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SYNTHESIS AND EVALUATION OF THE ANTI-INFLAMMATORY ACTIVITY OF SOME 2-(TRIMETHOXYPHENYL)-4-R_1-5-R_2-THIAZOLES

C. ARANICIU[a], A. E. PÂRVU[b*], B. TIPERCIUC[a], M. PALAGE[a], S. ONIGA[a], P. VERITÉ[c], O. ONIGA[a]
[a]*Faculty of Pharmacy,"Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*
[b]*Department of Physiopathology, Faculty of Medicine, Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*
[c]*Department of Analytical Chemistry, Faculty of Medicine-Pharmacy, University of Rouen, Rouen, France*

Introduction. There is an increasing need to obtain new anti-inflammatory molecules that act either as COX-2 selective inhibitors or as iNOS inhibitors. To that effect, we synthesized some 2-(trimethoxyphenyl)-4-R_1-5-R_2-thiazoles and evaluated their anti-inflammatory properties. Experimental. The main synthesis route involved the Hantzsch condensation of the 3,4,5-trimethoxy-benzothioamide with various α-halo-ketones. For all synthesized compounds, the anti-inflammatory activity was evaluated *in vivo* on an experimental acute inflammation at rats thought the acute phase bone marrow response, phagocytic capacity, and nitro-oxidative stress status. Results. A total of 13 new compounds were synthesized and all of them have various degrees of anti-inflammatory activity. All tested compounds reduced the acute phase bone marrow response, the phagocytic capacity and the nitro-oxidative stress. Conclusions. Two of the compounds had a more potent action than meloxicam in all studied areas.

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Keywords: Anti-inflammatory, Oxidative stress, iNOS, Aryl-thiazoles

1. Introduction

The treatment of acute and chronic inflammation, by non steroidal anti-inflammatory drugs (NSAIDs), is a field of great interest for researchers. The classic NSAIDs, molecules that act as non specific cyclooxygenase COX inhibitors, have long been blamed for a series of severe and frequent adverse reactions like gastric irritation, gastritis, ulcers, gastro-intestinal bleeding etc [1,2]. More recently, this therapeutic class has been associated with other adverse reactions, especially in the cardiovascular and renal system [3]. The selective COX 2 inhibitors, that only a decade ago seemed to be the perfect molecules, suffered massive image damage because of severe and sometimes fatal cardiovascular events, and most of them have been withdrawn from market [4,5]. In this context, the need for new COX 2 inhibitor molecules, with a better safety profile is becoming stringent.

In the same time, other molecular pathways for blocking inflammation are considered. A field that is rapidly gaining momentum, is that of selective inducible NO synthase (iNOS) inhibitors. NOS and COX share some similarities: both have a constitutive form and an inducible one. Both inducible forms of the two enzymes are up-regulated by inflammatory stimuli and are responsible for similar pathophysiological responses (pain, fever etc.). Moreover, a series of interaction between the two pathways have been reported. There are several proposed mechanisms for NO-mediated regulation of prostaglandin production. Nitric oxide produced by iNOS can

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[*] Corresponding author: parvualinaelena@yahoo.com

directly modify cysteine in the COXs and activate them. In the same time, peroxynitrite, formed by the interaction of NO with superoxide radicals, can interact with the heme residue in COX (activation) or with the tyrosine residue(s) in COX (inactivation) [6].

Inspired by the iNOS inhibiting molecules bearing the thiazole moiety [7] and consisting with our group's previous focus of research [8, 9, 10] we decided to synthesize a series of new potentially anti-inflammatory molecules with the 2-(trimethoxyphenyl)-thiazole scaffold. The compounds were evaluated for their effect in an acute experimental inflammation on rats, by measuring the cellular mediated systemic inflammatory response, and the nitro-oxidative status of the serum.

2. Experimental

Chemistry

General

Chemicals used for the biological determination such as: sulfanilamide (SULF), N-(1-Naphthyl) ethylendiamine dihydrochloride (NEDD), Vanadium (III) chloride (VCl_3) methanol, diethylether, xylenol orange [o-cresosulfonphthalein-3,3-bis(sodium methyliminodiacetate)], ortho dianisidine dihydrochloride (3-3′-dimethoxybenzidine), ferrous ammonium sulfate, hydrogen peroxide (H_2O_2), sulfuric acid, hydrochloride acid, glycerol, trichloroacetic acid (TCA), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Taufkirchen Germany).

Solvents and reagents used for synthesis and purification were purchased from Alfa Aesar (Karlsruhe, Germany). All chemicals were of analysis grade.

Thin layer chromatography was performed on Silica Gel sheets, with UV-light visualization. The melting points are uncorrected and were obtained by using an Electrothermal 9100 melting point apparatus. IR spectra were recorded by a Shimatzu IR 27G spectrometer. MS spectra were obtained by using a Varian Mat 111, 70 eV by directly introduction of the solid samples. The ^{1}H-NMR were recorded on a Bruker Avance NMR spectrometer, operating at 500MHz, in DMSO-d6 as solvent. Chemical shift values are reported in δ units, relative to TMS as internal standard. Microanalysis was performed by Vario El CHNS analyzer.

Synthesis of the 3,4,5-trimethoxybenzothioamide

A solution of 3,4,5-trimethoxybenzonitrile (1 mM) in ethanol (1.8 ml) and triethylamine (200µl) was maintained at room temperature while hydrogen sulfide was bubbled into the solution for 8h. The reaction mixture was poured on water, and the resulting solid was filtered and recrystallised from ethanol.

Synthesis of the 4-aryl-5-R_2-2-(3,4,5-trimethoxyphenyl)thiazoles(1-9)

A mixture of 3,4,5-trimethoxybenzothioamide (1 mM) and the corresponding α-bromoketone (1 mM) was dissolved in anhydrous acetone (5 ml) and stirred at room temperature for 24h. The resulting solid was filtered and washed with a solution of Na_2CO_3 5% until free of acid. The compounds were then recrystallised from methanol to yield the pure compounds.

Synthesis of the 4-R_1-5-R_2--2-(3,4,5-trimethoxyphenyl)thiazoles(10-13)

To a solution of 3,4,5-trimethoxybenzothioamide (1 mM) in 4 ml ethanol an equimolar quantity of α-chloroketone was added and it was refluxed for 5 h. The reaction mixture was cooled to room temperature and neutralized with Na_2CO_3. The solid obtained was filtered, washed with water and recrystallised from ethanol.

*5-methyl-4-phenyl-2-(3,4,5-trimethoxyphenyl)-thiazole(**1**)*

Yellow powder. m.p. 101°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 7.8 (m, 2H, CH Ar), 7.50 (m, 2H, CH Ar), 7.45 (m, 1H, CH Ar), 7.25 (s, 2H, CH Ar), 3.92 (s, 6H, -OCH_3), 3.73 (s, 3H, -OCH_3), 2.50 (s, 3H, -CH_3). Anal. calcd. (%) for $C_{19}H_{19}NO_3S$ (341,42): C, 66.84; H, 5.61; N, 4.10; S, 9.39. Found: C, 66.76; H, 5.60; N, 3.90; S, 9.45. MS (EI, 70eV): m/z 341 (M+).

*4-phenyl-2-(3,4,5-trimethoxyphenyl)-thiazole(**2**)*
Off-white powder. m.p. 92°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 7.8 (m, 2H, CH Ar), 7.65 (s, 1H, H-Thiazole), 7.50 (m, 2H, CH Ar), 7.45 (m, 1H, CH Ar), 7.25 (s, 2H, CH Ar), 3.93 (s, 6H, -OCH_3), 3.73 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{18}H_{17}NO_3S$ (327.39): C, 66.03; H, 5.23; N, 4.28; S, 9.79. Found: C, 66.15; H, 5.20; N, 4.18; S, 9.85. MS (EI, 70eV): m/z 327 (M+).

*4-(4-nitrophenyl)-2-(3,4,5-trimethoxyphenyl)-thiazole(**3**)*
Yellow powder. m.p. 220°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 8.08 (m, 2H, CH Ar), 8.02 (s, 1H, H-Thiazole), 7.98 (m, 2H, CH Ar), 7.24 (s, 2H, CH Ar),, 3.90 (s, 6H, -OCH_3), 3.75 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{18}H_{16}N_2O_5S$ (372.39): C, 58.05; H, 4.33; N, 7.52; S, 8.61. Found: C, 58.1; H, 4.30; N, 7.45; S, 8.72. MS (EI, 70eV): m/z 372 (M+).

*4-(4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-thiazole(**4**)*
Pale-yellow powder. m.p. 105°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 8 (m, 2H, CH Ar), 7.98 (s, 1H, H-Thiazole), 7.27 (s, 2H, CH Ar), 7.04 (m, 2H, CH Ar), 3.91 (s, 6H, -OCH_3), 3.81 (s, 3H, -OCH_3), 3.74 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{19}H_{19}NO_4S$ (357.42): C, 63.85; H, 5.36; N, 3.92; S, 8.97. Found: C, 63.75; H, 5.35; N, 3.90; S, 8.99. MS (EI, 70eV): m/z 357 (M+).

*4-(4-benzonitrile)-2-(3,4,5-trimethoxyphenyl)-thiazole(**5**)*
Orange-yellow powder. m.p. 200°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 8.30 (m, 2H, CH Ar), 8.10 (s, 1H, H-Thiazole), 7.75 (m, 2H, CH Ar), 7.28 (s, 2H, CH Ar) 3.93 (s, 6H, -OCH_3), 3.75 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{19}H_{16}N_2O_3S$ (352.41): C, 64.76; H, 4.58; N, 7.95; S, 9.10. Found: C, 64.80; H, 4.55; N, 7.35; S, 9.19.
MS (EI, 70eV): m/z 352 (M+).

*4-(naphthalen-1-yl)-2-(3,4,5-trimethoxyphenyl)-thiazole(**6**)*
Yellow powder. m.p. 198°C.^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 8.65 (s, 1H, CH Ar), 8.33 (s, 1H, H-Thiazole), 8.2 (m, 1H, CH Ar), 8.03 (m, 2H, CH Ar), 7.95 (m, 1H, CH Ar), 7.55 (m, 2H, CH Ar), 7.34 (s, 2H, CH Ar), 3.93 (s, 6H, -OCH_3), 3.75 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{22}H_{19}NO_3S$ (377.46): C, 70.00; H, 5.07; N, 3.71; S, 8.50. Found: C, 70.52; H, 5.05; N, 3.69; S, 8.46. MS (EI, 70eV): m/z 377 (M+).

*4-(2-hydroxybenzamid-5-yl)-2-(3,4,5-trimethoxyphenyl)-thiazole(**7**)*
Greenish-yellow powder. m.p. 235°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 12.8 (s, 1H, OH), 9.01 (s, 1H, CH Ar), 8.80 (s, 2H, $CONH_2$), 8.40 (s, 1H, H-Thiazole), 8.01 (d, 1H, CH Ar), 7.25 (s, 2H, CH Ar), 7.04 (d, 1H, CH Ar), 3.93 (s, 6H, -OCH_3), 3.81 (s, 3H, -OCH_3), 3.74 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{19}H_{18}N_2O_5S$ (386.42): C, 59.06; H, 4.70; N, 7.25; S, 8.30. Found:
C, 59.00; H, 4.76 N, 7.20; S, 8.32. MS (EI, 70eV): m/z 386 (M+).

*4-(4-chlorophenyl)-2-(3,4,5-trimethoxyphenyl)-thiazole(**8**)*
Yellow powder. m.p. 197°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 7.51 (m, 2H, CH Ar), 7.35 (s, 1H, H-Thiazole), 7.30 (m, 2H, CH Ar), 7.28 (s, 2H, CH Ar) 3.90 (s, 6H, -OCH_3), 3.75 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{18}H_{16}ClNO_3S$ (361.84): C, 59.75; H, 4.46; N, 3.87; S, 8.86. Found: C, 59.70; H, 4.44; N, 3.88; S, 8.96. MS (EI, 70eV): m/z 361 (M+).

*4-(4-methyl-2-phenyl-thiazol-5-yl)-2-(3,4,5-trimethoxyphenyl)-thiazole(**9**)*
Yellow powder. m.p. 195°C. 7.98 ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ 7.98 (m, 2H, CH Ar), 7.96 (s, 1H, H-Thiazole), 7.52 (m, 2H, CH Ar), 7.5 (m, 1H, CH Ar), 7.24 (s, 2H, CH Ar), , 3.90 (s, 6H, -OCH_3), 3.74 (s, 3H, -OCH_3), 2.69 (s, 3H, -CH_3). Anal. calcd. (%) for $C_{22}H_{20}N_2O_3S_2$ (424.54): C, 62.24; H, 4.75; N, 6.60; S, 15.11. Found: C, 62.14; H, 4.70; N, 6.68; S, 15.20. MS (EI, 70eV): m/z 424 (M+).

*4-(chloromethyl)-2-(3,4,5-trimethoxyphenyl)-thiazole(**10**)*
Yellow powder. m.p. 149°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ, 7.28 (s, 2H, CH Ar), 7 (s, 1H, H-Thiazole) 4.46 (s, 2H, -CH_2Cl) 3.94 (s, 6H, -OCH_3), 3.75 (s, 3H, -OCH_3). Anal. calcd. (%) for $C_{13}H_{14}ClNO_3S$ (299.273): C, 52.09; H, 4.71; N, 4.67; S, 10.70. Found: C, 52.20; H, 4.61; N, 4.59; S, 10.81. MS (EI, 70eV): m/z 299 (M+).

*5-acetyl-4-methyl-2-(3,4,5-trimethoxyphenyl)-thiazole(**11**)*
White-yellow powder. m.p. 100°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ, 7.2 (s, 2H, CH Ar), 3.91 (s, 6H, -OCH_3), 3.72 (s, 3H, -OCH_3), 2.68 (s, 3H, -CH_3), 2.50 (s, 3H, -$COCH_3$). Anal. calcd. (%) for $C_{15}H_{17}NO_4S$ (307.36): C, 58.61; H, 5.57; N, 4.56; S, 10.43. Found: C, 58.72; H, 5.50; N, 4.49; S, 10.55. MS (EI, 70eV): m/z 307 (M+).

*5-carboxyethyl-4-methyl-2-(3,4,5-trimethoxyphenyl)-thiazole(**12**)*

White powder. m.p. 119°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ, 7.2 (s, 2H, CH Ar), 4.90 (m, 2H,-COOCH$_2$-), 3.91 (s, 6H, -OCH$_3$), 3.72 (s, 3H, -OCH$_3$), 2.60 (s, 3H, -CH$_3$), 1.55 (m, 3H, -COOCH$_2$**CH$_3$**). Anal. calcd. (%) for $C_{16}H_{19}NO_5S$ (337.39): C, 56.96; H, 5.68; N, 4.15; S, 9.50. Found: C, 56.86; H, 5.60; N, 4.19; S, 9.62. MS (EI, 70eV): m/z 337 (M+).

*4-(ethyl-acetate)-2-(3,4,5-trimethoxyphenyl)-thiazole(**13**)*

Light-brown powder. m.p. 220°C. ^{1}H RMN (DMSO-d6, 500 MHz, ppm): δ, 7.25 (s, 2H, CH Ar), 7.1 (s, 1H, H-Thiazole) 4.50 (m,2H, -COOCH$_2$-), 3.91 (s, 6H, -OCH$_3$), 3.72 (s, 3H, -OCH$_3$), 3.55 (s, 2H, -CH$_2$-COO-), 1.35 (m, 3H, -CH$_3$). Anal. calcd. (%) for $C_{16}H_{19}NO_5S$ (337.39): C, 56.96; H, 5.68; N, 4.15; S, 9.50. Found: C, 56.90; H, 5.63; N, 4.09; S, 9.59. MS (EI, 70eV): m/z 337 (M+)

Biological evaluation

The anti-inflammatory activity was assessed for all synthesized compounds. This evaluation was performed by using an experimental acute inflammation model on rats. Inflammation was induced by i.m. administration of turpentine oil (0,6ml/100g b.w.). The anti-inflammatory reference drug used was meloxicam. The experimental model involved the evaluation of the acute phase bone marrow response, phagocytes activity, oxidative stress status and NO synthesis. All experiments were performed in triplicates using methods reported in our previous research [8,9,10].

Animals

All experiments were carried out on male albino rats (Wistar-Bratislava), fully matured and weighing 200-250 g. The animals were supplied by the University of Medicine and Pharmacy Cluj-Napoca breeding facilities. The experimental procedures performed complied to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. All study protocols were previously approved by the Institutional Animal Ethical Committee of the University of Medicine and Pharmacy Cluj-Napoca.

The animals were divided into 16 groups of ten. The group **C** **(**Control) consisted of healthy animals that did not receive any treatment. Animals in group **I** (Inflammation) received a pro-inflammatory substance administered i.m. (turpentine oil 0.6 ml/100g body weight). The other groups received the same amount of pro-inflammatory substance and also received intraperitoneal injection with either reference NSAID=meloxicam (**M**) at a 3.2 mg/kg dose, or the synthesized compounds in an equi-molar dose with meloxicam (**1-13**). The tested compounds were administered i.p. as suspensions in 1% carboxymethyl celullose in saline vehicle.

After the treatment animals were maintained for 24 h in a controlled environment, at 25° C with a 12 h light/dark cycle, and food and water *ad libidum*.

Anti-inflammatory activity

After 24 h animals were anesthetized with ketamine (i.p. 90mg/kg b.w.) and blood samples were harvested by retro-orbital sinus puncture on EDTA for the acute phase bone marrow response and *In vitro* phagocytosis test, and without anticoagulant for serum separation. Serum was separated by centrifugation at 1500×*g* for 10 min. The samples were assayed immediately or stored until analysis at -80 °C.

The acute phase bone marrow response

The acute phase bone marrow response was evaluated by determining the total leukocyte count and leukocyte count expressed as percentage [9, 11]).

In vitro phagocytosis test

The phagocytosis test was performed by incubating a blood sample harvested on EDTA with an *Escherichia coli* suspension (4 x 10^6 germs/ml, in saline solution 0,9%, in ratio 0,2 ml blood / 20 μl suspension E. coli) at 37°C, for 30 min. Afterwards, smears stained May-Grunwald-Giemsa were prepared and the count was done by optic microscopy (Olympus microscope).

The phagocytic capacity was evaluated by two parameters: The phagocytic activity (PA=the number of the E. coli germs phagocyted by 100 leukocytes) and the phagocytic index (PI%= percentage of leukocytes that phagocyted at least one germ) [9, 12, 13].

Serum nitric oxide evaluation

The general procedure was described in our previous papers [9, 14]. The Griess reaction was used as an indirect assay to determine the total serum nitrite (NO_2^-) and nitrate (NO_3^-) as a measure of the degree of NO production (NOx). Serum samples were passed through 10-KD filters (Sartorius AG, Goettingen, Germany) and deproteinized by methanol/diethyl ether (3/1, *v/v*) (sample: methanol/diethyl ether, 1:9, *v/v*) [15]. In brief, 100 µL of VCl_3 (8 mg/mL) was added to 100 µL of the supernatant for the reduction of nitrate to nitrite, followed by the addition of the Griess reagents, 50 µL of SULF (2%) and 50µL of NEDD (0.1%). After 30 min of incubation at 37 °C, the absorbance was read at 540 nm. Serum NOx was expressed as nitrite µmol/L [16].

Oxidative stress evaluation

Serum total oxidant status determination

Total oxidant status (TOS) of serum was measured using a colorimetric measurement method [10, 17]. In this method oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide (H_2O_2) and the results are expressed in µmol H_2O_2 Equiv./L.

Serum total antioxidant response determination

The total antioxidant status was measured in serum using a colorimetric method for the total antioxidant response (TAR). In this method the hydroxyl radical is produced by the Fenton reaction, and reacts with the colorless substrate o-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon addition of a serum sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction medium are suppressed by the antioxidant components of the serum, preventing the color change and thereby providing an effective measure of the total antioxidant capacity of the serum. The assay is calibrated with Trolox and results are expressed as mmol Trolox Equiv./L [10,18].

Calculation of oxidative stress index

The percent ratio of the total oxidative status to the total antioxidant response gave the oxidative stress index (OSI), an indicator of the degree of oxidative stress [10,19]. To perform the calculation, the result unit of TAR, mmol Trolox equivalent/L, was changed to µmol Trolox equivalent/L and OSI was calculated with the formula: OSI (Arbitrary Unit) = TOS (µmol H_2O_2 Equiv. /L) / TAC (mmol Trolox Equiv./L).

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical comparisons between the groups were made using one-way analysis of variance (ANOVA) test. P-values<0.05 were regarded as statistically significant. Pearson's and Spearman's correlation tests were performed in order to evaluate statistical correlation. Data was analyzed using the software: SPSS for Windows, version 16.

3. Results

Chemistry

The main synthesis route involved the Hantzsch condensation of the 3,4,5-trimethoxy-benzothioamide with various α-halo-ketones (fig. 1). 13 new compounds were obtained and were characterized using IR, ^{1}H-RMN, mass spectra and elemental analysis. The structures of all new compounds were confirmed.

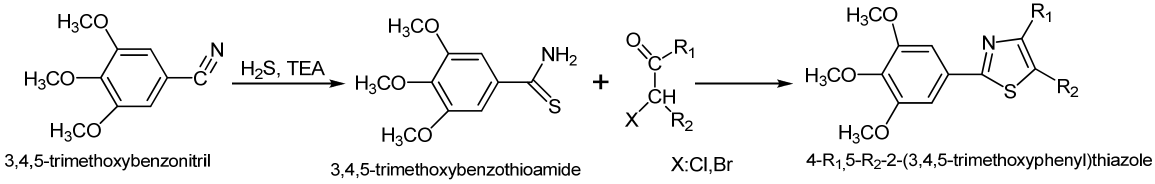

Fig.1. The synthesis and structures of the 2-(3,4,5-trimethoxyphenyl)thiazolyl compounds.

Compound	R_1	R_2
1	$-C_6H_5$	$-CH_3$
2	$-C_6H_5$	H
3	$-(4-NO_2-C_6H_4)$	H
4	$-(4-CH_3O-C_6H_4)$	H
5	$-(4-CN-C_6H_4)$	H
6	$-C_{10}H_7$	H
7	$-(3-OH,4-CONH_2-C_6H_3)$	H
8	$-(4-Cl-C_6H_4)$	H
9	$-(2-C_6H_5-4-CH_3$-Thiazol-5-yl)	H
10	$-CH_2Cl$	H
11	$-CH_3$	$-COCH_3$
12	$- CH_3$	$-COOC_2H_5$
13	$-CH_2-COOC_2H_5$	H

Biological evaluation

Effects on the acute phase bone marrow response

The effects of the studied compounds on the acute phase bone marrow response are shown in table 1.

When comparing with the inflammation **I** group, all tested compounds significantly reduce absolute leukocytes count ($p<0.001$), except compound **2** that had no significant influence ($p>0.05$). By comparing the same results with the meloxicam group **M**, two compounds have significantly stronger inhibitory effects than meloxicam: compound **3** ($p< 0.01$) and compound 8 ($p<0.001$).

In regard of neutrophils percentage, compounds **1, 3, 4, 8, 10** show a significant reduction ($p<0.001$), as do compounds **5, 6, 7, 13** ($p< 0.01$), when compared with the inflammation group **I**. By contrast, compounds **2, 9, 11, 12** show a significant increase in neutrophils percentage ($p<0.05$). When compared to meloxicam **M** group, compounds **1, 3, 4, 8, 10** showed a more potent decrease of neutrophils percentage ($p<0.001$).

Compounds **1, 3, 4, 5, 6, 7, 8, 10, 13** reduce absolute leukocyte count by reducing the percentage of neutrophils. This can be considered a systemic anti-inflammatory effect obtained by blocking cell mediated inflammation. Compounds **3** and **8** show a significantly higher inhibitory effect than meloxicam on both total leukocytes count and neutrophils percentage.

Table 1. Effects of the synthesized compounds on the acute phase bone marrow response

Compound	Leukocytes (no./mm^3)	Neutrophiles(%)	Monocytes(%)	Lymphocytes(%)
Control	5743.75 ± 1375.93	66.17 ± 6.79	8 ±1.05	32.8 ± 4.73
Inflammation	13575 ± 1216.15	69.5 ± 3.81	1.5 ± 0.53	22.2 ± 2.3
Meloxicam	4593.75 ± 314.46	65.6 ± 3.63	1.14 ± 0.31	31.8 ± 4.87
1	7220 ± 1789.05	56.4 ± 2.55	1.6 ± 0.7	43.6 ± 1.78
2	13255 ± 3437.69	82.4 ± 5.91	1.6 ± 0.7	15.2 ± 3.16
3	4255 ± 361.98	53.88 ± 3.18	1.8 ± 0.42	42.25 ± 6.63
4	4435.71 ± 184.2	57 ± 3.46	1.4 ± 0.52	42.4 ± 7.01
5	4578.57 ±318.67	61.6 ± 6.08	1.17 ± 0.41	35.6 ± 2.95
6	4368.75 ± 489.12	61.2 ± 8.8	1.29 ± 0.76	34.4 ±2.17
7	4062.5 ± 504.09	63.4 ± 5.8	1.29 ± 0.49	33.2 ± 3.74
8	4243.75 ± 132.12	60.6 ± 2.55	1.57 ± 0.53	37.6 ± 4.45
9	4406.25 ± 318.97	79 ± 11.91	1.6 ± 0.84	21.2 ± 6.3
10	4481.25 ± 334.81	56.8 ± 4.29	1.4 ± 0.52	40.8 ± 1.55
11	6712.5 ± 1605.91	81.22 ± 6.92	1.57 ± 0.79	18.44 ± 5.41
12	6718.75 ± 1813.82	79 ± 6.43	1.86 ± 0.69	21 ± 2.75
13	6306.25 ± 993.71	63.8 ± 5.43	1.29 ± 0.76	32.8 ± 2.86

*****All experiments were performed in triplicates. Results are expressed as mean ± standard deviation

Effects on the *in vitro* phagocytosis test

Comparison of the PA (fig.2)and PI% (fig. 3) or the tested compounds and the inflammation group reveals that all compounds significantly reduce the PA and PI ($p<0.001$). Compared to meloxicam, compound **10** has a more potent reducing effect on both PA ($p<0.001$) and PI ($p<0.01$) values. Compounds **3, 8** have similar PA with meloxicam, but exert a more potent inhibition of PI than meloxicam ($p<0.01$).

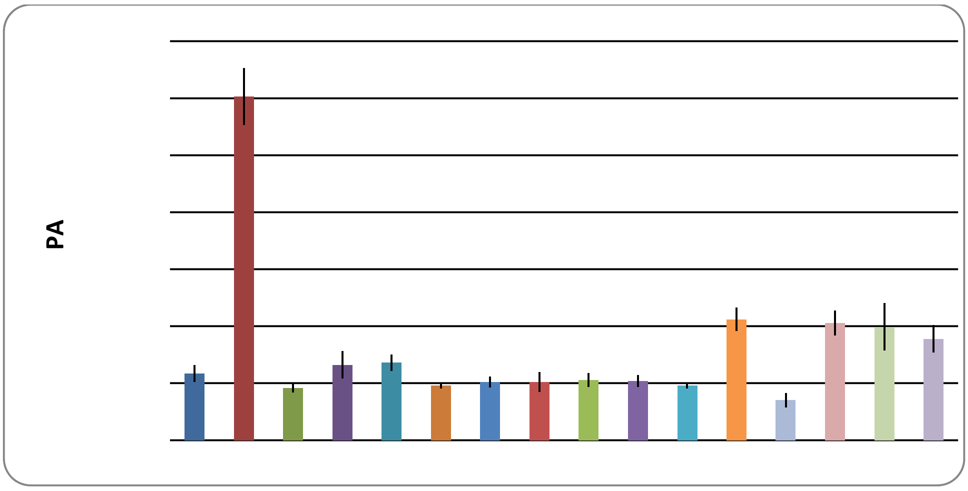

Fig. 2. The effects of the studied compounds on phagocytic activity PA.

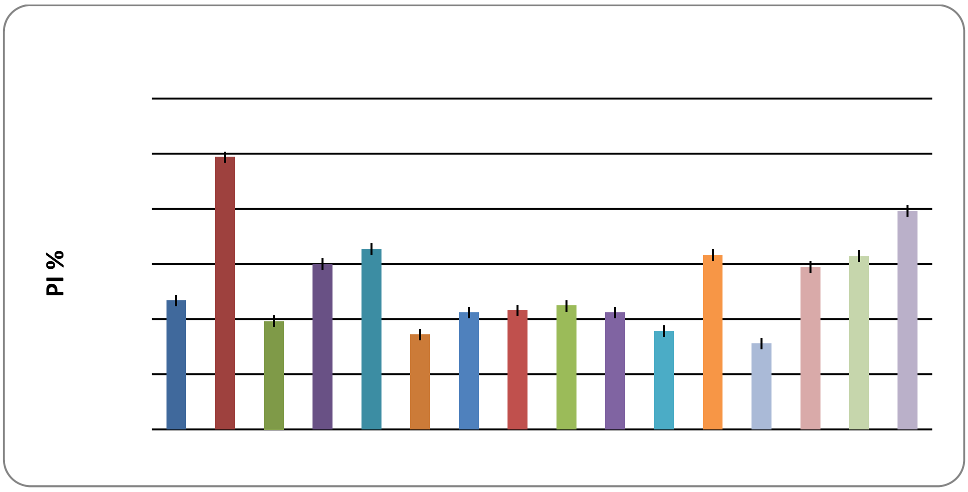

Fig. 3. The effects of the studied compounds on phagocytic index PI %.

Effects on the serum nitrite and nitrate levels

The serum concentrations of nitrites and nitrates are important markers of inflammation, due to the fact that in an acute inflammation iNOS is activated. Nitrites and nitrates are the main metabolic products of NO and thus relay information about the inflammatory state. All tested compounds produce a significant reduction of the serum nitrite/nitrate levels (all $p<0.001$ except compound **5** with $p<0.01$) (fig.4). When compared with meloxicam, compound **3** has a similar level, while compound **13** appears to be a more potent inhibitor of nitrite/nitrate formation ($p<0.001$). All other compounds inhibit the formation of NO to a lesser degree than meloxicam.

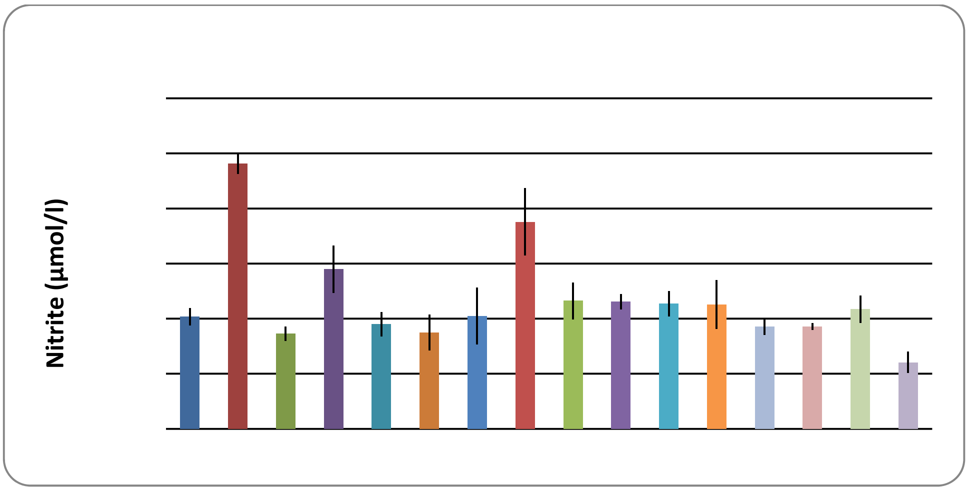

Fig. 4. The effects of the synthesized compounds on serum nitrite and nitrate values.

Effects on serum total oxidant status

TOS is a measure of the oxidizing capacity of the serum. TOS is higher in inflammation due to the release of reactive oxygen intermediates. TOS was significantly decreased by the compounds **3, 4, 5, 6, 7, 8, 13** ($p<0.001$), while the rest of the compounds showed an increase in TOS, by comparison with the **I** group (table 2). Compounds **6, 7, 8** have lowered TOS significantly more than meloxicam ($p<0.001$) while compounds **3, 5** decrease TOS levels ($p<0.05$) to a more similar extent with meloxicam.

Effects on total antioxidant response

TAR characterizes the antioxidant capacity of the serum. All the tested compounds have significantly increased TAR levels when compared with group **I** ($p<0.001$) (table 2). Compounds **5, 6, 7, 8** increased TAR significantly more than meloxicam ($p<0.05$) while compound **3** has a similar TAR value with meloxicam ($p>0.05$).

Table 2. The effects on serum total oxidant status (TOS) and total antioxidant response (TAR)

Compound	TOS (μmol H_2O_2 Equiv./l)	TAR (mmol Trolox Equiv./l)
Control	16.22 ± 4.41	5.4 ±0.05
Inflammation	31.7 ± 4.89	2.4 ±0.05
Meloxicam	22.92 ± 4.46	5.28 ±0.03
1	36.56 ± 8.84	5.23 ±0.32
2	42.48 ± 10.5	5.16 ±0.08
3	18.23 ± 3.57	5.3 ±0.12
4	21.37 ±4.41	5.26 ±0.14
5	19.02 ± 1.84	5.44 ±0.16
6	14.03 ± 2.52	5.43 ±0.12
7	16.23 ± 0.75	5.4 ±0.15
8	13.74 ± 1.12	5.42 ±0.18
9	40.19 ± 8.97	4.74 ±0.23
10	44.23 ± 4.53	4.55 ±0.15
11	46.28 ± 3.87	4.98 ±0.34
12	53.07 ± 9.47	5.24 ±0.23
13	19.97 ± 2.35	5.27 ±0.2

*All experiments were performed in triplicates. Results are expressed as mean ± standard deviation

Effects on oxidative stress index

OSI (fig. 5). is calculated using TAR and TOS, thus giving a better understanding of the oxidative stress in the serum. All studied compounds have an oxidative stress index significantly lower than the inflammation group **I** ($p<0.001$). Compounds **3-8** and **13** have demonstrated a significantly lower OSI than meloxicam ($p<0.001$)

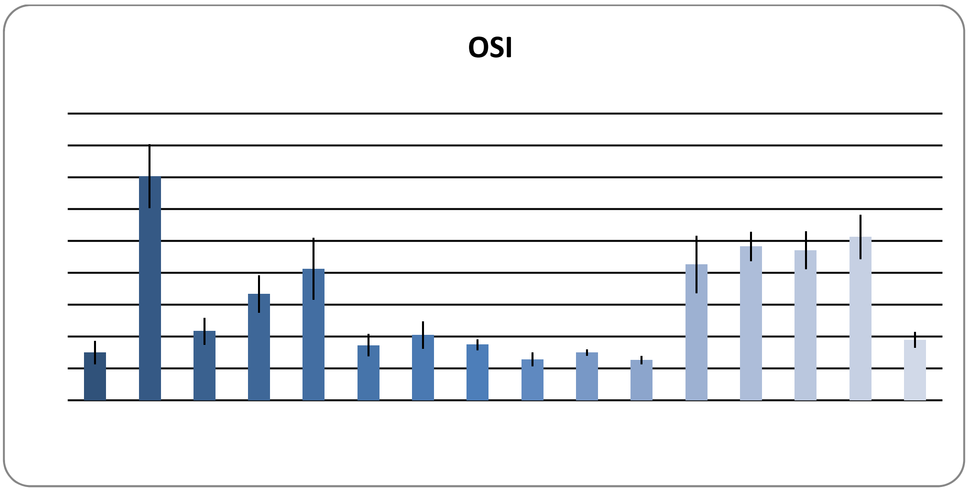

Fig. 5. The effects of the compounds on oxidative stress index OSI.

4. Discussions

In regard of cell mediated inflammatory response, assessed by the acute phase bone marrow response, for a good anti-inflammatory response absolute leukocyte count should be lowered by the drop of neutrophils. This result is true for all the studied compounds, except **2** (no significant reduction in leukocyte count) and **9, 11, 12** (the reduction of leukocytes count was not due to neutrophils drop). Compounds **3, 4, 8, 10** show a good correlation between reduction of neutrophils percentage and the decrease of nitrite and nitrate production (r=0.7). For these compounds the neutrophils percentage drop also exhibits a positive correlation with the reduction of phagocytic activity PA and phagocytic index PI (r=0.7-0.8). Compound **3, 4, 8** have a positive correlation between the absolute leukocyte count and the oxidative stress index OSI (r= 0.7).

The inhibition of NO production shows a positive correlation with the decrease of PI and PA for compounds **3** and **8** (r=0.8). In the same time NO inhibition correlates positively with reduction of oxidative stress and improvement of the oxidative status, as depicted by OSI, for the compounds **3, 4, 5, 6, 7, 8, 13**.

In general, all of the synthesized compounds have a degree of anti-inflammatory effects, causing the decrease in parameters under the values obtained for the **I** group. The most preeminent action can be seen in compounds **3** and **8** that show a more powerful anti-inflammatory effect than meloxicam, in all determined parameters.

Compounds **4, 5, 6, 7** also had a good overall anti-inflammatory effect, but it was less efficient than meloxicam, at equimolar dose. Further studies with higher concentration of compounds are needed to determine the true potency of their anti-inflammatory action.

Compounds **9**, **10**, **11**, **12** and **13** had modest anti-inflammatory effects, except compound 13, which had a good inhibitor of NO production and a potential *in vivo* anti-oxidant.

This data is consistent with the known structure activity relation in the class of NSAID. These postulate that a good inhibitor of COX 2 should have at least three aromatic cycles [20], which is true for compounds **1-8**. In the same time compounds with four rings (compound **9**) and with two rings (compounds **10-13**) have shown a less than important anti-inflammatory effect.

For the active compounds, the exact mechanism of action should be further investigated, in order to establish if they act directly on COXs, selectively of specifically on COX 2, or on iNOS. In the same time, studies regarding safety and kinetics are required in order to further develop this molecules as potential new drugs.

5. Conclusions

13 new molecules were synthesized, characterized and evaluated for their anti-inflammatory effects. All synthesized compounds had a degree of anti-inflammatory action. Compounds **1, 3, 4, 10, 8** had a better inhibitory effect on acute phase bone marrow response than the standard meloxicam. Compounds **3, 8, 10** reduced the phagocytic capacity more than meloxicam. All compounds reduced the serum levels of nitrite and nitrate, compound **13** had a significantly higher inhibition capacity than meloxicam, while compound **3** had a similar effect with meloxicam. All studied compounds improved the oxidative stress index, with compounds **3, 4, 5, 6, 7, 8, 13**, having a significantly better effect than meloxicam. The best anti-inflammatory compounds were **3** and **8**, which had a better effect than meloxicam, in all tested parameters.

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