Cytotoxic properties of the nitrosyl iron complex with phenylthiyl

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Nitrosyl compounds based on the iron-sulfur clusters are a promising class of inorganic nitric oxide donors. We studied cytotoxic properties of one of such NO donors, *viz.*, the nitrosyl [2Fe—2S] complex with phenyl ligands (Ph-complex). The Ph-complex induces the HeLa and H1299 tumor cell death. The Ph-complex and cisplatin used in combination exhibit synergistic cytotoxicity. During studies of the poly(ADP-ribose) polymerase degradation, it was found that the HeLa cell death induced by the Ph-complex proceeds presumably by the mechanism of apoptosis. In the MCF7 cells, the Ph-complex causes induction of p53 protein expression and changes in its apparent molecular weight.

Key words: nitrosyl iron-sulfur complex, nitric oxide, cytotoxicity, p53 tumor suppressor.

Iron-sulfur clusters are widely present in living nature. Proteins containing iron-sulfur clusters are found in all the living organisms, from bacteria to mammals. Functions of iron-sulfur proteins are mainly associated with metabolism of iron, redox reactions, and electron transfer in the mitochondrial respiratory chain.[1] The improper assembly of iron-sulfur clusters and malfunction of iron-sulfur proteins lead to disorders that limit energy production, mitochondrial and antioxidant dysfunction.[2]

A number of nitrosyl iron-sulfur complexes capable of donating nitric oxide have been synthesized earlier. As it was shown in the preceding studies, the obtained iron-sulfur complexes affect the bacterial DNA repair system,[3] can cause damage in DNA,[4] and exhibit antitumor properties.[5]

Nitric oxide possess a wide range of biological activity, since it reacts with various molecules and affects activities of many enzymes. When NO reacts with oxygen and its reactive species, an additional series of biologically active molecules is formed: from nitrogen dioxide to peroxynitrite.[6] Effects exerted on cells by compounds capable of generating nitric oxide depend on their structure, mechanism of the NO donation, and the type of cells. Nitric oxide can cause DNA damage and lead to apoptosis,[7—9] enhance the cytotoxic effect of antitumor agents,[9—11] or decrease susceptibility of cells to cytostatics.[12] The effect of NO on such processes as cell division and cell death, its role in the oxidative stress and intracellular signal transduction provide the basis for the possible use of NO donors in the therapy of different diseases including cancer.[13]

The present work is aimed at studying cytotoxic properties of a new NO donor from the class of nitrosyl iron-sulfur complexes. We studied a direct effect of the nitrosyl iron complex with phenylthiyl on tumor cells, its influence on the cell sensitivity to cisplatin, and effect on the p53 tumor suppressor. We showed that this iron-sulfur complex induces tumor cell death and poly(ADP-ribose) polymerase degradation, increases the cell sensitivity to cisplatin, and causes accumulation and changes in the apparent molecular weight of the p53 protein.

Experimental

The complex $[Fe_2(\mu_2\text{-}SPh)_2(NO)_4]$ (Ph-complex) (Fig. 1, *a*) was obtained according to the procedure described earlier;[14] DMSO was purified using the known procedure.[15]

The following reactants were used in the work: *cis*-dichlorodiammineplatinum(II) (cisplatin), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), 1,2-bis(dimethylamino)ethane (*N*,*N*,*N*´,*N*´-tetramethylethylenediamine, TEMED), β-mercaptoethanol, Bromophenol Blue, dithiothreitol (DTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *p*-cumaric acid, luminol, RNase A, protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, E-64, leupeptin, pepstatin), primary (rabbit) antibody against β-actin (Sigma, USA), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Tris, nonidet P-40 (NP-40) (Amresco, USA), acrylamide (Serva, Germany), primary (rabbit) antibodies against p53 protein and C-terminal fragment of poly(ADP-ribose) polymerase (PARP), anti-rabbit IgG secondary (goat) antibody conjugated with the horseradish peroxidase (Santa Cruz Biotechnology, USA),

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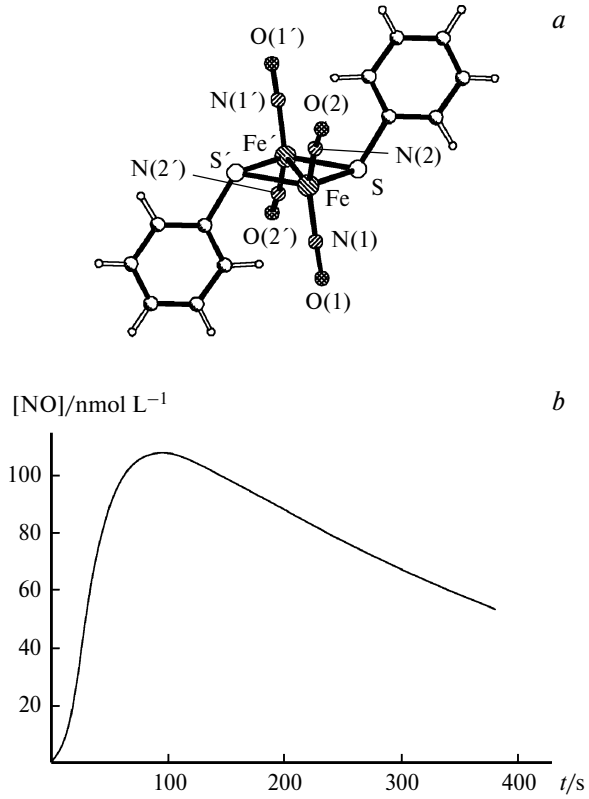

Fig. 1. The structure of the Ph-complex (*a*) and kinetics of the nitric oxide formation during dissolution of the Ph-complex (the final concentration was 8 μmol L^{-1}) in anaerobic 2% aqueous DMSO solution (*b*).

4-(1,1,3,3-tetramethylbutyl)phenyl-poly(ethylene glycol) (Triton X-100), polyoxyethylene (20) sorbitan monolaurate (Tween-20), glycine (Panreac, Spain), bovine serum albumin (BSA), *N*,*N*′-methylene-bis-acrylamide, ammonium persulfate (APS), phenylmethanesulfonyl fluoride (PMSF) (Dia-M, Russia), incubation medium DMEM (Institute of Poliomyelitis and Viral Encephalitis of the Russian Academy of Medical Sciences, Russia), foetal calf serum (BioWest, France), propidium iodide (Applichem, Germany).

Electrochemical determination of NO. The concentration of NO generated by the Ph-complex in solutions was measured using an inNO Nitric Oxide Measuring System (Innovative Instruments, USA). A measured amount of the Ph-complex ($0.4 \cdot 10^{-5}$ mol L^{-1}) was dissolved in DMSO (10 mL) for 3 min at 25 °C, and the solution (1 mL) was anaerobically placed into a temperature-controlled electrochemical cell filled with the phosphate buffer (49.0 mL). The final concentrations in the electrochemical cell were 8 μmol L^{-1} for the Ph-complex and 2% for DMSO. The concentration of NO was detected automatically for 400 s (with a step 0.2 s) from the moment when hydrolysis began. The amiNO-700 sensor (Innovative Instruments, USA) was calibrated with the standard aqueous $NaNO_2$ solution (100 μmol L^{-1}, coming with the electrode), which was added to a mixture consisting of KI (20 mg), 1 *M* aqueous H_2SO_4 (reagent grade) (2 mL), and water (20 mL).

Cell culture. Experiments were carried out on the HeLa (human cervical adenocarcinoma), MCF7 (human breast adenocarcinoma), and H1299 (human non-small cell lung carcinoma) cell cultures. The cells were cultured at 37 °C in the atmosphere of CO_2 (5%) in the DMEM medium containing 10% foetal calf serum.

Determination of the IC_{50} dose and combination index of cytotoxic effect. Cytotoxicity was studied using the MTT assay. The cells were plated in 24-well plates ($1.5 \cdot 10^4$ cells per well) in the standard incubation medium. The Ph-complex was dissolved in DMSO before use, and cisplatin was dissolved in water. The studied compounds were added into the incubation medium in 24 h after the plating. When cytotoxicity was studied for the individual compounds, cisplatin was added into the incubation medium in the concentrations from 2.5 to 30 μmol L^{-1}; the Ph-complex was added in the concentrations from 7.5 to 90 μmol L^{-1}. For the combined use, the compounds were added in the ratio of 0.9 : 1 (cisplatin : Ph-complex) in the concentrations from 0.9 to 36 μmol L^{-1} for cisplatin and from 1 to 40 μmol L^{-1} for the Ph-complex. The final concentration of DMSO was 0.1%. In 24 h after the addition of the compounds into the incubation medium, MTT was added to the final concentration of 0.45 mg mL^{-1}, and the cells were stained in the incubator for 4 h. After the staining, the incubation medium was removed, and crystals of the formed MTT formazane were dissolved in the acidified alcohol (50% isopropanol, 0.05 *M* HCl). The staining intensity was determined at 570 nm. The staining intensity of the cells treated with solvents (DMSO or water) was taken as 100%. The IC_{50} values for each cell line were calculated using the median effect analysis. The cytotoxic effect for the combination of the Ph-complex with cisplatin was analyzed by calculating the combination index.[16]

Preparation of cell lysates. The cells were plated in Petri dishes in the standard incubation medium (10^6 cells per point). The studied compounds were added into the incubation medium in 24 h after the plating. The incubation medium was removed through different periods of time after the addition of the compounds, the cells were washed thrice with the PBS buffer (137 m*M* NaCl, 2.68 m*M* KCl, 4.29 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4, pH 7.4). The cells were lysed in the buffer containing 500 m*M* HEPES (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 25 m*M* NaF, 10 μ*M* $ZnCl_2$, glycerol (10%), 1% Triton X-100, 1 m*M* DTT, 1 m*M* PMSF, and a protease inhibitor cocktail on ice for 20 min. The lysates obtained were clarified by centrifugation at 10000 *g* for 15 min. The content of proteins in lysates was measured by the Lowry method.[17]

Denaturing poly(acrylamide) gel (PAGE) electrophoresis of proteins. Electrophoresis was carried out in 0.75 mm thick gels using a Mini-PROTEAN Tetra system (Bio-Rad, USA). The separating gel contained 375 m*M* Tris (pH 8.8), SDS (0.1%), acrylamide (10%), and *N*,*N*′-methylene-bis-acrylamide (0.27%). Polymerization was initiated by the addition of APS to the final concentration of 0.033% and TEMED to the final concentration of 0.067%. After polymerization of the separating gel, a stacking gel was formed, which contained 125 m*M* Tris (pH 6.8), SDS (0.1%), acrylamide (3.98%), and *N*,*N*′-methylene-bis-acrylamide (0.106%). Polymerization was initiated by the addition of APS to the final concentration of 0.05% and TEMED to the final concentration of 0.1%. The cell lysates were mixed with the equal volume of the loading buffer containing 125 m*M* Tris (pH 6.8), SDS (4.1%), glycerol (20%), β-mercaptoethanol (0.2%), and Bromophenol Blue (0.0005%), and then heated at 96 °C for 3—5 min. Each lane of the gel was loaded with 20 μg of

protein except the cases specified in the Results and Discussion section. Marker protein kits containing either proteins with the weights of 116, 66.2, 45, 35, 25, 18.4, and 14.4 kDa or proteins with the weights of 97, 66.2, 45, 31, 21.5, and 14.4 kDa (Helicon, Russia) were used as molecular weight markers. Electrophoresis was carried out using the electrode buffer containing 25 m*M* Tris (pH 8.3), 192 m*M* glycine, and SDS (0.1%), with the electric field strength being 15—20 V cm^{-1}, for 2—3 h at ~20 °C.

Immunoblotting. After electrophoresis, the proteins were transferred from the gels onto a Hybond-C Extra membrane (Amercham-GE Healthcare, USA) in the transfer buffer (25 m*M* Tris, 19.3 m*M* glycine, methanol (20%)) at 4 °C and the current strength 300 mA for 3 h. After the transfer was completed, the membrane was incubated in a blocking solution containing 5% of BSA and 0.02% of NaN_3 in the TBST buffer (100 m*M* Tris (pH 7.5), 150 m*M* NaCl, Tween-20 (0.1%)) for 1 h at ~20 °C. After blocking, the membrane was incubated for 1 h with primary antibodies against p53, the C-terminal fragment of PARP, or β-actin, diluted in a ratio 1 : 1000 in the blocking solution, and then it was washed thrice with the TBST buffer. Then the membrane was incubated with secondary antibody diluted in a ratio 1 : 5000 in the blocking solution without NaN_3 and washed thrice with the TBST buffer. The chemiluminescence reaction was carried out using the following mixture of solutions: the solution *A* (140 μL) containing 0.68 m*M* *p*-cumaric acid in DMSO, the solution *B* (14 mL) containing 100 m*M* Tris (pH 8.5), 1.25 m*M* luminol, and 30% aqueous H_2O_2 (5 μL). After incubation in this mixture for 1 min, the membrane was exposed on an X-ray film.

Flow cytofluorimetry. The cells were plated in Petri dishes in the standard incubation medium (10^6 cells per point). In 24 h after the plating, the compounds under study were added into the incubation medium and, after 24 h, the cells were collected by trypsinization, washed thrice with PBS, and then fixed and permeabilized by a dropwise addition of 70% aqueous ethanol cooled to −20 °C. The samples were kept for 12 h at 4 °C. Ethanol from the fixed samples was removed by triple washing with a solution of BSA (1%) in PBS. The washed cells were resuspended in PBS containing 1% of BSA, propidium iodide (0.1 mg mL^{-1}), and RNase A (1 mg mL^{-1}). After incubation for 1 h at 37 °C, the samples were subjected to the flow cytofluorimetry on a FACSCalibur instrument (Becton Dickinson, USA) with an exitation laser (λ 488 nm).

Results and Discussion

NO-Donating ability. The Ph-complex generates NO; however, the rate of NO donation in solutions depends on the nature of the solvent. In 100% DMSO, the complex is stable, and no NO release is observed for several hours. In aqueous solutions of DMSO (1—10%) the NO release during hydrolysis of the Ph-complex is detected already within first seconds after dissolution. The maximum amount of NO (~110 nmol per mole of the complex) is observed 100 s after dissolution. The results of the direct anaerobic electrochemical measurements of the NO amounts released during hydrolysis of the Ph-complex are shown in Fig. 1, *b* and demonstrate an observed dependence between the NO amount in the aqueous solution at pH = 7 and the time that was calculated in terms of the scheme given below. The appearance of the maximum in the kinetic curve can be related to further transformations of NO. A simple two-step Scheme 1 suggested in the work[18] was used for its explanation.

Scheme 1

$$LFe(NO) \xrightarrow{k_1} LFe + NO$$

$$NO \xrightarrow{k_2} P$$

L = $[Fe(\mu_2\text{-}SR)_2(NO)_3]$, P are the products

It can be used for deriving the following functional dependence of the NO concentration on the time:

$$[NO] = [Ck_1/(k_2 - k_1)][(\exp(-k_1 t) - \exp(-k_2 t)].$$

The physical meaning of the constant *C* is the maximum concentration of NO that would be observed in the absence of its further transformation. Attention should be paid to the fact that the maximum number of NO molecules per nitrosyl iron complex is less than the minimum stoichiometric number which is equal to unity. The cause of this result and the nature of possible reactions leading to the disappearance of NO in the system have been discussed earlier.[18]

Cytotoxicity of Ph-complex. The Ph-complex causes the HeLa and H1299 tumor cell death. The results of MTT assay (Fig. 2) demonstrated that the Ph-complex decreases viability of the cells of both lines virtually to the

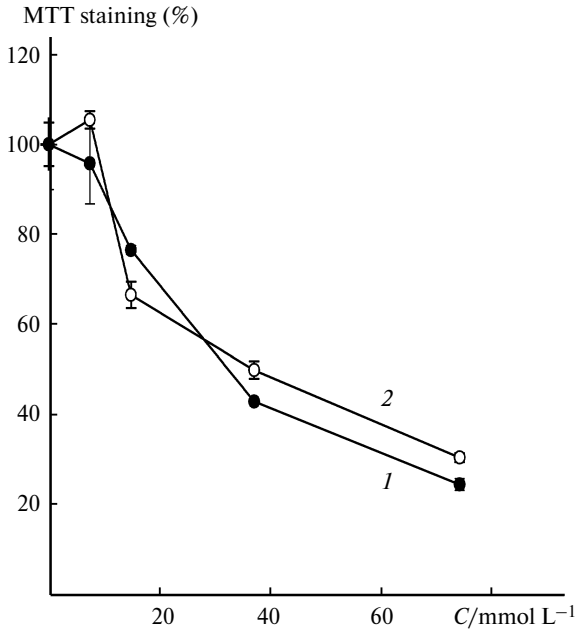

Fig. 2. The change in MTT staining of the cells upon the action of the Ph-complex on the HeLa (*1*) and H1299 (*2*) cells.

equal extent. The observed decrease in the MTT staining intensity is related to the cell death. The diagrams of the propidium iodide DNA staining in cells are shown in Fig. 3. Normally (see Fig. 3, *a* and *c*), the histograms exhibit two characteristic peaks corresponding to the content of cells in the G1 and G2 or M phases, and the intermediate region of the histogram representing the amount of cells in the S phase of the cell cycle. Virtually all the cells are viable, since almost no cells with the reduced DNA content (the subG1 region) are observed. The Ph-complex causes accumulation of the cells in the subG1 region (see Fig. 3, *b* and *d*). In the case of the HeLa line, reduction in the relative content of cells in the G2 and M phases of the cell cycle is also noticeable (see Fig. 3, *b*). It is possible that in the G2 or M phases, the HeLa cells are less sensitive to the complex and finish their division cycle in its presence. Dissimilar effect was observed in the case of the H1299 cells (see Fig. 3, *d*), and the cells in different phases of division are approximately equally sensitive to the action of the complex.

The Ph-complex is apparently capable of causing the tumor cell death by the mechanism of apoptosis. Apoptosis is characterized by the activation of the proteolytic enzymes, caspases, that hydrolyze proteins in the strictly specific sites. One of the caspase substrates, *viz.*, the protein poly(ADP-ribose) polymerase (PARP) with the molecular weight of ~116 kDa, is cleaved by caspases with the formation of the fragment with the molecular weight of ~85 kDa, which is characteristic of the cells undergoing apoptosis.[19] The results of immunoblotting of the cell lysates with antibody to the C-terminal fragment of PARP are shown in Fig. 4. The used antibody recognizes both the full-length PARP and the 85 kDa product of caspase-dependent degradation. It is seen that 18 h after the introduction of the Ph-complex, the 85 kDa fragment of PARP is clearly detected in the cell lysates, that can indicate activation of caspases in cells.

Synergistic cytotoxic effect of Ph-complex and cisplatin. Nitric oxide displays a wide range of biological activity, and it was shown that a variety of NO donors exhibit chemical sensitizing effect on tumor cells, which consists in the potentiation of cytotoxic properties of antitumor agents. We studied the cytotoxic effect of cisplatin when it is administered simultaneously with the Ph-complex. The

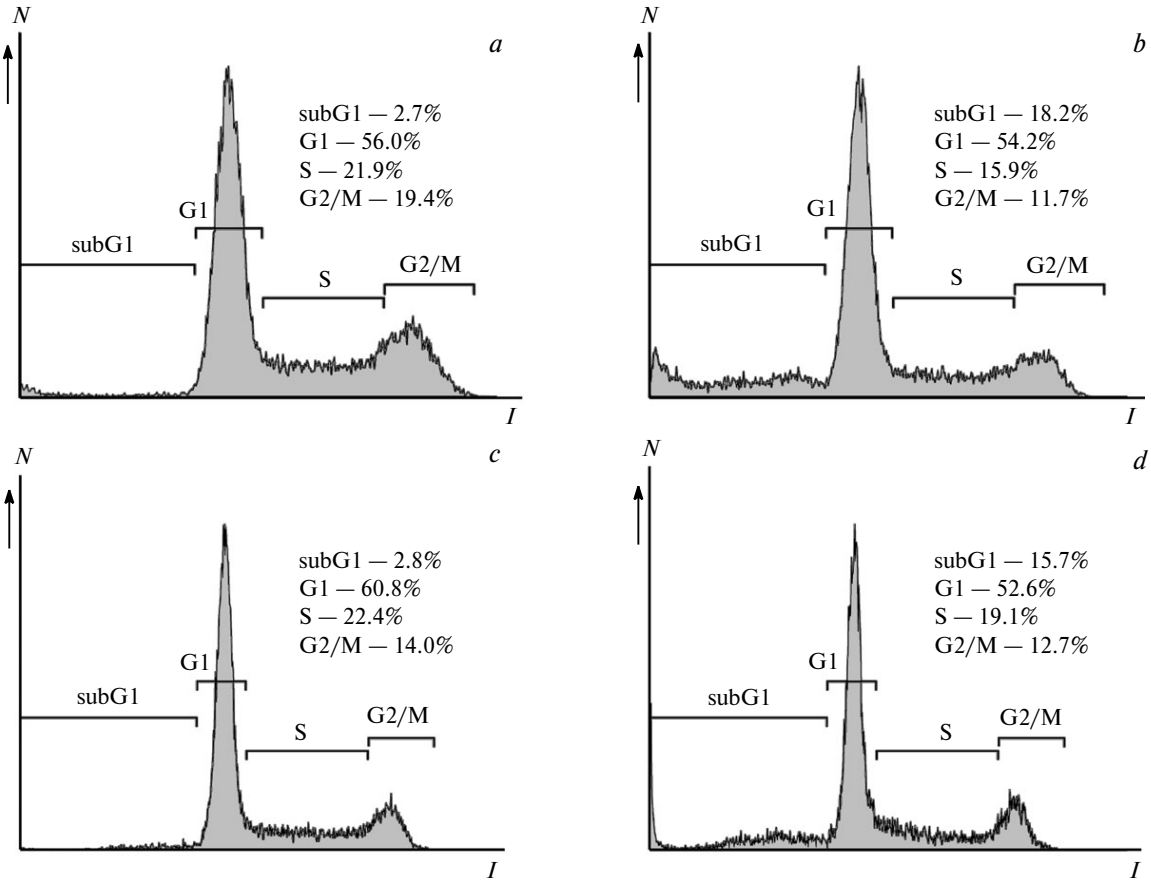

Fig. 3. The histograms of propidium iodide DNA staining in the HeLa (*a*, *b*) and H1299 cells (*c*, *d*) in the control with 0.1% DMSO (*a*, *c*) and upon the action of the Ph-complex in the concentration of 25 μmol L^{-1} (*b*, *d*); *I* is the fluorescence intensity, *N* is the number of cells.

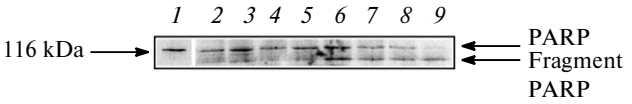

Fig. 4. The immunoblotting of lysates obtained from the HeLa cells in the control experiment (*1*) and after administration of the Ph-complex (*2*—*9*) in the concentration of 25 μmol L^{-1} with antibody to the C-terminal fragment of PARP (*3*). The time of the Ph-complex action: 10 (*2*), 12 (*3*), 14 (*4*), 16 (*5*), 18 (*6*), 20 (*7*), 22 (*8*), and 24 h (*9*).

obtained values of the combination index (Fig. 5) are lower than unity virtually within the whole range of the fractional effect (percentage of dead cells) for both the model of independent action of the compounds and the model suggesting their mutual inhibition. These data show the synergistic cytotoxicity of the Ph-complex and cisplatin. Possibly, the synergy observed is based on the sensitization of the cells with nitric oxide produced by the Ph-complex (see Fig. 1).

Effect of Ph-complex on p53 protein. The p53 protein plays one of the major roles in the cell response to the DNA-damaging factors of chemical, physical, and biological nature. The DNA damage activates p53, which induces a cascade of reactions associated with the cell cycle arrest or cell death. Activation of p53 consists in its accumulation, translocation from the cytosol to the nucleus, and induction of transcription of its target genes.[20] We studied the effect of the Ph-complex on the content of p53 in the MCF7 cells. These cells, unlike the H1299 cell line, contain an intact p53 gene and, unlike the HeLa line, contain no oncogenic viruses affecting the functions of the p53 protein. Results of the p53 protein immunodetection in the MCF7 cells in control and after exposure to cisplatin or the Ph-complex are shown in Fig. 6. Immunoblotting for β-actin, a constitutive cellular protein, was used as an internal control. Normally, the p53 level in the MCF7 cells is very low, but it increases in cells treated with cisplatin or the Ph-complex. For electrophoresis (see Fig. 6, *a*), the lysates were balanced with β-acin, for which the samples from the cells treated with the Ph-complex were used in the five-fold excess as compared to the other samples. Figure 6, *b* shows the results of immunoblotting of samples balanced with the total protein. The data obtained show that the Ph-complex causes accumulation of p53 and, at the same time, reduction in the β-acin expression. It is seen from Fig. 6 that the treatment with the Ph-complex, in addition to accumulation of the p53 protein, causes changes in its electrophoretic mobility. The apparent molecular weight of p53 is increased by 3.5—4 kDa. Such a behavior of the p53 protein can be caused by its covalent modifications, for example, phosphorylation.

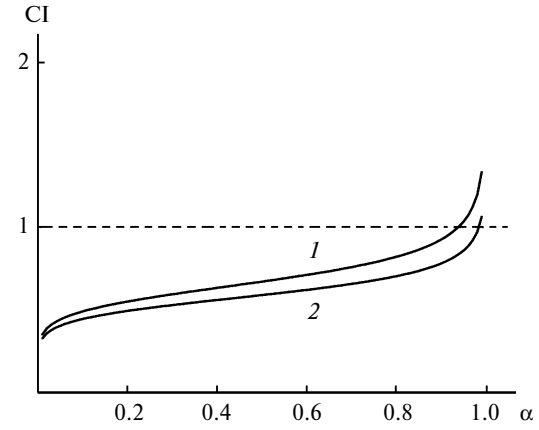

Fig. 5. The combination index (CI) of the cytotoxic effect for the combination of cisplatin and the Ph-complex (the molar ratio 9 : 10): *1*, the model suggesting an independent cytotoxic action of compounds; *2*, the model suggesting a mutual inhibition of the cytotoxic effects of the compounds;[11] α is the fraction of dead cells.

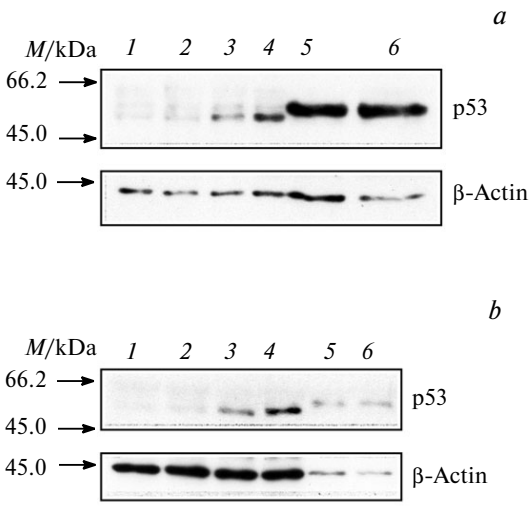

Fig. 6. The immunoblotting of lysates obtained from the MCF7 cells in the control experiment (*1*, *2*) and after administration of cisplatin in the concentration of 50 μmol L^{-1} (*3*, *4*) and the Ph-complex in the concentration of 100 μmol L^{-1} (*5*, *6*) with antibodies to p53 and β-actin; the time of action was 6 (*1*, *3*, *5*) and 12 h (*2*, *4*, *6*); the samples were balanced with β-actin (*a*) and the total protein (*b*).

From the results of our studies, it follows that the Ph-complex, like other NO donors,[21—24] causes the tumor cell death (see Figs 2 and 3). It was found that the Ph-complex causes degradation of PARP (see Fig. 4), which indicates its ability to induce the cell death by the mechanism of apoptosis.

It is known that the NO donors can significantly affect susceptibility of tumors to antitumor agents. The NO donors increase sensitivity of tumor cells *in vivo* and *in vitro* not only to chemotherapy drugs,[9—11] but also to radiation.[25] At the same time, it is known that many NO donors can reduce sensitivity of cells to cisplatin.[12] Presumably, one of the major mechanisms of cell sensitization to

cisplatin consists in the reduction of cellular glutathione pool by NO donors. At the same time, it was noted that the effect of NO on sensitivity of cells to chemical agents depends on the type of cells and realizes by different molecular mechanisms.[26] We showed that the combined use of the Ph-complex with cisplatin exhibits synergistic cytotoxic effects of these compounds (see Fig. 5). This reflects the chemical sensitizating properties of the Ph-complex apparently related to its ability to spontaneously generate NO.

Nitric oxide affects the functions of the p53 tumor suppressor. The p53 protein level in the cells is increased in response to both administration of chemical NO donors[24,27] and introduction into cells a gene which encodes the enzyme NO synthase.[28] It should be noted that activity of p53 is required for the cell cycle arrest by the NO donor, *N*-{4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]-butyl}-1,3-propanediamine, in the colorectal cancer cells HCT 116, since in the p53-deficient HTC 116 cell line, no cell cycle arrest in response to *N*-{4-[1-(3-aminopropyl)-2-hydroxy-2-nitrohydrazino]butyl}-1,3-propanediamine is observed.[27] Similar results were obtained for the RAW 264.7 macrophage cell line when treated with the NO donor, *S*-nitrosoglutathione.[8] In our case, no dependence of the cytotoxic effect on the activity of p53 was observed: the HeLa cells containing the wild-type p53 gene and the H1299 cells which do not express p53 exhibit similar sensitivity to the Ph-complex (see Figs 2 and 3).

We found that the Ph-complex causes an increase in the amount of the p53 protein in the MCF7 cells (see Fig. 6), that agrees with the literature data[27] on the induction of p53 in these cells exposed to the NO donors. In addition, we discovered an effect of an increase in the apparent molecular weight of the p53 protein by several kiloDaltons. This phenomenon can be related to the covalent modifications, since it is known that in process of activation, the p53 protein undergoes phosphorylation and acetylation.[29] Similar change in the electrophoretic mobility was observed for the amphibian p53 protein in response to the DNA-damaging factors, and, in this case, treatment with phosphatase led to the decrease in the apparent molecular weight of the protein.[30] However, in the work[30] no similar changes in the human p53 protein were observed, and an increase in the apparent molecular weight of p53 from *X.laevis* affected only part of the cellular protein pool. In our case, treatment of human MCF7 cells with the Ph-complex leads to entire transition of the p53 protein to the form with the increased molecular weight. The nature of the changes in properties of p53 treated with the Ph-complex remains unclear and requires additional studies.

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