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BIOPHYSICS OF COMPLEX SYSTEMS

The Antitumor Effect of Dinitrosyl Iron Complexes with Glutathione in a Murine Solid-Tumor Model

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Abstract—A significant antitumor activity of aqueous solutions of binuclear dinitrosyl iron complexes with glutathione was found when they were injected intravenously in a model of a solid malignant tumor, that is, Lewis carcinoma, in mice. Dinitrosyl iron complexes completely inhibited the tumor growth (by 100%) at doses of 20, 10, and 2 μmol/kg in the first 11 days after the beginning of experiment followed by tumor proliferation at a rate that was lowest for the lowest of the used doses. At day 16, the inhibition of tumor growth was 90% when a solution of dinitrosyl iron complexes was injected at a dose of 2 μmol/kg five times with an interval of 2 to 3 days between injections; whereas the inhibition of tumor growth did not exceed 70 and 30% at doses of 10 and 20 μmol/kg, respectively. Acceleration, rather than inhibition of carcinoma growth was observed at a dose of 100 μmol/kg. The tumor weight increased 1.5–2.0 times compared to the control values, depending on the time.

Keywords: nitric oxide, dinitrosyl iron complexes, Lewis lung carcinoma in mice

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In the recently published article by Prof. W.F. Liaw's research team from Taiwan, it was reported that water-soluble mononuclear dinitrosyl iron complexes (M-DNICs) containing two thiol ligands, $S(CH)_2OH$ and $S(CH)_2NH_3$, and one iron dinitrosyl $Fe(NO)_2$ fragment exhibit a significant antitumor effect in a model of xenografts of human prostate cancer in mice [1]. These complexes, when administered intravenously (in the tail vein) at a daily dose of ~0.8 μmol/kg every 3 days for 21 days, caused inhibition of tumor growth by 60 and 95% compared with the control on days 7 and 21 of tumor development, respectively. It was shown that the inhibition of tumor growth under the influence of the tested substance is due to the apoptotic death of tumor cells initiated by the release of nitric oxide (NO) from M-DNICs [1].

We obtained a similar result earlier while studying the biological effect of binuclear dinitrosyl iron complexes (B-DNICs–GS) with a thiol-containing ligand, that is, glutathione in an experimental rat endometriosis model of benign tumor. When administered intraperitoneally at a daily dose of 6 μmol/kg for 10 days, the studied agents, which are NO donors, completely suppressed the development of rapidly proliferating endometrium tissue that developed from fragments of the rat uterine epithelium transplanted onto the inner surface of the peritoneum. At 1 month after transplantation, the sizes of these tumors were increased fivefold in the control and reached a diameter of 10 mm; whereas tumors were not detected in the most animals treated with B-DNICs [2–4].

It was suggested that the inhibition of endometrium tissue development under the influence of B-DNICs–GS is caused by the destructive impact of iron chelators produced by tumors in order to provide tumor tissues with iron required for their growth [2–4]. By binding iron, the chelators could effectively decompose B-DNICs–GS that occurred near the tumors or inside them, which could ensure the selective cytotoxic effect of these complexes against the tumors. Nitric oxide released in a significant amount from B-DNIC–GS was converted by the reaction with superoxide to peroxynitrite, which had a cytotoxic effect against endometrioma cells.

Abbreviations: B-DNIC–GS, binuclear dinitrosyl iron complex with glutathione; M-DNIC, mononuclear dinitrosyl iron complex.

We further demonstrated a dose-dependent antitumor effect of B-DNIC–GS in a model of murine solid tumor, that is, Lewis lung carcinoma at intraperitoneal administration at single doses of 25, 50, 125, and 200 μmol/kg daily for 10 days [5–7]. The antitumor effect of these complexes was manifested in the inhibition of tumor growth by 60–80% compared to the control and varied depending on the dose and time of the effect evaluation. The inhibition of Lewis lung carcinoma growth under the action of dinitrosyl iron complexes was observed during administration of the agent for 7–10 days. At a later time, after the cessation of drug administration, the rates of tumor growth were practically the same in the treated and control animals; as a result, the tumor sizes in the treated animals reached the same level as in the control by day 20. The growth-inhibitory effect of the agents was increased with increasing doses of B-DNIC–GS and was most pronounced (80%) when using the agent at a dose of 200 μmol/kg (relative to one $Fe(NO)_2$ fragment in these complexes) [5–7].

Based on these data, it was hypothesized that the removal of the growth-inhibitory effect of B-DNICs–GS against tumor after cessation of intraperitoneal injection (days 7–10 after tumor transplantation) could be due to formation of a hypothetical antinitrosative defense system from nitric oxide released from B-DNICs–GS in the tumor cells by this time [6], similar to the defense system, which occurs in many types of bacteria in response to NO impact [8].

However, the results obtained by the research team of Prof. W.F. Liaw, according to which M-DNICs with thiol-containing ligands can almost completely suppress the development of the human prostate cancer in mice and cause death of the cultured tumor cells when administered intravenously at a dose of less than 1 μmol/kg [1], call the validity of our hypothesis about the existence of an antinitrosative defense system into question.

The difference between the results we obtained and those of the Chinese research team [1] may be due to a number of factors, including the different sensitivities of experimental tumor models to the applied impacts, the distinctive features of the tested compounds, and the change in the drug efficiency depending on the dosages and modes of B-DNIC administration to the animals (intraperitoneally or intravenously)

The present work is devoted to the verification of the significance of one of these factors for implementation of antitumor effect of dinitrosyl iron complexes, namely, the assessment of the influence of the method of B-DNIC administration on the agent efficacy. A study of the antitumor activity of B-DNICs–GS at intravenous administration at doses of 2, 10, 20, and 100 μmol/kg was performed in the Lewis lung carcinoma model.

MATERIALS AND METHODS

Reagents. For the synthesis of B-DNICs–GS, we used iron sulfate ($FeSO_4 \cdot 7H_2O$) (Fluka, Switzerland), reduced glutathione, and sodium nitrite (Sigma, United States).

Synthesis of B-DNICs with glutathione. Synthesis of B-DNICs–GS was carried out according to the described procedure [9]. Glutathione (40 mM), iron sulfate (20 mM), and sodium nitrite (20 mM) were added consecutively to a 15-mM HEPES buffer solution (10 mL) at the starting pH value of 7.4. Adding glutathione to a HEPES buffer solution resulted in a decrease in pH up to 3.5–4.0, which was sufficient for initiation of the S-nitrosoglutathione formation starting immediately after sodium nitrite was added to a solution. The solution gradually became of a dark red color as S-nitrosoglutathione formed. Measurements of optical absorbance at a wavelength of 334 nm, which corresponds to the absorption band of S-nitrosoglutathione, demonstrated that 1.5 h was sufficient for the conversion of almost all sodium nitrite to S-nitrosoglutathione by the reaction with glutathione. The pH of the reaction mixture was then increased to 7.4 by dropwise addition of a solution of strong alkali (NaOH); in this case, the reaction mixture took on a red–orange color caused by B-DNIC–GS formation. After keeping this solution in air, which resulted in the formation of a maximum amount of B-DNICs–GS, iron that was not incorporated in these complexes was precipitated in the form of hydroxide complexes and removed by filtration through a paper filter. The concentration of B-DNICs–GS was estimated by the intensity of characteristic absorption bands at 310 and 360 nm with extinction coefficients of 9200 and 7400 $M^{-1} cm^{-1}$, respectively [10]. At the initial concentrations of reagents given above, the B-DNIC concentration was ~9.5 mM (relative to one $Fe(NO)_2$ fragment in these complexes). This solution was diluted with a 15-mM HEPES buffer to a concentration of 4 mM and then used in experiments in animals.

Biological experiments were performed on 40 female BDF_1 mice weighing 18–20 g from the Stolbovaya nursery of the Russian Academy of Sciences (Moscow oblast). The tumor test system was a murine solid tumor, Lewis lung carcinoma, transplanted subcutaneously according to a standard protocol [11].

An aqueous B-DNIC–GS solution was injected intravenously in mice in the tail vein at daily doses of 20, 10, and 2 μmol/kg (which corresponds to 0.4, 0.2, and 0.04 μM of B-DNIC–GS relative to one $Fe(NO)_2$ fragment) on days 1, 4, 7, 10, and 14 after tumor transplantation.

In some experiments, B-DNICs–GS was injected intravenously at a daily dose of 100 μmol/kg (2 μM per animal) three times from day 1 to day 7 with a 2-day interval. A solution of B-DNIC–GS at a concentration of 20 μM was used.

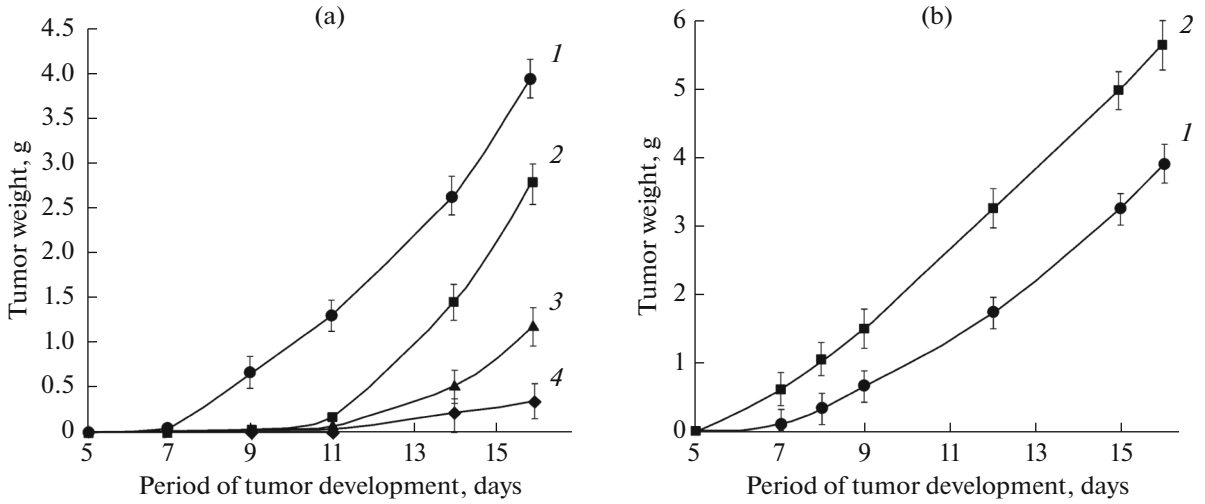

Fig. 1. The antitumor activity of B-DNIC–GS at intravenous injection in the Lewis lung-carcinoma model: (a) injection at daily doses of 20, 10, and 2 μmol/kg (curves *2–4*, respectively); (b) injection at a daily dose of 100 μmol/kg (curve *2*). In both graphs, curves *1* are the control. Doses of B-DNIC–GS are given relative to one $Fe(NO)_2$ fragment.

Evaluation of antitumor activity. The differences in the kinetics of tumor growth (tumor growth inhibition index) serve as an indicator the growth-inhibitory effect of the agent. The tumor growth inhibition index (TGI, %) was determined from the ratio $TGI = (P_C - P_T)/P_C\,\%$, where P_C and P_T are the volume (or weight) of the tumors in the control and treated animals, respectively. When studying the kinetics of tumor growth, we measured two mutually perpendicular dimensions of a tumor node throughout the tumor-development period. The tumor volume was calculated according to the formula for an ellipsoid as $V = ab^2/2$, where a is the length and b is the width and height of the tumor node. When determining the weight of this node, we used the tumor density value equal to 1 g/cm^3 [11].

Each group of animals subjected to this therapeutic treatment consisted of six–eight mice; the control group consisted of eight–ten animals. Observation of the animals was continued throughout the period of tumor development, until the death of the animals.

Statistical data processing. The estimates of tumor sizes (weights) in ten animals were processed using the Statistica 6.0 software package.

RESULTS

As can be seen from the kinetics of the Lewis lung carcinoma weight changes, when B-DNICs–GS were injected intravenously to mice at daily doses of 20, 10, and 2 μmol/kg five times for 14 days with an interval of 2 to 3 days between injections, complete (100%) inhibition of tumor growth was observed in the first 11 days for all used doses (Fig. 1a). Subsequently, the effect of inhibition became weaker and decreased to the values of 30, 70, and 90% on day 16 for doses of 20, 10, and 2 μmol/kg, respectively (Fig. 1a, Table 1).

For the B-DNIC–GS action at a daily dose of 100 μmol/kg, in this case, stimulation of carcinoma growth was observed during the entire period of observation (Fig. 1b). On day 9, the tumor weight in the experiment exceeded the control value by approximately two times; on day 16, by 1.6 times. In this case,

Table 1. The antitumor effect of the binuclear dinitrosyl iron complex (B-DNIC) with glutathione at a number of doses at intravenous injection in the Lewis lung carcinoma model

Group	Daily dose, μmol/kg	Average tumor weight, g	Tumor growth inhibition, %
B-DNIC–GS	100	5.61 ± 0.35	Stimulation of growth by ~50%
B-DNIC–GS	20	2.76 ± 0.54	30
B-DNIC–GS	10	1.19 ± 0.14	70
B-DNIC–GS	2	0.37 ± 0.08	90
Control	–	3.93 ± 0.65	–

Administration of the agent on days 1, 4, 7, 10, and 14 after tumor transplantation. *, Administration of the agent on days 1, 4, and 7 after tumor transplantation. Evaluation of the effect was performed on day 18 of tumor development.

some of the mice (up to 30%) died, especially in the first week after the beginning of the complexes administration.

DISCUSSION

Our studies fully confirmed the results obtained by the research team of Prof. Liaw, who demonstrated the high antitumor effect of M-DNICs with thiol-containing ligands for the first time when they were administered intravenously to animals [1]. Moreover, as in the experiments of this research team, who found 95% inhibition of tumor growth in the model of xenografts of human prostate cancer in mice at a comparatively low dose of the used complexes (~1 μM/kg), we also observed the most efficient inhibition of tumor growth under the action of B-DNICs–GS when they were administered intravenously at approximately the same low dose (2 μM/kg). However, the kinetics of this effect turned out to be different: the development of Lewis lung carcinoma was completely suppressed by B-DNICs–GS during the first 11 days after tumor transplantation followed by a tendency to restore its growth rate (Fig. 1a, curve *4*), whereas the rate of human prostate growth under the action of M-DNICs with thiol-containing ligands continuously decreased from the first to the third week after tumor transplantation [1].

When B-DNICs with glutathione were administered intravenously to mice at higher doses, 10 and 20 μmol/kg, the character of the kinetics of the Lewis lung carcinoma size changes was found to be the same as at injection of these complexes at a dose of 2 μmol/kg, namely, the complete inhibition of tumor growth during the first 11 days followed by restoration of tumor growth rate during 5 days for a dose of 20 μmol/kg and a noticeable similar tendency for a lower dose of 10 μmol/kg (Fig. 1a, curves *2* and *3*; Table 1).

The stimulatory action of B-DNICs–GS that we found when administered intravenously to mice at a high dose of 100 μmol/kg (Fig. 1b, curve *2*) is very intriguing. This was unexpected, because earlier in our experiments in the Lewis lung carcinoma model, the same complexes initiated an effective reduction of tumor growth when they were administered intraperitoneally to mice at a single dose of 100 μmol/kg [5–7].

What could cause such a paradoxical phenomenon as an in vivo stimulatory effect of high doses of B-DNICs–GS on the Lewis lung carcinoma development upon intravenous administration in mice? One would expect the opposite effect, namely, an increase in the inhibitory effect of B-DNICs–GS against carcinoma cells with increasing doses of these complexes, as it was in experiments in vitro in the culture of various malignant cells; the results of these experiments were reported in [1, 12, 13].

To answer this question, first of all, it should be noted that at high doses, B-DNICs as NO donors should have a depressing effect on the functional activity of immunocompetent cells (macrophages, neutrophils, T-helpers, etc.) and thereby weaken the cell-mediated immunity in animals. This kind of negative effect of M- and B-DNICs with different thiol-containing ligands (as well as other NO donors) on various cell cultures of animals, including immunocompetent cells, was previously demonstrated in [12–23]. Moreover, the administration of M- and B-DNICs at a concentration of 1 mM in different cell cultures was sufficient for a 100% lethal effect against the cultured cells. It was this concentration of B-DNICs–GS in the blood of mice that was set when these complexes were administered at a dose of 100 μmol/kg (2 μmol of B-DNICs–GS entering in the blood of one animal at a volume of ~ 2 mL). In this case, the cytotoxic effect of B-DNICs–GS was extended not only on immunocompetent cells, but also on the entire body of animals, as evidenced by the death of a portion of mice in the first days after intravenous administration of B-DNICs at a dose of 100 μmol/kg.

It is interesting that on the background of the apparent weakening of cell-mediated immunity in these animals under the B-DNIC–GS action, the administered agents did not exert a cytotoxic growth-inhibitory effect on the tumor (Lewis lung carcinoma) development. In contrast to in vitro experiments in cultures of malignant cells [1, 12, 13], the complexes ceased to suppress the development of Lewis lung carcinoma. Moreover, they enhanced the tumor growth! Thus, a direct correlation between the activity of the cell-mediated immunity system and the cytotoxic antitumor effect of the B-DNICs–GS injected to the blood was found. This means that the tested B-DNIC-GS agents could exhibit such an antitumor effect only via immunocompetent cells.

The latter could be determined by the fact that B-DNICs entering the body can be included in immunocompetent cells, which directionally transferred them to a malignant tumor with the subsequent transfer of these complexes to tumor tissues. Such a transfer was demonstrated earlier [24]. The DNICs that were transferred into tumor tissue could be destroyed (as we suggested earlier for benign tumors, that is, endometriomas [2–4]) under the influence of endogenous iron chelators produced in malignant tumors to provide them with iron necessary for tumor proliferation. As a result of these processes, a considerable amount of free nitric oxide occurred in the tumor. Its oxidation to peroxynitrite in the reaction with superoxide led to the appearance of cytotoxic products of decomposition of protonated peroxynitrite, such as hydroxyl radicals and nitrogen dioxide, in the tissue [25], which caused the death of tumor cells. If B-DNICs entered the normal slowly proliferating tissues located near the tumor, then B-DNICs were

not decomposed in them to release a significant amount of NO into the intracellular medium because of the absence of endogenous iron chelators, and thus did not exert a cytotoxic effect on these tissues.

According to these ideas, the efficiency of delivery of B-DNICs to the targets of their action, malignant tumors, should be reduced with deterioration of the state of immunocompetent cells, i.e., the weakening of cellular immunity with increasing content of these complexes in the environment.

Such, a paradoxical regularity, at first glance, was found in experiments in mice during intravenous administration of B-DNICs–GS at doses of 20, 10, and 2 μmol/kg (Fig. 1a). Characteristically, during the first 11 days of tumor development, the growth-inhibiting effect was observed under the influence of all tested doses of B-DNICs–GS, and only later the recovery of tumor growth under the influence of all studied doses of B-DNICs–GS was observed; and, which is very paradoxical, with a higher rate than the higher dose of these complexes was administered intravenously (Fig. 1a). Obviously, B-DNIC–GS administration to animals five times even in small doses, which resulted in the accumulation of adverse events in immunocompetent cells and thus to the above-mentioned dysfunction of cellular immunity, provided complete suppression of the cytotoxic effect against tumors after 11 days of the experiment. In this case, the subsequent tumor growth was provided by a fraction of the surviving tumor cells, the size of which was the smallest in animals receiving B-DNICs–GS at a daily dose of 2 μmol/kg.

We consider a proposed mechanism of the antitumor effect of the tested B-DNICs–GS on the Lewis lung carcinoma development, which is associated with the functioning of the system of cellular immunity in mice, as a working hypothesis that requires further experimental verification.

At the same time, the results we obtained do not allow us to abandon the idea of the role of the antinitrosative defense system, as mentioned in the introduction, and could arise in tumor cells in response to the appearance of NO released from B-DNICs–GS in them. It is obvious that the expression of the corresponding genes that provide (as in the case of bacteria [8]) the synthesis of antinitrosative defense proteins could be more effective at higher doses of B-DNICs–GS. As a result, tumor cells became resistant to the damaging effect of NO, and not only exogenous NO (coming from B-DNICs–GS), but also endogenous NO produced from L-arginine by an enzymatic route. This led to the fact that the carcinoma growth not only did not weaken in comparison with the control, but even increased (figure b) at administration of B-DNICs–GS at a dose of 100 μmol/kg to mice.

In conclusion, it should be noted that in the present study we found the same changes in the kinetics of the Lewis lung carcinoma development at intravenous administration of B-DNIC–GS to mice as in our previous experiments with intraperitoneal administration of the agent [5–7]. The only difference was that B-DNIC–GS when administered intraperitoneally acted most efficiently on the tumor, not in low but at higher doses, 100–200 μmol/kg [6, 7]; and with intravenous administration at a low dose of 2 μmol/kg (Fig. 1, curves *2–4*).

The antitumor effect of complexes during their intraperitoneal administration at doses of 25–50 μmol/kg was slightly expressed [5]. It is not impossible that only a small part of B-DNICs–GS could reach circulating blood when administered intraperitoneally, by "sticking" on the peritoneum and intestinal tissue proteins to form the corresponding protein-bound DNICs as a result of the transfer of $Fe(NO)_2$ fragments from B-DNICs–GS to the thiol groups of proteins. In fact, in the previous experiments with intravenous and intraperitoneal administration of B-DNICs–GS to rats, it was shown that the concentration of protein-bound DNICs found in the blood of animals is three to four times higher for the intravenous route of administration than for intraperitoneal application [26].

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