Synthesis of oxalic acid derivatives and their antitumor activity in experiments *in vivo*

B. S. Fedorov,† *S. A. Goncharova, T. A. Rajevskaya,*★ *A. B. Eremeev, A. N. Utenyshev, M. A. Fadeev, G. N. Bogdanov,*† *N. P. Konovalova, and S. M. Aldoshin*

Institute of Problems of Chemical Physics, Russian Academy of Sciences, 1 prosp. Akad. Semenova, 142432 Chernogolovka, Moscow Region, Russian Federation. Fax: +7 (496) 522 3507. E-mail: tara@icp.ac.ru

A series of amino acid derivatives of oxalic acid were obtained. In combination therapy with conventional cytostatics used in lower doses, the new compounds substantially increase the efficacy of the drugs in the treatment of experimental P388 murine leukemia and some its drug-resistant strains.

Key words: hydroxamic acids, anticancer drugs, adjuvant effect, combination therapy, murine leukemia, drug-resistance, *in vivo*.

Anticancer drugs are known to have severe side effects on normal tissues and organs due to their high toxicity, resulting in that the patients, as a rule, cannot undergo the complete course of treatment. Therefore, of particular interest are attempts to reduce the toxic effect of conventional cytostatics by using low-toxic adjuvant compounds based on metabolically active substances, thereby significantly reducing therapeutic doses of cytostatics (and, consequently, decreasing their toxicity) with retention of efficacy.

Over the years, our research interest has focused on the synthesis of dicarboxylic acid derivatives and evaluation of their antitumor activity.[1,2] We have prepared[3] oxalic, tartaric, aspartic, and maleic acid hydroxamides. These compounds, when used as chemosensitizers in combination therapy of transplanted tumors with cisplatin or cyclophosphamide, completely inhibit B-16 melanoma metastasis and metastatic Lewis lung carcinoma, resulting in 100% survival of experimental leukemic animals. We believe that these compounds can also be effective sensitizers of conventional anticancer drugs in drug-resistant tumors.

Drug resistance is a serious impediment to cancer chemotherapy because it is developed in response to the treatment with drugs, to which cancer cells were highly sensitive at the beginning of the treatment.[4] In the clinics, the diversity of tumors relapsing after the treatment with different drugs is due particularly to outgrowth of drug-resistant cell clones. Currently, there are no drugs, including the latest and targeted pharmaceuticals, to which no resistance is developed.[5]

There are two main types of drug resistance — drug resistance (DR) and multidrug resistance (MDR). Drug resistance is acquired in response to alkylating agents and antimetabolites and is associated with the mechanisms of their action. Multidrug resistance arises simultaneously against numerous drugs, mainly of natural origin (anthracycline antibiotics, vinca alkaloids, epipodophyllotoxins, taxol, actinomycin D, colchicine, *etc.*), in addition to a single drug initially administered.[6] The development of MDR is not related to the mechanisms of action of drugs but is attributed to amplification (or overexpression) of closely related genes (mdr) encoding transmembrane glycoprotein P-gp that mediates energy-dependent efflux from the cells particularly of those agents, against which resistance is developed.

Many details of the mechanisms of DR and MDR have been elucidated. Although these data are helpful in finding ways to overcome these types of drug resistance,[7] there are no strategies for reversing DR and MDR. Therefore, the development of new treatments of cancer cells with acquired DR or MDR is a challenging task that has received great attention in cancer research.

In this study, we examined the possibility of using oxalic acid derivatives to increase the efficacy of conventional cytostatics applied in lower doses in experimental P388 leukemia and its drug-resistant strains *in vivo*.

Results and Discussion

The goal of this study was to synthesize new oxalic acid derivatives of the general formula RC(O)—C(O)R, where R = —$NHCH_2C(O)OMe$ (**1**) or —NHCH(Me)C(O)OMe (**2**) and also (2,2,6,6-tetramethylpiperidin-4-yl)amino (**3**). The method for the synthesis of compounds **1**—**3** is based on the treatment of oxalyl dichloride with amino acid methyl ester followed by the separation of oxalylbis(amino acid) dimethyl ester and its subsequent treatment with an

† Deceased.

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 4, pp. 0694—0699, April, 2018.

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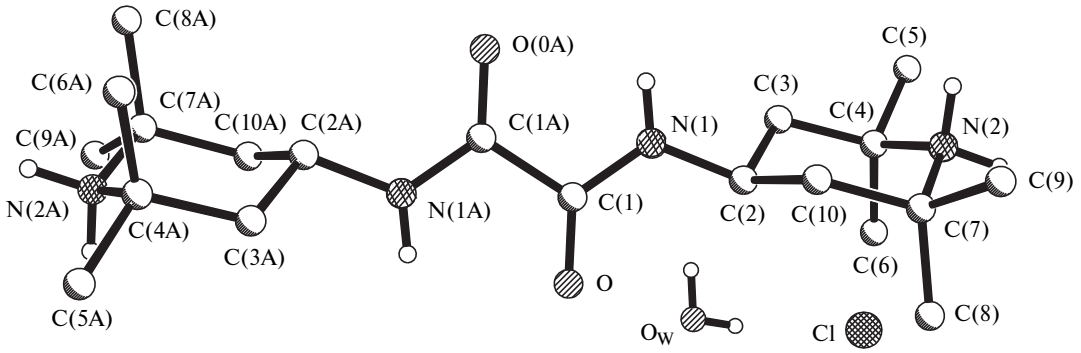

Fig. 1. Overall view of the molecular structure of compound **3** (hydrogen atoms are omitted).

aqueous sodium hydroxide solution in methanol. The target product was isolated by filtration. It should be noted that the use of sodium salts ensures good solubility of the compounds in water, which is necessary for their administration to animals as aqueous solutions.

Compound **3** was prepared as a dihydrochloride salt. The nitrogen atoms N(2) and N(2A) are protonated (Fig. 1). The bond lengths between the N atom and carbons are 1.528 Å (N(2)—C(4)) and 1.525 Å (N(2)—C(7)). The nitrogen-containing heterocycle adopts a chair conformation. The angles between the plane of the central part of the ring formed by the C(3)C(10)C(4)C(6) atoms and the planes through the C(3)C(10)C(2) and C(4)C(7)N(2) atoms are 48.1° and 49.1°, respectively. The bond lengths in the central moiety of molecule **3** are as follows: C(1)—C(1A), 1.541 Å; C(1)—N(1), 1.337 Å; C(1)—O, 1.229 Å; N(1)—C(2), 1.456 Å. The crystal structure of compound **3** contains a water molecule of crystallization, the oxygen atom of which is involved in an intermolecular N—H...O hydrogen bond having the following parameters: HN...O_W(A), 2.261 Å; N(1)...O_W(A), 2.997 Å; N(1) HNO_W(A), 143.7°. The hydrogen atoms of the water molecule of crystallization form contacts with chloride anions: H_W(1A)...Cl(A) = 2.536 Å and H_W(2A)...Cl(B) = = 2.448 Å (Fig. 2).

We examined the possibility of using the synthesized oxalic acid derivatives to increase the efficacy of conventional cytostatics, when applied in lower doses, in experimental P388 leukemia and its drug-resistant strains *in vivo*.

The evaluation of acute toxicity of the new compounds in mice demonstrated that compounds **1** and **2** have low toxicity. The administration in increasing doses up to 1250 mg kg^{-1} did not lead to death of animals. In this case, the therapeutic dose was taken as 400 mg kg^{-1}. For compound **3**, LD_{50} was 138 mg kg^{-1} and the therapeutic dose was 46 mg kg^{-1}.

In combination therapy experiments, the synthesized compounds were combined with conventional cytostatics, the doses of the latter being substantially decreased compared to their therapeutic doses: fivefold for cyclophosphan (CP), 6.8-fold for cisplatin (cPt), 1.5-fold for mitomycin C (MitC), sixfold for etoposide, and fourfold for doxorubicin (DOX). The use of lower doses of anticancer drugs is essential in order to reduce their high toxicity, which often requires treatment interruption much earlier than necessary.

The results of evaluation of the efficacy of compound **2** in the initial P388 tumor (drug-sensitive) and in a number of its drug-resistant strains are summarized in Table 1.

As can be seen in Table 1, the use of compound **2** in the initial P388 strain led to a 109% and 110% increase in median life span (ILS), when used in combination with cisplatin and cyclophosphan, respectively; with etoposide, the increase in median life span was 137%. In combination with etoposide, the survival rate of animals increased from 17% to 50%. In the P388/cPt strain, compound **2** caused a 85% increase in the efficacy of etoposide; in the P388/CP strain, the efficacy of doxorubicin (DOX) was enhanced by 61%.

The data on the effect of compound **3** on the efficacy of therapy with conventional cytostatics of drug-resistant strains of P388 leukemia are given in Table 2.

As can be seen in Table 2, compound **3** did not have intrinsic antitumor activity against DR and MDR strains. However, compound **3** can, in some cases, increase the efficacy of conventional cytostatics. For instance, in the P388/rub strain compound **3** enhanced the efficacy of mitomycin C by 64%. In the P388/CP strain, the use of compound **3** in combination with etoposide resulted in a 43% increase in ILS. In the P388/cPt strain, compound **3** enhanced the efficacy of etoposide by 48%.

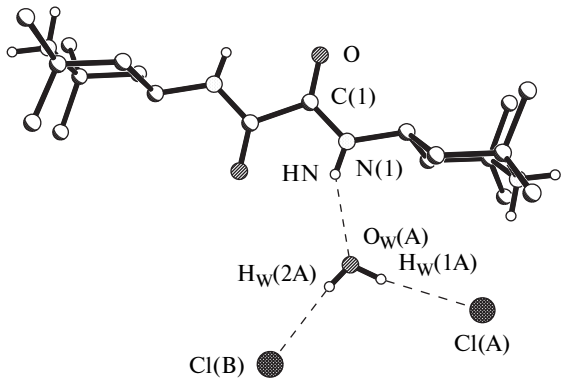

Fig. 2. System of hydrogen bonds formed by a water molecule of crystallization in the crystal of compound **3**.

Table 1. Efficacy of compound **2** in P388 leukemia and its drug-resistant strains *in vivo* in combination therapy with conventional cytostatics used in lower doses

Agent	Dose /mg kg^{-1}	Dosage regimen (days after transplantation)	Number of survivals (%)	ILS (%)
		P388		
2	400	1—7	0	28
cPt	0.6	1—7	0	169
CP	20	1, 6	0	163
DOX	1.0	1, 7	0	145
Etoposide	2.5	1, 5, 9	17	273
MitC	1.0	1, 6	0	126
2 + cPt	400 + 0.6	(1—7) + (1—7)	0	278
2 + CP	400 + 20	(1—7) + (1, 6)	17	273
2 + DOX	400 + 1.0	(1—7) + (1, 7)	0	96
2 + etoposide	400 +2.5	(1—7) + (1, 5, 9)	50	410
2 + MitC	400 + 1.0	(1—7) + (1, 6)	0	108
Control	—	—	0	—
		P388/CP		
2	400	1—7	0	7
cPt	0.6	1—7	0	32
DOX	1.0	1, 7	0	89
Etoposide	2.5	1, 5, 9	0	98
MitC	1.0	1, 6	0	62
2 + cPt	400 + 0.6	(1—7) + (1—7)	0	32
2 + DOX	400 + 1.0	(1—7) + (1, 7)	17	150
2 + etoposide	400 + 2.5	(1—7) + (1, 5, 9)	0	128
2 + MitC	400 + 1.0	(1—7) + (1, 6)	0	75
Control	—	—	0	—
		P38 8/cPt		
2	400	1—7	0	0
CP	20	1, 6	0	3
DOX	1,0	1, 7	0	88
Etoposide	2.5	1, 5, 9	17	200
MitC	1.0	1, 6	0	19
2 + CP	400 + 20	(1—7) + (1, 6)	0	5
2 + DOX	400 + 1.0	(1—7) + (1, 7)	50	90
2 + etoposide	400 + 2.5	(1—7)+(1, 5, 9)	0	285
2 + MitC	400 + 1.0	(1—7) + (1, 6)	0	14
Control	—	—	0	—
		P388/rub		
2	400	1—7	0	0
cPt	0.6	1—7	0	143
CP	20	1, 6	17	230
MitC	1.0	1, 6	0	174
2 + cPt	400 + 0.6	(1—7)+(1—7)	0	101
2 + CP	400 + 20	(1—7) + (1, 6)	0	126
2 + MitC	400 + 1	(1—7) + (1, 6)	0	100
Control	—	—	0	—

Compound **1** was tested in four drug-resistant strains of P388 leukemia *in vivo* (Table 3).

As can be seen in Table 3, the monotherapy with compound **1** was in general inefficient, but in some cases compound **1** somewhat enhanced the efficacy of conventional cytostatics.

Therefore, no one of the tested compounds had intrinsic antitumor activity in all the tumors used. However the synthesized compounds, when used in combination therapy with known cytostatics in very low doses, had, in some cases, significant adjuvant effect. For instance, compound **2** in the initial P388 strain increased the efficacy of cPt, CP, and etoposide by more than 100%. Besides, in combination with etoposide, the number of surviving animals increased from 17% to 50%. In two drug-resistant strains, the adjuvant effect was observed with the use of compound **2**. Thus, in the P388/cPt strain, compound **2** enhanced the efficacy of etoposide by 85%, and it caused

Table 2. Efficacy of compound **3** in drug-resistant strains of P388 leukemia *in vivo* in combination therapy with conventional cytostatics used in lower doses

Agent	Dose /mg kg^{-1}	Dosage regimen (days after transplantation)	Number of survivals (%)	ILS (%)
		P388/vcr		
3	46	1—7	0	0
CP	20	1, 5	0	87
cPt	0.6	1—7	0	90
MitC	1	1, 6	0	82
3 + CP	46 + 20	(1—7) + (1, 5)	0	102
3 + cPt	46 + 0.6	(1—7) + (1—7)	0	91
3 + MitC	46 + 1	(1—7) + (1, 6)	0	88
Control	—	—	0	—
		P388/rub		
3	46	1—7	0	4
CP	20	1, 5	0	119
cPt	0.6	1—7	0	176
Mit.C	1	1, 6	0	117
3 + CP	46 + 20	(1—7) + (1, 5)	0	121
3 + cPt	46 + 0.6	(1—7) + (1—7)	0	179
3 + MitC	46 + 1	(1—7) + (1, 6)	0	181
Control	—	—	0	—
		P388/CP		
3	46	1—7	0	1
cPt	0.6	1—7	0	36
DOX	1.0	1, 7	50	203
Etoposide	2.5	1, 5, 9	0	112
MitC	1	1, 6	0	64
3 + cPt	46 + 0.6	(1—7) + (1—7)	0	69
3 + DOX	46 + 1	(1—7) + (1, 7)	17	108
3 + etoposide	46 + 2.5	(1—7) + (1, 5, 9)	0	155
3 + MitC	46 + 1	(1—7) + (1, 6)	0	62
Control	—	—	0	—
		P388/cPt		
3	46	1—7	0	1
CP	20	1, 5	0	10
DOX	1.0	1, 7	0	106
Etoposide	2.5	1, 5, 9	0	127
MitC	1	1, 6	0	62
3 + CP	46 + 20	(1—7) + (1, 5)	0	26
3 + DOX	46 + 1	(1—7) + (1, 7)	0	116
3 + etoposide	46 + 2.5	(1—7) + (1, 5, 9)	0	175
3 + MitC	46 + 1	(1—7) + (1, 6)	0	59
Control	—	—	0	—

a 61% increase in the efficacy of doxorubicin in the P388/CP strain. In the P388/rub strain, compound **1** enhanced the efficacy of CP by 44%; in the P388/cPt strain, it caused a 40% increase in the activity of DOX. Compound **3** had adjuvant effect in three drug-resistant tumors: it enhanced the efficacy of mitomycin C in the P388/rub strain by 64% and caused a 43% and 48% increase in the efficacy of etoposide in the P388/CP and P388/cPt strains, respectively.

Once again, it should be emphasized that in all combination therapy experiments, the known anticancer drugs were used in doses substantially lower than therapeutic one. These drugs are commonly known to be highly toxic. Therefore, their use in lower doses (and consequently, with lower toxicity) and with high efficacy will make it possible to increase the duration of treatment and achieve better therapeutic outcome.

The results of the present study demonstrate that this line of research holds promise.

Experimental

Elemental analysis was performed on an Euro EA-3000 analyzer. The ^{1}H NMR spectra were measured on an Avance III spectrometer (Bruker, 500 MHz).

Table 3. Efficacy of compound **1** in drug-resistant strains of P388 leukemia *in vivo* in combination therapy with conventional cytostatics used in lower doses

Agent	Dose /mg kg^{-1}	Dosage regimen (days after transplantation)	Number of survivals (%)	ILS (%)
		P388/vcr		
1	400	1—7	0	0
CP	20	1, 6	0	112
cPt	0.6	1—7	0	137
1 + CP	400 + 20	(1—7) + (1, 6)	0	87
1 + cPt	400 + 0.6	(1—7) + (1—7)	0	79
Control	—	—	0	—
		P388/rub		
1	400	1—7	0	0
CP	20	1, 6	0	98
cPt	0.6	1—7	0	210
1 + CP	400 + 20	(1—7) + (1, 6)	0	142
1 + cPt	400 + 0.6	(1—7) + (1—7)	0	135
Control	—	—	0	—
		P388/CP		
1	400	1—7	0	0
cPt	0.6	1— 7	0	47
DOX	1.0	1, 7	0	122
Etoposide	2.5	1, 5, 9	0	128
MitC	1.0	1, 6	0	50
1 + cPt	400 + 0.6	(1—7) + (1—7)	0	7
1 + DOX	400 + 1.0	(1—7) + (1, 7)	0	84
1 + etoposide	400 + 2.5	(1—7) + (1, 5, 9)	17	165
1 + MitC	400 + 1.0	(1—7) + (1, 6)	0	64
Control	—	—	0	—
		P388/cPt		
1	400	1—7	0	3
CP	20	1, 6	0	12
DOX	1.0	1, 7	0	**77**
Etoposide	2.5	1, 5, 9	0	190
MitC	1.0	1, 6	0	58
1 + CP	400 + 20	(1—7) + (1, 6)	0	12
1 + DOX	400 + 1.0	(1—7) + (1, 7)	0	117
1 + etoposide	400 +2.5	(1—7) + (1, 5, 9)	0	185
1 + MitC	400 + 1.0	(1—7) + (1, 6)	0	47
Control	—	—	0	—

***N,N′*-Bis(2-methoxy-2-oxoethyl)oxalamide (1).** Triethylamine 26.43 mL, 0.19 mol) was added with vigorous stirring to a suspension of glycine methyl ester hydrochloride (11.92 g, 0.095 mol) in chloroform (150 mL) at ~20 °C. The reaction mixture was cooled to 0 °C, and oxalyl dichloride (6.02 g, 0.048 mol) was added. Then the reaction mixture was warmed to ~20 °C, stirred for 1.5 h, transferred into a separatory funnel, and washed with a 2% aqueous solution of $NaHCO_3$ (75 mL). The aqueous solution was extracted with chloroform (4×50 mL). The chloroform extracts were combined and dried over $MgSO_4$. The chloroform was removed under reduced pressure, and colorless crystals were isolated. The crystals were washed with diethyl ether and dried in air. The yield of compound **1** was 8.7 g (78%), m.p. 155—157 °C. Found (%): C, 41.34; H, 4.96; N, 12.26. $C_8H_{12}N_2O_6$. Calculated (%): C, 41.44; H, 5.17; N, 12.07. ^{1}H NMR (DMSO-d_6), δ: 9.12 (2 H, NH, J = 6.2 Hz); 3.92 (d, 4 H, CH_2, J = 6.2 Hz); 3.65 (s, 6 H, OCH_3).

***N,N′*-Bis(1-methoxy-1-oxoprop-2-yl)oxalamide (2).** Triethylamine (16.72 g) was added to a suspension of DL-alanine methyl ester hydrochloride (8.35 g) in chloroform (150 mL). The reaction mixture was cooled to 0 °C, and oxalyl dichloride (2.6 mL) was added. Then the reaction mixture was warmed to ~20 °C, stirred for 1.5 h, transferred into a separatory funnel, and washed with a 2% aqueous solution of $NaHCO_3$ (75 mL). The aqueous solution was extracted with chloroform (4×50 mL). The extracts were combined and dried over $MgSO_4$. The chloroform was removed under reduced pressure. Colorless crystalline compound **2** was obtained in a yield of 4.54 g (58%). M.p. 125—126 °C. Found (%): C, 45,95; H, 6.02; N, 11.09. $C_{10}H_{16}N_2O_6$. Calculated (%): C, 46.15; H, 6.15; N, 10.76. ^{1}H NMR (DMSO-d_6), δ: 9.02 (d, 2 H, NH, J = 6.2 Hz); 4.38 (m, 2 H, CH); 3.64 (s, 6 H, OCH_3); 1.35 (d, 6 H, CH_3, J = 7.3 Hz).

***N,N′*-Bis(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide dihydrochloride (3).** Oxalyl dichloride (2.03 g) was added to a solution of 4-amino-2,2,6,6-tetramethylpiperidine (5 g) in dichloromethane (50 mL). The reaction mixture was stirred for 2 h at 10 °C. The colorless precipitate that formed was filtered off, washed with diethyl ether (20 mL), and dried at ~20 °C. Crystals

of compound **3** were obtained in a yield of 7.24 g, m.p. 296 °C. According to the X-ray diffraction data, the crystals are compound **3** dichloride containing water of crystallization.

X-ray diffraction study. The unit cell parameters for compound **3** were measured and the three-dimensional X-ray diffraction intensity set was collected on a Kuma-4 automatic diffractometer (Mo-Kα radiation, graphite monochromator) at 293 K. Transparent single crystals of compound **3** ($C_{20}H_{40}N_4O_2 \cdot 2H_2O \cdot 2Cl$, M = 475.5) are monoclinic: a = 11.048(2) Å, b = 9.893(2) Å, c = 11.784(2) Å, V = 1267.5(4) Å^3, Z = 2, d_{calc} = 1.246 g cm^{-3}, μ(Mo-Kα) = 0.287 mm^{-1}, space group $P2(1)/n$. The intensities of 7640 reflections were measured in the 2θ angle range ≤50.0° using the ω-scanning technique from a single crystal of dimensions 0.22×0.14×0.11 mm. The empirical absorption correction was applied using the Multiscan method. After rejection of systematic absences and merging of equivalent reflections, the working set of measured F^2(hkl) and σ(F^2) consisted of 3749 unique reflections, of which 3299 reflections were with $F^2 > 2\sigma(F^2)$. The structure was solved by direct methods and refined by the full-matrix least-squares method based on F^2 with anisotropic displacement parameters for nonhydrogen atoms using the SHELXTL program package.[8] In the crystal structure of the complex, most H atoms were located in difference Fourier maps. The coordinates and isotropic displacement parameters of all H atoms were refined by the least-squares method using a riding model.[9] In the final cycle of the full-matrix refinement, the absolute shifts of all 202 variable parameters were less than 0.001σ. The final parameters of the refinement were $R_1 = 0.037$, $R_w = 0.13$; GOOF 0.613 based on the observed reflections. After the refinement, the maximum and minimum difference electron density residuals were 0.531 and −0.267 e A^{-3}, respectively.

All structural data were deposited with the Cambridge Crystallographic Data Centre (CCDC 1564933).

Biological assays. Tests in animals were performed in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1997) and the guidelines for implementation of preclinical studies of pharmaceutical agents.[10—13] The P388 murine leukemia was maintained in the DBA/2 mouse line; its drug-resistant variants — the strains resistant to rubomycine (P388/rub), vincristine (P388/vcr), cyclophosphan (P388/CP), and cisplatin (P388/cPt) — were maintained in BDF_1 mice by intraperitoneal transplantation of 10^6 cells per animal. Besides, P388 leukemia was transplanted into BDF_1 mice. The increase in median life span (ILS) served as the activity criterion

$$\text{ILS}\ (\%) = [(\text{MLSt} - \text{MLSc})/\text{MLSc}] \cdot 100,$$

where MLSt and MLSc is the median life span (in days) of treated and control animals, respectively. Each experimental group contained six—eight animals.

Drug-resistant strains of P388 leukemia were produced and characterized in our previous studies.[14—16] The P388/rub and P388/vcr strains carried MDR phenotype and genotype. Cells of resistant strains were stored in glass vials in liquid nitrogen. The vials were thawed when required, and the cells were administered intraperitoneally to BDF_1 mice. The tumors in the third transplant generation were used in experiments.

The general toxicity of the tested compounds was evaluated in BDF_1 hybrid mice after a single intraperitoneal administration at different doses. The doses that cause death in 50% and 100% of animals (LD_{50} and LD_{100}, respectively) were determined from the plot of mortality of animals *versus* the dose. The therapeutic dose corresponds to 1/3 of LD_{50}.

Dilution of preparations. The synthesized compounds were dissolved in sterile distilled water. All drugs and compounds were administered intraperitoneally in a volume of 0.2 mL per 20 g of body weight.

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Received May 18, 2017;
in revised form July 28, 2017;
accepted December 20, 2017