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The Effects of Nitroazolopyrimidines on the A_1 Adenosine Receptor and Intraocular Pressure in Rats

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Abstract—Six compounds of the 5(7)-alkylamino-6-nitroazolopyrimidine and 8-alkylazolo[5,1-*b*]purine series were selected based on the structural analysis of A_1 adenosine receptor inhibitors and the role of this biological target in the modulation of intraocular pressure, an important factor in the pathogenesis of glaucoma. These heterocycles were shown to exhibit a weak affinity towards the A_1 adenosine receptor on an in vitro model of the adenosine-dependent change of the chronotropic effect on isolated atria of white mice. On the other hand, thiadiazolo[3,2-*a*]pyrimidines and triazolo[5,1-*b*]purine displayed an in vivo hypotensive effect in rats. The leading compound, 5-methyl-8-(hydroxyethyl)triazolo[5,1-*b*]purine) (0.2% solution), caused a 34% reduction of ophthalmotonus in 3 h without an adverse resorptive effect. In addition, using the MTT-test it was shown on the human HepG2 cell line that the heterocycles affecting the intraocular pressure were by one to two orders of magnitude less cytotoxic than the reference doxorubicin.

Keywords: adenosine, adenosine receptors, azolopyrimidines, azolopurines, glaucoma, intraocular pressure

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INTRODUCTION

Currently, glaucoma is one of the major reasons for irreversible blindness worldwide. The number of patients with glaucoma is going to continuously grow according to the most optimistic projections [1], which correlates well with the trend towards an increase in people with visual impairments due to glaucoma [2].

Despite the certain success in studying pathogenesis of this disease and development of treatment protocols, IOP reduction remains the priority in glaucoma therapy [3].

For topical glaucoma therapy drugs with a different mode of action are used including IOP production-reducing agents (β-blockers, carboanhydrase II inhibitors, and α-adrenomimetics) and agents improving the IOP production efflux (prostaglandin analogs, m-cholinomimetics, and α-adrenomimetics). In recent years, new classes of drugs, which can reduce IOP by impacting certain biological targets, are under discussion. Among them there are agents reducing ophthalmotonus by trabecular efflux, particularly, Rho kinase inhibitors [4], compounds with an affinity to subtype III melatonin receptors, which can reduce IOP in a concentration-dependent mode [5], nitric oxide donors, and agents affecting globular proteins of the trabecular meshwork and Schlemm's canal, as well as antagonists of adenosine receptors.

Adenosine receptor A_1 was shown to be expressed in the anterior segment of the human eye and is an attractive target for IOP control [6]. New studies in this area demonstrated that this receptor is widely spread in the drainage system in the eye, particularly, in the ducks with aqueous fluid, and plays an essential role in IOP modulation [7, 8]. In particular, an excess of adenosine, an endogenous A_1 receptor agonist, in the anterior eye chamber causes eye hypertension [9]. Therefore, the search for new inhibitors of this subtype of adenosine receptors, is a promising approach for IOP reduction.

The goal of this work was the synthesis of heterocycles of the azoloazine series and evaluation of their impact on the A_1 AR and IOP in rats in order to assess a potential relationship between the inhibitory activity and the hypotensive effect.

RESULTS AND DISCUSSION

The synthesis of heterocycles of the azoloazine series. We showed previously that nitrogen-containing azoloazines are close structural analogs of the known antagonists of the A_{2a} adenosine receptor [10]. It is known that binding sites of A_{2a} and A_1 receptors are

Abbreviations: A_1 AR, A_1 adenosine receptor; IOP, intraocular pressure; HR, heart rate.

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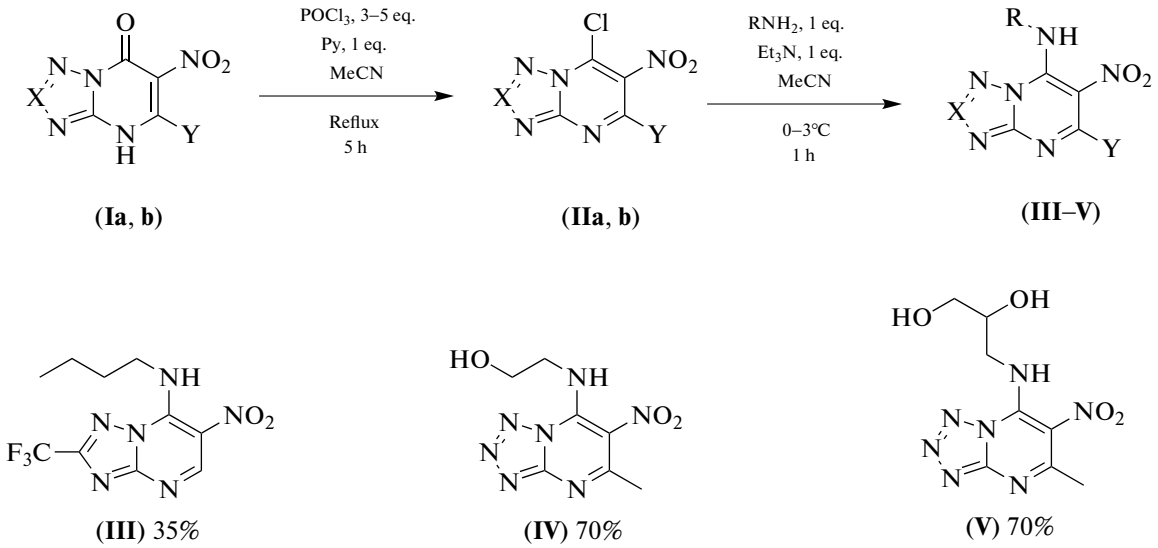
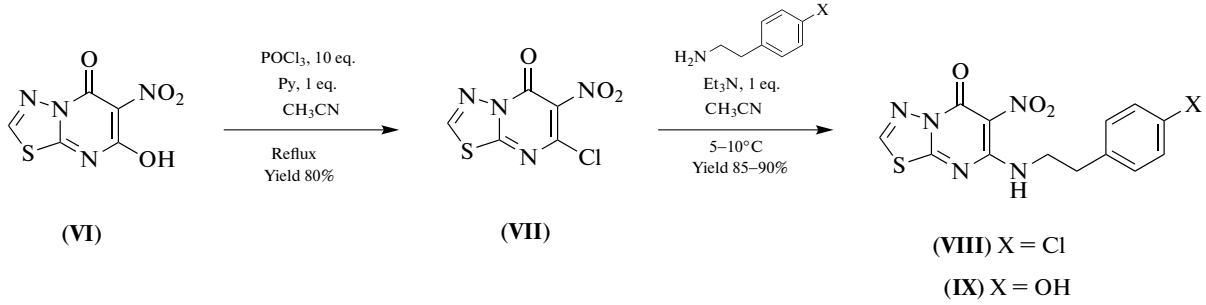
structurally close, which, on the one hand, calls for the search for selective effectors and, on the other hand, suggests that effectors of the A_{2a} receptor would be affine towards the binding site of the A_1 receptor. With the goal of studying the antagonistic effect we synthesized an alkylamine bearing nitroazolopyrimidines (**III**–**V**) using successive chlorodeoxygenation with a mixture of phosphoryl chloride and pyridine and nucleophilic substitution of a halogen atom by alkylamines (Scheme 1) [11–13]. This strategy was also used for the synthesis of 5-alkylamino-6-nitro-1,3,4-thiadiazolo[3,2-*a*]pyrimidin-8-ones containing fragments of *p*-chlorophenyl ethylamine (**VIII**) and tyramine (**IX**) (Scheme 2).

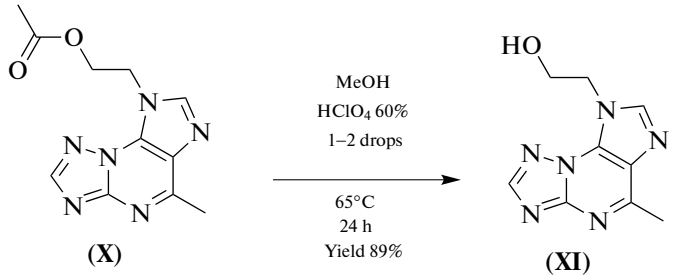


Scheme 1. Two-step synthesis of 6-nitro-7-alkylaminoazolo[1,5-*a*]pyrimidines (**III**–**V**).



Scheme 2. Synthesis of 5-alkylamino-6-nitro-1,3,4-thiadiazolo[3,2-*a*]pyrimidin-7-ones (**VIII**) and (**IX**).

In addition, 5-methyl-8-(2-hydroxyethyl)triazolo[5,1-*b*]purine (**XI**) was obtained, which is a structural analog of tricyclic inhibitors of adenosine receptors. To this end, an acyl protective group of the previously obtained triazolo[5,1-*b*]purine (**X**) was removed in the presence of a catalytic amount of chloric acid under refluxing in methanol (Scheme 3).



Scheme 3. Acyl deprotection of the hydroxyl group of heterocycle (**X**) resulting in 5-methyl-8-(2-hydroxyethyl)-1,2,3-triazolo[5,1-*b*]purine (**XI**).

Pharmacological Properties of the Synthesized Heterocycles

In vitro antagonistic activity towards the A_1 AR. The study of the antagonistic effect of azoloazine analogs (**III**–**V**), (**VIII**), (**IX**), and (**XI**) at 10 μM concentrations was performed on a model of the adenosine-dependent change of the chronotropic effect in vitro using isolated atria of white mice. It was found that heterocycles of the nitroazolopyrimidine series (**III**–**V**) displayed the highest antagonistic activity, whereas the corresponding thiadiazolopyrimidines (**VIII**), (**IX**), and triazolopurine (**XI**) barely affected the A_1 AR. Although all the compounds under study were inferior in this activity to the reference caffeine (Table 1), the results implied that a triazolo[1,5-*a*]pyrimidine scaffold could serve a promising backbone for the search of the structures with a higher affinity to this biological target.

In vitro ophthalmohypotensive properties. The effects of the compounds on IOP values were studied on intact outbred rats by tonometry. All the compounds under study were shown to display ophthalmohypotensive properties. Particularly, for heterocycle (**VIII**) a 10% increase in ophthalmotonus for 60 min was observed followed by an insignificant IOP reduction to the initial values in both tested and control eyes, which indicated an undesirable resorptive effect. However, by hour 3 the IOP of the tested eye reduced by 20%, whereas in the control eye it did not change.

The studies of compound (**IX**) demonstrated that 60 min after instillation into the tested eye, IOP remained nearly the same. However, the IOP value fell by 13% by hour 2 and by 18% by hour 3 of the study (Fig. 1b). The IOP in the control eye was within the normal range, which implied the lack of systemic effects of the compound under study.

Table 1. A_1 antagonistic activity of the synthesized heterocycles

Compound or drug	Inhibition of the negative chronotropic adenosine effect, Δ%
(III)	36.9 ± 5.4
(IV)	32.5 ± 3.8
(V)	45.9 ± 3.7
(VIII)	0.5 ± 0.5*
(IX)	2.5 ± 2.5*
(XI)	2.5 ± 1.1*
Caffeine	60.5 ± 3.7

Concentrations of the tested compounds and caffeine were 10 μM. The data are given as arithmetic mean ± standard error of the arithmetic mean.

* $p < 0.05$ if compared with the values for caffeine (Kruskel–Wallis test followed by Dunn's test).

Installation into the tested eye of azolo[5,1-*b*]purine (**XI**) reduced ophthalmotonus by 26% as early as by hour 1 of the study and by 30% by hour 2. The maximal effect (34%) was observed in 3 hours (Fig. 1c). In the control eye IOP changed in the range of 1 to 2 mm Hg, which evidenced the lack of systemic effects of the tested compound (**XI**).

It was found that the most active towards A_1 AR heterocycles (**III**–**V**) did not reduce IOP at the tested concentrations. Probably, the effects of compounds (**VIII**), (**IX**), and (**XI**) on IOP are not associated with A_1 AR inhibition but with another mode of biological action.

Cytotoxicity of the compounds under study. At the final step, with the goal of preliminary evaluation of toxicological properties of the most active (**VIII**), (**IX**), and (**XI**) we studied their cytotoxicity in the MTT test on human HepG2 cells. The LC_{50} values of 6-nitrothiadiazolo[3,2-*a*]pyrimidines (**VIII**) and (**IX**) were 0.073 and 0.072 mM respectively, which is 30–40 times lower than that of reference doxorubicin. The lowest cytotoxic effect was found for triazolo[5,1-*b*]purine (**XI**), whose LC_{50} exceeded 1 mM and fell outside the limits of maximal concentrations under study (Table 2). Thus, its safety was 500 times higher than that of doxorubicin.

1,2,4-Triazolo[1,5-*a*]pyrimidine (**III**) and tetrazolo[1,5-*a*]pyrimidine scaffolds (**IV**, **V**) were shown to have the highest affinity towards A_1 AR, whereas 1,3,4-thiadiazolo[3,2-*a*]pyrimidines (**VIII**), (**IX**) and 1,2,4-triazolo[5,1-*b*]purine (**XI**) did not demonstrate a statistically reliable effect towards this receptor. On the other hand, the affinity to A_1 AR of the synthesized 6-nitroazolo[1,5-*a*]pyrimidines was lower than that of the reference caffeine, which evidences the necessity of the next structural modifications to develop more powerful inhibitors.

A reverse dependency was observed in the studies of ophthalmohypotensive properties of the synthesized heterocycles: azolo[1,5-*a*]pyrimidines (**III**–**V**) did not impact the rat IOP and thiadiazolo[3,2-*a*]pyrimidines (**VIII**), (**IX**) and triazolo[5,1-*b*]purin (**XI**) demonstrated hypotensive properties. The leading compound 5-methyl-8-(hydroxyethyl)triazolo[5,1-*b*]purine (**XI**) reduced ophthalmotonus by 34% in 3 h without the resorptive effect. In addition, the heterocycles affecting IOP were shown to be considerably less toxic than the reference doxorubicin.

EXPERIMENTAL

Synthesis of azoloazine heterocycles. ^1H and ^{13}C NMR spectra were registered on an Avance II spectrometer (400 and 100 MHz respectively, Bruker, Germany) at 25°C using TMC as an internal standard and DMSO-d_6 and $CDCl_3$ as solvents. Element analysis was performed on a 2400 CHN analyzer (Perkin-Elmer, United States). The reactions were monitored

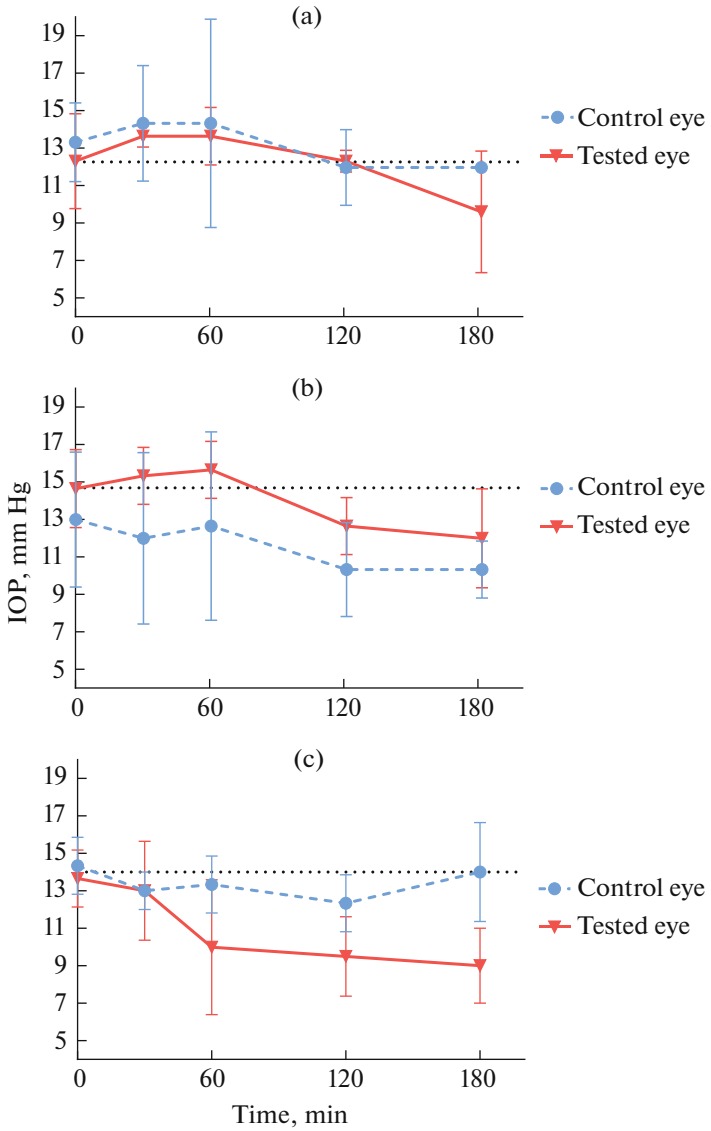


Fig. 1. Effects of compounds **(VIII)** (a), **(IX)** (b), and **(XI)** (c) on IOP of intact rats.

by TLC on Silufol UV-254 plates (Imid Ltd., Russia). Melting points were measured on a Stuart SMP3 apparatus. Heterocycles **(III)** [14], **(IV)** and **(V)** [12], **(VIII)** and **(IX)** [13], and **(X)** [11] were synthesized as described previously.

Table 2. Cytotoxicity of **(VIII)**, **(IX)**, and **(XI)** versus doxorubicin on human hepatocellular carcinoma HepG2 cells (MTT test)

Compound or drug	LC_{50}, mM
(VIII)	0.073
(IX)	0.072
(XI)	>1
Doxorubicin	0.002

5-Methyl-8-(2-hydroxyethyl)triazolo[5,1-*b*]purine (XI). 60% Perchloric acid (0.5 mL) was added to a solution of 5-methyl-8-(2-acetocyethyl)triazolo[5,1-*b*]purine (2.18 g, 0.01 mol) in MeOH (40 mL). The reaction mixture was refluxed for 24 h, evaporated in vacuum at 35°C, and the residue was recrystallized from *iso*-butanol.

White powder. R_f (EtOAc) 0.4; mp 214–216°C. Yield 89%. Found, %: C 49.44; H 4.50; N 38.80. Calculated for $C_9H_{10}N_6O$, %: C 49.54; H 4.62; N 38.51. ^{1}H NMR (400 DMSO-d_6), δ (ppm): 2.84 (3H, s, CH_3), 3.87 (2H, t, *J* 4.0, OCH_2), 4.69 (2H, t, *J* 4.0, NCH_2), 8.40 (1H, s, C7H), 8.84 (1H, s, C2H). ^{13}C ($CDCl_3$), δ (ppm): 20.2 (CH_3), 49.0 (NCH_2), 59.8 (OCH_2), 127.5 (C5a), 134.3 (C8a), 142.6 (C7), 151.2 (C3a), 152.3 (C2), 159.4 (C5).

Pharmacological Studies

Antagonistic activity towards A_1 AR. Antagonistic activity of compounds (**III**–**V**), (**VIII**), (**IX**), and (**XI**) towards A_1 AR was studied on isolated atria of 20 4-month-old white mice of both sexes (Rappolovo nursery, Leningrad region) using Brigadirova's modification of the model of the adenosine-dependent change of the chronotropic effect in vitro [15] in Krebs–Henseleit buffer composed of NaCl (118.0 mM); KCl (4.7 mM); KH_2PO_4 (1.18 mM); $MgSO_4$ (1.2 mM); $CaCl_2$ (2.5 mM); $NaHCO_3$ (25.0 mM); and glucose (5.55 mM; pH 7.4) with permanent oxygenation (95% O_2 and 5% CO_2) and thermostatic control at 37°C. The compounds under study at a concentration of 10 μM were placed into a dish with isolated atria and adenosine (10 μM; Sigma-Aldrich, United States) was added. The activity was evaluated by the inhibition degree of the adenosine-induced reduction of chronotropism of isolated atria operating in the routine mode (without stimulation) versus the control adenosine effect (in Δ%). Atrial contractions were registered using an isometric TSD125C transducer in a 4-channel DA100C (Biopac Systems, Inc., United States) system of life support of isolated tissues with the isometric load of 1 g and the AcqKnowledge 4.0 (Biopac Systems, Inc., United States) software. The number of contractions of isolated atria was measured with 30-second intervals followed by calculations of the heart rate. Caffeine (10 μM; Sigma-Aldrich, United States), a nonselective antagonist of $A_{1/2a}$ adenosine receptors, was used as a comparative.

The value of atrial chronotropism inhibition (Δ%) was calculated by the formula:

$$\Delta\% = 100 - \left(\Delta HR_{contr}/\Delta HR_{exp} \times 100\right),$$

where ΔHR_{contr}, is the difference between the initial HR and HR calculated after the adenosine addition in the control experiment; ΔHR_{exp}, the difference between the initial HR and HR calculated after the adenosine addition against the background of the exposition of the compounds under study.

The statistical processing was performed using a nonparametric Kruskal–Wallis critical value and Dunn's test for multiple comparisons in the GraphPad Prism 7.0 program (GraphPad Software, United States).

In vitro ophthalmohypotensive properties. The effect of the compounds on the IOP level was studied on 48 adult 2-months-old outbred intact rats of both sexes (Rappolovo nursery, Leningrad region) by tonometry using a TonoVet tonometer (Finland) [16]. The compounds were studied by the method described by Marcus et al. [17]. Azolopyrimidine derivatives (**III**–**V**), (**VIII**), (**IX**), and (**XI**) were once instilled into the right eye of the animal at a concentration of 0.2% (50 μL) and, in the left eye, distilled water was instilled at the same volume. Timolol (Timolol-SOLOpharm, 0.5%, Grotex, Russia) and brimonidine (Santibrim, 0.1%, Sentiss Pharma Ltd, India) eye drops were used as reference compounds. Their IOP-reducing effect has been confirmed and they are used in clinical practice. They were also instilled into the right (tested) eye of the animals (50 μL), whereas distilled water of the same volume was instilled in the left eye. The left eye was used for the evaluation of the potential systemic effect of the compounds under study. IOP was measured at five time points (0, 30, 60, 120, and 180 min), where 0 min was the starting point. The IOP-reducing activity was assessed by the maximal IOP reduction against initial values.

Cytotoxicity of the compounds. Cytotoxicity of the most active compounds (**VIII**), (**IX**), and (**XI**) was evaluated by the MTT test [18] on HepG2 (human liver cancer) cell line (ATCC® HB-8065™). The cells were cultivated in the F-12 medium (Gibco, United States) supplemented with 10% embryonal calf serum (Gibco, United States), 1% penicillin–streptomycin (Gibco, United States), 1% essential amino acids (NEAA) (Sigma-Aldrich, United States), and 2 mM sodium pyruvate (Sigma-Aldrich, United States) in the CO_2 incubator in the atmosphere of 5% CO_2. The compounds and doxorubicin (Sigma-Aldrich, United States), the most widely used reference cytostatic, were studied in the range of 0.1 to 1.0 mM under a 48-h incubation.

The cell viability, which correlated with the ability of mitochondrial dehydrogenases to transform the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, was assessed by the values of optical density at 555 nm (the reference wavelength 650 nm) using a plate CLARIOstar reader (BMG LABTECH, United States). The data treatment and LC_{50} (concentration inhibiting cell viability by 50% vs. the intact control) calculation were performed using MARS Data Analysis Software (BMG LABTECH, United States) and GraphPad Prism v.7.0 (GraphPad Software, United States).

CONCLUSIONS

To summarize, we synthesized six compounds of the azoloazine series. It was shown on an in vitro model of the adenosine-dependent change of chronotropic effect on isolated atria of white mice that 1,2,4-triazolo[1,5-*a*]pyrimidine (**III**) and tetrazolo[1,5-*a*]pyrimidine (**IV**) and (**V**) scaffold demonstrated the highest affinity towards A_1 AR, whereas 1,3,4-thiadiazolo[3,2-*a*]pyrimidines (**VIII**) and (**IX**) and 1,2,4-triazolo[5,1-*b*]purine (**XI**) did not display a statistically reliable inhibitory effect towards this receptor. On the other hand, the synthesized 6-nitroazolo[1,5-*a*]pyrimidines were inferior to the comparative caffeine in the affinity towards A_1 AR, which indicates the necessity of further structural modifications for the development of more powerful inhibitors. An inverse relationship was observed in the studies of ophthalmohypotensive properties of the synthesized heterocycles: azolo[1,5-*a*]pyrimidines (**III**–**V**) did not affect the IOP in rats and thiadiazolo[3,2-*a*]pyrimi-

dines (**VIII**), (**IX**) and triazolo[5,1-*b*]purine (**XI**) displayed a hypotensive activity. The leading 5-methyl-8-(hydroxyethyl)triazolo[5,1-*b*]purine (**XI**) was shown to reduce ophthalmotonus by 34% in 3 h, a negative resorptive effect not being observed. In addition, the heterocycles affecting IOP were one to two orders of magnitude less cytotoxic than the reference doxorubicin in the MTT test on human hepatocellular carcinoma HepG2 cell line.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

The studies were performed in compliance with the guidelines on preclinical studies of drugs (ed. Mironov), part 1, M.:Grif & Co, 2012; European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes CETS 123, Strasbourg, 1986; and Directive 2010/63/EU of the European Parliament and of the Council of 22 September, 2010, on the protection of animals used for scientific purposes.

Conflict of Interests

The authors declare no conflict of interest.

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