Cytostatic action of triazole and oxazolidinone derivatives

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We synthesized a number of derivatives of 1,2,3-benzotriazole, 1,2,4-triazole, and oxazolidine-2-one derivatives, among which compounds suppressing the growth of the tumor cells HeLa and H1299 were revealed. The studies of the effect of these compounds on the cell cycle and activity of caspase dependent degradation of poly(ADP-ribose) polymerase showed that these compounds at the IC_{50} doses exhibit cytostatic effect on tumor cells. In the MCF7 cells, these compounds induce an increase in expression of the tumor suppressor p53.

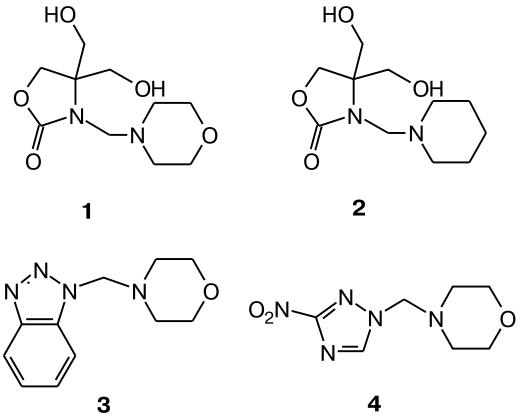
Key words: triazole, oxazolidin-2-one, cytotoxicity, tumor suppresor p53.

The biological activity of triazoles is known for a long time. For example, inhibition of catalase, the enzyme of antioxidant system, by 3-amino-1,2,4-triazole have been found in 1958.[1] Nowadays, triazole derivatives are commonly used as antimicrobial, antiviral, antiinflammatory, and antihelminthic agents;[2] they are also being studied intensively as potential antitumor agents. A variety of the triazole compounds, namely, heterocycle-conjugated,[3] glycosyl,[2] and aryl hydroxamic[4] derivatives of 1,2,3-triazole, novobiocin analogs,[5] as well as ribonucleoside,[6] dichlorofluorophenyl[7] and diaryl[8,9] derivatives of 1,2,4-triazole have been shown to possess cytotoxicity towards the tumor cell culture. A high antitumor potential has been demonstrated for platinum and palladium triazole-containing complexes.[10]

Many studies were aimed at the effect of triazole derivatives on specific molecular targets. For example, it has been shown that triazoles suppress selectively the activity of the tyrosine protein kinases involved in the regulation of cytokine synthesis[11] or cell division[12], as well as the activity of the serine-threonine protein kinases involved in the regulation of cell cycle.[13] It has been shown that the triazole derivatives inhibit histone deacetylases, which are currently regarded as promising antitumor compounds.[4] The triazole derivatives of combretastatin A-4, which is tubulin polymerization inhibitor, have been shown to possess cytotoxic activity.[14,15] 1,2,4-Triazole ribosides suppress expression of the Hsp27, the heat shock protein defining the tumor resistance towards chemotherapeutic agents, which, in turn, induces the death of therapy resistant tumor cells.[6]

High antimicrobial and fungicidal activity of oxazolidinones as well as their effects on monoaminooxidase has been shown, which demonstrates their potential for the therapy of neurodegenerative diseases.[16,17] The studies on the antitumor activities of oxazolidinone derivatives are also being performed.[18] It has been shown that the phenyl derivatives of oxazolidinone exhibit cytotoxic activity.[19] As in the case of triazoles, tubulin polymerization inhibitors are being designed based on oxazolidinones.[20] In addition, it is known that an oxazolidinone derivative is one of the metabolites of the well known antitumor agent, cyclophosphamide.[21]

The aim of the present work is to synthesize triazole and oxazolidinone derivatives and to study their cytotoxic properties. It was shown that compounds **1**—**4** have cytostatic effect on tumor cells and induce expression of the tumor suppressor p53.



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Experimental

Compounds **1**—**4** were prepared by *N*-aminomethylation of 4,4-bis(hydroxymethyl)oxazolidin-2-one, benzotriazole, and 3-nitro-1,2,4-triazole. Compound **3** has been described,[22] compounds **1**, **2**, and **4** were synthesized for the first time. 4,4-Bis-(hydroxymethyl)oxazolidin-2-one and 3-nitro-1,2,4-triazole were prepared by known procedures.[23,24] The ^{1}H NMR spectrum of compound **4** was recorded on a Bruker Avance III-500 instrument in DMSO-d_6 and the ^{1}H NMR spectra of compounds **1** and **2** were recorded on a Bruker DPX-200 instrument in $CDCl_3$ using Me_4Si as the internal standard.

4,4-Bis(hydroxymethyl)-3-morpholinomethyloxazolidin-2-one (1). To a mixture of 4,4-bis(hydroxymethyl)oxazolidin-2-one (0.74 g, 5 mmol), water (7 mL), 30% formalin (0.5 g, 5 mmol), and a solution of morpholine (0.44 g, 5 mmol) in water (4 mL) was added dropwise with stirring at room temperature. The solution obtained was stirred for 1 h at room temperature and 1 h at 60 °C. The volatile products were removed in air stream. The residue was washed with Pr^{i}OH (4 mL) to obtain compound **1** in a yield of 0.88 g (71.5%), m.p.131—133 °C; after recrystallization from Pr^{i}OH m.p. 133—134 °C.

4,4-Bis(hydroxymethyl)-3-piperidinomethyloxazolidin-2-one (2) was prepared analogously from 4,4-bis(hydroxymethyl)-oxazolidin-2-one (0.74 g, 5 mmol), 30% formalin (0.5 g, 5 mmol), and piperidine (0.43 g, 5 mmol). The yield was 0.3 g (26%), m.p. 119—121 °C (from toluene).

1-Morpholinomethyl-3-nitro-1,2,4-triazole (4) was prepared analogously from 3-nitro-1,2,4-triazole (0.57 g, 5 mmol), 30% formalin (0.5 g, 5 mmol), and morpholine (0.43 g, 5 mmol). The reaction mixture was kept for 24 h at room temperature. The yield was 0.66 g (62%), m.p. 134—136 °C (from Pr^{i}OH).

1-Morpholinomethylbenzotriazole (3) was prepared according to a known procedure,[22] m.p. 104—106 °C (*cf.* Ref. 22, m.p. 104—105 °C).

The properties of the compounds synthesized are given in Table 1.

Ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *p*-cumaric acid, luminol, ribonuclease A, protease inhibitors cocktail (AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstatin), and anti-β-actin primary (rabbit) antibodies were obtained from Sigma (USA). 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane sulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), and Nonidet P-40 (NP-40) were obtained from Amresco (USA). Acrylamide and actinomycin D were obtained from Serva (Germany). Anti-p53 and anti-poly(ADP-ribose) polymerase (PARP) primary (rabbit) antibodies, as well as anti-rabbit IgG secondary (goat) antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology (USA). 4-(1,1,3,3-Tetramethylbutyl)-phenyl-polyethylene glycol (Triton X-100), polyoxyethylene (20) sorbitan monolaurate (Tween20), and glycine were obtained from Panreac (Spain). Bovine serum albumin (BSA), *N*,*N*′-methylenebisacrylamide and phenylmethylsulfonyl fluoride (PMSF) were obtained from Dia-M (Russia). The incubation medium (DMEM) was obtained from the Institute of Poliomyelitis and Viral Encephalites of the Russian Academy of Medical Sciences. Fetal bovine serum was obtained from BioWest (France). Propidium iodide was obtained from ApplicHem (Germany).

Cell culture. The experiments were carried out on the cell lines HeLa (human cervical carcinoma), MCF7 (human breast adenocarcinoma), and H1299 (human non-small cell lung carcinoma). Cells were maintained at 37 °C under atmosphere containing 5% CO_2 in DMEM with addition of 10% fetal bovine serum.

Effect of compounds on cell viability. MTT test was applied for the study of cytotoxicity. Cells were plated in a 24-well plate ($1.5 \cdot 10^4$ HeLa or H1299 cell per well) in the standard incubation medium. The compounds under study were added at different concentrations into the incubation medium 24 h after plating. After 24 h of exposure, MTT was added into the incubation medium to final concentration 0.45 mg mL^{-1} and cells were stained in an incubator for 4 h. After staining, the incubation medium was removed, the crystals of MTT-formazan that formed were dissolved in 50% isopropyl alcohol with 0.05 *M* HCl. The optical density of MTT-formazan solutions was determined at the wavelength of 570 nm. The staining of untreated cells was taken as 100%.

Table 1. Elemental analysis data and ^{1}H NMR spectra of compounds **1**, **2**, and **4**

Compound	Found / Calculated (%)			Molecular formula	^{1}H NMR, δ, ($\Delta\nu_{AB}$/Hz, *J*/Hz)
	C	H	N		
1	48.70 48.77	7.10 7.37	11.20 11.37	$C_{10}H_{18}N_2O_5$	2.65 (br.t, 4 H, N$\underline{CH_2}CH_2O$, ^3J = 4.4); 3.68 (AB system, 4 H, CC$\underline{H_2}$OH, $\Delta\nu_{AB} \approx 27.1$; $\lvert^2J_{AB}\rvert \approx 12.3$); 3.73 (br.t, 4 H, $NCH_2C\underline{H_2}O$, ^3J = 4.4); 3.94 (s, 2 H, NCH_2N); 4.13 (s, 2 H, CH_2, cycl.); 4.91 (br.s, 2 OH)
2	54.30 54.08	8.40 8.25	11.50 11.47	$C_{11}H_{28}N_2O_4$	1.40—1.68 (м, 6 H,$CH_2CH_2CH_2$, piperidine); 2.57 (m, 4 H, NCH_2C, piperidine); 3.67 (AB system, 4 H, CCH_2OH, $\Delta\nu_{AB} \approx 24.8$; $\lvert^2J_{AB}\rvert \approx 12.2$); 3.87 (s, 2 H, NCH_2N); 4.10 (s, 2 H, OCH_2C); 5.41 (br.s, 2 H, OH)
4	39.39 39.43	5.31 5.20	32.80 32.85	$C_7H_{11}N_5O_3$	2.56, 3.58 (both m, 4 H each, NCH_2, OCH_2, morpholine); 5.22 (s, 2 H, NCH_2N); 8.84 (s, 1 H, N=CHN)

Preparation of cell lysates and nuclear extracts. Cells were plated in Petri dishes in the standard incubation medium (10^6 cells per dish). After 24 h of plating, the compounds under study were added into the incubation medium. At different time intervals of exposure, the incubation medium was aspirated, cells were washed three times with the PBS buffer (137 mmol L^{-1} NaCl, 2.68 mmol L^{-1} KCl, 4.29 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4).

For the preparation of lysates, cells were lysed for 20 min at 0 °C in the buffer containing 500 mmol L^{-1} HEPES (pH 7.5), 150 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 25 mmol L^{-1} NaF, 10 μmol L^{-1} $ZnCl_2$, 10% glycerol, 1% Triton X-100, 1 mmol L^{-1} DTT, 1 mmmol L^{-1} PMSF and the protease inhibitor cocktail. The resulted lysates were clarified by centrifugation at 10000 g for 15 min.

For the preparation of nuclear extracts, cells were lysed for 20 min with constant stirring at 0 °C in the buffer containing 10 mmol L^{-1} HEPES (pH 7.9), 1.5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} KCl, 0.1% NP-40, 1 mmol L^{-1} DTT, 1 mmol L^{-1} PMSF, and the protease inhibitor cocktail. After 20 min of centrifugation at 10000 g, the pellet of nuclei was washed with the same buffer and suspended in the buffer containing 20 mmol L^{-1} HEPES (pH 7.9), 25% glycerol, 420 mmol L^{-1} NaCl, 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} EDTA, 1 mmol L^{-1} DTT, 1 mmol L^{-1} PMSF, and the protease inhibitor cocktail. The suspension was stirred for 45 min at 4 °C. The resulting nuclear extracts were separated from the pellet by centrifugation for 20 min at 10000 g.

The protein content in the lysates and nuclear extracts was measured according to the Lowry method.[25]

Immunoblotting. The proteins of lysates and nuclear extracts were separated with 10% PAGE and transferred on a Hybond-C Extra (AmercHam-GE HealtHcare, USA) membrane in transfer buffer (25 mmol L^{-1} Tris, 19.3 mmol L^{-1} glycine, 20% methanol) at 4 °C at the current strength of 300 mA for 3 h. After transferring, the membrane was incubated for 1 h at room temperature in a blocking solution containing 5% BSA and 0.02% NaN_3 in the TBST buffer (100 mmol L^{-1} Tris (pH 7.5), 150 mmol L^{-1} NaCl, 0.1% Tween20). The membrane was incubated for 1 h with the anti-p53, anti-PARP or anti-β-actin primary antibodies diluted in the 1 : 1000 ratio in a blocking solution and washed three times with the TBST buffer. The membrane was then incubated with secondary antibodies diluted in the 1 : 5000 ratio in a blocking solution (without NaN_3) and washed three times with the TBST buffer. The chemiluminescence reaction was carried out using a mixture of solutions as follows: solution A (140 μL) containing 0.68 mmol L^{-1} *p*-cumaric acid in DMSO and solution B (14 mL) containing 100 mmol L^{-1} Tris (pH 8.5), 1.25 mmol L^{-1} luminol, and 30% H_2O_2 (5 μL). After 1 min of incubation in this mixture, the membrane was exposed on an X-ray film.

Flow cytofluorometry. For the study of the cell cycle phases by flow cytofluorometry, the cells were plated in Petri dishes in the standard incubation medium (10^6 cells per dish). After 24 h of plating, the compounds under study were added into the incubation medium. After 24 h of exposure, the cells were harvested by tryspinization, washed three times with PBS, fixed and permeabilized by dropwise addition of 70% ethanol cooled to −20 °C. The samples were kept for 12 h at 4 °C. Ethanol was removed from the fixed samples by three times washing with a 1% solution of BSA in PBS. The washed cells were resuspended in PBS containing 1% BSA, 0.1 mg mL^{-1} propidium iodide, and 1 mg mL^{-1} ribonuclease A. After 1 h of incubation at 37 °C, the samples were analyzed by flow cytofluorometry on a FACSCalibur device (Becton Dickinson, USA) with a 488 nm exciting laser.

Results and Discussion

Effect of compounds on the tumor cell viability and cell cycle. All compounds under study affect the HeLa and H1299 cell viability (Fig. 1). The HeLa cells are more sensitive to compounds **1** and **3** compared to the H1299 cells; the differences in the sensitivities of the HeLa and H1299 cells towards compounds **2** and **4** are less pronounced. The IC_{50} doses for the HeLa cells were about 130 μmol L^{-1} for **1** and **2** and about 110 μmol L^{-1} for **3** and **4**, and those for the H1299 cells were about 200 μmol L^{-1} for **2** and **4** and about 250 μmol L^{-1} for **1** and **3**. Additional studies revealed that at concentrations of 150 μmol L^{-1} 4,4-bis(hydroxymethyl)oxazolidin-2-one and morpholine had no effect on the HeLa cell growth.

The study of the cell division phases upon the action of compounds **1**—**4** showed that these compounds at the IC_{50} doses affect weakly the cell cycle (Fig. 2, Table 2). Small changes were observed upon the action of the compounds under study on the H1299 cells in the S and G2/M phases of the cell cycle. Compounds **1**, **2**, and **4** have virtually no effect on the HeLa cell cycle. At the same time, the viability of the HeLa cell population decreased by 50% at the used doses (see Fig. 1), which leads typically to the change in the cell cycle profile and cell accumulation in a certain phase, wherein cells are more sensitive to the action of compounds. The absence of significant changes in the HeLa cell cycle profile under the conditions of the decrease in the cell viability suggests that they are sensitive to the action of compounds **1**, **2**, and **3** in all phases of the cell division. The H1299 cells are less sensitive to the action of the compounds when passing through the S, G2, and M phases; as a result, they finish these phases and stop division mainly in the G1 phase. It is also important to note that upon the action of the compounds under study the cell accumulation was virtually not observed in the subG1 region. This indicates that at these doses the compounds do not induce the cell death.

The inability of the compounds to cause the cell death is confirmed by the study using the molecular marker of apoptosis, poly(ADP-ribose) polymerase (PARP) (Fig. 2). This protein is hydrolyzed during apoptosis, whereby its molecular weight decreases from 116 to 85 kDa.[26] From Fig. 2 follows that exposure of cells to compounds **1**—**4** at the IC_{50} doses results in no increase in the amount of the low molecular weight fragment of PARP, whereas actinomycin D (ActD) at the IC_{50} dose (45 nmol L^{-1}) causes notable degradation of PARP after 24 h of its introduction.

Thus, the oxazolidinone derivatives **1** and **2** and triazole derivatives **3** and **4** used at the IC_{50} doses have

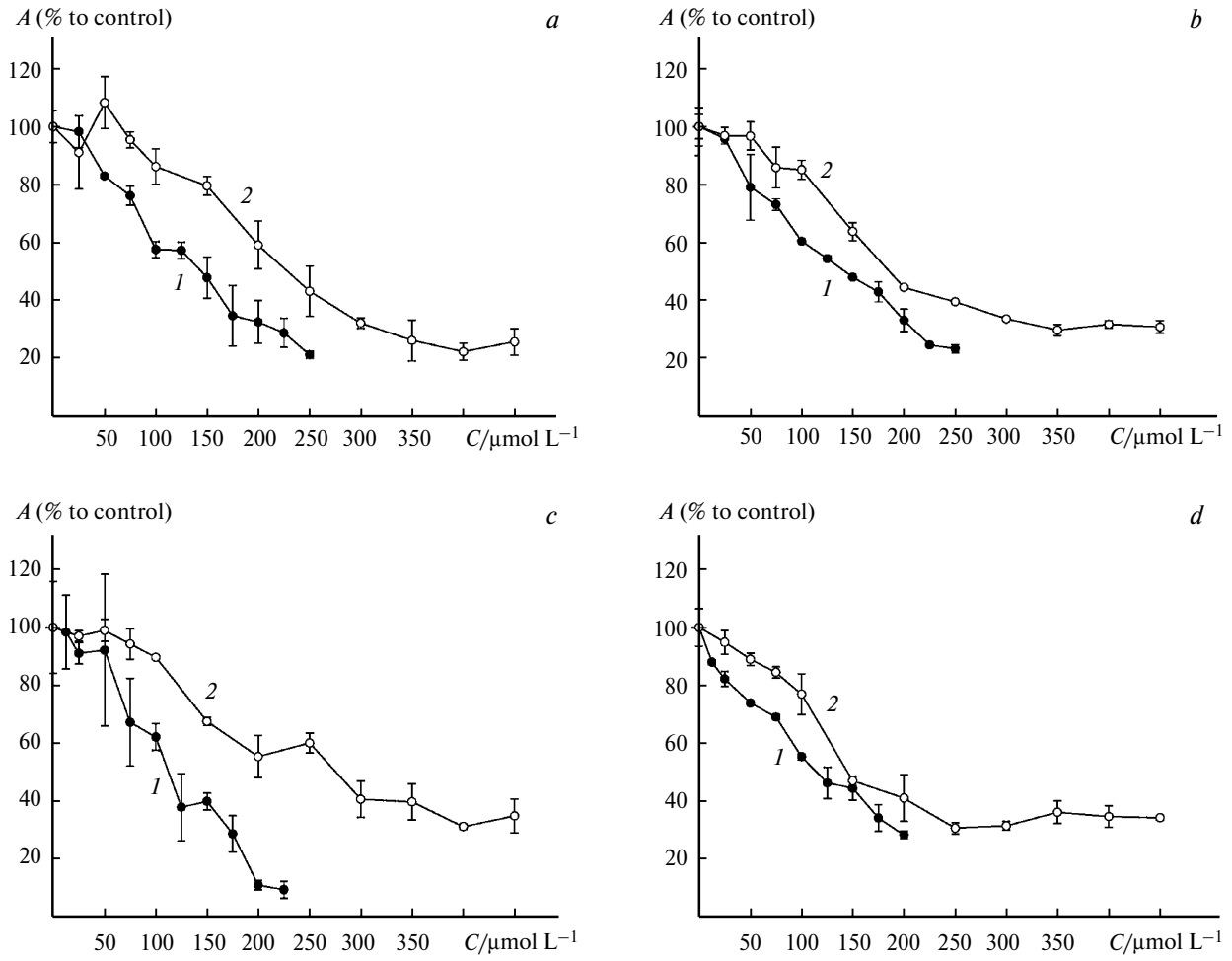

Fig. 1. The effect of compounds **1** (*a*), **2** (*b*), **3** (*c*), and **4** (*d*) on the intensity of MTT staining of the HeLa (*1*) and H1299 (*2*) cells.

the cytostatic effect on the cells and do not induce cell death.

Effect of compounds 1—4 on the protein p53. The protein p53 is involved in the regulation of cell division and defines the cell responses on stress factors of different nature.[27] To study the effect of compounds **1—4** on the protein p53, we used the MCF7 cells of human breast adenocarcinoma, since the p53 function in these cells is affected weakly in contrast to the HeLa and H1299 cells. It is seen from Fig. 3 that all compounds under study cause significant p53 accumulation in the cells. After the analysis of p53 expression, in the same samples, we determined the content of β-actin that is a constitutively expressed cell protein. Slight differences in the signal intensity for β-actin shows that the changes in p53 expression indicate its real accumulation in cells and are not related to the differences in loading of the cell lysates to a gel. The increase in the p53 protein level upon the action of oxazolidinone and triazole derivatives is comparable with its reaction to the action of cytotoxic compound actinomycin D. The molecular mechanism of such reaction requires further stud-

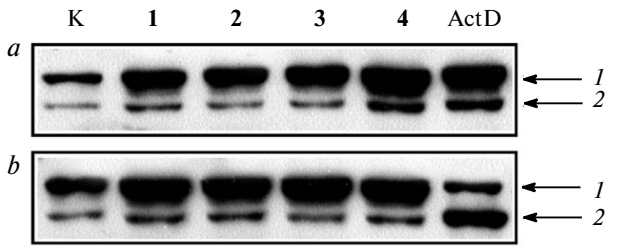

Fig. 2. Immunoblotting with the antibody against PARP of the HeLa cell nuclear extracts after 12 h (*a*) and 24 h (*b*) of the introduction of compounds **1—4** at the IC_{50} doses for the HeLa cells: 130 μmol L^{-1} for compounds **1** and **2**, 110 μmol L^{-1} for compounds **3** and **4**, and 45 nmol L^{-1} for actinomycin D. The arrows show PARP with the molecular weights of 116 (*1*) and 85 kDa (*2*). Here and in Fig. 3, K is a control and ActD is actinomycin D.

Table 2. The relative contents of the HeLa and H1299 cells in the phases G1, S, G2 and M, and in the subG1 region of the cell cycle upon the action of compounds **1—4** according to the data from flow cytofluorometry

Cell line	Sample (IC_{50}/μmol L^{-1})	Relative content of cells (%)			
		subG1	G1	S	G2/M
HeLa	Control	2.0	61.6	20.8	15.6
	1 (130)	1.2	63.8	19.1	15.8
	2 (130)	1.7	64.4	18.2	15.8
	3 (110)	4.4	54.6	23.0	17.9
	4 (110)	2.1	61.9	20.2	15.7
H1299	Control	1.1	52.7	30.6	15.6
	1 (250)	3.7	62.2	25.9	8.2
	2 (200)	4.1	65.9	21.6	8.4
	3 (250)	5.3	58.5	26.9	10.2
	4 (200)	2.4	64.7	24.7	8.2

ies. It is known[28] that the protein p53 is activated not only upon the action of DNA-damaging factors, but also under the conditions being not related directly with DNA damages, for example, upon depletion of nucleotide pool, hypo- and hyperoxia, and *etc*. To date, the mechanism of the p53 response to the action of oxazolidinone and triazole derivatives it is not known.

It is of note that the p53 protein activation can explain the differences in behavior of the HeLa and H1299 cells in the response to the action of the compounds under study. The HeLa cells express p53, although its functioning is hampered by the action of oncovirus proteins,[29] whereas the H1299 cells contain mutations in both alleles of p53 gene, which results in its complete shutdown.[30] The observed changes in the profile of the H1299 cell cycle indicating the ability of the cells to go through the S, G2, and M phases upon the action of the oxazolidinone and triazole derivatives under study can be explained by the lack of the protein p53 in the cells.

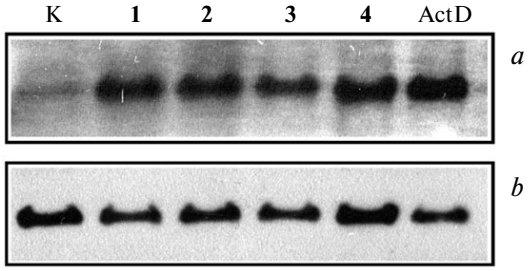

Fig. 3. Immunoblotting with the antibodies against the protein p53 (*a*) and β-actin (*b*) of the MCF7 cell lysates 6 h after introduction of compounds **1—4** at the IC_{50} doses for the MCF7 cells: 450 μmol L^{-1} for compounds **1** and **3** and 400 μmol L^{-1} for compounds **2** and **4**.

Thus, the studied 1,2,3- and 1,2,4-triazole and oxazolidinone derivatives **1—4** exhibited cytostatic effect on the tumor cells, which is accompanied with induction of the p53 protein expression.

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