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The Relationship between NO-Synthase Inhibitory Activity of N,S-Containing Heterocycles and Their Radioprotective and Antileukemic Properties

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Abstract—The effect of NO-synthase (NOS) activator and inhibitors on leukemic cell lines HL-60, K-562, and MOLT-4 and bone marrow cells of untreated patients diagnosed with B-cell acute lymphoblastic leukemia compared with lymphocytes from healthy donors is examined. The obtained data on the relationships between the radioprotective, NOS inhibitory, and cytotoxic properties of a number of thiazine, thiazoline, and thiourea derivatives indicates their potential for use as agents for complex radio- and chemotherapy.

Keywords: acute leukemia, thiazine, thiazoline, thiourea derivatives, NO-synthase inhibitors, radioprotectors
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INTRODUCTION

The processes of spontaneous and radiation-induced cancer development are very similar both in mechanism of development and clinical manifestations. As a rule, this mechanism includes the enhancement of oxidative stress and lipid peroxidation, disturbance of the p53 response and phosphorylation, damage and/or exhaustion of possibilities of antioxidant systems, impaired expression of transcription factors, NF-κB activation, and others. In some cases, one of the factors is an increase in the expression of inducible NO-synthase and the NO level. Certainly, the causes of various cancers may have a specific initiation (including polymorphism) and line of growth, but all of the above-mentioned factors are present in the process of their development. Since hematopoiesis is the most radiosensitive process in the body, radiation exposure primarily promotes the development of various leukemias, i.e., disruption of proliferation and differentiation of blood cells. The high sensitivity of hematopoietic cells to radiation, prooxidative, and other leukemogenic effects is associated with their morphological and energetic features. At the same time, oxidative stress is a necessary condition for the emergence and development of leukemogenesis, and the ability of leukemic cells to continue interrupted differentiation can be explained by the capacity of differentiation agents to directly or indirectly affect the level of reactive oxygen species (ROS) and change the ROS-dependent transcription of differentiation genes. The possibilities in antioxidant therapy partly confirm the peroxide–oxygen concept of leukemogenesis [1].

Nitric oxide, being one of the most important signaling molecules, is included into the system of active oxidative agents. NO is able to form peroxynitrite with a superoxide radical anion, which is one of the most potent cytotoxic products. It is assumed [2] that NO, along with other signaling molecules, may be an important regulator of homeostasis for early progenitors of hematopoiesis. The expression of various isoforms of NOS (inducible (*i*NOS), endothelial (*e*NOS), and neuronal (*n*NOS)) is found in human bone marrow and blood cells [3, 4]. Increased NO activity at the early stage of hematopoiesis contributes to the emergence of cancer stem cells and carcinogenesis, which occurs when the body is exposed to ionizing radiation. *i*NOS and NO molecules are included in the acute radiation response [5], the consequence of which is the development of leukemia. Overexpression of *i*NOS is observed in patients diagnosed with acute myeloid leukemia (AML) [6] and under laminar hemodynamic shock activation of *e*NOS (and nuclear factor NFκB, a leukemogenesis participant) occurs. Hydrogen peroxide (an active product of water radiolysis and an oxidative agent) also mediates increased *e*NOS expression [7]. It has been shown in vitro [8] that an increase in the level of NO in blood lympho-

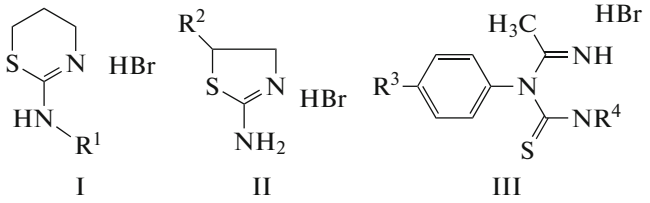

Fig. 1. Hydrobromide derivatives of 2-amino-5,6-dihydro-4H-1,3-thiazine (I), 2-amino-2-thiazoline (II), and thiourea (III).

cytes of healthy patients leads to genomic instability and increased risk of cancer and metastasis.

However, NO has dual pro- and antiapoptotic functions; the manifestation of each of them is determined by its concentration and a number of biochemical processes associated with the change (disruption) of the NO amount. The relationship between these functions is not completely understood as yet. A serious role of transcription factor upregulation, associated with the level of *i*NOS expression, is seen in carcinogenesis.

Obviously, if the radiation causes an increase in NO (NOS expression), the enzyme inhibitors should be radioprotectors. Indeed, for some heterocyclic inhibitors containing sulfur and nitrogen atoms, inhibitory activity against different NOS isoforms has been demonstrated [9]. Some representatives of these classes have shown antitumor and other biological activity.

The question arises whether there is a relationship in the chain NOS inhibition–survival of leukemic cells of different types–radioprotective properties of substances. The question also arises of how such a relationship can be predicted, if it exists. We have considered this question on the example of heterocyclic derivatives of thiourea, which is an aliphatic precursor of five- or six-membered heterocycles containing nitrogen and sulfur atoms.

MATERIALS AND METHODS

In this work, derivatives of 2-amino-5,6-dihydro-4H-1,3-thiazine (I), 2-amino-2-thiazoline (II), and thiourea (III) were synthesized (Fig. 1, Table 1). The procedures of synthesis were previously described [10–13]. The composition and structure of the drugs was verified by elemental analysis and ^{1}H and ^{13}C NMR. The choice of drugs was determined by their different NOS-inhibitory activity [14], in order to create a chain of different levels of enzyme inhibition (NOS activator–inert drug (towards *i*NOS)–NOS inhibitors) with increasing degree of inhibition.

The cell lines HL-60 (human promyelocytic leukemia cells), K-562 (chronic myeloid leukemia cells), and MOLT-4 (human T-cell acute lymphoblastic leukemia), cultured using standard procedures, as well as bone marrow cells of patients (aged 4–16 years) diagnosed with B-cell acute lymphoblastic leukemia (hereinafter B-ALL) before the start of chemotherapy, were used in this study. Blood sampling and preparation of donor cell material was performed according to the procedure described previously [15]. Lymphocytes from healthy donors of the same age range were used as a control. In all cases, hematological assay showed the content of blast cells in the mononuclear fraction >80%.

Determination of cell viability was performed by the modified MTT method [16]. At least ten samples were used for each case. The results were processed by the Mann–Whitney U test ($p < 0.05$). The LC_{50} value was estimated by the median and Student's t statistics.

The values of inhibitory activity of the compounds in vitro were taken from the literature [9]. Experiments in vivo were performed according to Vanin's method using EPR spectroscopy with a Fe^{2+}-diethyldithiocarbamate complex ($Fe^{2+}(DETC)_2$) as a spin trap [17]. EPR spectra were recorded at 77 K on a Bruker device (model ESP-300E). The measurement results of NO content in the liver were expressed as the ratio of the EPR signal amplitude of the complex NO–Fe^{2+}–$(DETC)_2$ in samples of mouse liver exposed to lipopolysaccharides (LPSs) + the substance to the signal amplitude in mouse liver samples treated only with LPSs. Thus, the measured NO value in vivo refers to the residual (after inhibition) production of the total pool of NOSs, i.e., all enzyme isoforms.

RESULTS AND DISCUSSION

Table 1 shows the survival of different types of leukemic cells compared with the cells of healthy donors under the action of NOS effectors in order of reducing activity of the administered inhibitor. The relationship of NOS-activity (in vivo) of the compounds and cell survival in patients with B-ALL is shown in Fig. 2. It can be seen that there is a relationship between the inhibition of NO production and leukemic cell survival, which is different for different types of leukemia.

The NOS activator possesses the highest cytotoxicity, with cytotoxicity against leukemic cells 4–10 times higher for all of the studied cases. The highest cytotoxicity and therapeutic index TI = LC_{50} (for healthy cells)/LC_{50} (for leukemic cells) was observed for B-ALL cells.

The curve of the dependence of healthy donor cell survival (LC_{50}) on the level of NOS inhibition in vivo (Fig. 2) increases under the transition from the activator to inhibitors and does not change up to the 80% level of inhibition (LC_{50} at the level of ~2 pmol/mL). Further decrease in the amount of NO (at the level of NOS inhibition from 80 to 90%) causes a sharp increase in survival. Further, the LC_{50} value is practically unchanged (within the error) at a new higher level (~10 mmol/mL). Two exceptions are observed: for compounds 4 and 6. Compound 4 has a long

Table 1. Dependence of cell survival (LC_{50}) on inhibitory activity against NO-synthases of administered compounds (in vitro and in vivo)

Number of compound	Compound	Inhibitory activity, %		LC_{50}, μmol/mL				
		in vitro* (lit. data)	in vivo**, residual NO	healthy donors	B-ALL	HL-60	K-562	MOLT-4
1	III ($R^3 = iC_3H_7$; $R^4 = C_5H_{10}$)	Weak activator	Activator. 170 ± 10	0.5 ± 0.2	0.05 ± 0.02	0.11 ± 0.03	0.14 ± 0.05	0.10 ± 0.03
2	I ($R^1 = C_6H_4F$)	–	80 ± 20	2.6 ± 0.9	1.5 ± 0.3	0.5 ± 0.2	0.8 ± 0.3	1.1 ± 0.3
3	II, III ($R^3 = CH_3$; $R^4 = C_4H_8$)	*i*NOS: 0 *n*NOS: 0	20 ± 4	2.0 ± 0.4	1.5 ± 0.3	1.0 ± 0.2	3.2 ± 0.5	2.0 ± 0.6
4	I ($R^1 = C_{12}H_{25}$)	*i*NOS: 3 ± 1 *n*NOS: 58 ± 4	15 ± 4	0.04 ± 0.01	0.004 ± 0.001	0.33 ± 0.09	2.50 ± 0.5	1.5 ± 0.4
5	I, II ($R^2 = CH_2OH$)	–	11 ± 3	8.0 ± 2.1	4.5 ± 0.9	0.4 ± 0.1	11 ± 4	5.0 ± 1.5
6	II, III ($R^3 = iC_3H_7$; $R^4 = C_2H_6$)	*i*NOS: 2.0 ± 0.5 *n*NOS: 25 ± 5	10 ± 3	3.0 ± 0.5	0. 3 ± 0.1	–	–	–
7	I (R^1 = H, counterion – Sal)	–	6 ± 2	10 ± 2	2.4 ± 0.5	0.5 ± 0.2	1.6 ± 0.2	1.3 ± 0.3
8	II ($R^2 = CH_3$)	*i*NOS: 90 ± 5	5 ± 2	15 ± 3	1.9 ± 0.5	1.1 ± 0.4	2.2 ± 0.7	2.0 ± 0.5
9	I(R^1 = H)	*i*NOS: 68 ± 7 *n*NOS: 66 ± 6	3 ± 1	13 ± 3	4.3 ± 0.5	1.0 ± 0.2	15.5 ± 5.5	2.8 ± 0.2
10	I ($R^1 = COC_6H_5$)	*i*NOS: 92 ± 9 *n*NOS: 88 ± 7	3 ± 1	10.6 ± 2.3	0.9 ± 0.3	0.5 ± 0.2	1.0 ± 0.3	1.2 ± 0.4

* A (native enzyme)–A (enzyme in the presence of the drug); ** relative to NO in liver of control mice (%) at a dose of the drug 10 μmol/kg; Sal—salicylate ion.

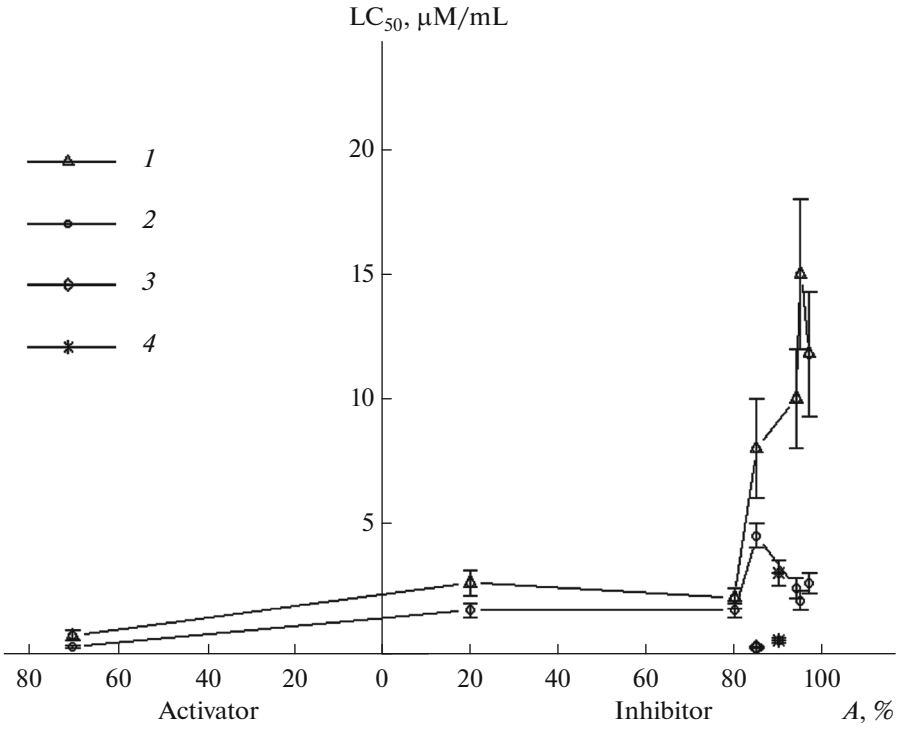

Fig. 2. Dependence of survival (LC_{50}) of healthy (*1*) and leukemic (*2*) cells on NOS-inhibitory activity of administered compounds. Activator on the left, inhibitors on the right of the vertical axis; (*3*) compound 4 (forms nanoparticles); (*4*) compound 6 (radiosensitizer).

hydrophobic tail and, as was shown by the method of dynamic light scattering, in solution forms nanoparticles 10–100 nm in size, which cyclically change their size depending on the time of solution standing. Inclusion of this compound in the considered dependence is impossible due to its much greater toxicity toward healthy cells. Compound 6 has two features: 1) a very low percentage of *i*NOS inhibition in vitro, which indicates its higher inhibitory ability towards *e*NOS (in vivo); 2) it is a radiosensitizer (Table 2), i.e., it has the opposite effect on the products of oxidative stress (or this effect is absent).

When the amount of NO is reduced by a strictly defined value (~85–89% *i*NOS inhibition), a sharp (trigger) change in the mechanism of its effects on healthy cells occurs; the behavior of the cells after the jump weakly depends on the NO concentration (within the measurement error) at a new higher level of survival. Thus, healthy cells have a system for response to increases and decreases in the NO concentration. This response system can be compared with a buffer system: when the system "fails" at strictly defined NO concentration, cell viability "jumps" to a new higher level.

In the case of the cells from patients with B-ALL, their survival curve qualitatively coincides with the dependence observed for healthy cells up to the 80% level of NOS inhibition, but cytotoxicity against leuke-

Table 2. Correlation of radioprotective, cytotoxic and NOS-inhibitory properties as exemplified by representatives of studied compound classes

Number of compound	DMF	Inhibitory activity towards *i*NOS, in vitro % (lit. data)/ in vivo (towards all NOSs)	TI* = LC_{50} (healthy donors)/LC_{50} (leukemic cells, B-ALL)
6	~0.8	2/90	3.0/0.3 = 10
3	1.0	0/80	2.0/1.5 = 1.3
9	1.3 [21]	68/97	13/4.3 = 3
8	1.3 [21]	90/95	15/1.9 = 7.9
10	1.5	92/97	10.6/0.9 = 11.8

Designations: TI—therapeutic index; DMF—dose modifying factor.

mic cells is quantitatively slightly higher (approximately 1.5–2.0 times). Subsequently, as with healthy cells, a jump of survival growth is observed, but it subsequently returns to the previous level, where NOS inhibition is higher than 90%. A similar regularity, both qualitatively and quantitatively, is observed for the cell line MOLT-4 (T-ALL). At the same time, no explicit dependence of survival on NOS inhibition is observed for the chronic myeloid leukemia cell line K-562; moreover, there are a number of substances that cause significant increases in the survival of these leukemic cells.

The strength of NOS inhibitors has almost no effect on the cell line HL-60 (except the NOS activator), but the therapeutic indexes in this case were the highest, reaching 20. It is known that the effect of cytokines as proinflammatory stimuli on NO production for the HL-60 cells is different from that in the case of K-562 and MOLT-4 [18].

Aspirin, salicylates, and their derivatives have been actively tested as antileukemic agents, particularly in pediatric oncology [19, 20]. However, the substitution of the counterion, hydrobromide, by salicylate (compound 7) did not show an increase in cytotoxicity. The use of salicylates requires a special study.

Comparison of the NOS-inhibitory and antileukemic (against B-ALL cells) properties of the compounds and their radiomodifying effect (Table 2) showed some correlation of TI with the increase in the radioprotective activity. In the case when the DMF values were equal (compounds 8 and 9), TI increased with increasing *i*NOS inhibition. A radiosensitizer (compound 6) and compound 3 (which is not a radiomodifier) equally affect the healthy cells, but their TI differs tenfold due to the greater cytotoxicity of the radiosensitizer against B-ALL cells.

The data for compound 5 are somewhat surprising (89% NOS inhibition). In this case, for all the cells except healthy donor cells and the cell line HL-60, there was an increase (a peak of varying magnitude) in LC_{50}. This can be explained by the specific properties of the drug, whose effect on leukemic cells is stronger than that of NO. However, it seems more likely that in this area of inhibition level values, i.e., under strictly defined NO concentrations caused by the inhibitor, the intersection (overlapping) of opposite mechanisms of NO action (pro- and antiapoptotic) occurs, and this may lead to instability of the results, which begin to depend on the specific properties of the compounds affecting the leukemic cells.

Thus, the increase in the strength of the radioprotector, as well as the increase in the activity of NOS inhibitors, contributes to the increase in TI when exposed to cancer cells of patients with B-ALL. This means that the role of oxidative stress, which is one of the main targets of radioprotectors, and ER-stress, occurring as a result of NO (NOS) exposure, may be adjusted by the representatives of thiazine-thiazolines and thioureas with various DMF parameters and NOS-inhibitory activity. These compounds may be useful in combination therapy with radio- and chemotherapy.

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