% in the 1st, 2nd and 3rd hour of the experiment, respectively. Its dose of 10 mg/kg b.w. reduced edema by 47.2, 83.2, and 91.8%. A statistically significant antiedematous effect was observed after the administration of compound **7** at two doses. Doses of 100 mg/kg b.w. and 50 mg/kg b.w. reduced edema by 79.8-64.4% and 97.5-81.9% in the 2nd and 3rd hour of observation, respectively. Compound **8** when administered at doses 25 mg/kg b.w. and 10 mg/kg b.w. significantly lowered edema throughout the whole observation period by 88.8-93.8% and 80.5-87.7%, respectively.

Compound **9** showed anti-inflammatory effect in a dose range from 5 mg/kg b.w. to 25 mg.kg b.w. but only two highest doses significantly reduced edema formation by 68.7-92.7% (Table 1). The theophylline derivative **10** at a dose of 25 mg/kg b.w. produced a strong anti-inflammatory effect inhibiting edema of the mouse hind paw by 80.5, 88 and 91.8% in the 1st, 2nd and 3rd hour after carrageenan injection, respectively. When the dose was lowered, the activity of this compound slightly decreased. Compound **11** administered at doses of 25 mg/kg b.w. and 10 mg/kg b.w. demonstrated a significant anti-inflammatory effect. This effect increased with elapsing time of the experiment reaching the maximum in the 3rd hour. Dose lowering caused extinction of the anti-inflammatory activity.

The effects of the compounds on vascular permeability during zymosan-induced peritonitis

The effect of the investigated compounds on vascular permeability was tested at the dose of 50 mg/kg b.w. Indomethacin at the dose of 50 mg/kg b.w. was used as a reference compound. The intensity of early vascular permeability was significantly inhibited in the groups receiving compounds **1**, **3** and **4** compared to the control group (Table 2). These compounds decreased the vascular permeability by 94.3-81.2%. The reducing effect on the vascular permeability of these compounds was greater

than that of indomethacin. The early vascular permeability was significantly inhibited also in the groups receiving compounds **5**-**11** compared to the control group (Table 2). These compounds decreased the vascular permeability by 77.3 to 61.1%. In turn, compound **2** influenced vascular permeability during zymosan-induced peritonitis, but the effect was not statistically significant.

The effects of the compounds on infiltration of neutrophils during zymosan-induced peritonitis

The effect of the investigated compounds on infiltration of neutrophils was tested at the dose of 50 mg/kg b.w. The early infiltration of neutrophils measured at 4 hours following zymosan injection was significantly stronger than that of indomethacin and was inhibited in the group receiving compound

Table 2. Percent inhibition of vascular permeability in zymosan-induced peritonitis in mice.

Compound	Dose [mg/kg]	Absorbance	Inhibition %
Control	-	2.230 ± 0.180	-
1	50	0.138 ± 0.050	93.8***
2	50	1.400 ± 0.190	37.2
3	50	0.126 ± 0.070	94.3***
4	50	0.418 ± 0.040	81.2**
5	50	0.739 ± 0.021	66.8*
6	50	0.728 ± 0.028	67.3*
7	50	0.700 ± 0.030	68.6*
8	50	0.825 ± 0.039	63.0*
9	50	0.588 ± 0.060	77.3**
10	50	0.798 ± 0.046	64.2*
11	50	0.867 ± 0.063	61.1*
Indomethacin	50	0.456 ± 0.120	79.5**

Data are presented as the means ± SEM of 6–8 mice per group. The results were analyzed by Student *t*-test, * $p < 0.05$,** $p < 0.01$,*** $p < 0.001$ *vs.* control.

Table 3. Percent inhibition of neutrophil infiltration in zymosan-induced peritonitis in mice.

Compound	Dose [mg/kg]	Count of neutrophils	Inhibition %
Control	-	$6.4 \times 10^6 \pm 0.76$	-
1	50	$8.3 \times 10^5 \pm 0.92$	87.0**
2	50	$4.4 \times 10^6 \pm 1.36$	31.2
3	50	$2.1 \times 10^5 \pm 0.18$	96.7***
4	50	$8.4 \times 10^5 \pm 0.28$	86.8**
5	50	$2.1 \times 10^6 \pm 0.16$	67.1*
6	50	$1.9 \times 10^6 \pm 0.26$	70.3*
7	50	$2.4 \times 10^6 \pm 0.15$	62.5*
8	50	$2.9 \times 10^6 \pm 0.62$	54.6*
9	50	$1.7 \times 10^6 \pm 0.85$	73.4*
10	50	$2.6 \times 10^6 \pm 0.74$	59.3*
11	50	$1.9 \times 10^6 \pm 0.62$	70.3*
Indomethacin	50	$9.8 \times 10^5 \pm 0.29$	84.6**

Data are presented as the means ± SEM of 6-8 mice per group. The results were analyzed by Student *t*-test, * $p < 0.05$,** $p < 0.01$,*** $p < 0.001$ *vs.* control.

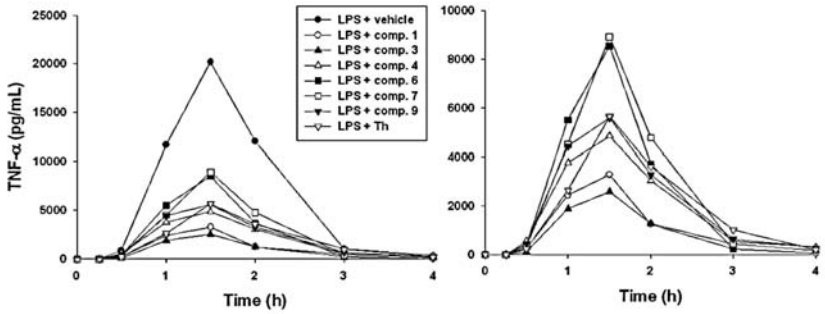

Figure 2. TNF-α concentration as a function of time following intraperitoneal administration of selected xanthine derivatives (50 mg/kg) to rats with endotoxemia (n = 4–5), (left panel: 0–25000 pg/mL, right panel: 0–10000 pg/mL)

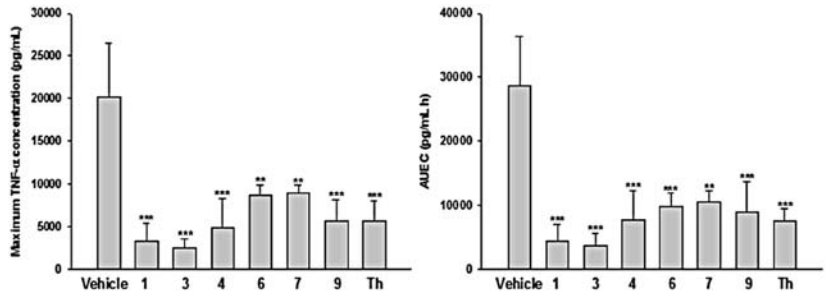

Figure 3. Maximum TNF-α concentration (left panel) and AUEC (right panel) observed following administration of LPS (1 mg/kg, *i.v.*) simultaneously with vehicle only or studied compounds given *i.p.* at a dose of 50 mg/kg to rats (n = 4–5). * < 0.05 *vs.* LPS + vehicle, (one-way ANOVA with *post-hoc* Tukey test)

1 (decrease by 87.0%) and in the group treated with **3** (decrease by 96.7%), and **4** (decrease by 86.8%).

The early infiltration of neutrophils was significantly inhibited also in the groups receiving compounds **5**-**11** compared to the control group. These compounds decreased the count of neutrophils by 73.4 to 59.3% (Table 3). Compound **2** influenced infiltration of neutrophils during zymosan-induced peritonitis, but the effect was statistically non-significant.

Antioxidant activity measured by the FRAP assay

Investigation of the effect of the test compounds on the total antioxidant power revealed that 9 of them: **1**, **2**, **4** and **6-11** at a concentration of 10^{-5} M increased the total ferric reducing antioxidant ability by 0.29–3.7% of the maximum value obtained for ascorbic acid at the same concentration. The remaining compounds did not show an antioxidant effect in this test.

Inhibition of TNF-α production *in vivo*

Administration of compounds **1**, **3**, **4**, **6**, **7**, and **9** to rats with LPS-induced endotoxemia caused a significant inhibition of TNF-α production in plasma (Fig. 2). The most pronounced effect was observed in the presence of compounds **3** and **1**. The maximum concentration of TNF-α achieved at 1.5 h following LPS administration decreased significantly from 20157 pg/mL (LPS + vehicle) to 2579 and 3269 pg/mL, respectively (Fig. 3). At the same time, compounds **4** and **9** decreased the maximum concentration of TNF-α by 75.9 and 71.8%, respectively, and these differences were also statistically significant. The weakest inhibitory effect exhibited compounds **6** and **7**. For these compounds TNF-α cytokine concentrations at 1.5 h were more than 3 times higher when compared with the strongest inhibitor of TNF-α that is compound **3** (a decrease by 57.6 and 55.7% compared to the control group, i.e., LPS + vehicle). In addition, these concentrations were 1.5 times higher than that observed following

Th administration (5665 pg/mL) used as a reference compound in this study. Similar differences were observed between the areas under effect curve (AUEC) calculated based on the individual TNF-α - time profiles in the vehicle group and all study groups (Fig. 3).

DISCUSSION AND CONCLUSION

The aim of the study was to evaluate of anti-inflammatory activity of new methylxanthine derivatives. For this purpose we used zymosan-induced peritonitis and the carrageenan-induced hind paw edema models in animals (11). The carrageenan test is used to evaluate the anti-inflammatory effect; injection of this compound into the hind paw of an animal induces a long-lasting edema (12, 13).

The carrageenan-induced paw edema model demonstrated that compounds **6** and **8**-**11** containing amide substituent showed the greatest anti-inflammatory (antiedematous) effect. The effect of all these compounds was comparable and increased with time elapsing from the beginning of the experiment reaching the maximum in the 3rd hour. The effect was dose-dependent. The above compounds produced the highest statistically significant effect at the dose of 25 mg/kg b.w. All these methylxanthine derivatives revealed a stronger action compared with ketoprofen. The administration of a lower dose of 10 mg/kg b.w. only slightly diminished anti-inflammatory activity.

The remaining compounds showed also anti-inflammatory activity but at higher doses as they were administered at 100-10 mg/kg b.w. in this test. A statistically significant effect was observed (except for compound **2)** only in the 2nd and 3rd hour of the experiment. The administration of a lower dose of 50 mg/kg b.w. induced only a minute lowering of anti-inflammatory activity.

Murine zymosan-induced peritonitis was described as a suitable model of acute inflammation, characterized by vascular changes and production of inflammatory mediators leading to leukocyte accumulation in the inflammatory focus (14, 15). Two major events are critical for development of zymosan-induced peritonitis, namely the early increase in vascular permeability (< 1 h) and the infiltration of neutrophils into the peritoneum that follows after some hours (9). The mechanisms operating during the above stages have been investigated and these studies revealed that early vascular permeability depends mostly on cysteinyl-leukotrienes released by resident peritoneal macrophages and, to lesser extent, on mast cell histamine and prostaglandins (PGE_2, prostacyclin) of multiple cellular origins (9).

This study focused on two major events leading to the development of inflammation, i.e., the early increase in vascular permeability and neutrophil infiltration into peritoneum. The results indicated that the compounds under investigation demonstrated anti-inflammatory activity in both tests. In the study of cellular infiltration they significantly limited the migration of leukocytes to site of inflammation, which was the peritoneum.

Compounds **1** (with ester terminal moiety) as well as **3** and **4** (containing carboxylic group) showed the strongest anti-inflammatory activity in the zymosan-induced peritonitis.

Compounds **3** and **4** with terminal carboxylic moiety significantly reduced the vascular permeability by 94.3 and 81.2% and inhibited infiltration of neutrophils by 96.7 and 86.8%, respectively, compared to the control group. In turn, 8-alkoxy-1,3-dimethyl-2,6-dioxopurin-7-yl derivative with ester terminal group, i.e., compound **1** reduced the vascular permeability by 93.8% and inhibited infiltration of neutrophils by 87.0%, while 8-oxo-purine-2,6-dione analogue (compound **7**) reduced the vascular permeability by 68.6% and inhibited infiltration of neutrophils by 62.5%. The statistical analysis showed that compounds **6** and **8**-**11** (containing amide and benzylamide) significantly reduced the vascular permeability by 61.1-77.3% compared to the control group. The obtained data also demonstrated that administration of these compounds at the same dose (50 mg/kg b.w.) significantly inhibited infiltration of neutrophils by 54.6-73.4%.

Summing up, all compounds **1**-**11** showed statistically significant anti-inflammatory activity in both tests: the carrageenan-induced edema test and the zymosan-induced peritonitis (except for compound **2**). They significantly reduced the early vascular permeability, inhibited infiltration of neutrophils and tempered paw edema formation. In the carrageenan-induced paw edema model, compounds **6** and **8**-**11** showed the greatest anti-inflammatory (antiedematous) effect. In the case of the zymosan-induced peritonitis, compounds **3** and **1** followed by **4** and **9** revealed the greatest anti-inflammatory activity.

The mechanism of tissue damage due to inflammatory processes has been partly linked to the release of reactive oxygen species (ROS) from activated neutrophils and macrophages. Excessive ROS production leads to tissue damage by degradation of macromolecules and peroxidation of membrane lipids. On the other hand, reactive oxygen species

support and spread inflammation by the stimulation of cytokine production (IL-1, TNF-α, INF-γ) which increase further neutrophil and macrophage influx (16). Thus, free radicals are indispensable mediators of inducing and maintaining inflammation while their neutralization by antioxidants and free radical scavengers can limit its severity (17).

In order to elucidate anti-inflammatory activity of theophylline derivatives, we determined the total ferric reducing antioxidant power (FRAP). The pharmacological studies demonstrated that the test compounds were practically almost completely devoid of antioxidant activity. Therefore, their anti-inflammatory activity does not result from the influence on the total antioxidant potential.

Due to the important role of TNF-α in inflammation, further attempts to clarify the mechanism of action of the most active test compounds involved determination of TNF-α levels after their administration to rats with LPS-induced endotoxemia.

TNF-α is one of the major proinflammatory cytokines that stimulates the release of other mediators of inflammation, thereby inciting further inflammatory responses (18). The TNF-α expression is mainly under the regulatory control of nuclear factor-κB (NF-κB).

LPS-induced models of sepsis and septic-shock are commonly used to evaluate efficacy of anti-inflammatory drugs (19). *In vitro* methods employing LPS-stimulated whole blood or murine macrophages, although less cost- and time-consuming, may provide results not entirely reflecting *in vivo* drug activity. It has been shown that IC_{50} values assessed *in vitro* were up to 10 times higher than those estimated *in vivo* (20). Thus, it seems that inhibition of TNF-α production following LPS administration to animals may be more appropriate to study the true efficacy of new compounds than *in vitro* methods.

The results of the *in vivo* experiment on a series of the investigated purine-2,6-dione derivatives in rats with endotoxemia indicate that all compounds studied significantly inhibited TNF-α production in rat plasma (Figs. 3 and 4). The results of the *in vivo* study indicate that among compounds studied **1** and **3** and also **4** and **9** are the strongest inhibitors of TNF-α production in rat plasma. For these compounds TNF-α concentrations were lower than that observed for theophylline used in this study as a reference compound. In addition, these compounds most strongly inhibited infiltration of neutrophils in the zymosan-induced peritonitis. Based on the results of preliminary study performed in our laboratory, compounds **1** and **3** are the weakest phosphodiesterase (PDE) inhibitors as assessed using rat liver homogenates (5). The strongest inhibition of PDE in the liver homogenates revealed compounds **6** and **7** (5). For these compounds TNF-α concentrations at 1.5 h were higher than that observed for theophylline.

This discrepancy between TNF-α and PDE-inhibition by studied compounds may be explained by differences in distribution pattern and relative abundance of PDEs in plasma and liver. For example, in CD4+ and CD8+ T-lymphocyte homogenates, substantial PDE 4 and PDE 3 and only low PDE 1, 2 and 5 activities were observed (21). Recently, it has been shown that PDE 4A, PDE 4B, PDE 4D and PDE 7A mRNA are present in similar quantities in both CD4+ and CD8+ lymphocytes (22). Monocytes exclusively contain PDE 4 but their *in vitro* maturation leads to a PDE isoenzyme profile similar to that of alveolar macrophages (23). In turn, in the liver, PDE 2-4, 8, 9, and PDE 11 show relatively high expression, whereas PDE 7 is not detected.

Thus, TNF-α inhibition by new methylxantine derivatives observed in the study seem to be related to the increased levels of cAMP (an inhibitor of the NF-κB pathway) that is assumed to be the most important mechanism of action of most xanthines with anti-inflammatory activity (24, 25) or to a direct inhibition of NF-κB activation. The mechanism of compounds in reducing inflammation is thought to be due to inhibition of TNF-α, a cytokine that has been shown to increase leukocyte adhesion and to disrupt intercellular junctions of postcapillary venular endothelium, leading to plasma extravasation. In the carrageenan-induced paw edema model, derivatives of theophylline reduced the edema response and in addition, inhibited infiltration of neutrophils in the zymosan-induced peritonitis. Probably the test compounds by reducing TNF-α act on endothelial cells inhibiting the expression of adhesion molecules and chemokines necessary for the accumulation of white blood cells at the site of inflammation.

In the next stage of research on these compounds it is planned to test whether they have the ability to inhibit histone deacetylase, cyclooxygenase and Transient Receptor Potential (TRP) ankyrin 1 (TRPA1). Review of world literature provides data confirming that the anti-inflammatory effect of some drugs (including theophylline) results from the influence on histone deacetylase and ion channels TRPA1 (26, 27).

In summary, the study indicates that the 8-alkoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl derivatives (**1**-**11**) are a new class of compounds with anti-

inflammatory activities. The carrageenan-induced paw edema model demonstrated that compounds **6** and **8**-**11** containing amide substituent showed the greatest anti-inflammatory (antiedematous) effect. The compounds with carboxylic (**3, 4**) and ester moiety (**1**) showed the highest anti-inflammatory activity in the zymosan-induced peritonitis. They significantly decreased neutrophils count and inhibited intensity of early vascular permeability. In addition, especially compound **3** and also compound **1** significantly inhibited of TNF-α production in plasma of rats with endotoxemia and this effect was stronger than that of theophylline. Compounds did not show significant antioxidant properties. Therefore, their beneficial effects observed in the models of inflammation used in this study are not related to the influence on the total antioxidant potential. The results obtained indicate that the mechanism of anti-inflammatory activity of compounds is probably related to the inhibition of TNF-α release.

REFERENCES

1. Całkosiński I., Dobrzyński M., Całkosińska M., Seweryn E., Bronowicka-Szydełko A. et al.: Postepy Hig. Med. Dosw. 63, 395 (2009).
2. Talik P., Krzek J., Ekiert R.J.: Sep. Purif. Rev. 41, 1 (2012).
3. Barnes P.J.: Am. J. Respir. Crit. Care Med. 188, 901 (2013).
4. Kaneko T.: US 4,599,337 (1985).
5. Zygmunt M., Chłoń-Rzepa G., Sapa J., Pawłowski P.: Pharmacol. Rep. 67, 9 (2015).
6. Winter C.A., Risley E.A., Nuss G.W.: Proc. Soc. Exp. Biol. Med. 111, 544 (1962).
7. Lence P.: Arch. Int. Pharmacodyn. Ther. 136, 237 (1962).
8. Kolaczkowska E., Grzybek W., van Rooijen N., Piccard H., Plytycz B. et al.: J. Leukoc. Biol. 85, 374 (2009).
9. Kolaczkowska E., Barteczko M., Plytycz B., Arnold B.: Inflamm. Res. 57, 272 (2008).
10. Benzie I. F., Strain J.J.: Anal. Biochem. 239, 70 (1996).
11. Abdel-Salam O.M., Baiuomy A.R., El-Shenawy S.M., Arbid M.S.: Pharmacol. Res. 4, 47 (2003).
12. de Barros B.S., da Silva J.P., de Souza Ferro J.N., Agra I.K., de Almeida Brito F. et al.: J. Nat. Med. 65, 526 (2011).
13. Ramos-Zepeda G., Herrero J.F.: Neurosci. Lett. 384, 177 (2005).
14. Santos E.N., Lima J.C., Noldin V.F., Cechinel-Filho V., Rao V.S. et al.: An. Acad. Bras. Cienc. 83, 557 (2011).
15. Zygmunt M., Dudek M., Bilska-Wilkosz A., Bednarski M., Mogilski S. et al.: Acta Pol. Pharm. Drug Res. 70, 899 (2013).
16. Lee H.N., Kundu J.K., Cha Y.N., Surh Y.J.: J. Cell. Sci. 126, 4037 (2013).
17. Conforti F., Sosa S., Marrelli M., Menichini F., Statti G.A. et al.: J. Ethnopharmacol. 116, 144 (2008).
18. Borges V.M., Vandivier R.W., McPhillips K.A., Kench J.A., Morimoto K. et al.: Am. J. Physiol. Lung. Cell. Mol. Physiol. 297, L586 (2009).
19. Buras J.A., Holzmann B., Sitkovsky M.: Nat. Rev. Drug. Discov. 4, 854 (2005).
20. Magee M.H., Blum R.A., Lates C.D., Jusko W.J.: Br. J. Clin. Pharmacol. 53, 474 (2002).
21. Tenor H., Staniciu L., Schudt C., Hatzelmann A., Wendel A. et al.: Clin. Exp. Allergy 25, 616 (1995).
22. Jones N.A., Leport M., Holand T., Vos T., Morgan M. et al.: Pulm. Pharmacol. Ther. 20, 60 (2007).
23. Tenor H., Hatzelmann A., Kupferschmidt R., Stanciu L., Djukanovic R. et al.: Clin. Exp. Allergy 25, 625 (1995).
24. Sinha B., Semmler J., Eisenhut T., Eigler A., Endres S.: Eur. J. Immunol. 25, 147 (1995).
25. Semmler J., Gebert U., Eisenhut T., Moeller J., Schonharting M.M. et al.: Immunology 78, 520 (1993).
26. Chen H.Y., Li L., Fu Z.J.: Pharmazie 69, 55 (2014).
27. Zygmunt M., Zmudzki P., Chlon-Rzepa G., Sapa J., Pawlowski M.: Lett. Drug Des. Discov. 11, 1204 (2014).

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