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New 2-(4-(4-bromophenylsulfonyl)phenyl)-4-arylidene-oxazol-5(4H)-ones: analgesic activity and histopathological assessment

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Abstract

This paper reports the synthesis, analgesic activity, acute toxicity and histopathological (HP) assessment of four new compounds from oxazol-5(4H)-ones class that contain in their molecule a diarylsulfone moiety. The new 2-(4-(4-bromophenylsulfonyl)phenyl)-4-arylidene-oxazol-5(4H)-ones were obtained by reaction of 2-(4-(4-bromophenylsulfonyl)benzamido)acetic acid intermediate with aromatic aldehydes (benzaldehyde, 4-methoxy, 4-nitro or 4-bromobenzaldehyde), in acetic anhydride and in the presence of anhydrous sodium acetate. The new compounds have been characterized by spectral techniques, such as: Fourier-transform infrared spectroscopy (FT-IR), mass spectrometry (MS), proton nuclear magnetic resonance (¹H-NMR) and by elemental analysis. The acute toxicity of the new oxazol-5(4H)-ones in mice was assessed through "acute toxic class" method, according to Organization for Economic Co-operation and Development (OECD) Guidelines. The HP assessment of some preserved organs collected from mice has been performed. The analgesic activity of all new synthesized compounds was carried out with two pharmacological tests: the writhing test and the hot plate test. In order to predict the binding affinities of the synthesized oxazol-5(4H)-ones derivatives against molecular targets involved in pain and inflammation, molecular docking simulations were performed. The results of the writhing test indicated that the most active compound was the oxazolone that contains in the molecule a methoxy group. The acute oral toxicity study revealed no lethal effect of new compounds. The HP assessment of the preserved organs collected from mice did not indicate any cytohistopathological aspects that can be linked to any inflammatory, neoplastic or cytotoxic process, demonstrating the low toxicity of new compounds.

Keywords: oxazol-5(4H)-ones, acute toxicity, analgesic activity, histopathological assessment.

Introduction

Oxazol-5(4H)-ones (azlactones) are 1,3-oxazole derivatives that possess a ketone group at the 5th position of the pentaatomic nucleus and a double bond between the carbon from the 2nd position and the nitrogen atom. These compounds can be classified as saturated and unsaturated, the unsaturated derivatives having an exocyclic double bond at the 4th carbon atom of the nucleus.

There are many reports that mention various oxazoles and oxazolones derivatives for their biological actions, such as antimicrobial [1–3], antitumoral [3–5], analgesic and anti-inflammatory [3, 6–10], including the antagonistic action upon transient receptor potential cation channel subfamily V member 1 (TRPV1) channel [11]. Moreover, there are drugs with anti-inflammatory activity containing the oxazole nucleus, being currently used in therapy. For example, *oxaprozin* is a non-steroidal anti-inflammatory drug with an 1,3-oxazolic nucleus that blocks prostaglandin

synthesis by non-selective inhibition of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), that is used in the treatment of many musculoskeletal inflammatory diseases [6, 12, 13].

Another representative of the 1,3-oxazole class with anti-inflammatory properties is *deflazacort* that has a steroidal skeleton and it is used in the therapy of the Duchenne muscular dystrophy, a fatal disease, characterized by the absence of dystrophin, progressive muscle degeneration and cardiorespiratory failure [14, 15].

Also, *ditazole*, a drug containing an oxazole nucleus, is a non-steroidal anti-inflammatory agent, that is used as platelets aggregation inhibitor [3, 16, 17].

Therefore, with the purpose to enrich the pain medication class, the researchers investigated the analgesic and anti-inflammatory potential of the benzyldiene-oxazolones [6, 7, 10].

Based on the molecular model of the coxibs (rofecoxib, valdecoxib, celecoxib), a series of unsaturated oxazolones,

derivatives of 4-(arylidene)-2-(pyridyl-3-yl)-oxazol-5(*H*)-ones (I, Figure 1), was synthesized and evaluated for its anti-inflammatory activity. The results indicated that most of derivatives exhibited a good inhibition of COX-2 enzyme, in some cases better than celecoxib, the drug used as standard. The derivatives containing the 4-hydroxybenzylidene and 4-*N,N*-dimethylaminobenzylidene fragments, presented half maximal inhibitory concentration (IC_{50}) values of 0.024 μ M and 0.019 μ M, lower compared to celecoxib, which has an IC_{50} of 0.05 μ M [6].

Another study showed that a series of benzylidene-oxazolones presents anti-inflammatory activity, the 4-

(3,5-dimethoxybenzylidene)-2-phenyl-oxazol-5(*H*)-one derivative (II, Figure 1) having a remarkable effect, decreasing the edema with 50.6% opposed to 41.5% (effect of aspirin) [10].

Also, Mariappan *et al.* [7] synthesized a series of benzylidene-oxazolones and screened their analgesic and anti-inflammatory effects. The results indicated that some derivatives showed promising analgesic activity when compared to the standard, pentazocine, the oxazole (III) (Figure 1) having the best effect. Also, all compounds possessed anti-inflammatory activity, the same oxazolone (III) being more active than aspirin.

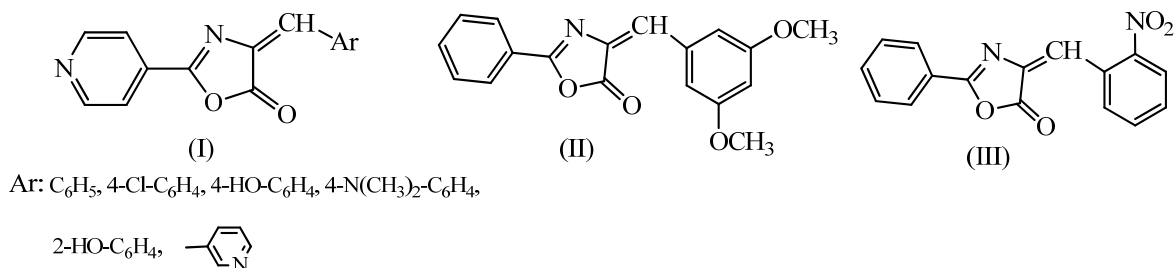


Figure 1 – Benzylidene-oxazolones (I), (II) and (III) with analgesic/anti-inflammatory activity.

The sulfone group, is another pharmacophore, usually incorporated in many compounds with biological activity [18] and in the structures of some anti-inflammatory drugs, such as celecoxib, piroxicam [19] or dapsone [20, 21]. *Dapsone*, 4,4'-diaminodiphenylsulfone, is an anti-bacterial drug with anti-inflammatory properties, that has been used for treating inflammatory diseases characterized by abnormal infiltration of neutrophils or eosinophils (*i.e.*, *dermatitis herpetiformis*, *erythema elevatum diutinum*, *pustular psoriasis*, etc.) [20, 21].

Previously, we have synthesized various heterocyclic compounds that possess a diarylsulfone fragment with biological potential, that have been investigated for their cytotoxicity, antimicrobial and analgesic activity [22–26].

Aim

All this data determined us to unite in the same molecule the oxazol-5(*H*)-one nucleus and the 4-(4-bromophenylsulfonyl)phenyl fragment with the aim of obtaining new analgesic and anti-inflammatory agents. In order to assess the therapeutic potential of the new synthesized compounds, we firstly established the median lethal dose (LD_{50}) range according to the *Organization for Economic Co-operation and Development* (OECD) Protocols. The histopathological (HP) examination of some preserved organs from mice has been performed. The analgesic activity of all compounds was evaluated through two pharmacological tests, the writhing and hot plate tests. Furthermore, molecular docking studies have been carried out in order to investigate the likelihood of possessing inhibitory activities against COX-2 and other pain-related biological targets.

Materials and Methods

Chemistry

The melting points of the compounds were determined with a Bötius apparatus and are uncorrected and the elemental analysis with a PerkinElmer 2400 Series II

CHNS/O Elemental Analyzer (Waltham, MA, USA). The Fourier-transform infrared spectroscopy (FT-IR) spectra were registered in potassium bromide (KBr) pellets with a Bruker Vertex 70 spectrometer. The proton nuclear magnetic resonance (¹H-NMR) spectra were recorded on a Varian Gemini 300BB spectrometer, at 300 MHz, using deuterated dimethyl sulfoxide (DMSO-*d*₆) as solvent. The chemical shifts (δ) are reported in ppm related to the tetramethylsilane (TMS) internal standard and the coupling constants (*J*) are expressed in Hz. The abbreviations for multiplicities of signals are the following: s – singlet, d – doublet, t – triplet, m – multiplet. The mass spectra were acquired with an atmospheric pressure chemical ionization (APCI) mass spectrometer, using a protocol previously described for similar compounds [22].

Synthesis of new 2-(4-(4-bromophenylsulfonyl)phenyl)-4-arylidene-oxazol-5(*H*)-ones 2a–d

Into a round-bottomed flask a mixture of the 2-(4-(4-bromophenylsulfonyl)benzamido)acetic acid **1** [27] (15 mmol), aromatic aldehyde (15 mmol), sodium acetate (1.23 g, 15 mmol) and acetic anhydride (28.5 mL) were added. The mixture was refluxed for four hours. After cooling, cold ethanol was added to the mixture and was the flask left overnight, at 2°C. The resulted yellow precipitates were filtered off, washed alternatively with boiling water and cold ethanol. The new oxazolones were recrystallized from an ethanol–chloroform mixture (1:2, *v/v*).

Animals

Laboratory animals (NMRI adult female mice) were supplied by the rodent farm of Carol Davila University of Medicine and Pharmacy, Bucharest, Romania. All animals were housed in plexiglas cages, with *ad libitum* drinking water and food, in 12-hour light/dark cycle, at a temperature between 21°C and 24°C, and the relative humidity of 35–45%. The habituation of animals to the experimental environment has been done for five days.

By using an electronic hygro-thermometer, the temperature and relative humidity were daily monitored. The study was performed according to the Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes [28]. The Bioethics Commission of the Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest approved the experimental protocol (Approval No. 2267/05.02.2020).

Statistical analysis

The Microsoft Excel 2010 and GraphPad Prism software were used for the statistically results processing. The results were expressed as mean (M) \pm standard deviation (SD). The evaluation of Gaussian distribution was performed with D'Agostino & Pearson test ($n=9-10$) or Kolmogorov-Smirnov ($n=6$). For group comparisons, Student's *t*-test was used. The obtained results with $p<0.05$ were considered in our study to have statistical significance.

Acute oral toxicity study

The determination of acute toxicity used the *OECD Acute Toxic Class Method Guideline*. This method is a step-by-step procedure that uses a small number of animals, which allows substances to be placed in toxicity classes, depending on the presence or absence of animal lethality [29].

The animals were fasted four hours prior to treatment and two hours after, with free access to drinking water. A single dose of 300 mg/kg body weight (b.w.) of each tested substance (3% aqueous suspensions) was administered by gavage to three mice/substance. Another three mice (the control group) received 10 mL/kg b.w. of distilled water. After 48 hours, another 15 mice received the same treatment. According to the *Guideline*, due to the lack of lethality in the previous stages of the research, another three animals/substance received, by gavage, 2000 mg/kg b.w. of each substance (10% aqueous suspensions). Another control group ($n=3$) received 20 mL/kg b.w. of distilled water.

All mice were observed individually for any indications of toxic effects within the first four hours after dosing and daily thereafter, for a period of 14 days. The animals were weighed initially, seven days and 14 days after the beginning of the experiment. Behavioral pattern, changes in physical appearance, pain, signs of illness, injury, and mortality were recorded daily by visual observation.

At the end of the 14 days, the animals that received 2000 mg/kg b.w. and their control group were sacrificed, the brain, liver, lungs, heart, spleen and kidneys were removed and preserved in 10% neutral buffered formalin solution [30].

Histopathological assessment

The preserved organs (brain, liver, lungs, heart, spleen and kidneys) of 15 animals, stored in 10% neutral buffered formalin solution, have been prepared by the histological technique, sectioned and stained using the Hematoxylin-Eosin (HE) protocol. The histological sections were examined and photographed with an Olympus BX5 microscope, with the aim of observing HP aspects of interest [30].

Analgesic effect

Acetic acid-induced writhing test

Six groups ($n=10$) received the following treatments, by gavage: tested compounds 150 mg/kg b.w. (1.5% aqueous suspensions), diclofenac sodium 30 mg/kg b.w. (0.3% aqueous suspension) and 10 mL/kg b.w. distilled water. After one hour, all mice received intraperitoneally 0.6% acetic acid (10 mL/kg b.w.). Five minutes after acetic acid injection, writhings were counted for 20 minutes.

Hot plate test

For this test, seven groups were formed ($n=9-10$). Tested compounds were administered orally, 150 mg/kg b.w. (1.5% aqueous suspensions). Diclofenac sodium and tramadol hydrochloride, both 30 mg/kg b.w. (0.3% aqueous suspensions), were used as reference drugs, whereas the control group was treated with 10 mL/kg b.w. distilled water. Animals were placed on a hot plate (Ugo Basile, Italy) and lick time was evaluated at 53°C, initially and at one hour following the administration of the tested compounds [24].

Molecular docking protocol

Molecular docking simulations were performed in order to predict the binding affinities of the synthesized oxazol-5(4H)-one derivatives against molecular targets involved in pain and inflammation. Thus, crystal structures of COX-1 (PDB:5WBE), COX-2 (PDB:3LN1) isoforms, and transient receptor potential cation channel, subfamily A, member 1 (TRPA1) (PDB:3J9P) and TRPV1 (PDB:5IS0) calcium channels were retrieved from *Research Collaboratory for Structural Bioinformatics Protein Data Bank* (RCSB PDB) database and were hypothesized as potential targets for the tested oxazol-5(4H)-ones [31-34]. Both COX isoforms were taken into consideration in order to predict the selectivity on the inducible isoform. TRPA1 and TRPV1 pain receptors are calcium channels sensitive to noxious environmental stimuli and proton concentration variations [35-37].

The three-dimensional (3D) crystal structure of TRPA1 receptor was refined by adding missing residues, building missing loops and optimizing sidechain conformations, as reported in a previous paper [38]. Cocrystallized ligands and water molecules were removed and polar hydrogens were added to all the crystal structures which were thereafter energetically minimized using AMBER ff14SB force field with Chimera v.1.13.1 [39].

The ligand preparation methodology consisted in generating the 3D coordinates for the oxazol-5(4H)-one derivatives, polar hydrogens addition matching protonation states which correspond to the physiological pH and energy minimization with MMFF94s force field, using Open Babel v2.4.1 software [40]. The molecular docking study was carried out with PyRx v0.8 virtual screening interface [41], which runs AutoDock Vina v1.1.2 as a docking algorithm [42]. The experiment was performed with rigid residues and the searching space of the algorithm was set to include the known active binding sites of the proteins

and the docking results were retrieved as binding energy (ΔG), expressed as kcal/mol. The molecular docking algorithm was validated by docking TRPV1, COX-1 and COX-2 cocrystallized ligands (capsazepine, mofezolac and celecoxib) into their specific binding sites, as positive controls, in order to evaluate the superimposability between the predicted and experimentally determined conformations. Protein–ligand interactions were evaluated using BIOVIA Discovery Studio Visualizer (BIOVIA, Discovery Studio Visualizer, Version 17.2.0, Dassault Systèmes, 2016, San Diego, CA, USA).

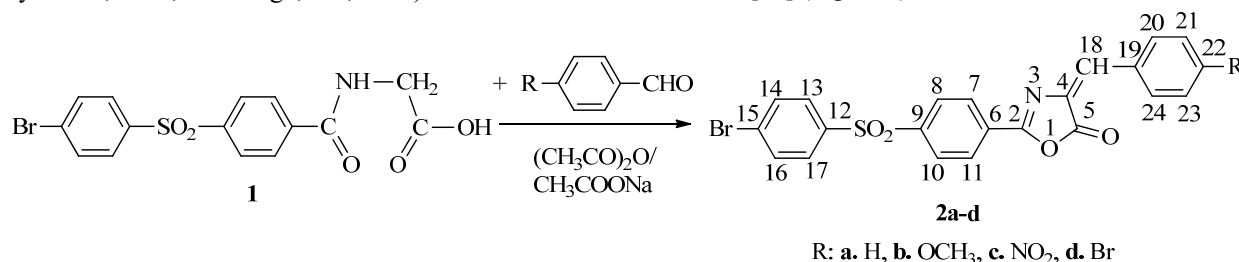


Figure 2 – The new 2-(4-(4-bromophenylsulfonyl)phenyl)-4-arylidene-oxazol-5(4H)-ones **2a–d** synthesis.

The chemical structures of new compounds were confirmed by FT-IR, ¹H-NMR, mass spectrometry (MS) spectra and elemental analysis.

2-(4-(4-bromophenylsulfonyl)phenyl)-4-benzylidene-oxazol-5(4H)-one 2a

Melting point (m.p.) 242–244°C; yield 37%; FT-IR (KBr, ν cm⁻¹): 3090, 3061, 3002, 1796, 1774, 1653, 1596, 1573, 1553, 1331, 1295, 1227, 1162, 613, 575; ¹H-NMR (DMSO-*d*₆, δ ppm, *J* Hz): 8.25–8.29 (m, 4H, H-7, H-11, H-20, H-24), 8.18 (d, 8.5, 2H, H-8, H-10), 7.96 (d, 8.4, 2H, H-13, H-17), 7.86 (d, 8.4, 2H, H-14, H-16), 7.5 (m, 2H, H-21, H-23), 7.41 (s, 1H, H-18), 7.51 (m, 1H, H-22); Anal. (%): Calcd. for C₂₂H₁₄BrNO₄S (468.32 g/mol): C, 56.42; H, 3.01; N, 2.99; S, 6.85. Found: C, 56.03; H, 2.67; N, 3.09; S, 6.81; APCI, *m/z* (%): 468 [⁷⁹Br M+H]⁺, 470 [⁸¹Br M+H]⁺.

2-(4-(4-bromophenylsulfonyl)phenyl)-4-(4-methoxybenzylidene)-oxazol-5(4H)-one 2b

m.p. 254–256°C; yield 56%; FT-IR (KBr, ν cm⁻¹): 3098, 3041, 3011, 2934, 2838, 1790, 1770, 1651, 1603, 1580, 1548, 1510, 1332, 1292, 1262, 1159, 613, 577; ¹H-NMR (DMSO-*d*₆, δ ppm, *J* Hz): 8.29 (d, 8.6, 2H, H-7, H-11), 8.29 (d, 8.4, 2H, H-20, H-24), 8.18 (d, 8.6, 2H, H-8, H-10), 7.94 (d, 8.6, 2H, H-13, H-17), 7.87 (d, 8.6, 2H, H-14, H-16), 7.42 (s, 1H, H-18), 7.11 (d, 8.4, 2H, H-21, H-23), 3.86 (s, OCH₃); Anal. (%): Calcd. for C₂₃H₁₆BrNO₅S (498.35 g/mol): C, 55.43; H, 3.24; N, 2.81; S, 6.43. Found: C, 55.51; H, 3.33; N, 2.85; S, 6.63; APCI, *m/z* (%): 498 [⁷⁹Br M+H]⁺, 500 [⁸¹Br M+H]⁺.

2-(4-(4-bromophenylsulfonyl)phenyl)-4-(4-nitrobenzylidene)-oxazol-5(4H)-one 2c

m.p. 284–287°C; yield 66%; FT-IR (KBr, ν cm⁻¹): 3090, 3073, 3042, 1795, 1769, 1657, 1597, 1572, 1552, 1519, 1347, 1290, 1227, 1158, 613, 574; ¹H-NMR (DMSO-*d*₆, δ ppm, *J* Hz): 8.52 (d, 8.5, 2H, H-21, H-23), 8.37 (d, 8.4, 2H, H-7, H-11), 8.35 (d, 8.5, 2H, H-20, H-24), 8.21 (d, 8.4, 2H, H-8, H-10), 7.95 (d, 8.4, 2H, H-13, H-17), 7.87 (d, 8.4, 2H, H-14, H-16), 7.57 (s, 1H, H-18); Anal. (%): Calcd. for C₂₂H₁₃BrN₂O₆S (513.32 g/mol): C, 51.48;

Results

Chemistry

The new 2-(4-(4-bromophenylsulfonyl)phenyl)-4-arylidene-oxazol-5(4H)-ones **2a–d** resulted through the simultaneous cyclo-condensation of the 2-(4-(4-bromophenylsulfonyl)benzamido)-acetic acid **1** with benzaldehyde, 4-methoxy-, 4-nitro- or 4-bromobenzaldehyde, in the presence of acetic anhydride and anhydrous sodium acetate. The intermediate acid **1** was obtained according to literature [27] (Figure 2).

H, 2.55; N, 5.46; S, 6.25. Found: C, 51.31; H, 2.51; N, 5.58; S, 6.50; APCI, *m/z* (%): 513 [⁷⁹Br M+H]⁺, 515 [⁸¹Br M+H]⁺.

2-(4-(4-bromophenylsulfonyl)phenyl)-4-(4-bromobenzylidene)-oxazol-5(4H)-one 2d

m.p. 275–277°C; yield 64%; FT-IR (KBr, ν cm⁻¹): 3090, 3063, 3043, 1794, 1773, 1653, 1575, 1547, 1328, 1291, 1161, 1229, 614, 574; ¹H-NMR (DMSO-*d*₆, δ ppm, *J* Hz): 8.32 (d, 8.5, 2H, H-7, H-11), 8.03 (d, 8.5, 2H, H-8, H-10), 8.02 (d, 8.5, 2H, H-20, H-24), 7.80 (d, 8.5, 2H, H-14, H-16), 7.70 (d, 8.5, 2H, H-13, H-17), 7.60 (d, 8.5, 2H, H-21, H-23), 7.20 (s, 1H, H-18); Anal. (%): Calcd. for C₂₂H₁₃Br₂NO₄S (547.22 g/mol): C, 48.29; H, 2.39; N, 2.56; S, 5.86. Found: C, 48.39; H, 2.15; N, 2.66; S, 5.80; APCI, *m/z* (%): 546 [⁷⁹Br M+H]⁺, 548 [⁸¹Br M+H]⁺.

Acute oral toxicity study

There was no lethal effect following the administration by gavage of the tested substances (300 mg/kg b.w. and 2000 mg/kg b.w., respectively). Body weight increased similar to the untreated animals (Figure 3). For all groups, no changes in the external appearance or motor behavior of the animals were recorded.

Histopathological assessment

The HP assessment of some preserved organs (brain, liver, lungs, heart, spleen, and kidneys) collected from mice did not reveal any cytohistopathological aspects that can be linked to any inflammatory, neoplastic or cytotoxic process. Also, no significant differences concerning lesional or topostructural elements between the control group and the groups treated with compounds **2a–d** were observed, thus demonstrating the low toxicity of the new compounds. Some images of the organs of mice treated with new compounds are presented in the Figures 4–9.

Analgesic effect

Acetic acid-induced writhing test

In the writhing test, the lowest number of writhings was observed for the animals that received the reference substance, diclofenac 30 mg/kg b.w. (–65.31% vs. control

group, $p < 0.001$), a non-steroidal anti-inflammatory, non-selective COX inhibitor. All four compounds decreased the number of writhings, when compared to the control group. This decrease was statistically significant, according to the Student's *t*-test, only for oxazolone **2b** (-41.98%, $p < 0.05$) (Table 1).

Hot plate test

For the hot plate test, the results showed an increased licking time, one hour after the administration of the substances, only for the animals from the groups that received oxazolone **2a** (19.39%), diclofenac (24.75%) and tramadol (42.29%, $p < 0.001$) (Table 2).

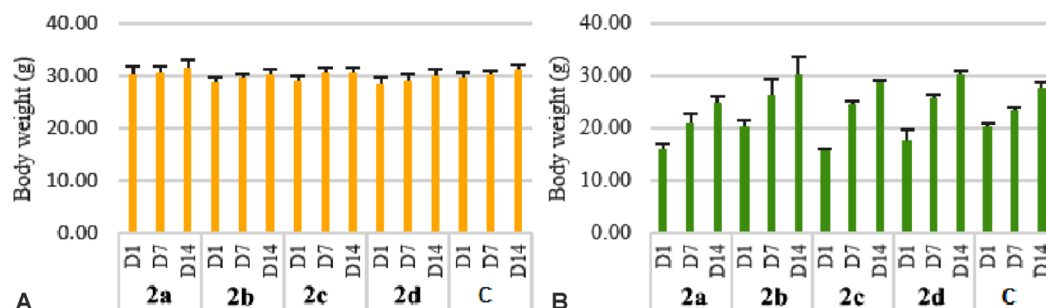


Figure 3 – Average change in body weight (b.w.) of animals treated by gavage, with the tested substances: (A) Stage I, 300 mg/kg b.w.; (B) Stage II, 2000 mg/kg b.w.

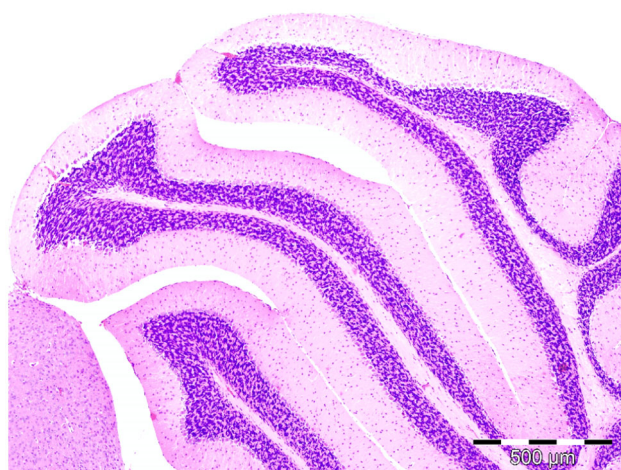


Figure 4 – Cerebellum. Normal appearance of the topographical elements which form the cerebellar hemispheres. Mouse treated with compound **2a**. HE staining, $\times 40$.

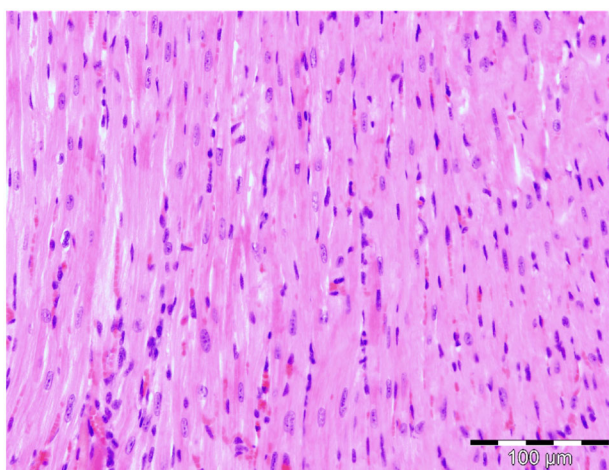


Figure 5 – Heart. Normal aspects of cardiomyocytes in the left ventricle. Mouse treated with compound **2b**. HE staining, $\times 200$.

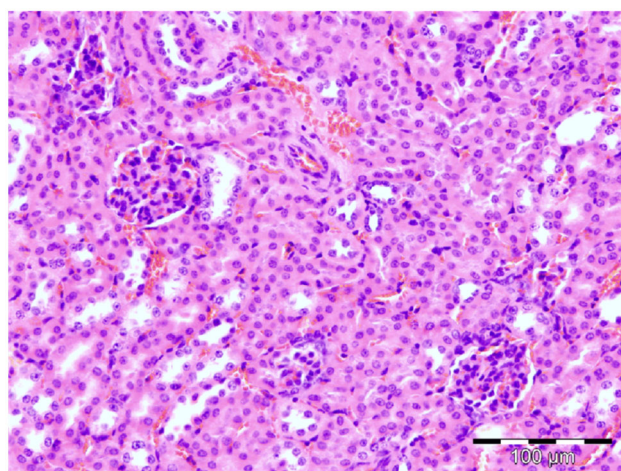


Figure 6 – Kidneys. Normal aspects of the proximal renal tubules; hyperemia in the mesangial and interstitial capillaries. Similar aspects with control group and compound **2a**. Mouse treated with compound **2b**. HE staining, $\times 200$.

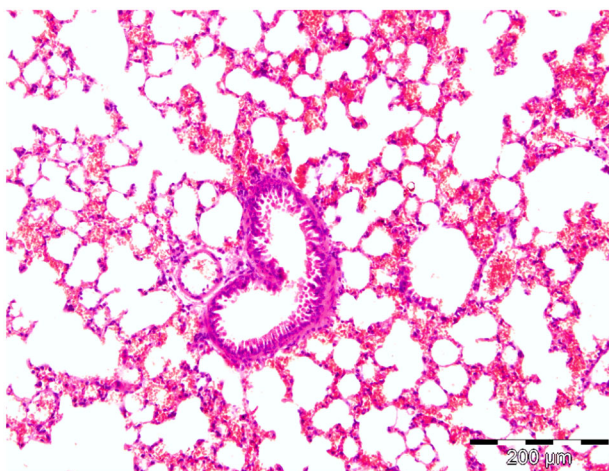


Figure 7 – Lungs. Histological modifications similar to the control group and compounds **2a**, **2b** and **2c**; hyperemia and microhemorrhages in the interstitial areas and in the interalveolar spaces. Mouse treated with compound **2d**. HE staining, $\times 100$.

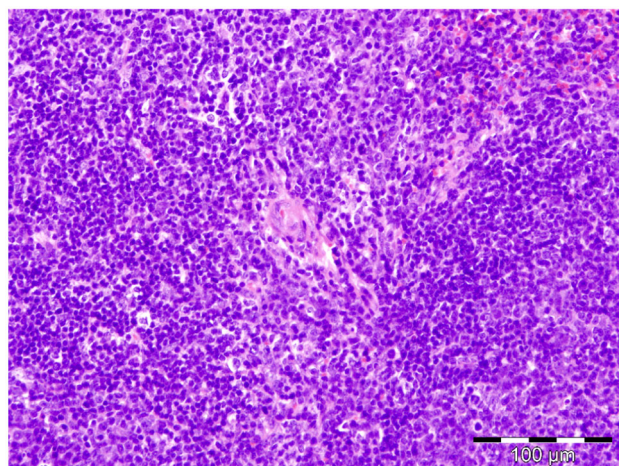


Figure 8 – Spleen. Lymphoid tissue well represented in the lymphocyte sheaths and in the paracortical areas. No toxic changes. Mouse treated with compound 2c. HE staining, $\times 200$.

Table 1 – Acetic acid-induced writhing test: analgesic effect [%] vs. control group for the tested compounds 2a–d

Group	M \pm SD [%]	$\Delta\%$ vs. control
Control	34.3 \pm 11.24	–
Diclofenac sodium	11.9 \pm 10.44	-65.31***
2a	26.1 \pm 9.05	-23.91
2b	19.9 \pm 10.99	-41.98*
2c	25.2 \pm 12.96	-26.53
2d	32.4 \pm 7.3	-5.54

M: Mean; SD: Standard deviation; * $p < 0.05$, *** $p < 0.001$ – Student's *t*-test.

Table 2 – Hot plate test: analgesic effect [%] vs. initial for the tested compounds 2a–d

Group	Lick time (initial) M \pm SD [s]	Lick time (at 1 hour) M \pm SD [s]	$\Delta\%$ vs. initial
Control	10.35 \pm 2.41	9.94 \pm 2.73	-3.96
Diclofenac sodium	10.06 \pm 2.66	12.55 \pm 4.62	24.75
Tramadol hydrochloride	10.01 \pm 2.71	14.24 \pm 3.25	42.29***
2a	10.11 \pm 2.85	12.07 \pm 3.21	19.39
2b	10.19 \pm 3.55	10.26 \pm 2.94	0.69
2c	10.17 \pm 2.55	9.88 \pm 1.88	-2.84
2d	10.39 \pm 1.52	10.22 \pm 3.06	-1.6

M: Mean; SD: Standard deviation; *** $p < 0.001$ – Student's *t*-test.

Molecular docking

A molecular docking study was performed in order to establish possible molecular mechanisms of action for oxazol-5(4*H*)-one derivatives. The 3D structures of oxazol-5(4*H*)-ones were docked against COX-1, COX-2 enzymes and TRPA1, TRPV1 calcium channels. The docking protocol was successfully validated by analyzing the superimposition between the conformations of docked positive controls and the cocrystallized complex, the differences between conformers being negligible. The binding energies for each compound are shown in Table 3.

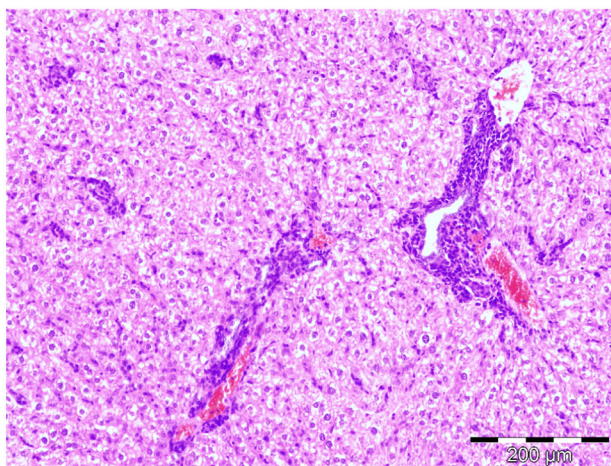


Figure 9 – Liver. Capillaries and venules with peri-vascular lymphoid infiltrations; hepatocyte vacuolar degeneration of higher intensity compared to control group and compounds 2a, 2b and 2c. Mouse treated with compound 2d. HE staining, $\times 100$.

Table 3 – Molecular docking results expressed as binding energies for compounds 2a–d and positive controls

Compound	Binding energy [kcal/mol]			
	COX-1	COX-2	TRPA1	TRPV1
2a	-8.6	-3.4	-8.8	-9.3
2b	-8.1	-3.6	-8.6	-9.6
2c	-8.5	-3.7	-8.6	-9.6
2d	-8.1	-3.1	-8.4	-9.3
Mofezolac	-7.5	–	–	–
Celecoxib	–	-12	–	–
HC-030031	–	–	-7.4	–
A-967079	–	–	-8.1	–
Capsazepine	–	–	–	-7.8

COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2; TRPA1: Transient receptor potential cation channel, subfamily A, member 1; TRPV1: Transient receptor potential cation channel subfamily V member 1.

Interactions between compound **2b** and TRPA1 and TRPV1 calcium channels were chosen for further discussion due to their high binding affinities. The interacting forces between **2b** and TRPA1 are depicted in Figure 10.

Compound **2b** bound to the HC-030031 specific binding site and formed hydrogen bonds between oxazolone moiety and THR869 and GLN968 residues, respectively. Moreover, another hydrogen bond was formed between the sulfone group and ASP1037. The protein–ligand complex is further stabilized by weak hydrophobic interactions, such as amide– π stacked interactions between phenyl moiety and GLN968, π –anion interaction between oxazolone and ASP963, alkyl and π –alkyl interactions with ALA965, LYS969 and PRO1034, and van der Waals interactions with several residues from the binding site. Notably, **2b** interacts with ARG872 residue from the transmembrane 5 domain through van der Waals forces, which is considered as a relevant residue for TRPA1 inhibition [33].

The docked conformation and protein–ligand interactions between **2b** and TRPV1 are shown in Figure 11.

Oxazol-5(4*H*)-one derivative **2b** formed one carbon–hydrogen bond between the methoxy group and GLU570, a weak π – σ interaction between the phenyl moiety and ALA665, and between the oxazolone substructure and LEU515, alkyl and π -alkyl interactions with LEU553,

ALA566 and ILE668, a π -sulfur interaction with MET547 and van der Waals interactions with other residues situated in the binding pocket. Moreover, it was previously reported that the interacting residue GLU570 from the active site is involved in TRPV1 activation [34].

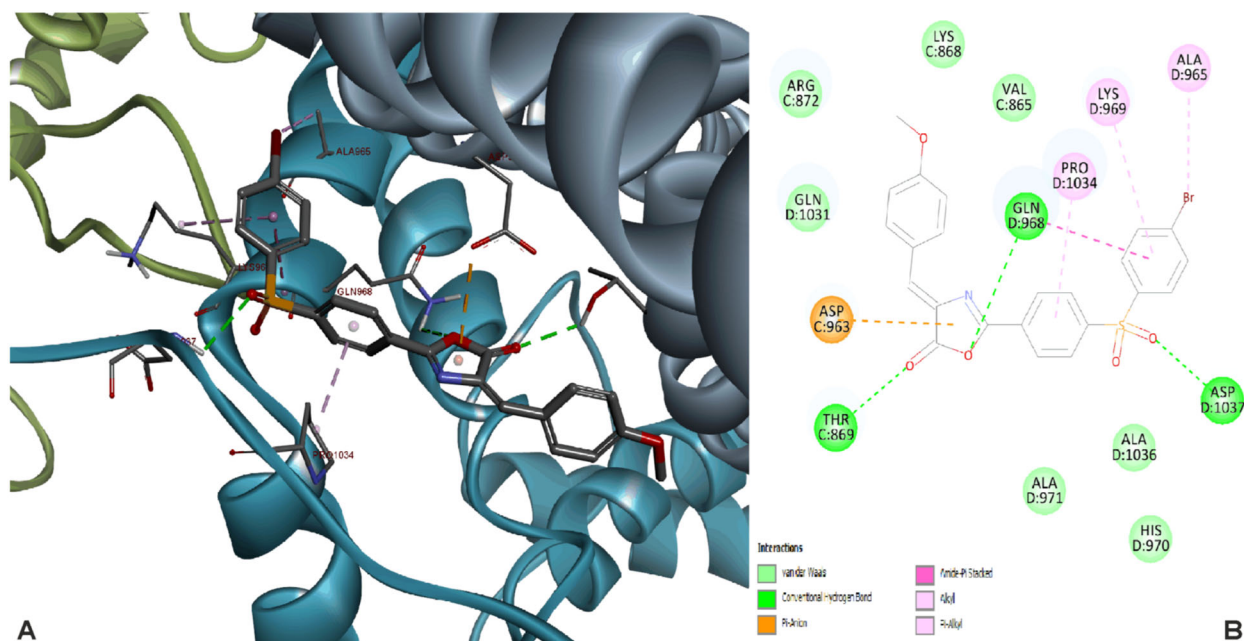


Figure 10 – Three-dimensional docked conformation of the protein–ligand complex (A) and two-dimensional depiction of molecular interactions between compound **2b** and TRPA1 crystal structure (B). TRPA1: Transient receptor potential cation channel, subfamily A, member 1.

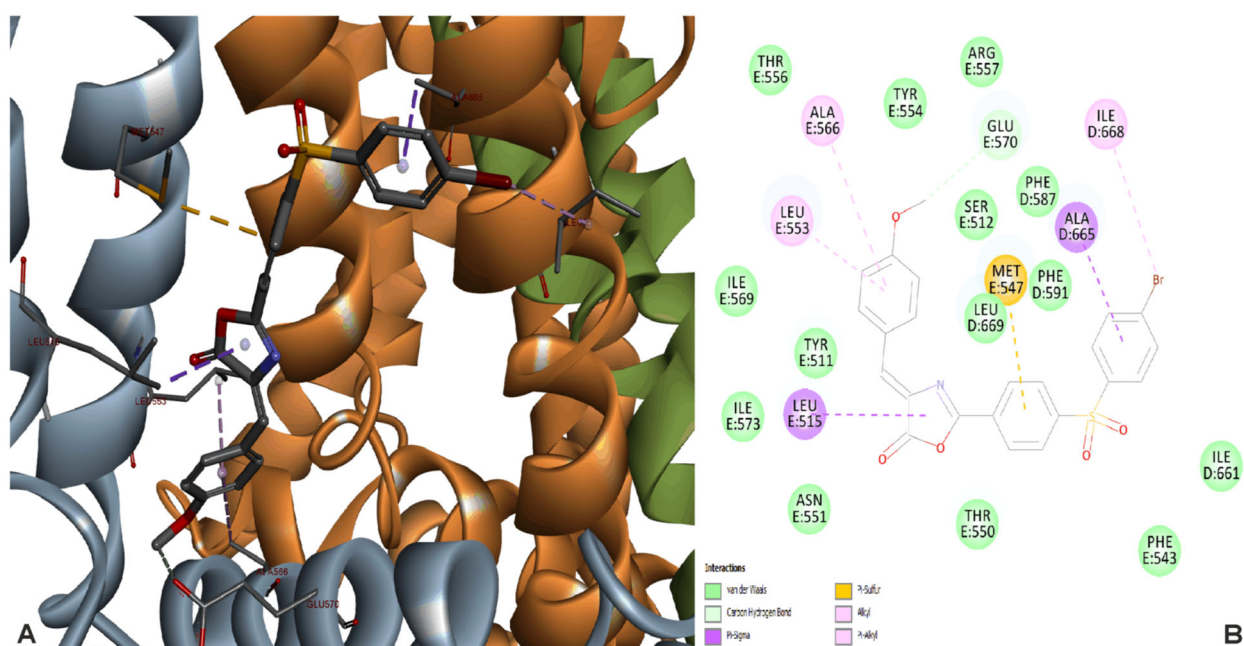


Figure 11 – Three-dimensional docked conformation of the protein–ligand complex (A) and two-dimensional depiction of molecular interactions between compound **2b** and TRPV1 crystal structure (B). TRPV1: Transient receptor potential cation channel subfamily V member 1.

Discussions

Chemistry

The first argument that confirms the structure of oxazolones is the disappearance of the NH absorption band from the IR spectra of the new compounds that was present at 3341 cm^{-1} in the spectrum of precursor **1** [27].

The absorption bands due to the stretching vibration $\nu_{\text{C=O}}$ from 1794–1796 cm^{-1} and 1769–1774 cm^{-1} range correspond to the C=O lactonic group, and are similar to others reported for this class of heterocycles [22, 43]. In the ^1H -NMR spectra of new compounds the absence of the NH signal (8.20 ppm) from acid **1** [27] and the appearance of a new singlet characteristic to =CH–

benzylidene proton with δ 7.20–7.57 ppm is the main proof that the condensation reaction of the aromatic aldehydes with *N*-acylated glycine **1** occurred.

In the mass spectra, the molecular ions that contain ^{79}Br and ^{81}Br isotopes correspond to the molecular mass of the new compounds.

Acute oral toxicity study

The determination of the oral toxicity after a single dose, was performed according to the *OECD 423 Guideline*. The study consisted in the administration of a dose of 300 mg/kg b.w. to a group of three animals. The test was repeated with the same dose and the same number of animals only if no lethality was recorded. In the end, if no lethality was recorded, then a dose of 2000 mg/kg b.w. was given to a group of three animals. The tested substances were placed in the Category 5 (LD_{50} 2000–5000 mg/kg b.w.) because the toxicity and mortality at doses of 300 mg/kg b.w. and 2000 mg/kg b.w. were absent [29].

Histopathological assessment

The anatomical-clinical and HP aspects were assessed according to the current protocols. The studied organ sections were compared to the control group sections.

In all the examined cases, the most relevant cytohistopathological changes were highlighted at microvascular and cellular level, in the form of circulatory or degenerative changes, with a low intensity, zonal location and no pathological significance was observed when compared to the control group.

The highest frequency of circulatory changes in the form of hyperemia and interstitial hemorrhagic microfoci was found in the lungs and kidneys (Figures 6 and 7).

The cellular degeneration was characterized by focal infiltration with very low intensity, having hepatocellular localization in Kiernan spaces or in perivenous spaces. In only one case, in mice treated with compound **2d**, there was an increase in the intensity and distribution of degenerative liver lesions (Figure 9).

The results of the HP examinations did not reveal significant differences regarding the intensity and distribution of the cytohistopathological changes at the level of all the organs examined between the cases from the experimental groups and the cases from the control group.

Compared to the control group, the intensity and distribution of cytohistopathological lesions registered a slight increase (2% of the examined cases), in the case of the group treated with **2d**.

The studies regarding to the toxic effects of the heterocycles from benzylidene-oxazolones class are poorly represented in the literature data. The study regarding to acute toxicity in mice and HP evaluation of some benzylidene-oxazol-5(4*H*)-ones with antidiabetic potential reported by Mariappan *et al.* indicated a mild toxicity of these. The HP assessment of an oxazolone derivative [4-(3-chlorobenzylidene)-2-phenyl-oxazol-5-one] showed a slightly elongation of sinusoids at hepatic level and a minor destruction of tubular cells and glomeruli of kidneys. Nevertheless, there were no changes of the cellular structures in the cardiac tissue [44].

Analgesic effect

Non-clinical studies are commonly used to determine the therapeutic potential of xenobiotics [45, 46]. The analgesic effect was quantified by two pharmacological tests, widely used to investigate the analgesic action of xenobiotics: acetic acid-induced writhing test and hot plate test. The hot plate test evaluates the reflexes of the animals after paws contact with a heated surface. This method selectively detects the central analgesic action [47]. In the writhing test, acetic acid, an irritating substance is used to induce visceral pain. Substances with peripheral analgesic action, such as nonsteroidal anti-inflammatory drugs, decrease the number of contortions induced in laboratory animals [48].

Acetic acid-induced writhing test

By using this method, the peripheral analgesic action is detected, mediated by endogenous substances, such as arachidonic acid metabolites and prostaglandins, following the COX pathway [49]. All four compounds decreased the number of writhing, when compared to the control group, with oxazolone **2b** (–41.98%, $p < 0.05$) having the most intense analgesic effect. For this reason, we can say that a possible mechanism of action of the tested compounds could be the COX inhibition.

Hot plate test

In the hot plate test, tramadol, an opioid analgesic, had the most intense analgesic effect (42.29%, $p < 0.001$). As expected, the analgesic effect of diclofenac has no statistical significance because this test is selective for central action analgesics. The only tested substance that an increased licking time was oxazolone **2a** (19.39%, $p > 0.05$). According to these results, the tested compounds **2a–d** do not exhibit central analgesic action, after oral administration of 150 mg/kg b.w.

Molecular docking simulations

None of the oxazol-5(4*H*)-ones exhibited satisfactory binding affinities for COX-2 isoform, showing binding energies between –3.7 kcal/mol and –3.1 kcal/mol, while celecoxib bound to the enzyme with an energy equal to –12 kcal/mol. Thus, inhibition of COX-2 as a possible analgesic and anti-inflammatory mechanism of action is unlikely. However, all four ligands showed higher binding affinities for COX-1 isoform than mofezolac, but these results are not relevant for explaining a possible mechanism of analgesic action.

All tested compounds showed high binding affinities for both TRPA1 and TRPV1 receptors. Moreover, the predicted binding energies were lower than those observed for the positive controls in all cases, suggesting that the oxazol-5(4*H*)-ones could have greater potencies than “golden standard” inhibitors HC-030031 and A-967079 for TRPA1, and than capsaizepine for TRPV1, respectively. However, only oxazolone **2b** exhibited an analgesic effect in the mouse writhing test, implying that in reality, if the compounds would indeed inhibit TRPA1 and TRPV1, the differences in binding affinities would be much greater than those predicted with the docking algorithm. TRPA1 and TRPV1 antagonism are plausible molecular mechanisms for **2b** since both receptors are stimulated by protons,

which in our experiment were generated by acetic acid ionization in physiological medium after intraperitoneal injection. Moreover, TRPA1 is also activated by reactive oxygen species, which can be generated during the inflammatory process triggered by acetic acid [50].

Conclusions

New 2-(4-(4-bromophenylsulfonyl)phenyl)-4-arylidene-oxazol-5(4H)-ones have been synthesized and tested for their analgesic activity. The acute oral toxicity study indicated that compounds had no lethal effect and the HP assessment of the preserved organs collected from mice did not reveal any cytohistopathological aspects that can be linked to any inflammatory, neoplastic or cytotoxic process, demonstrating the low toxicity of new compounds. In the writhing test, the best analgesic activity was observed for compound **2b** that has a methoxy group in the molecule. A possible mechanism of action of these compounds could be the antagonism of TRPA1 or TRPV1 calcium channels, rather than COX inhibition, as indicated by the molecular docking simulations. However, further studies need to be performed for the evaluation of the analgesic and anti-inflammatory activities and for the determination of the mechanism of action.

Conflict of interests

The authors declare that they have no conflict of interests.

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