

METFORMIN ENHANCES CYTOTOXIC ACTION OF DICHLOROACETATE AGAINST LEWIS LUNG CARCINOMA CELLS IN VITRO

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Tumor cell metabolism is considered one of the hallmarks of cancer. This concept is exploited in the development of new ways of anticancer therapy based on the use of substances capable of changing drastically bioenergetic metabolism of tumor cells. Among them, sodium dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase, and metformin (MTF), an antidiabetic hypoglycemic drug, an inhibitor of the mitochondrial respiratory chain (complex I), both have been long used in clinical non-oncological practice, and presently are considered promising candidates in oncology. **Aim:** To study the capability of MTF to enhance the antitumor action of DCA against Lewis lung carcinoma cells *in vitro*. **Materials and Methods:** LLC/R9, a low metastatic variant of Lewis lung carcinoma cells, was used. Effects of 30 mM DCA in combination with 2 mM MTF on cell survival, cell cycle distribution, apoptosis, mitochondrial potential, intracellular ATP level, glucose consumption, and lactate production rates were determined *in vitro*. **Results:** MTF was shown to enhance the cytotoxic/cytostatic action of DCA against LLC/R9 cells *in vitro*. Treatment of LLC/R9 cells with 30 mM DCA in combination with 2 mM MTF resulted in a 39% decrease in the number of viable cells ($p < 0.05$), a 2.8-fold increase of the number of dead cells ($p < 0.05$), a near 2-fold decrease in the proportion of cells at the S-phase ($p < 0.05$), a 4-fold increase in the apoptosis ($p < 0.05$) and significant reduction ($p < 0.05$) of the mitochondrial membrane potential of tumor cells as compared to corresponding values in control. DCA alone reduced glucose consumption and lactate production rates by more than 26% ($p < 0.05$) and 34% ($p < 0.05$), respectively, whereas MTF counteracted these effects. Nevertheless, in the cells treated with both DCA and DCA in combination with MTF, the intracellular adenosine triphosphate increased by 33–35% compared with that in the control ($p < 0.05$). **Conclusion:** MTF enhanced the cytotoxic/cytostatic action of DCA against LLC/R9 cells *in vitro*, which points on their possible synergistic antitumor action *in vivo*.

Key Words: sodium dichloroacetate, metformin, Lewis lung carcinoma.

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It is known that metabolic therapy in oncology has been used for a long time since the development of the first chemotherapy drugs targeting mainly nucleotide synthesis, that is, the anabolic component of the metabolism of a malignant cell, which provides the realization of its basic function — uncontrolled proliferation [1]. The idea to exploit the energy metabolism of malignant cells as a new target of metabolic antitumor therapy has emerged recently, after understanding that the proliferation, survival, and metastasis of malignant cells require their constant potent supply with energy and large amounts of metabolites for the synthesis of proteins, lipids, and nucleic acids in the conditions of an almost constant metabolic deficit [2–5]. The overarching and critical role of energy metabolism in the course of the malignant process became more apparent after the discovery of metabolic reprogramming of the malignant cell, which is realized, in particular, through the regulation of oncogenes and oncosuppressor genes, and is involved in the reduction of susceptibility to apoptosis and convenient chemotherapeutic agents [6–8]. That is why compounds capable of influencing the energy metabolism of malignant cells are being actively investigated as potential antitumor agents. Agents with

a proven antitumor potential, which targets energetic metabolism of tumor cells, include sodium dichloroacetate (DCA), a pyruvate dehydrogenase kinase inhibitor that enhances oxidative phosphorylation [9–12], and metformin (MTF), an inhibitor of the mitochondrial respiratory chain (complex I), an antidiabetic hypoglycemic drug, capable of blocking oxidative phosphorylation [13–15], both of which have long been used in clinical non-oncological practice.

However, the high adaptive metabolic plasticity of malignant cells, which contributes to their survival and high proliferation in adverse conditions of the tumor microenvironment, significantly reduces the chances of obtaining high antitumor efficacy from agents that inhibit only one of the adenosine triphosphate (ATP) generation pathways [16, 17]. Effective metabolic targeted therapy requires the combined use of several inhibitors capable of inhibiting various components of the energy metabolism of malignant cells [18]. To date, it has been shown that DCA is capable of exerting a synergistic antitumor effect when used in combination with MTF for some types of malignant cells, such as breast cancer [19], ovarian cancer [20], etc. Previously, we have also demonstrated the high antitumor efficacy of DCA in combination with MTF against C6 glioma *in vitro* and *in vivo* [21]. This work is aimed to investigate the ability of MTF to potentiate DCA cytotoxic/cytostatic activity against Lewis lung carcinoma (LLC) cells *in vitro*.

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Abbreviations used: ATP — adenosine triphosphate; DCA — sodium dichloroacetate; LLC — Lewis lung carcinoma; MTF — metformin.

MATERIALS AND METHODS

Tumor cell culture. A low metastatic variant of LLC cells (LLC/R9) was obtained from the National Bank of Cell Lines and Tumor Strains of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine.

LLC/R9 cells were maintained *in vitro* in RPMI 1640 medium with 10% fetal calf serum (Sigma, USA) and 40 µg/ml gentamicin at 37 °C in a humidified atmosphere with 5% CO₂.

The cytotoxic/cytostatic effects of DCA (Sigma, USA) and MTF (Sigma, USA) alone and in combination were assessed by the IC₅₀ index — concentration of the agent, which causes a 50% reduction in the number of viable cells as compared to control due to its cytotoxic and/or cytostatic action.

Tumor cells were seeded into 96-well plates at a density of 10⁵ cells/ml (to determine IC₅₀) or in a 35 mm Petri dish with a density of 0.3 · 10⁶ cells/ml (to evaluate the effects of selected concentrations of DCA (30 mM) and MTF (2 mM). At the end of the preincubation period (16–18 h), the medium was replaced with a fresh one with the addition of test agents at corresponding concentrations, alone or in combination, and incubated for 1 day.

In all cases, the cells that were incubated under analogous conditions, but without the addition of any agents, served as a control.

Each agent concentration and combination of these agents were evaluated in 3 replicates (for IC₅₀ determination) or 5 replicates (for determining effects of 30 mM DCA and 2 mM MTF).

The number of viable cells was evaluated using sulphorhodamine B (Sigma, USA) [22] or by their direct counting in a hemocytometer using trypan blue.

The distribution of cells by the cell cycle phases and the level of apoptosis were assessed by flow cytometry according to [23]. The number of apoptotic cells was evaluated by the sub-G0/G1 peak.

The rates of glucose consumption and lactate production by tumor cells were estimated based on the contents of the substrates in the incubation medium and the number of viable cells at the beginning of the incubation period and after 1 day.

The glucose and lactate levels were determined in the samples of supernatants using a biochemical analyzer and commercial kits for their determination.

The membrane potential of the internal membrane of the mitochondria (Δψ_m) of tumor cells was determined using the cationic dye JC-10 (Sigma, USA) by the ratio of fluorescence intensity in the red (FL2) and green (FL1) regions measured by flow cytometry.

The level of intracellular ATP was determined using the luciferin-luciferase method and the ATP assay kit (Sigma, USA) according to the manufacturer's protocol.

Statistical analysis of the data was performed using descriptive statistics, Student's *t*-test and Mann — Whitney *U*-test, linear and nonlinear regres-

sion analysis using Microsoft Excel and Microcal Origin software. Data are presented as M ± m, where M is the mean value; m is the standard error of the mean value.

RESULTS AND DISCUSSION

MTF was found to enhance the sensitivity of LLC/R9 cells to the cytotoxic action of DCA *in vitro*. In the case of their combined use, MTF even in low non-cytotoxic concentrations reduced IC₅₀ for DCA in a concentration-dependent manner. In particular, MTF was not cytotoxic when used alone at a concentration of 2.5 mM but in combination MTF + DCA, IC₅₀ for DCA was reduced by 36% (*p* < 0.05) (Figure, Table 1).

To evaluate the synergistic action of the test compounds against LLC/R9 cells, a sub-cytotoxic DCA concentration of 30 mM and MTF at a concentration of 2 mM, which is a slightly cytotoxic but enhances the cytotoxic action of DCA, were used (IC₅₀ for MTF equaled 12.1 ± 0.6 mM). The obtained data are presented in Table 2.

As can be seen from Table 2, the treatment with 30 mM DCA and 2 mM MTF resulted in a decrease of the number of viable cells by almost 39% (*p* < 0.05) and 19% (*p* < 0.05), an increase of the number of dead cells by 2.8 times (*p* < 0.05) and twice (*p* < 0.05), as well as a decrease of the proportion of cells at the S-phase by almost half (*p* < 0.05) and 1.4 times (*p* > 0.05) compared with that in the control and with 30 mM DCA, respectively.

The sharp increase (more than 4-fold, *p* < 0.05) of the level of apoptosis detected after the treatment with the combination of DCA and MTF was most likely

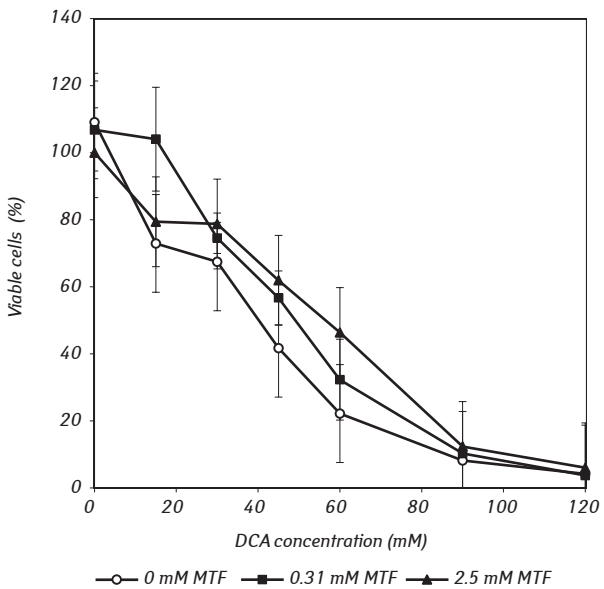


Figure. The cytotoxic action of DCA in combination with MTF in low concentrations upon LLC/R9 cells *in vitro*

Table 1. The effect of MTF in low concentrations on IC₅₀ value for DCA

DCA (mM)	MTF (mM)	IC ₅₀ (mM) for DCA
0–120	0	50.8 ± 7.6
0–120	0.3	49.1 ± 3.4
0–120	2.5	32.5 ± 2.1*

Note: **p* < 0.05 as compared to the corresponding value in the case of single-use.

Table 2. The action of DCA, MTF and their combination upon LLC/R9 cell growth and metabolic features *in vitro*

Index	Control	30 mM DCA	30 mM DCA + 2 mM MTF
Total number of cells (mln)	1.21 ± 0.08	0.94 ± 0.04*	0.80 ± 0.03*.*
Number of viable cells (mln)	1.16 ± 0.07	0.89 ± 0.04*	0.72 ± 0.02*.*
Necrosis (%)	3.7 ± 0.8	5.0 ± 1.6	10.3 ± 0.9*.*
Apoptosis (%)	1.9 ± 0.4	7.7 ± 0.8*	8.0 ± 0.6*
G0/G1 (%)	61.4 ± 1.9	56.5 ± 2.2	61.7 ± 1.9
G2/M (%)	23.2 ± 2.0	31.4 ± 1.3*	29.8 ± 2.2
S (%)	15.4 ± 1.7	12.0 ± 2.8	8.5 ± 1.9*
Mitochondrial membrane potential (FL2/FL1)	1.51 ± 0.01	1.47 ± 0.006*	1.44 ± 0.005*
Glucose consumption rate (μmol/10 ⁶ cells* <i>h</i>)	0.49 ± 0.02	0.36 ± 0.04*	0.55 ± 0.03 [#]
Lactate production rate (μmol/10 ⁶ cells* <i>h</i>)	0.76 ± 0.03	0.50 ± 0.05*	0.81 ± 0.04 [#]
ATP level (nmol/10 ⁶ cells)	12.8 ± 1.0	17.1 ± 0.4*	17.3 ± 1.6*

Note: *.**p* < 0.05 as compared to control and cells treated by DCA alone, respectively.

due to the action of DCA itself. This was evidenced by virtually the same high percentage of apoptotic cells induced by the action of DCA alone. Similarly, a statistically significant decrease (*p* < 0.05) of the membrane potential of the mitochondrial inner membrane, recorded in the case of a combination of DCA and MTF, was due to the action of DCA alone.

Interestingly, the rate of glucose consumption and lactate production by LLC/R9 cells treated with DCA in combination with MTF were not significantly different from those in control. Instead, the effect of DCA alone on the energy metabolism of LLC/R9 cells was manifested by a statistically significant decrease in the rate of glucose consumption and, accordingly, the rate of lactate production by these cells. Thus, the rate of glucose consumption by the cells due to the action of DCA alone decreased by almost 27% (*p* < 0.05) and by almost 35% (*p* < 0.05) compared to the control and combination with MTF, respectively. At the same time, the rate of lactate production due to the action of DCA alone was by 34% (*p* < 0.05) and more than 38% (*p* < 0.05) lower than in the control and combination with MTF, respectively.

Although DCA in combination with MTF did not significantly alter both glucose consumption rate and lactate production rate, intracellular ATP levels in LLC/R9 cells increased by 35% (*p* < 0.05) compared with control. The same statistically significant increase of ATP levels in tumor cells was observed in the case of DCA alone, despite the significant decrease of both glucose consumption and lactate production rate due to DCA action on these cells.

Therefore, *in vitro* studies have shown that MTF, even at a relatively low concentration, is capable of potentiating the cytotoxic/cytostatic action of DCA against LLC/R9 cells. Moreover, only the combination of DCA and MTF resulted in a sharp decrease in the number of viable cells due to the simultaneous enhancement of both the cytotoxic and cytostatic effects of these two compounds. The first was confirmed, in particular, by a significant increase of the level of necrotic cells, the second — by a twofold (*p* < 0.05) decrease of the proportion of cells in the S-phase.

Instead, the synergistic proapoptotic action of DCA and MTF against LLC/R9 cells was not revealed. Usually, the induction of apoptosis is considered as one of the main mechanisms of antitumor action for both DCA [9, 10] and MTF [15, 24, 25] and their

combinations [19, 20]. In the case of LLC/R9 cells treated with DCA alone and in combination with MTF, high levels of apoptosis and a decrease of their mitochondrial membrane potential were observed only because of DCA action. Given the direct link between the induction of tumor cell apoptosis and the activity of oxidative phosphorylation [26], DCA-induced increase in LLC/R9 cell apoptosis was associated with its ability to activate oxidative phosphorylation. Activation of mitochondrial respiration and indirect inhibition of glycolysis activity in LLC/R9 cells treated with DCA were evidenced by the decrease in glucose consumption rate (by almost 27%, *p* < 0.05) and lactate production rate (by 34%, *p* < 0.05).

MTF neutralized the indicated effects of DCA on glucose metabolism in LLC/R9 cells. In these cells, MTF, despite the presence of DCA, increased the rate of glucose consumption and lactate production to practically the levels recorded in the control, i.e. it exerted its ability to block oxidative phosphorylation [13, 14]. The lack of a modulatory effect of MTF on the level of DCA-induced apoptosis in LLC/R9 cells was related to the ability of MTF to attenuate oxidative phosphorylation function and to induce the compensatory glycolysis activation in tumor cells. This was also indicated by a decrease in the percentage of cells in the S-phase because, as we know, S-phase entry is directly dependent on their mitochondrial activity [27, 28].

It is also possible that the ability of MTF to activate AMP-activated protein kinase-dependent signaling cascades in tumor cells has been implicated in its effects [29]. Blocking oxidative phosphorylation, as well as activation of AMPK-dependent signaling pathways by this agent can limit the apoptotic death of tumor cells, in particular, due to decreased level of ROS production, a major inducer of apoptosis, and due to AMPK-dependent inhibition of many energy-dependent intracellular processes, including apoptosis. In the case of LLC/R9 cells, the indicated effects of MTF can explain not only the absence of an increase in the level of DCA-induced apoptosis but also a significant increase in their necrotic death.

Synergistic action of DCA and MTF *in vitro* against LLC/R9 cells in our study gives a reason to hope for their possible synergistic antitumor action *in vivo* too. However *in vivo* the tumor microenvironment can play a dramatic role through its capability to change tumor

cell metabolism drastically, especially energetic metabolism. Such influence of tumor microenvironment should modify the cytotoxic action of DCA and MTF because their cytotoxicity is largely dependent on their action on the energetic metabolism of tumor cells.

It is generally recognized that the main features of tumor cell microenvironment are hypoxia, nutrient substrate deficiency, lactic acidosis, tumor infiltration with conditionally healthy host cells. To predict tumor microenvironment effects on tumor cells sensitivity to the action of chemotherapy and energy metabolism modifiers is too hard because these effects are formed from simultaneous action of numerous factors every of which can affect tumor cells in a multidirectional manner. Moreover, it is well known that the tumor cell microenvironment is very heterogeneous within a tumor.

Hypoxia can significantly reduce tumor cell sensitivity to proapoptotic stimuli due to activation of the expression of hypoxia-inducible factor-1 alpha, one of the key metabolic regulators, and subsequent significant change in energetic metabolism, i.e. drastic activation of glycolysis [30, 31]. Nutritional substrate deficiency caused by tumor microvasculature inconvenience can also affect the tumor cell sensitivity to energy metabolism modifiers such as DCA and MTF via reduction of their adaptive potential or by the strong effects of metabolic stress on their energy metabolism and gene expression profiles. For example, glucose deficiency can both increase the sensitivity of tumor cells to DCA and MTF [32, 33] and induce resistance [34]. Lactic acidosis, as a consequence of lactic acid accumulation in the tumor microenvironment, is also capable of significantly modifying the sensitivity of tumor cells to both nutrient substrate deficiency and apoptosis, and therefore to the action of antitumor drugs [35]. Besides these agents, in particular, MTF can exert systemic action via modification of glucose metabolism not only in tumor cells but also in normal cells [36].

In this study, we established the ability of MTF even in a slightly cytotoxic concentration to enhance the cytostatic/cytotoxic action of DCA against LLC/R9 tumor cells *in vitro*. *In vivo* such synergistic action of DCA and MTF also seems to be operative although drastically affected by tumor cells microenvironment.

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