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ORIGINAL CONTRIBUTIONS

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HYPOXIA ENHANCES ANTITUMOR ACTIVITY OF DICHLOROACETATE

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It is known that glycolysis contributes to the survival of tumor cells by providing them with energetic and plastic substrates. Dichloroacetate (DCA) as inhibitor of kinase pyruvate dehydrogenase shifts balance of energy metabolism of tumor cells from aerobic glycolysis towards oxidative phosphorylation. *The aim* of the study was to investigate cytostatic/cytotoxic effect of DCA on glioma C6 cells at the conditions of different oxygenation of the cell incubation medium. *Materials and Methods:* DCA action on glioma C6 cells was investigated upon the conditions of normoxia, hypoxia (1% of oxygen) and hyperoxia (30% and 95% of oxygen) *in vitro*. The number and viability of tumor cells were assessed using trypan blue dye-exclusion test. Apoptosis was determined using dye Hoechst 33258. Lactate production by tumor cells was determined by enzymatic method using lactate dehydrogenase. Cell cycle distribution was studied using flow cytometry. Reactive oxygen species (ROS) content was evaluated using 2′,7′-dichlorofluorescein diacetate. *Results:* By the data of *in vitro* cytotoxicity, upon hypoxia IC_{50} value of DCA was three times lower ($p < 0.05$) than that upon normoxic conditions (18.2 ± 3.9 mM *vs.* 51.2 ± 8.1 mM). Hypoxia itself enhanced the ROS production in glioma cells by 113.5% ($p < 0.05$) that correlated with increase of apoptosis by 292% ($p < 0.05$). In hypoxic glioma C6 cells DCA did not significantly influence the ROS production, but decreased hypoxia-induced apoptosis by 3.5–6.5 times ($p < 0.05$) and significantly increased cell death rates via necrosis ($p < 0.05$). In contrast to hypoxia, upon the conditions of hyperoxia IC_{50} values for DCA did not differ from the corresponding values upon the normoxia conditions and at 30% and 95%oxygen content were equal to 35.8 ± 7.2 mM and 42.3 ± 5.1 mM respectively. *Conclusion:* According to the obtained results, hypoxia enhances cytostatic/cytotoxic effects of DCA in glioma C6 cells via high level of DCA-induced necrosis of tumor cells and hypoxia-induced ROS hyperproduction.

Key Words: **dichloroacetate, glioma C6, hypoxia, hyperoxia, reactive oxygen species, apoptosis.**

It is known that in most of malignant tumors, ATP is produced by means of glycolysis even in the presence of oxygen (Warburg effect). Despite aerobic glycolysis is less effective way of ATP generation compared with oxidative phosphorylation, it provides high proliferative potential of tumor cells and contributes to their survival decreasing risks of the formation of apoptotic stimuli [1–3]. Therefore, in recent times, tumor metabolism, which provides tumor cells with such growth advantages, is actively considered as new target for the antitumor therapy, and compounds able to inhibit glycolysis in malignant cells are considered to have potential of effective antitumor drugs. Such anti-metabolic agent, which has showed antitumor activity in the respect of many types of cancer cells [4–7], proved to be dichloroacetate (DCA), inhibitor of pyruvate dehydrogenase kinase (PDK) [8]. Capability of DCA to inhibit PDK is considered to underlie its antitumor activity. PDK is negative regulator of enzymes of mitochondrial pyruvate dehydrogenase (PDH) complex, which plays key role in regulation of tricarbonic acid cycle and oxidative phosphorylation. In case of inactivation (phosphorylation) of PDH complex by PDK, energy metabolism of tumor cell switches to glycolysis, but in catalytically active (non-phosphorylated) state, PDH complex regulates entrance of pyruvate in mitochondria and catalyzes its transformation in acetyl-CoA — substrate for the tricarbonic acid cycle. DCA, by inhibiting activity of PDK, is able to stimulate indirect activation of enzymes of PDH complex and, therefore, cause shift of the energy balance of cell from glycolysis towards activation of oxidative phosphorylation.

In case of activation of oxidative phosphorylation and decrease of intensity of glycolysis caused by action of DCA, the inhibition of tumor cell proliferation, in particular due to the significant decrease of hexose monophosphate shunt metabolites and pyruvate, which are critical for the synthesis of proteins and nucleic acids, may be expected. DCA-induced activation of mitochondrial metabolism can cause not only blocking of tumor cell proliferation, but also evoke intensification of mitochondrial-derived reactive oxygen species (ROS) generation and induce apoptosis. However, despite theoretical clearness of antitumor effect of DCA, its efficacy is not guaranteed. Activation of oxidative phosphorylation evoked by action of DCA can cause not only antitumor effect, but also can contribute to the survival of tumor cells, in particular due to the increase of ATP synthesis. For instance, in some studies, significant proapoptotic effect of DCA on tumor cells was determined [4–7], while in the other studies actually antiapoptotic effect of DCA was registered [9].

Nevertheless, taking into account the fact that DCA switches energy metabolism of tumor cell to the mainly oxidative phosphorylation, it should be expected that hypoxia, by limiting the activity of cellular respiration, can essentially amplify its antitumor effect. Therefore, the aim of this study was to investigate cytostatic/cytotoxic effect of DCA on glioma C6 cells at different content of oxygen in the incubation medium.

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Abbreviations used: DCA – dichloroacetate; PDH – pyruvate dehydrogenase; PDK – pyruvate dehydrogenase kinase; ROS – reactive oxygen species.

MATERIALS AND METHODS

Studies have been carried out using glioma C6 cells taken from National Bank of Cell Lines and Tumor Strains of IEPOR of NAS of Ukraine. Glioma cells were maintained *in vitro* in nutrient medium DMEM adding 10% ETC (Sigma, USA), 2 mM L-glutamine and 40 mkg/ml of gentamicin at 37 °C in the humidified environment, 5% CO_2.

Cytostatic/cytotoxic effect of DCA on glioma C6 cells was evaluated upon the conditions of incubation of cells in normoxia (21% O_2), hypoxia (1% O_2), and hyperoxia (30% and 95% O_2). Various content of oxygen in incubation chamber was generated by adjusting of partial oxygen pressure using gases nitrogen and oxygen in corresponding ratios. Level of oxygen in incubation chamber was controlled using oximeter ISO2 (World precision instruments, USA).

The number of tumor cells and their viability were assessed by the dye-exclusion method using 0.4% trypan blue.

Glioma cells were seeded in 24-well plates at a density of $1,5 \cdot 10^5$ cells/well or in 6-cm dish at a density of $0,6 \cdot 10^6$ cells/dish and incubated under standard conditions overnight. After preincubation medium was replaced with fresh growth medium with DCA and after 24 h incubation upon corresponding conditions needed assays were performed.

Cell cycle distribution was examined by staining cells with propidium iodide using flow cytometry according [10].

The apoptosis was evaluated using dye Hoechst 33258 (Sigma, USA). For this, cells were stained by Hoechst 33258 in the final concentration 1 μg/ml at 37 °C during 20 min and were washed out by salt solution. After that, changes of morphology of nuclei were assessed by counting not less than 500 cells/sample. Apoptotic cells were determined by presence of fragmented nucleus or condensed chromatin and were expressed as a percentage from the total number of cells.

ROS production in tumor cells was assayed using 2′,7′-dichlorfluorescein-diacetate (Sigma, USA) [11]. The fluorescence was monitored by a fluorophotometer. The excitation wavelength was set at 485 nm, while the emission wavelength was 520 nm.

To assess the effect of DCA on tumor cell lactate production the supernatants were analyzed for lactate concentration using enzymatic spectrophotometric method with lactate dehydrogenase (Sigma, USA) [12]. Lactate production rates were expressed relative to the number of viable cells.

Statistical analysis of the obtained data was carried out using descriptive methods, *t*-test, and nonlinear regression analysis.

RESULTS AND DISSCUSSION

Study of impact of DCA on glioma C6 cells was carried out at different content of oxygen in incubation medium: normoxia (21% of oxygen), hypoxia (1% of oxygen) and hyperoxia (30% and 95% of oxygen). According to the obtained data, not only DCA, but also hypoxia actually shows cytostatic/cytotoxic effect. For instance, hypoxia alone significantly decreased proliferation of glioma C6 cells that was evident by significant 48% decrease ($p < 0.05$) of the number of the viable cells upon the conditions of hypoxia compared with the same upon the conditions of normoxia. Decrease of the number of viable cells upon the conditions of hypoxia took place, in particular, both due to the induction of apoptosis and tumor cell blocking in G0/G1. For instance, hypoxia caused the 23% increase of cells in phases G0/G1 ($p < 0.05$) and significant decrease (almost in half, $p < 0.05$) of their quantity in the S-phase. Significant increase of level of apoptosis observed upon the conditions of hypoxia correlated with significant increase of generation of ROS by tumor cells. The number of apoptotic cells upon the conditions of hypoxia increased on 292% ($p < 0.05$), and level of generation of ROS increased on 113.5% ($p < 0.05$) compared with the corresponding indices upon the conditions of normoxia. No significant increase of number of necrotic cells upon the conditions of hypoxia was observed. It should be mentioned that expected increase of activity of glycolysis in glioma cells caused by hypoxia is confirmed by statistically significant increase of lactate production rate by glioma cells on 207% ($p < 0.05$).

Thus, strong decrease of tumor cell proliferation, high level of generation of ROS and, therefore, high level of hypoxia-induced apoptosis were the evidence of high sensitivity of glioma C6 cells to hypoxia. Hyperoxia (30% and 95% of oxygen), in the contrast to hypoxia, did not essentially influence the studied indices.

Data on the impact of DCA on glioma C6 cells showed that its cytostatic/cytotoxic effect was significantly enhanced upon the conditions of hypoxia. For instance, concentration of compound, which caused up to the 50% decrease of the number of viable cells due to its cytostatic and/or cytotoxic action (IC_{50}), was almost threefold lower upon the conditions of hypoxia compared with the same upon the conditions of normoxia and equaled 18.2 ± 3.9 mM and 51.2 ± 8.1 mM respectively (Fig. 1).

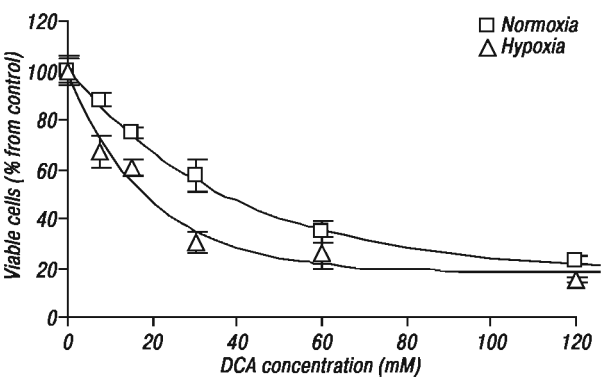

Fig. 1. The action of DCA on glioma C6 cell viability upon the conditions of hypoxia

The fact attracts attention that upon the conditions of normoxia, DCA decreases the number of viable cells mainly due to the realization of its cytostatic effect. As seen in Fig. 2, the number of necrotic cells was

not changed significantly and did not exceed 10% from the total number of tumor cells even at maximal concentrations of the compound. Predominance of cytostatic effect of DCA on glioma cells upon the conditions of normoxia is confirmed also by its low proapoptotic activity. According to the literature data, one of the mechanisms of antitumor effect of DCA is its capability to enhance ROS production and, therefore, to induce apoptosis [4–7].

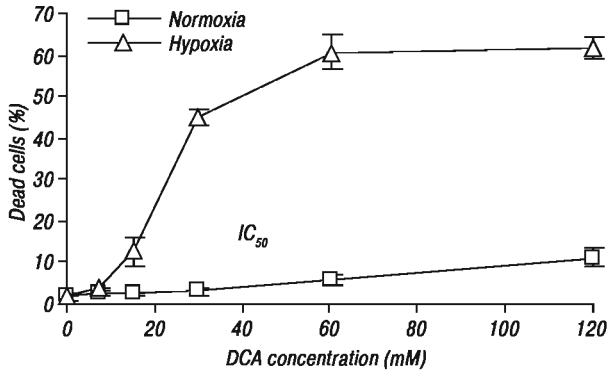

Fig. 2. DCA-induced necrosis in glioma C6 cells upon the conditions of hypoxia

DCA-induced apoptosis was observed in many types of tumor cells, in particular colorectal cancer [7], human glioblastoma cells *in vitro* and *in vivo* [6], endometrial cancer cells [5], etc. Meanwhile, in this study, DCA has not significantly influence the level of apoptosis upon the conditions of normoxia. The number of apoptotic glioma cells after their incubation with DCA in the wide range of concentrations, did not statistically differ from that in control (Table 1).

Table 1. Effect of DCA on apoptosis in glioma C6 cells upon the conditions of hypoxia

DCA, mM	Normoxia, %	Hypoxia, %
0	3.8 ± 1.7	14.9 ± 0.05*
7.5	3.2 ± 0.2	2.3 ± 0.08*,#
15	3.4 ± 0.2	4.2 ± 0.72
30	4.5 ± 0.4	4.3 ± 0.72
60	14.1 ± 2.8	3.5 ± 0.32

Note: *$p < 0.05$ *vs.* corresponding index upon the conditions of normoxia; #$p < 0.05$ *vs.* corresponding index upon the conditions of hypoxia itself.

In contrast to normoxia, it was determined that DCA upon the conditions of hypoxia showed mainly significant cytotoxic effect (see Fig. 2). In particular, DCA in concentration 15 mM caused more than fivefold increase of number of dead cells compared with the same upon the conditions of normoxia (12.6 ± 3.4% against 2.3 ± 0.3%, $p < 0.05$). In concentration of DCA 60 mM, number of dead cells increased up to 60.4 ± 4.2%, which was tenfold higher than corresponding index upon the conditions of normoxia.

On the background of enhancement of necrotic death of cells as a result of DCA action upon the conditions of hypoxia, significant decrease of the level of hypoxia-induced apoptosis was registered (Table 1). As it was previously showed, hypoxia caused the fourfold ($p < 0.05$) increase of the number of apoptotic cells compared with the corresponding index upon the conditions of normoxia. Upon hypoxic conditions, DCA regardless of its concentration fully inhibited hypoxia-induced apoptosis causing the decrease of the number of apoptotic cells 3.5–6.5 times ($p < 0.05$) compared with the corresponding index upon the hypoxic conditions itself. It was shown that DCA decreased apoptosis in some cell lines of human colorectal cancer upon the conditions of hypoxia that is considered by authors to be connected with HIF-1a-dependent signal cascades [9].

In contrast to hypoxia, hyperoxia did not significantly enhance cytostatic/cytotoxic effect of DCA on C6 cells. The calculated IC_{50} both upon the conditions of 30% and 95% of the content of O_2 in the incubation medium did not significantly differ from the corresponding index upon the conditions of normoxia (35.8 ± 7.2 mM and 42.3 ± 5.1 mM correspondingly against 51.2 ± 8.1 mM) (Fig. 3). If upon the conditions of hypoxia decrease of the number of viable cells was caused mainly by the cytotoxic effect of DCA, then upon the conditions of hyperoxia — due to the realization of the mainly cytostatic effect of DCA (Fig. 4). For instance, number of dead cells even at extremely high concentrations of DCA (120 mM) upon the conditions of 30% oxygen content in the incubation medium did not markedly differ from the corresponding index upon the conditions of normoxia. Only upon the conditions of 95% oxygen content DCA in the same high concentration significantly ($p < 0.05$) increased cell death up to 30 ± 0.1%. DCA did not increase apoptosis in glioma cells upon the conditions of hyperoxia (Table 2).

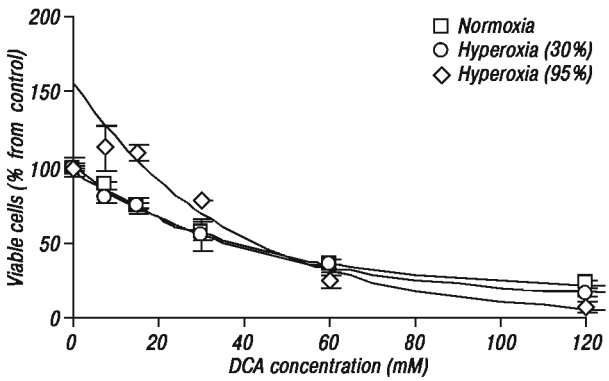

Fig. 3. The action of DCA on glioma C6 cell viability upon the conditions of hyperoxia

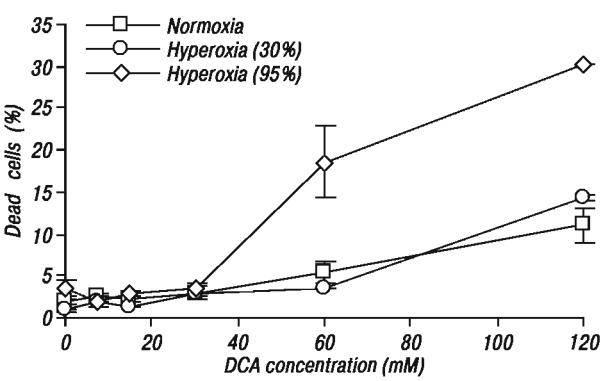

Fig. 4. DCA-induced necrosis in glioma C6 cells upon the conditions of hyperoxia

Table 2. Effect of DCA on apoptosis in glioma C6 cells upon the conditions of hyperoxia

DCA, mM	Normoxia	Hyperoxia (30%)
0	2.3 ± 0.3	3.0 ± 1.1
7.5	2.3 ± 0.2	3.0 ± 0.3
15	2.0 ± 0.2	2.9 ± 0.01
30	6.8 ± 3.8	4.8 ± 1.5
60	5.0 ± 1.3	5.7 ± 1.7

It was shown that DCA did not cause significant action on the cell cycle (Tables 3, 4). According to the obtained data, upon the conditions of normoxia only treatment of tumor cells with DCA in concentrations higher than IC_{50} resulted in statistically significant increase ($p < 0.05$) of cells in phase G0/G1 and decrease ($p < 0.05$) of cells in S-phase. Lack of capability of DCA to redistribute tumor cells by phases of cell cycle is the evidence that its cytostatic action, apparently, was conditioned by its ability to block their cell cycle progression into the all phases.

Table 3. Effects of DCA on the cell cycle of glioma C6 cells upon the conditions of hypoxia

DCA, mM	Normoxia			Hypoxia		
	G0/G1	G2/M	S	G0/G1	G2/M	S
0	56.9 ± 0.3	16.6 ± 0.6	26.5 ± 0.3	70.2 ± 0.9*	15.8 ± 0.6	14.0 ± 0.2*
7.5	55.2 ± 0.7	14.9 ± 0.6	29.9 ± 0.1	71.2 ± 1.1	15.2 ± 0.3	13.6 ± 1.4
15	54.5 ± 1.0	17.5 ± 0.2	28.0 ± 0.8	67.2 ± 1.5	16.4 ± 0.5	16.4 ± 0.6
30	57.3 ± 1.1	20.1 ± 0.7	22.7 ± 0.4	68.0 ± 0.7	19.4 ± 0.8	12.7 ± 0.3
60	63.8 ± 1.3*	18.1 ± 0.4	18.1 ± 0.8*	66.6 ± 0.7	22.3 ± 1.4	11.1 ± 0.7

Note: *$p < 0.05$ *vs.* corresponding index upon the conditions of normoxia.

Table 4. Effects of DCA on the cell cycle of glioma C6 cells upon the conditions of hyperoxia

DCA, mM	Normoxia			Hyperoxia (30%)		
	G0/G1	G2/M	S	G0/G1	G2/M	S
0	51.2 ± 1.6	18.7 ± 1.1	30.1 ± 0.5	47.8 ± 0.3*	22.3 ± 0.5	29.9 ± 0.9
7.5	51.2 ± 1.0	20.8 ± 0.2	28.0 ± 0.8	46.7 ± 0.7	23.1 ± 0.1	30.2 ± 0.8
15	50.6 ± 0.5	21.0 ± 0.2	28.3 ± 0.7	46.4 ± 1.2	21.7 ± 1.2	31.9 ± 0.0
30	48.7 ± 0.5	22.3 ± 0.6	29.0 ± 0.1	45.8 ± 0.1	23.0 ± 1.0	31.2 ± 1.1
60	54.8 ± 1.5	19.5 ± 1.2	25.7 ± 1.1	50.5 ± 1.7	18.0 ± 1.5	31.5 ± 0.9

Note: *$p < 0.05$ *vs.* corresponding index upon the conditions of normoxia.

Data concerning with DCA action on the ROS production by tumor cells as one of the possible ways of realization of its cytostatic/cytotoxic effect are represented in Fig. 5. In this study, statistically significant increase of intracellular ROS level in glioma C6 cells as a result of DCA treatment was observed only upon the conditions of extreme hyperoxia. In particular, as seen in Fig. 5, upon the conditions of 95% hyperoxia, DCA in concentration of 30 mM caused up to 66% ($p < 0.01$) of increase of ROS production by tumor cells. Upon the conditions of normoxia, DCA in the range of concentrations 20–60 mM, i.e., in concentrations lower and closer to IC_{50}, did not cause significant increase of ROS production instead. DCA ability to increase of ROS level on 85–91% in glioma cells upon the normoxia conditions was observed only in extremely high concentrations (120–240 mM), which significantly exceed IC_{50} (data are not presented).

Lactate production rate was assessed as surrogate marker of DCA inhibition of glycolysis. Obtained results are represented in Table 5. According to the obtained data, only upon the conditions of hypoxia, DCA in the range of concentrations close to IC_{50} slightly slowed down lactate production by glioma cells. At least, DCA treatment in concentration of 15 mM (which was almost equal to its IC_{50} upon the conditions of hypoxia) and 60 mM caused 21% and 32% decrease ($p < 0.05$) of lactate production rate by glioma cells compared with the same in the conditions of hypoxia itself. At the same time, upon the conditions of normoxia and hyperoxia, no essential impact of DCA on lactate production rate was detected.

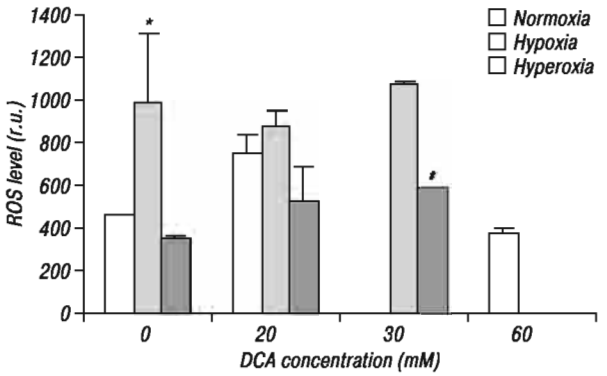

Fig. 5. Effect of DCA on ROS production by glioma cells. *Note:* *$p < 0.05$ *vs.* corresponding index upon the conditions of normoxia; #$p < 0.05$ *vs.* corresponding index upon the conditions of hyperoxia itself.

Table 5. Effect of DCA on lactate production rate by glioma cells under different oxygen contents

DCA, mM	Lactate production rate (mkmol/10^6 cells per hour)		
	Normoxia	Hypoxia (1% O_2)	Hyperoxia (95% O_2)
0	0.45 ± 0.03	1.38 ± 0.06	0.63 ± 0.15
15	0.48 ± 0.04	1.09 ± 0.11	0.44 ± 0.18
30	0.49 ± 0.09	1.18 ± 0.43	0.62 ± 0.04
60	0.52 ± 0.07	0.94 ± 0.08*	0.89 ± 0.18

Note: *$p < 0.05$ *vs.* corresponding index upon the conditions of hypoxia itself.

Thus, in this study, no apparent ability of DCA to increase ROS production by glioma C6 cells and induce apoptosis upon the conditions of hypoxia, as well as normoxia and hyperoxia was detected. Obviously, cytostatic/cytotoxic activity of DCA on glioma C6 cells realizes not due to the hyperproduction of ROS and initiation of apoptosis, but rather due to its ability to modify energy metabolism in tumor cells. It is confirmed by data on the action of DCA upon hypoxia, to be exact, high level of necrotic death of tumor cells. By amplifying activity of PDH complex and, therefore, rendering impossible compensatory activation of glycolysis in tumor cells, DCA, apparently, essentially limited their metabolic adaptation to hypoxia that caused fast decrease of ATP and emergence of metabolic catastrophe. Meanwhile, it is known that both rapid decrease of ATP in tumor cell due to the inhibition of energy metabolism and activation of proapoptotic signals upon the conditions of significant limitation of the level of intracellular ATP induce necrosis [13, 14]. Ability of DCA to cause dramatic decrease of intracellular ATP has been showed, at least for cervical carcinoma cells [15].

Impact of DCA on the energy metabolism of glioma C6 cells is confirmed not only by increase of level of their necrotic death, but also decrease of lactate production rate. Upon the conditions of hypoxia, DCA caused 21–32% decrease of the lactate production by glioma cells that was the evidence of DCA-induced inhibition of glycolysis on the background of the ex-

pected decrease of glutaminolysis. Concerning normoxia conditions, lack of inhibiting impact of DCA on lactate production by glioma C6 cells can be explained by compensatory activation of glutaminolysis (as the main supplier of ATP in the most of tumor cells cultured *in vitro*) as a result of activation of oxidative phosphorylation.

Thus, it has been established that hypoxia significantly increases cytostatic/cytotoxic action of DCA on glioma C6 cells — tumor cells, which turned out to be sensitive to hypoxia alone. Obtained data on hypoxia-induced hyperproduction of ROS in tumor cells, as well as high level of DCA-induced necrotic death of these cells, show that enhancement of its cytostatic/cytotoxic effect on glioma C6 cells upon the conditions of hypoxia was connected both with its impact on the tumor metabolism and high level of hypoxia-induced ROS.

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