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Effect of Dinitrosyl Iron Complexes (NO Donors) on the Metabolic Processes in Human Fibroblasts

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Abstract—The results of the study of the effect of mononuclear dinitrosyl iron complexes (DNICs) with functional sulfur-containing ligands (NO donors) on the cell viability and metabolism of human lung fibroblasts are presented, and the efficiency of their action is evaluated. It was shown that cationic DNICs increased the cell viability of fibroblasts and demonstrated the cytoprotective properties. Fluorescent analysis revealed that the DNICs compounds decrease the mitochondrial membrane potential but do not have a significant effect on the level of glutathione and reactive oxygen species in fibroblasts. It is assumed that the DNICs have the therapeutic potential for treating cardiovascular diseases.

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Signaling molecule nitric oxide (NO) is a bioregulator that mediates a wide range of physiological and pathological processes in the cell [1]. Nitric oxide plays an important role in protection against the occurrence and progression of the cardiovascular disease (CVD): NO regulates the tonus of small and medium-sized blood vessels, vasodilation, promotes relaxation of smooth muscles, exhibits anticoagulant properties, suppresses adhesion of monocytes and platelets and production of vasoconstrictors, inhibits oxidation of low-density lipoproteins and synthesis of cytokines, and affects immune response and neurotransmission [2]. The concentration of NO is the key factor that determines its biological effect. At low concentrations, NO exhibits cytoprotective properties and maintains the homeostasis of the cardiovascular and nervous systems, whereas at high concentrations NO has a cytotoxic effect due to formation of the highly reactive compound peroxynitrite [3, 4].

Any disturbance in NO bioavailability leads to the loss of the cardioprotective effect and, in some cases, may even enhance the progression of the disease. It is known that a decrease in the production or bioavailability of NO is one of the causes of endothelial dysfunction in arterial hypertension, heart failure, cardiomyopathy, myocarditis, etc. [5]. Cardiac failure often develops during the treatment of cancer patients because of the high toxicity of anticancer drugs. Therefore, the synthesis of drugs that reduce the toxic effects of chemotherapy and enhance the viability of normal cells (fibroblasts and cardiomyocytes) is a relevant problem in cardiology and oncology.

In recent years, the interest in the study of nitrosyl complexes of transition metals, particularly iron complexes possessing cytoprotective properties, increases exponentially [6]. However, such compounds are often poorly soluble in water, short-lived, and toxic to the body [7].

The purpose of this study was to synthesize water-soluble cationic dinitrosyl iron complexes (DNICs), to study their cytoprotective properties in vitro (in particular, the effect on the viability and metabolic processes in human lung fibroblasts), and assess the efficiency of the therapeutic action of DNICs.

In the study, we used human lung embryonic fibroblasts (HLEFs), strain HLEF-104, which were kindly provided by S.V. Kostyuk (Medical Genetics Research Center, Moscow). The cells were incubated in DMEM supplemented with 10% (vol/vol) fetal calf serum and 10 mM HEPES (pH 7.2) in a humidified 5% CO_2 atmosphere at 37°C. After the cells in the monolayer reached 90% density, they were treated with 0.25% trypsin and EDTA and centrifuged at 3000 *g* for 5 min. The resulting supernatant was discarded, the cell pellet was resuspended in the growth medium, and the cells were placed in wells of a 96-well plate. In experiments, we studied the effect on the cells of the following mononuclear DNICs with functional sulfur-containing ligands, thiourea, and its derivatives: $[Fe(SC(NH_2)_2)_2(NO)_2]_2Fe_2(S_2O_3)_2NO_4$ (compound no. 3),

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$[Fe(SC(NH_2)(NHC_2H_5))_2(NO)_2]Cl[Fe(SC(NH_2)(NHC_2H_5))Cl(NO)_2]$

(compouund no. 4), and $[Fe(SC(NH_2)_2)_2(NO)_2]ClO_4Cl$ (compound no. 6). When dissolved in protic solvents, these DNICs release NO as a result of dissociation [8]. The synthesis and identification of compounds nos. 3, 4, and 6 were carried out according to [8].

The effect of DNICs on the viability of fibroblasts was analyzed using the alamarBlue® Cell Viability Assay (ThermoFisher Scientific, United States). This method makes it possible to determine the activity of mitochondrial NADH dehydrogenases, which cleave NADH to NAD and H^+, and the formed proton reduces the dye resazurin to fluorescent rezofurin [9].

Fibroblasts (4000 cells per well) were supplemented with DNICs (2×10^{-4} M), which were dissolved in water immediately prior to adding, and an equal volume of phosphate buffered saline (PBS, 100 mM, pH 7.2) was added to the control wells. Then, doxorubicin (1.4×10^{-4} M) was added to the cells. The samples were incubated at 37°C for 20 min in quadruplicate. Then, the alamarBlue® reagent (10 μL) was added to the wells, and the fluorescence intensity was measured for 34 h at E_{ex}/E_{em} = 570/590 nm with a Varian Cary Eclipse spectrofluorometer (Agilent Technologies, United States).

The DNIC-induced changes in the membrane potential were determined using the fluorescent dye JC-1 (Invitrogen, United States). This method is based on the fact that JC-1 accumulates in the mitochondria proportionally to the change in the membrane potential ($\Delta\Psi_m$), forming aggregates that fluoresce in the red range [12]. In the cytoplasm, JC-1 exists as a monomer, which fluoresces in the green range. The red-to-green fluorescence ratio (F_{red}/F_{green}) is proportional to the change in the membrane potential ($\Delta\Psi_m$). Fibroblasts (10000 cells per well) were supplemented with a solution of DNIC (2.0×10^{-4} M) or doxorubicin (1.4×10^{-4} M), the control wells were supplemented with an equal volume of PBS, and the plates were incubated for 15 min. Then, JC-1 (2.6×10^{-5} M) was added in the dark, and the plates were incubated at 37°C for 30 min. The red fluorescence intensity was measured at E_{ex}/E_{em} = 570/595 nm, and the green fluorescence intensity was measured at E_{ex}/E_{em} = 485/535 nm.

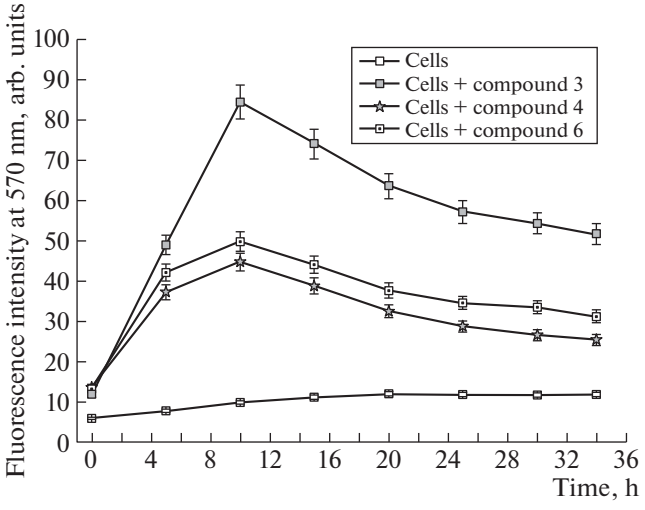

Fig. 1. Effect of DNICs on the viability of human lung embryonic fibroblasts. Here and in Figs. 2–4, data are represented as $M \pm m$, $n = 4$.

Changes in the level of reactive oxygen species (ROS) in fibroblasts after the addition of DNICs were evaluated using the nonfluorescent dye 2',7'- dihydrodichlorofluorescein diacetate (H2-DCFDA, Molecular Probes, United States) [11]. When H2-DCFDA gets into the cell, endogenous esterases cleave acetate groups to form the reduced form of DCHF in the cell. It is known that a sample can be easily oxidized in DCF in the presence of H_2O_2 or OH^- to form a fluorescent compound. Fibroblasts (4000 cells per well) were supplemented with a DNIC solution (2.0×10^{-4} M), the control samples were supplemented with an equal volume of PBS, and the plates were incubated for 15 min. The cells were then washed twice with PBS, supplemented with H2-DCFDA (10 μmol, 10 μL), and incubated at room temperature for 30 min. After the incubation, the cells were washed twice with PBS for 5 min. The fluorescence of the oxidized form of DCF was measured at E_{ex}/E_{em} = 488/525 nm.

The effect of DNIC on the level of intracellular reduced glutathione was assessed using *o*-phthalaldehyde as described in [10]. This method is based on the fact that the amino and sulfhydryl groups of glutathione react with *o*-phthalaldehyde and reduce it to form a fluorescent product.

Data were statistically processed using the Graph Pad Prizm software. Differences were considered statistically significant at $p \le 0.05$.

The results showed that DNIC compounds (nos. 3, 4, and 6) stimulated the viability of fibroblasts (Fig. 1). Compound no. 3 increased the cell viability approximately 8 times, and compounds 4 and 6 increased it 5 times compared to the control cells that were not treated with DNICs.

As can be seen in Fig. 1, the maximum increase in the cell viability after the treatment with DNICs was observed in 10 h. Then, the cell viability decreased; however, even 30 h later it remained 3–5 times higher than in the control cells, which were not treated with DNICs. Thus, DNIC increased the activity of mitochondrial NADH dehydrogenases, which are responsible for the ATP synthesis in the cell and thereby increase the cell viability [13]. The results indicate that

DNICs are cytoprotective agents. To confirm this result, we pretreated cells with DNICs and then supplemented them with doxorubicin, a well-known cytostatic agent that kills cancer cells, which is used to treat breast cancer. Figure 2 shows that doxorubicin caused a decrease in the cell viability, whereas the cells that were treated with compounds 3, 4, and 6 retained a higher viability: their survival was two- to threefold higher than that of the untreated cells for 500 min. Compounds 3 and 4 had the most pronounced protective effect, which lasted for 30 h. These data indicate that compounds 3 and 4 are effective cytoprotectors with a long-term effect, which protect cells from the toxic action of doxorubicin.

Since DNICs increased the viability of fibroblasts, we investigated the effect of DNICs on the mitochondrial membrane potential. It is known that NO inhibits the mitochondrial respiratory pathways [14]. It was found that certain chemical agents selectively accumulate in mitochondria and reduce their membrane potential and, thus, exhibit cytoprotective properties. These compounds can be used to prevent severe neurological disorders after ischemic stroke. Indeed, our results showed that the treatment of fibroblasts with DNICs (compounds 3, 4, and 6) decreased the mitochondrial membrane potential 2–3 times (Fig. 3). A decrease in the membrane potential was also observed when the cells were treated with DNICs in the presence of doxorubicin (Fig. 3). Doxorubicin is a well-known anticancer antibiotic of the anthracycline series and has antimitotic and antiproliferative effects on cells. The mechanism of action of doxorubicin includes the cooperation with DNA, generation of free radicals, and direct effect on cell membranes with the suppression of the synthesis of nucleic acids. Therefore, the treatment of cancer with doxorubicin is accompanied by an increase in the production of free radicals in the mitochondria, which have a deleterious effect on the structure of both tumor and normal cells as a result of oxidative stress. This is accompanied by numerous side effects of doxorubicin, which are toxic to the whole body. For example, the cardiovascular system disorders include cardiomyopathy, heart failure, and arrhythmia. This process directly depends on the mitochondrial membrane potential: the higher the latter, the greater amounts of free radicals are produced by the mitochondria. The adverse effect of doxorubicin on the normal cells, which is associated with the oxidative stress, can be reduced by providing the leakage of excess charge through the mitochondrial membrane. This protective mechanism is called the uncoupling of oxidative phosphorylation. It is used by cells in vivo but can also be induced by synthetic compounds. The results of our study showed that DNICs decrease the mitochondrial membrane potential and, possibly, induce the uncoupling of oxidative phosphorylation, thereby increasing the viability of fibroblasts.

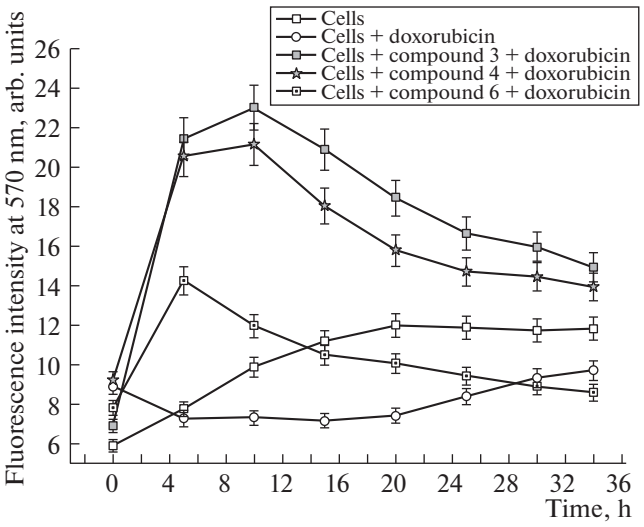

Fig. 2. Cytoprotective effect on DNICs on human lung embryonic fibroblasts.

Reactive oxygen species (ROS) play an important role of signaling molecules; however, their accumulation in pathological states leads to oxidative stress. The main source of ROS in cells is oxidative phosphorylation. Mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of many diseases. Therefore, the determination of ROS can provide important information about the physiological state of the cell and the function of mitochondria. We studied the effect of DNICs on the level of ROS in fibroblasts (Fig. 4). The results showed that, during the incubation of cells with DNICs, compounds 3 and 4 significantly increased the level of ROS, whereas compound 6 had no effect on the level of ROS in fibroblasts. This finding indicates that DNICs caused no physiological disorders in fibroblasts.

It is known that glutathione determines the redox characteristics of the intracellular environment and protects the cell from toxic free radicals [15]. The ratio of the reduced and oxidized forms of glutathione in the cell is one of the most important parameters that indicates the level of oxidative stress. We studied the effect of DNICs on the level of the reduced glutathione. The results showed that DNICs 3, 4, and 6 did not affect the level of the reduced glutathione (data not shown), which indicates the absence of oxidative stress in the cell.

Thus, the results of our study showed that DNICs can be regarded as cationic uncouplers, whose chemical structure apparently allows them to selectively accumulate in mitochondria and reduce excess mitochondrial potential. These compounds not only do not disturb the vital metabolic processes in the cell but, on the contrary, have a protective effect and increase the viability of fibroblasts. This indicates that these compounds are promising for the treatment of

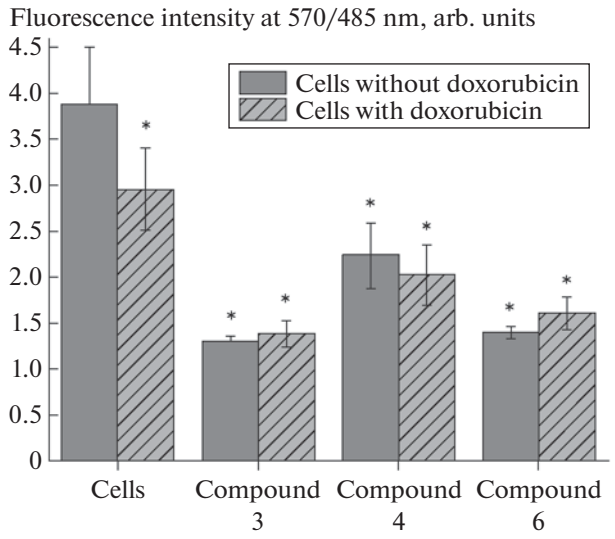

Fig. 3. Effect of DNICs on the mitochondrial membrane potential of human lung embryonic fibroblasts. Here and in Fig. 4, * $p < 0.05$.

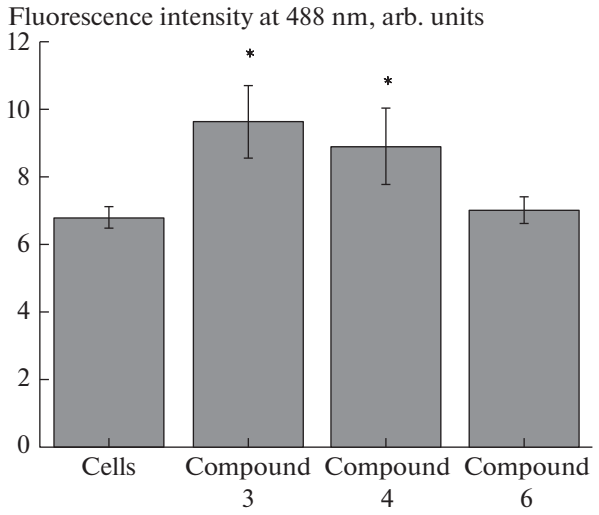

Fig. 4. Effect of DNICs on the level of reactive oxygen species in human lung embryonic fibroblasts.

cardiovascular disease (ischemia and stroke) and heart diseases in cancer patients.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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