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Antiaggregant activity of water-soluble furoxans

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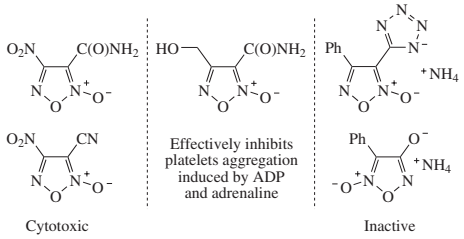
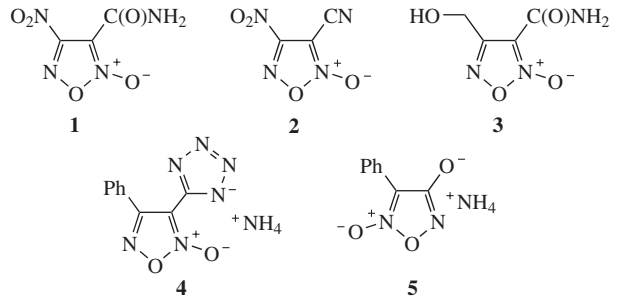
The effect of the water-soluble furoxan derivatives on platelet aggregation has been estimated using a series of inducers. The most active compound, 3-carbamoyl-4-(hydroxymethyl)furoxan (CAS-1609), effectively inhibits aggregate formation induced by adenosine diphosphate and adrenaline. This result is a good basis for the search of new structures with antiaggregant activity among furoxancarboxylic acid amides.



One of key goals of current organic chemistry is the design of molecular systems with target types of biological activity.[1] In recent years, significant attention has been brought to the search for novel drug candidates capable of releasing nitric oxide (NO) under physiological conditions.[2] It was revealed that high levels of NO can induce apoptosis leading to tumor cell death while low concentrations of NO are responsible for vasodilating and antiaggregant properties.[3] Among the variety of nitrogen-oxygen organic moieties which may serve as a source of exogenous NO, the furoxan (1,2,5-oxadiazole 2-oxide) scaffold has attracted considerable attention,[4] mainly as a structural motif in the design of complex NO-donor molecular hybrids[5] due to high stability of furoxan cycle under ambient conditions and absence of nitrate tolerance under continuous therapy.[6]

Recently,[7] we reported that 3-cyano-4-phenylfuroxan and 3-nitro-4-phenylfuroxan inhibit platelet agglutination induced by adenosin diphosphate and adrenaline. These results encouraged us to investigate a series of water-soluble furoxans as potential antiaggregant agents. For this aim, various furoxans containing functional groups (amidic, hydroxymethyl and tetrazolyl ammonium salts) promoting for solubility in water, were chosen, *viz.*, 3-nitrofuroxans **1**[8] and **2**,[9] known vasodilator 3-carbamoyl-4-(hydroxymethyl)furoxan CAS-1609 **3**[10] and ammonium salts **4**[9] and **5**. CAS-1609 has been a subject of thorough biological investigations,[11] however the measurements of its antiaggregant properties as well as those of the furoxans **1**, **2**, **4** and **5** were not performed so far. Hence, here we report the impact of water-soluble furoxans **1**–**5** on platelet aggregation studied with the use of a series of inducers.[†]



As furoxans are known to be cytotoxic compounds,[12] the ability of derivatives **1**–**5** to influence cell proliferation was estimated first.[‡] We found that furoxans **1** and **2** bearing nitro group at the 4-position possessed significant cytotoxic activity in a concentration of 38 nmol ml^{-1} causing death of LnCap cancer cells (Figure 1). This result is correlated with recent data demonstrating high cytotoxic activity of nitrobifuroxans against five human cancer cell lines: A549, HCT116, HeLa, MCF7 and RD.[13] Simultaneously, it was found by the Griess reaction using a spectrophotometric technique that nitrobifuroxans showed high levels of NO release. At the same time, compounds **3**–**5** did not

[†] All reactions were carried out in well-cleaned oven-dried glassware with magnetic stirring. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200.13 and 50.32 MHz, respectively) spectrometers and referenced to residual solvent peak. ^{14}N NMR spectra were measured on a Bruker AM-300 (21.69 MHz) spectrometer using $MeNO_2$ (δ_N 0.0 ppm) as an external standart. The IR spectra were recorded on a Bruker Alpha spectrometer in the range 400–4000 cm^{-1} (resolution 2 cm^{-1}) as pellets with KBr. Elemental analyses were performed using a Perkin-Elmer 2400 CHN Analyzer. The melting points were determined on a Kofler melting point apparatus and are uncorrected.

4-Nitrofuroxans **1**[8] and **2**,[9] CAS-1609 **3**[10] and ammonium salt **4**[9] were synthesized according to published procedures.

4-Hydroxy-3-phenylfuroxan ammonium salt **5**. A mixture of aqueous ammonia (22%, 0.2 ml) in MeOH (5 ml) was added dropwise to a magnetically stirred solution of 4-hydroxy-3-phenylfuroxan (303 mg, 1.7 mmol) in MeOH (5 ml) at room temperature.[17] The mixture was stirred for 2 h, then the volatiles were evaporated, the residue was triturated with cold EtOAc (3 ml), the white solid formed was filtered, washed with cold EtOAc and dried in air. Yield 172 mg (52%). IR (KBr, ν/cm^{-1}): 3320, 3112, 2856, 1610, 1532, 1314, 1210, 1096, 980. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 7.38–7.49 (m, 7H, Ph + NH_4), 8.52 (d, 2H, Ph, ^3J 7.7 Hz). ^{13}C NMR (50.3 MHz, DMSO-d_6) δ: 108.8, 125.6, 126.4, 128.1, 128.7, 167.2. ^{14}N NMR (21.7 MHz, DMSO-d_6) δ: –358.7 (NH_4). Found (%): C, 49.04; H, 4.88; N, 21.26. Calc. for $C_8H_9N_3O_3$ (%): C, 49.23; H, 4.65; N, 21.53.

[‡] *Cytotoxic activity*. A furoxan solution (0.0375 μmol ml^{-1}) in aqueous NaCl (40 μl, 0.9%) was added to 400 μl of a suspension of LnCap cells (ATCC, 10^6 per ml) in RPMI-1640 medium (Paneco, Russia) with 10% of fetal bovine serum. A 100 μl portion of the mixture was put in 96-well plate in triplicate and incubated at 37 °C. After 24 h the medium was aspirated and hematoxylin/eosin dye was added. The picture was taken using a microscope (×200).

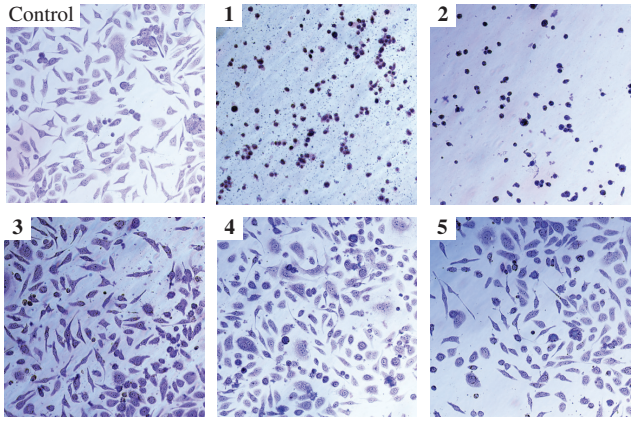


Figure 1 Effect of compounds **1**–**5** on proliferation of cancer cells (line LnCap).

affect cell proliferation indicating the lack of toxic properties. Further only furoxans **3**–**5** were examined in the experiments with platelets.

Ability of furoxans **3**–**5** to inhibit platelets aggregation was studied *in vitro* using platelet rich plasma (PRP). Adenosine diphosphate (ADP), adrenaline, collagen, ristocetin, and arachidonic acid were applied as inducers in the experiments. The measurement of the effects of the samples was performed according to the procedure described previously.[7] PRP with a sample was heated at 37 °C for 2 min, then an inducer was added and light transmission was measured.[§]

Furoxans **4** and **5** were shown to be inactive in the experiments with all types of inducers. The curves were similar to the control one (Figure 2). Furoxan **3** demonstrated significant activity in the experiments with ADP and adrenaline, but not in the presence of ristocetin and arachidonic acid (see Figure 2). Thus, in the control group the reaction started immediately after ADP or adrenaline addition, while in the presence of **3** no platelets aggregation was observed. It is remarkable that addition of ristocetin at 10th minute of the experiments switched on agglutination immediately (see Figure 2), which indicated the remaining ability of platelets to cell–cell interaction after treatment with furoxan **3**. These results testify that compound **3** acts according to selective mechanism of inhibition of platelets aggregation. It is noteworthy that ADP and adrenaline are considered to be the main agents causing thrombus formation.[14,15]

In the experiments with collagen, only delay in aggregates formation was observed. Thus, in the control group, platelets agglutination started at 5th minute of the experiment, while in the presence of **3** in a concentration of 0.0375 μmol ml^{-1}, the reaction started at about 6th minute (Figure 3). Increasing the concentration of **3** led to a bigger delay in the reaction, but the levels of agglutination were similar (~70% of light transmission was observed).

The previously[7] investigated 3-cyano-4-phenylfuroxan and 3-nitro-4-phenylfuroxan displayed the high antiaggregant properties. Replacement of phenyl fragments in these compounds with nitro groups caused the increase in their toxicity (compounds **1** and **2**). On the other hand, the replacement of cyano and nitro groups

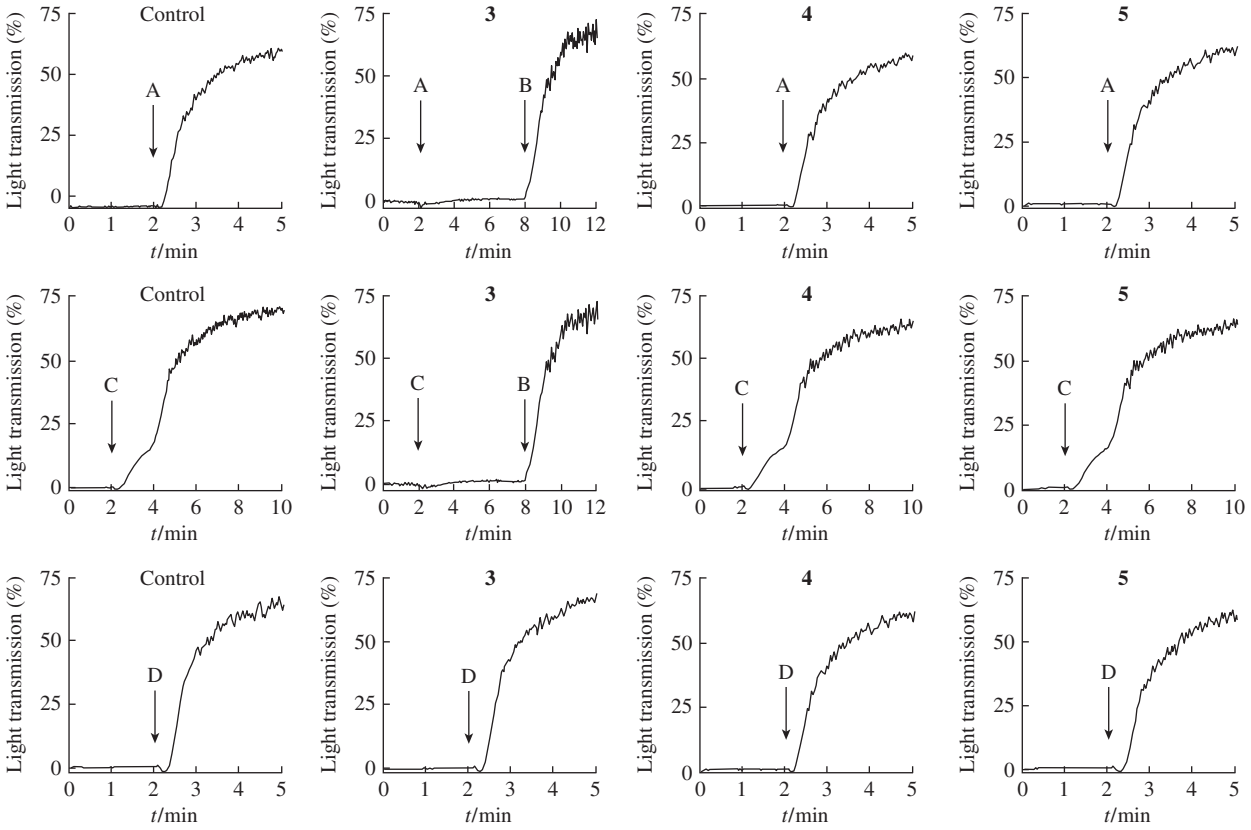


Figure 2 Effect of furoxans **3**–**5** on platelets aggregation *in vitro*. A – ADP, B – ristocetin, C – adrenaline, D – arachidonic acid.

[§] *Antiaggregant activity*. The test was performed using a Biola platelet aggregation analyzer (Russia) according to the established procedure. ADP, adrenaline, collagen, ristocetin and arachidonic acid (Helena, GB) kits were used for the experiments. Blood with citrate buffer (9:1) was centrifuged at 100 g for 10 min, and platelets rich plasma (PRP) was collected. To 300 μl of this heated at 37 °C PRP, 10 μl of a 1.25 mM water solution of a test sample was added, and the mixture was incubated at 37 °C for 2 min under stirring. Then a solution (30 μl) of an inducer was added, and light transmission was measured for 6–10 min at 37 °C. Purified water was used in control experiments. If aggregation did not occur, another inducer of aggregation was added and light transmission was measured for 5 min at 37 °C.

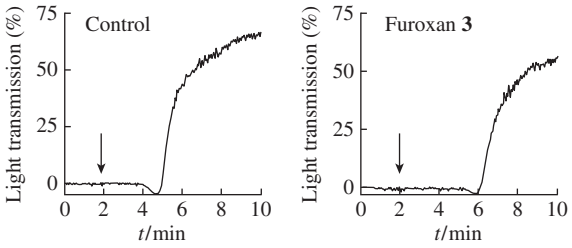


Figure 3 Delay in platelets aggregation induced by collagen in the presence of furoxan **3**.

with tetrazolyl ammonium salt fragment (compounds **4** and **5**) resulted in loss of antiaggregant capacity with any inducers. CAS-1609 (compound **3**) containing neither nitro nor cyano groups unexpectedly demonstrated significant antiaggregant activity induced by adenosine diphosphate and adrenaline and partially by collagen. As mentioned above,[10,11] CAS-1609 bearing amide substituent had very good cardiovascular effect, served as NO donor and exhibited very low toxicity. It is interesting to note that other furoxans containing amide groups also reveal a good cardiovascular activity.[16] It could be assumed that cardiovascular and antiaggregant properties are connected with each other although the mechanisms of these kinds of activity are quite different.

In conclusion, a series of substituted water-soluble furoxans was investigated to find new agents with antiaggregant properties. It was established that the properties of test furoxans were dependent on the nature of substituents. In contrast to previous data, the most active compound proved to be CAS-1609 containing amide and hydroxymethyl fragments. Therefore, the results obtained here afford a good basis for the search of new more effective agents with antiaggregant activity in series of water-soluble carboxamide and hydroxymethyl furoxan derivatives.

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