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6-Nitrotriazolo[1,5-*a*]pyrimidines as Promising Structures for Pharmacotherapy of Septic Conditions

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Abstract—Promising 6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidine analogues, structural analogues of synthetic inhibitors of adenosine receptors, were sorted out on the basis of quantum-chemical calculations. The compounds were synthesized by nitration and chlorodeoxygenation reactions. The in vivo activity of 6-nitroheterocycles was studied and the affinity to adenosine receptor A_{2A} was demonstrated in regard to septic conditions.

Keywords: adenosine receptors, chlorodeoxygenation, nitroderivatives, sepsis, triazolopyrimidines

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INTRODUCTION

In most countries, sepsis is among ten major factors causing patient death in departments of resuscitation and intensive care [1]. The frequency of incidents of severe sepsis among these patients is 2–18% and that of septic shock, about 4% [2]. The mortality due to sepsis can achieve 30%, and due to septic shock, more than 50%. Since microorganisms have been considered major causative agents of sepsis so far, antibiotics still play the leading role in its therapy in spite of the development of new drugs [3]. However, with all positive results of antibiotics therapy, it cannot affect the advanced inflammatory response, which plays one of the key roles in sepsis pathogenesis [4, 5]. It is necessary to develop a complex approach for sepsis treatment including the search for new targets for antiseptic drugs.

Recently, the adenosine receptor A_{2A} (A_{2A}AR) is regarded as a promising target. It was shown previously that the activation of this receptor reduced inflammation and the use of synthetic adenosine analogues for sepsis therapy increased viability [6, 7]. The A_{2A}AR activation in animal models in vivo resulted in the reduction of the effect associated with chronic inflammation, probably, due to the intensification of cAMP synthesis [6, 8–11]. Moreover, mice defective in the A_{2A}AR gene were sensitive to minimal effects stimulating inflammation [5]. It suggested that the attack on A_{2A}AR could result in the reduction of the inflammatory reaction via mediated signal cascades.

The therapeutic potential of the A_{2A}AR interaction with small molecules is generally accepted [12]. A series of selective A_{2A}AR agonists and antagonists are being tested in clinical trials as potential drugs for the treatment of Parkinson's disease, inflammation, cancer, ischemia, diabetic nephropathy, infections, and disorders of the central nervous system [12]. At the same time, a similar arsenal of drugs for the therapy of sepsis is practically absent. Condensed azoloazines, structural purine analogues, are among antiseptic agents involved in the regulation of adenosine receptor functioning [13, 14]. Azoloazines containing a bridge nitrogen atom, such as 1,2,4-azolo[1,3,5]triazines of the ZM-241385 type [16], SCH 442416 analogues, 1,2,4-triazolo[1,5-*a*]pyrimidines, [16], and others occupy a specific place among effectors of A_{2A} receptors.

In this work, we proposed 2,5-substituted-6-nitro-1,2,4-triazolo[1,5-a]pyrimidine-7(4H)-ones of type (**A**) and 2,5-substituted-6-nitro-7-amino-1,2,4-triazolo[1,5-*a*]pyrimidines of type (**B**), structural analogues of purines and ZM-241385, as new antiseptic agents taking into account that the electron effect of the nitro group in the azine cycle was equivalent to that of the pyridine nitrogen (Scheme 1).

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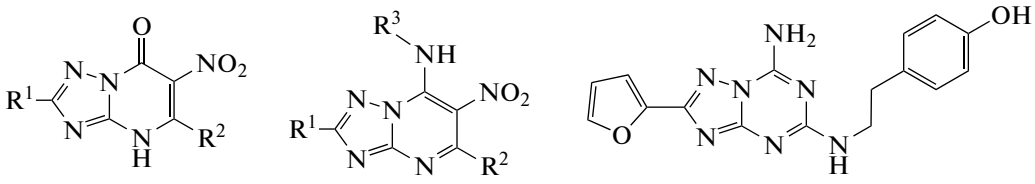


(A) (B) ZM-241385

Scheme 1. The structure of the known ZM-241385 and related 5-substituted 6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidine-7(4H)-ones of type (A) and 2,5-substituted-6-nitro-7-amino-1,2,4-triazolo[1,5-*a*]pyrimidines of type (B).

In this work, we reported the results of the structural analysis of the affinity of a series of triazolo[1,5-*a*]pyrimidines (A, B**)** to the A_{2A} receptor, the selection of promising molecules, their synthesis, and biological activity in regard to septic conditions.

RESULTS AND DISCUSSION

The choice of molecular structures relevant for adenosine receptors

At the first stage, we estimated the affinity of target compounds toward the A_{2A} receptor using computer modelling. As a comparative model we used ZM-241385.

The process of preparation of the receptor to docking involved the removal of different cofactors, inorganic ions, small inorganic molecules, and all water molecules outside the active site. Three-dimension protonation with consideration of pH (7.4) and characteristic ionization states of amino acid side chains as well as geometric optimization using molecular mechanics (the AMBER99 force field specially parametrized for macromolecules) were performed. The protein molecule was validated using a Ramachandran diagram [17] and estimation of permissible bond lengths and valence angles.

All the structures were minimized by the DFT (Density Functional Theory) method using a M06-2X meta hybrid functional [18] and a 31G(d) basic set of functions. The correctness of stationary points was confirmed by the lack of imaginary frequencies. Quantum chemical calculations were carried out using the Gaussian 09 program [19].

The docking procedure was performed using a Surflex-Dock flexible algorithm implemented in the Sybyl-X 1.1 software [20]. The method uses the empiric Hammerhead scoring function [21, 22] and employs an idealized active site ligand (called a Protomol) for the generation of ligand positions by the stepwise cross-sectional design, which can link fragments from different positions. Being one of the most effective algorithms for docking [23], Surflex-Dock is especially effective for sorting out false positive docking algorithms upon screening. With the exception of the options of the active site conformation adaptation to the ligand conformation and consideration of the cycle conformational flexibility (when relevant), standard settings were used by default.

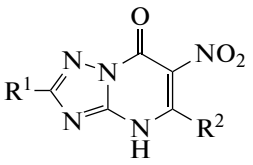
The pK_i values were calculated by the equation pK_i = –log[K_i], where K_i was the equilibrium constant of the (L*R) complex formation of the ligand (L) with the receptor (R) (K_i = [R*L]/[R]*[L] (Table 1)).

The results of docking clearly demonstrated that most of the compounds under study were involved in the complex formation with the receptors by means of hydrogen bonds with a moderate affinity to the receptor. The exception was compound (**Ia**) having pK_i > 7 and compound (**V**), 7-*N*-[2'-(4''-chlorophenyl)]ethylamino-6-nitro[1,2,4]triazolo[1,5-*a*]pyrimidine, which showed the most effective binding within the triazolopyrimidine series. The three-dimensional pattern of compound (**V**) interaction with the A_{2A} receptor is given in Fig. 1. The sp^2-hybrid nitrogen atom in the heterocycle position 3 formed a hydrogen bond with the amide proton of asparagine 253 side chain, whereas oxygen atoms of the nitro group served acceptors of the hydrogen bond with the hydroxyl proton of threonine 88. An essential structural feature of triazolopyrimidine (**V**) is an advantageous position of a stretched hydrophobic 2-(4'-chlorophenyl)ethyl group in the receptor active site (the receptor structure was taken from the PDB, Protein Data Bank, www.rcsb.org, PDB code: 3EML).

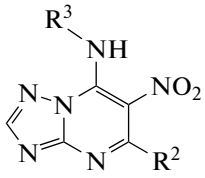
However, despite rather high p*Ki* values, all the designed triazolopyrimidines were inferior in their affinity to the reference ZM-241385. It is known that both polar (hydrogen bonds, etc.) and hydrophobic interactions are important for optimal binding. A visual analysis of two-dimensional ligand formulas and three-dimensional receptor–ligand complexes demonstrated that the shape of the referent antagonist was better adjusted to the active site pocket. In addition, it bears bulk hydrophobic groups providing effective nonpolar interactions and contributing a considerable energetic effect to the complex formation.

To summarize, the 1,2,4-triazolo[1,5-*a*]pyrimidines under study are promising molecules for the development of their synthesis and evaluation of their antiseptic activities.

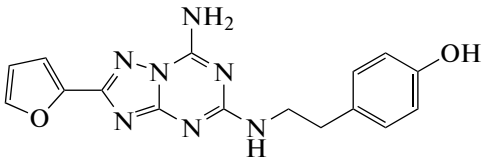
Table 1. The results of the docking test of compounds **(I)**–**(VI)** and ZM-241385 in respect to the $A_{2A}A$ receptor



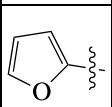
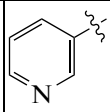
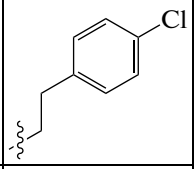
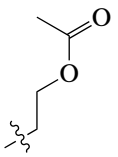
(Ia, c), (IIb, c, d)



(III)–(VI)



ZM-241385

No.	(Ia)	(Ic)	(IIb)	(IIc)	(IId)	(III)	(IV)	(V)	(VI)	ZM-241385
R^1		H		H	Me	H	H	H	H	–
R^2	H	H	Me	Me	Me	OH	H	H	Me	–
R^3	–	–	–	–	–	–	*n*-Bu			–
pK_i	7.14	5.23	5.99	5.27	5.11	5.34	6.53	7.54	6.48	10.00

Synthetic Part

The first stage of the synthetic part was dedicated to the development of methods of synthesis of 2-R^1-5-R^2-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-ones **(I)** and **(II)** (Scheme 2).

A general procedure for the synthesis of 2-R^1-5-R^2-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-ones consisted in the coupling of 5-R^1-3-amino-1,2,4-triazoles **(VII)** with β-ketocarboxylic acids or their derivatives. Particularly, heating of aminotriazole **(VII)**

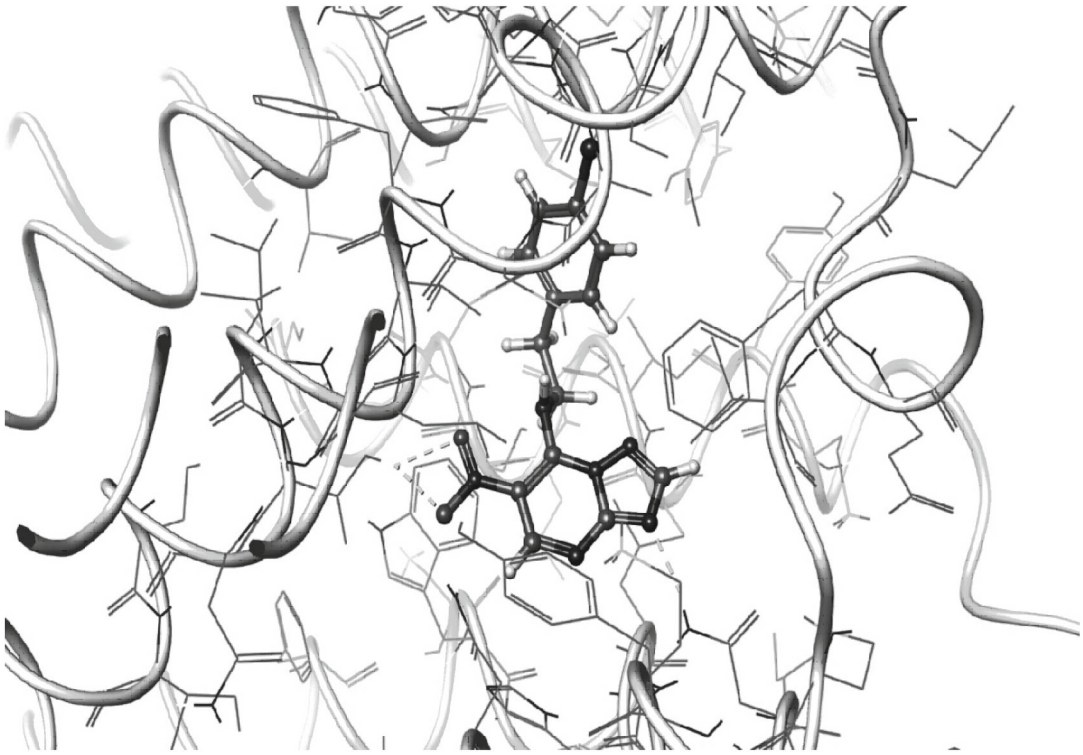


Fig. 1. The A_{2A} receptor complex with triazolopyrimidine **(V)**.

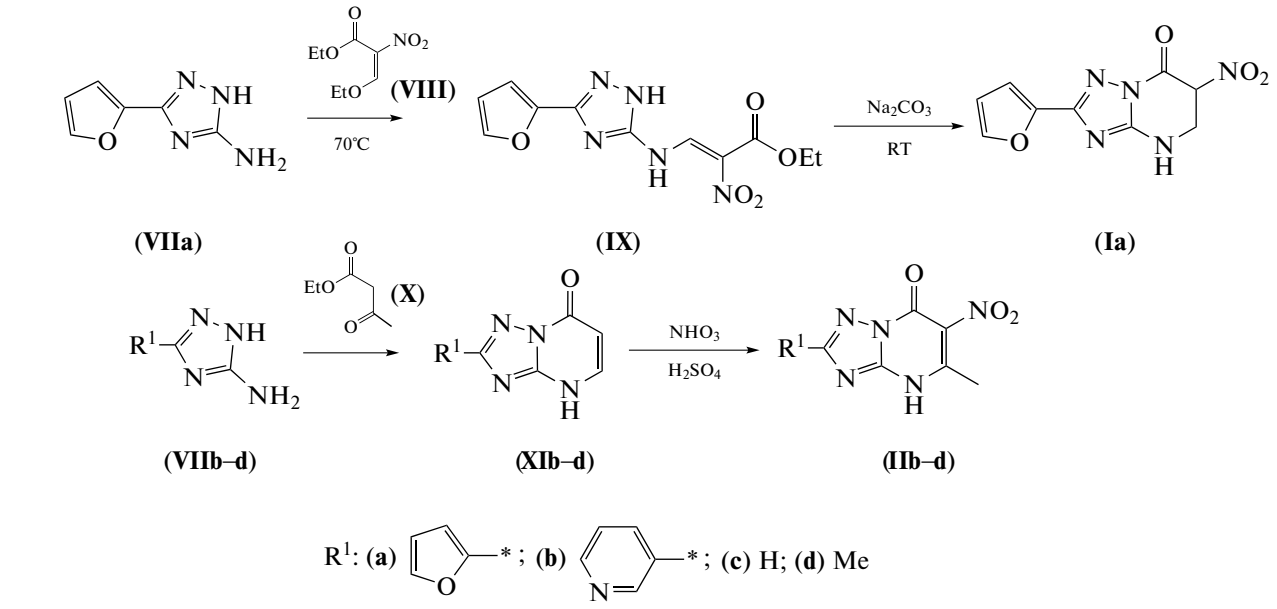
Table 2. The impact of triazolopyrimidine derivatives **(I)**–**(VI)** on the viability of mice with septic shock induced by an LPS injection

Group/Injected compound	Effect, died/totally	Viability, %	Injected compound	Effect, died/totally	Viability , %
Intact mice	0/5	100	**(V)**	2/5*	60
Placebo (LPS only)	5/5	0	**(IIb)**	5/5	0
Hydrocortisone, 15 mg/kg	2/5*	60	**(Ia)**	1/5*	80
(Ic)	4/5	20	**(IV)**	4/5	20
(IIc)	5/5	0	**(III)**	2/5*	60
(IId)	5/5	0	**(VI)**	2/5*	60

* $p < 0.05$.

with ethyl 2-nitro-3-ethoxyacrylate **(VIII)** resulted in ethyl 2-nitro-3-(5'-(furyl-2'')-1,2,4-triazol-3'-yl)amino-2-nitroacrylate **(IX)**, which was successively treated with 2M sodium carbonate and concentrated hydrochloric acid to give 2-(fur-2'-yl)-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-one **(Ia)** (Scheme 2). The structure of compound **(Ia)** was ascribed according to IR and ^{1}H NMR spectroscopy and element analysis.

If α-nitro-β-ketoester was unavailable due to its instability, as it took place in the case of 2-nitroacetoacetate [24, 25], an alternative synthetic scheme was used [26]. At the first stage, 5-R^1-3-amino-1,2,4-triazole **(VII)** was coupled with ethyl acetoacetate **(X)** in acetic acid to give 2-R^1-5-methyl-1,2,4-triazolo[1,5-*a*]pyrim7idin-7-ones **(XI)** (Scheme 2). Nitration, most commonly with a mixture of nitric and sulfuric acids [26], resulted in 2-R^1-5-methyl-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-ones **(II)** (Scheme 2).

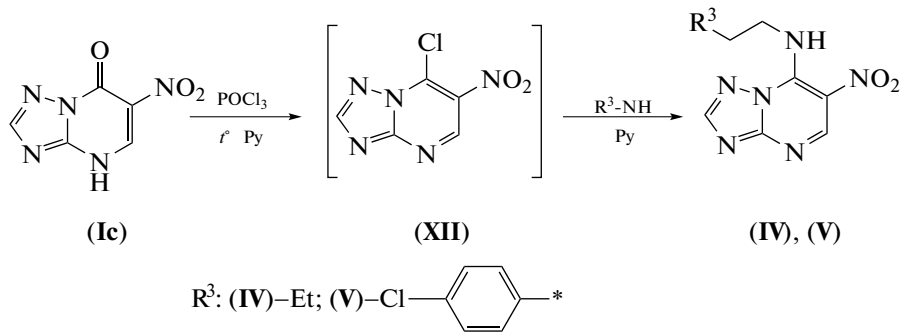


Scheme 2. The synthesis of 2-R^1-5-R^2-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-ones **(I)** and **(II)**.

In general, the method is multipurpose for both reaction stages but there are some exceptions. For, example, nitration of 2-furyltriazole pyrimidine **(XIa)** resulted in the dinitration product, namely, 2-(5'-nitrofur-2'-yl)-5-methyl-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-one. The use of other nitrating agents, such as acetyl nitrate in acetic acid or trifluoroacetyl nitrate in trifluoroacetic acid, did not lead to the regioselective formation of mononitration product **(IIa)**. The structure of 2-R^1-5-methyl-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-ones **(II)** was ascribed according to IR and ^{1}H NMR spectroscopy and element analysis.

The next stage of the synthetic part involved the development of synthesis of ZM-241385 structural analogues on the basis of 1,2,4-triazolo[1,5-*a*]pyrimidines. At the first step, 6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidin-7-one

(**Ic**) was treated with phosphoryl chloride in pyridine to give 6-nitro-7-chloro-1,2,4-triazolo[1,5-*a*]pyrimidine (**XII**) (Scheme 3) [27]. Since the chloro derivative (**XII**) was unstable and quickly hydrolyzed to the starting triazolopyrimidone (**Ic**), it was used at the next stage immediately after isolation without purification [28].



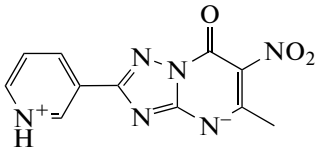
Scheme 3. The synthesis of 7-alkylamino-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidines (**IV**), (**V**).

The reaction of chlorotriazolopyrimidine (**XII**) with primary amines in the presence of pyridine resulted in 7-alkylamino-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidines (**IV**), (**V**) (Scheme 3). The data of element analysis and ^{1}H, ^{13}C NMR and IR spectra of amines (**IV**) and (**V**) supported the proposed structures. A characteristic feature of the ^{1}H NMR spectra of these compounds was a broad signal (δ 9.83–10.07 ppm) corresponding to the resonance of the NH proton and a two-proton multiplet (δ 4.39–4.48 ppm) of the N-CH_2 group. Such a spectral pattern unambiguously evidenced the presence of a (–NH–CH_2–) fragment.

Results of Biological Tests

The results of the in vivo testing of the compounds under study partly agreed with the calculated data. Screening of their biological activity was performed on a model of septic shock induced in mice by the injection of *Salmonella typhosa* lipopolysaccharide (LPS) (Sigma-Aldrich). For the experiment, 130 mice were taken. The compounds under study (**Ia**), (**Ic**), (**IIb–d**), and (**III**)–(**VI**) were dissolved in DMSO and injected intraperitoneally twice a day at a daily dose of 50 mg/kg (a previously found effective dose of the prototype, triazavirin) [29]. Hydrocortison hemisuccinate was used as a reference drug. The dynamics of mouse viability in the test and control groups was monitored for 3 days. The results were presented as a portion of survived mice (%) (Table 2).

Compound (**Ia**), which according to the results of quantum chemical calculations was only inferior to ZM-241385 and (**V**), demonstrated the highest activity (80% viability of mice with induced septic shock) (Table 1). Compound (**V**) displayed the best affinity among the heterocycles tested (with the exception of triazolotriazine-based ZM-241385) was also significantly active in animals preventing the death from septic shock in 60% animals. However, heterocycles (**IIb**) and (**IV**), which had a satisfactory computed affinity (pK_i = 5.99 and 6.53 respectively), did not demonstrate in vivo a significant antiseptic activity. Particularly, when compound (**IIb**) was injected in mice with septic shock, we did not observe an increase in the viability if compared with placebo. Possibly, these results can be explained by a Zwitter ion structure of the heterocycles, which prevented the formation of stable hydrogen bonds with the receptors (Scheme 4).



Scheme 4. Heterocycle (**IIb**) as a Zwitter ion.

On the basis of the analysis of viability of mice with septic shock in screening experiments we sorted out some promising compounds for the assessment of their effect on pathological symptoms of acute respiratory distress syndrome upon *Klebsiella pneumoniae* infection. This inflammatory injury of lungs is a severe life threatening syndrome, which often accompanies septic inflammation.

All the selected compounds were studied in combination with meropenem, which displays an antibacterial effect. Particularly, upon the injection of heterocycles (**III**) and (**VI**) (to a larger degree, $p = 0.03$) and (**Ia**) ($p < 0.05$) we observed a decrease in the extravascular lung water, which correlated with a weaker systemic inflammation associated with the migration of a larger quantity of leukocytes into the inflammation lesion.

To summarize, ZM-241385 structural analogues, triazolo[1,5-*a*]pyrimidine derivatives, were synthesized, a virtual prognosis model of their action was developed in respect to the A_{2A} receptor, and their sig-

nificant biological activity against the septic condition was found.

EXPERIMENTAL

Chemical Part

^{1}H and ^{13}C NMR spectra (δ, ppm, *J*, Hz) were registered on a Bruker Avance II spectrometer (400 and 100 MHz respectively) at 25°C; Me_4Si as an internal standard; DMSO-d_6 and $CDCl_3$ as solvents. Element analysis was performed on a Perkin Elmer 2400 CHN analyzer. The reactions were monitored by TLC on Silufol UV-254 plates, EtOAc as an eluent. Melting points were measured on Staffordshire ST15 0SA apparatus. Compounds (**Ic**), (**IIc**), (**IId**), (**III**), and (**VI**) were synthesized as described in [26, 28, 30–32]. Heterocycle **ZM-241385** was used as a virtual structure for the calculation of the affinity to the A_{2a} adenosine receptor in silico. However, it was not used as a reference in the in vivo experiments.

2-(3'-Pyridyl)-5-methyl-6-nitrotriazolo[1,5-*a*]pyrimidin-7-one (IIb). 2-(3'-Pyridyl)-5-methyltriazolo[1,5-*a*]pyrimidin-7-one (2.27 g, 0.01 mol) was added to a vitriol oil (10 mL) under cooling with ice and the suspension was stirred for 15 min under cooling with ice. Seventy-percent nitric acid (0.83 mL, 0.013 mol) was added to the suspension at 5–10°C. Cooling was removed and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was kept overnight at room temperature and then poured onto ice upon stirring. The resulting light yellow precipitate was filtered off and recrystallized from DMSO to give 95% of the product; mp 293–295°C. ^{1}H NMR (CF_3COOD): 2.76 (s, 3 H, CH_3), 8.26 (dd, 1 H, J_1 6.0, J_2 6.0, H5'), 8.95 (d, 1 H, *J* 6.0, H4'), 9.23–9.30 (m, 1 H, H6'), 9.52 (s, 1 H, H2'). ^{13}C NMR (CF_3COOD): 20.4 (CH_3), 130.2 (C5), 130.5 (C5'), 131.8 (C2), 142.4 (C2'), 145.1 (C4'), 147.5 (C6'), 151.8 (C7), 153.0 (C3a), 155.8 (C6), 160.0 (C3'). Found, %: C 45.49 H 3.41 N 28.80. $C_{11}H_8N_6O_3 \cdot H_2O$. Calc., %: C 45.52 H 3.47 N 28.96.

2-(2'-Furyl)-6-nitrotriazolo[1,5-*a*]pyrimidin-7-one (Ia). 3-(2'-Furyl)-5-amino-1,2,4-triazole (1.5 g, 0.01 mol) was mixed with ethyl 2-nitro-3-ethoxyacrylate (1.7 mL, 0.0105 mol) and the mixture was heated at 70°C for 30 min. The reaction mixture was cooled to room temperature and 2M sodium carbonate (5 mL, 0.01 mol) was added. The suspension was stirred for 45 min at room temperature, and the precipitate was filtered off, suspended in water (7 mL), and 12 M hydrochloric acid (0.8 mL, 0.01 mol) was added. The gray precipitate was filtered off and dried on air to give 87% of the product; mp 286–287°C. ^{1}H NMR (DMSO-d_6): 6.71 (dd, 1 H, J_1 3.4, J_2 1.7, H3'), 7.20 (d, 1 H, *J* 3.4, H2'), 7.94 (d, 1 H, *J* 1.7, H4'), 9.27 (s, 1 H, H5). ^{13}C NMR (DMSO-d_6): 112.3 (C3'), 112.4 (C2'), 125.1 (C3a), 144.5 (C1'), 145.4 (C4'), 148.0 (C5), 149.1 (C7), 152.5 (C6), 154.0 (C2). Found, %: C 43.65 H 2.01 N 28.30. $C_9H_5N_5O_4$. Calc., %: C 43.73 H 2.04 N 28.33.

7-Alkylamino-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidines (IV), (V). Phosphoryl chloride (4.6 mL, 0.05 mol) was added to a suspension of 6-nitrotriazolo[1,5-*a*]pyrimidine-7-one (1.81 g, 0.01 mol) in absolute MeCN (30 mL) and the mixture was heated to 60°C. Dry pyridine (1.64 mL, 0.02 mol) was added and the reaction mixture was refluxed for 3 h and evaporated in vacuum at 35°C. The residue was washed with hexane cooled to 5°C and dissolved in CH_2Cl_2 cooled to 5°C. To the resulting solution, triethylamine (1.53 mL, 0.011 mol) was added at 5–7°C and followed by the addition of the corresponding alkylamine (0.01 mol) at a temperature below 10°C. The mixture was stirred for 2 h at room temperature and washed with water (3 × 50 mL). The organic phase was dried with Na_2SO_4 and evaporated to dryness. The residue was dissolved in refluxing EtOAc (3 × 30 mL) and the combined extracts were washed with water (3 × 50 mL). The organic phase was dried with Na_2SO_4 and the product was purified by flash chromatography eluting with EtOAc.

7-*n*-Butylamino-6-nitro-1,2,4-triazolo[1,5-*a*]pyrimidine (IV). Light yellow powder. Yield 57%. mp 118–120°C. ^{1}H NMR ($CDCl_3$): 0.96 (t, 3 H, *J* 7.2, CH_3), 1.43–1.49 (мм, 2 H, CH_2), 1.75–1.81 (m, 2 H, CH_2), 4.39 (dt, 2 H, J_1 6.8, J_2 6.8, N–CH_2), 8.28 (s, 1 H, H2), 9.24 (s, 1 H, H5), 9.83 (br m, 1 H, NH). ^{13}C NMR ($CDCl_3$): 13.4 (CH_3), 19.5 (CH_2), 32.6 (CH_2), 46.6 (N-CH_2), 118.6 (C6), 146.1 (C7), 153.0 (C5), 156.2 (C2), 157.5 (C3a). Found, %: C 45.70 H 5.26 N 35.41. $C_9H_{12}N_6O_2$. Calc., %: C 45.76 H 5.12 N 35.57.

6-Nitro-7-(2'-(4"-chlorophenyl)ethylamino)-1,2,4-triazolo[1,5-*a*]pyrimidine (V). Yellow crystals. Yield 40%. mp 189–191°C. ^{1}H NMR (DMSO-d_6): 3.06 (t, 2 H, *J* 7.6, Ph-CH_2), 4.45–4.51 (br m, 2 H, N-CH_2), 7.32 (d, 2 H, *J* 8.4, 2 × CH), 7.35 (d, 2 H, *J* 8.4, 2 × CH), 8.64 (s, 1 H, H2), 9.19 (s, 1 H, H5), 10.07 (br m, 1 H, NH). ^{13}C NMR (DMSO-d_6): 35.9 (Ph-$\underline{C}H_2$), 46.9 (N-CH_2), 119.6 (C6), 128.3 (C2', C6'), 130.6 (C3', C5'), 131.2 (C4'), 137.2 (C1'), 146.1 (C7), 152.7 (C5), 156.2 (C2), 157.4 (C3a). Found, %: C 48.89 H 3.63 N 26.45. $C_{13}H_{11}ClN_6O_2$. Calc., %: C 48.99 H 3.48 N 26.37.

Biological: In Vivo Experiments

Mice were taken from a certified nursery "PLZh Rappolovo." During the quarantine the animal material was studied in a state veterinary laboratory GGBU LMVL to give a negative response to salmonellosis. Manipulations with the animals were performed in accordance with the guidelines on humane treatment of laboratory animals in biological experiments.

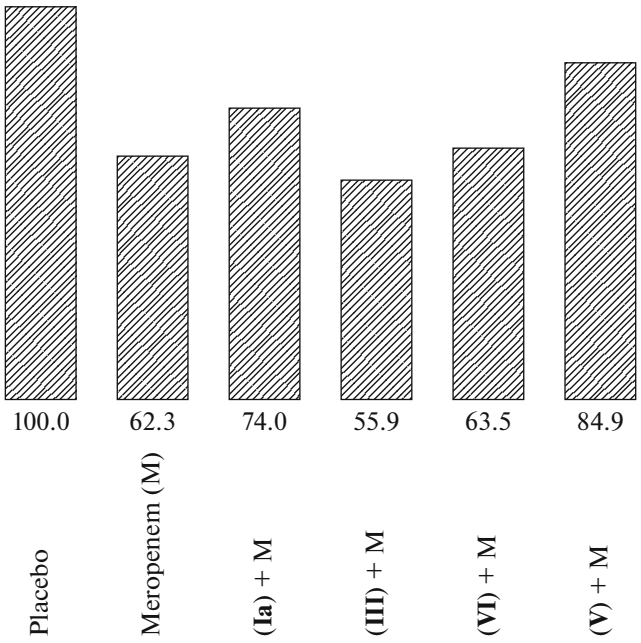


Fig. 2. The effect of experimental therapy with meropenem (M) and compounds under study (**Ia**), (**III**), (**V**), (**VI**) on the value of the weight coefficient (MC) of mouse lungs upon *K. pneumonae* infection.

Screening of biological activity of the compounds under study was carried out on a model of septic shock [33] induced by injections of 10 mg/kg *S. typhi* LPS. The experiment was carried out on 130 mice.

The compounds under study (I- were dissolved in 10% DMSO) and injected intraperitoneally twice a day at a daily dose of 50 mg/kg. As a reference drug, hydrocortisone hemisuccinate was injected intraperitoneally at a dose of 15 mg/kg once a day 2 h before modelling. LPS was injected 2 h after the injection of the tested compounds at a dose of 10 mg/kg once a day.

The dynamics of mouse viability in the test and control groups was monitored. The results were presented as a portion of survived mice (%).

Sepsis modeling was performed by infecting mice with *K. pneumoniae* strain NCTC5054 at a dose of 5×10^{10} CFU/kg body weight. The dose was injected intrapleurally at a volume of 0.02 mL per animal (20 g). The experiment was conducted on 35 mice.

The compounds under study were dissolved in 10% DMSO and injected intraperitoneally for 3 days at a daily dose of 50 mg/kg upon the simultaneous use of meropenem. As a reference, meropenem was injected intraperitoneally at a dose of 200 mg/kg.

On day 7, mice were subjected to euthanasia and the lung weight coefficient (the ratio of the wet lung weight to body weight before euthanasia expressed in mg/g) in the control and test groups. The results were reported on a histogram (Fig. 2).

Statistical analysis

For the statistical processing, the data was entered to a MSExcel 2010 program for accumulation and preparation for the analysis. The analysis was performed using statistical package programs Statistica (StatSoft, United States). The data was presented as a mean (M) and error in mean ($\pm m$). Nonparametric ANOVA test was used. The differences were regarded as significant at $p \leq 0.05$.

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