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# TUMOR MICROENVIRONMENT CHANGES TUMOR CELL SENSITIVITY TO ACTION OF ENERGY METABOLISM MODIFIERS

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***Background:*** **Taking into account differences in the bioenergetics between malignant and normal cells a search of antitumor drugs among the modifiers of tumor metabolism has a reasonable excuse. Earlier it was found that the cytotoxic/cytostatic action of sodium dichloroacetate (DCA) against Lewis lung carcinoma (LLC) cells *in vitro* was enhanced in the case of its combination with metformin (MTF). *Aim:* To study the antitumor action of DCA in combination with MTF against LLC *in vivo*. *Materials and Methods:* LLC/R9, a low metastatic variant of LLC cells, was used. LLC/R9 bearing mice were treated with MTF (at a total dose 0.15 g/kg b.w.) alone or in combination with DCA (at a total dose of 0.75 g/kg b.w.). LLC/R9 growth kinetics and the primary tumor growth and metastasis indices on the 23rd day after tumor cell inoculation were evaluated by routine procedures. The state of the electron transport chain of mitochondria in tumor cells was studied using electron paramagnetic resonance. The content of lactate and glucose in blood plasma from mice was measured by enzymatic methods using biochemical analyzer. The number of tumor-associated macrophages (TAMs) and their distribution by M1/M2 phenotype were estimated by flow cytometry using antibodies against CD68 and CD206. *Results*: In LLC/R9-bearing mice treated with DCA in combination with MTF, tumor growth and metastasis indices, as well as circulating glucose and lactate levels were not significantly different from those in the control group. The level of nitrosylation of non-heme and heme proteins and the content of iron-sulfur centers in the mitochondria of tumor cells in LLC/R9-bearing mice administered with DCA in combination with MTF did not also differ from the corresponding indices in control. Instead, in tumors treated with MTF alone and in combination with DCA the total $CD68^+$ TAMs count was almost 27% ($p < 0.05$) and 43% lower ($p < 0.05$) correspondingly than that in control, but this decrease was not accompanied by redistribution of $CD68^+/CD206^+$ and $CD68^+/D206^-$ subsets. *Conclusion*: DCA in combination with MTF, at least in doses applied, did not affect LLC/R9 growth and metastasis *in vivo*. The complete absence of an antitumor effect of DCA in combination with MTF was simultaneously associated with the absence of significant changes in the functional state of electron transport chain of mitochondria in tumor cells, circulating glucose and lactate levels, and the decrease of the TAMs amount in tumors. It suggests that the antitumor activity of DCA and MTF could be determined by both their local effects within a tumor and their multiple systemic impacts.**
***Key Words*: sodium dichloroacetate, metformin, tumor growth and metastasis.**

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In the last years tumor bioenergetics is considered an attractive target for antitumor therapy [1–3]. This approach is being developed actively after understanding that tumor metabolism features are critical for tumor cell survival, proliferation and metastasis. Among the drugs targeting the tumor cell metabolism sodium dichloroacetate (DCA) has been intensively researched the last decade as a promising antitumor agent. DCA is a small molecule well known since its use for the treatment of metabolic disorders associated with the development of lactic acidosis [4]. The mechanism of antitumor action of DCA is associated with its ability to intensify the oxidative phosphorylation and inhibit indirectly glycolysis [5]. This is due to the ability of DCA to inhibit a pyruvate dehydrogenase (PDH) kinase and thus activate PDH, which leads to the enhancement of mitochondrial bioenergetics and ROS-induced apoptotic death of the tumor cells [3]. Although DCA is considered as a promising antitumor drug, there is some heterogeneity of its antitumor action [3, 6–8]. As one of the ways of strengthening its antitumor efficiency as well as reducing its possible toxicity risks the combination DCA with traditional chemotherapeutic drugs or other modifiers of the tumor cell energy metabolism is exploited [9–11].

We also accumulated data on the antitumor action of DCA, alone and in combination with other agents [12–16]. Earlier it was found that the cytotoxic/cytostatic action of DCA against Lewis lung carcinoma cells *in vitro* was enhanced in the case of its combination with metformin (MTF) [16]. MTF is a hypoglycemic drug widely used for the treatment of type 2 diabetes, which is an inhibitor of the mitochondrial electron transport chain, and as well as DCA exerts its antitumor potential [17, 18]. The work aimed to study the antitumor efficacy of DCA in combination with MTF against Lewis lung carcinoma (LLC) *in vivo*.

## MATERIALS AND METHODS

***Animals and tumor models***. The studies were performed on C57Bl/6 mice 2–2.5 months old, weighing 18.5–21.5 g, bred in the vivarium at the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine. The research on animals was carried out in accordance with the provisions of the General Ethical Principles of Animal Experiments adopted by the First Congress on Bioethics (Kyiv, 2001) and international requirements in accordance with the European

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*Abbreviations used:* DCA – sodium dichloroacetate; DNICs – dinitrosyl iron complexes; EPR – electron paramagnetic resonance; ETC Mt – electron transport chain of mitochondria; LLC – Lewis lung carcinoma; LLC/R9 – low metastatic variant of Lewis lung carcinoma; MTF – metformin; TAMs – tumor-associated macrophages.

Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes (Strasbourg, 1986).

As an experimental tumor model, a low metastatic variant of Lewis lung carcinoma (LLC/R9) was used, which was obtained from the National Bank of Cell Lines and Tumor Strains of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine.

LLC/R9 cells were maintained *in vitro* in RPMI 1640 medium with 10% fetal calf serum (Sigma, USA) and 40 mg/ml gentamicin at 37 °C in a humidified atmosphere with 5% $CO_2$.

For *in vivo* experiments, tumor cells were grown *in vitro* under standard conditions. LLC/R9 cells were inoculated to the mice intramuscularly ($10^6$ cells in 0.1 ml of Hanks solution per animal).

***Agents, doses and administration mode***. DCA (Sigma, USA) and MTF (Sigma, USA) were used as compounds under study.

Taking into account that drug combination allows reducing drug doses for minimization of possible side effects in this work DCA was administered at a dose lower than the dose that caused antitumor action but had some kidney toxicity in LLC/R9-bearing mice [12].

To assess the antitumor activity of DCA in combination with MTF mice after the tumor cell inoculation were randomized by weight and distributed into 3 groups: i) mice administered with MTF alone at a total dose of 0.3 g/kg b.w. (n = 10); ii) mice administered with MTF at a total dose of 0.3 g/kg b.w. in combination with DCA at a total dose of 0.75 g/kg b.w. (n = 12); and iii) mice administered with water (control, n = 12).

Animals were administered with DCA orally via the drinking water daily from 6 pm to 9 am. MTF was administered orally with a feeding tube in 0.3 ml of water daily approximately at 2 pm. Both agents were administered 5 times a week for 3 weeks.

Control animals received water orally with a feeding tube in the same volume and by the same scheme as MTF.

Both agents were prepared *ex tempore*.

***The antitumor action of tested drugs*** was evaluated by their effect on the tumor growth kinetics, as well as the primary tumor volume, the number and volume of lung metastases in mice on the 23rd day after tumor cell inoculation, the day after the end of therapy.

Primary tumor diameter in tumor-bearing mice was measured with a caliper, starting from the appearance of the palpable tumor.

Primary tumor volume ($V_1$) was calculated on the basis of its diameter (d) measured using caliper three times per week starting from the appearance of the palpable tumor by the formula (1):

$$V_1 = 0.52*d^3 \quad (1)$$

The number of visible lung metastases and their diameter were evaluated according to the conventional method using a binocular microscope and a millimeter scale.

Total volume of metastasis ($V_2$) was calculated by the formula (2):

$$V_2 = \sum \frac{n_i * \pi * (d_i)^3}{6} \quad (2)$$

where $n_i$ — number of metastases with the diameter of $d_i$ (mm).

***Glucose and lactate contents*** in plasma from the animals were determined on a ChemWell® 2910 (Combi) automatic analyzer by enzymatic methods. For that, the peripheral blood of the mice was collected in heparinized tubes. After centrifugation, the plasma samples were stored at –20 °C before using.

***The functional activity of the components of the electron transport chain of mitochondria (ETC Mt) of tumor cells*** was investigated using the electron paramagnetic resonance (EPR) method. Using a standard press-form, from the removed tumor tissue cylindrical specimens of calibrated dimensions (d = 4.0 mm, l = 25–35 mm) were prepared, frozen and stored at a temperature of liquid nitrogen. The electron spin resonance spectra of the samples were recorded under low-temperature conditions at 77 K on an E-109 Varian spectrometer (USA) with modulation amplitude of $0.8 \cdot 10^{-4}$ Tl, a microwave emission power of 10.0 mW, and a steady-state instrument time of 1.0 s. According to the EPR spectra, the levels of restored non-heme iron-sulfur (Fe–S) centers (g = 1.94), nitrosyl heme iron complexes ($g_{ser}$ = 2.01), and nitrosyl non-heme iron complexes (dinitrosyl iron complexes — DNICs, $g_{ser}$ = 2.03) of mitochondrial ETC were evaluated [19].

***The number of tumor-associated macrophages (TAMs) and their distribution*** by M1/M2 phenotype were determined by flow cytometry using antibodies against CD68 and CD206.

For this purpose, tumor cells after mechanical disaggregation of tumor tissues were stained using following antibodies: a rat anti-mouse monoclonal CD68 labeled with PE (1:160, BD Farmingen, USA) and a rat anti-mouse monoclonal CD206 labeled with Alexa Fluor 647 (1:160, BD Farmingen, USA) with simultaneous permeabilization using saponin. Total TAM count was determined by the percentage of CD68 positive staining cells ($CD68^+$), whereas the number of cells with the M2 phenotype was determined by the percentage of the cells with CD206 positive staining among CD68 positively stained cells ($CD68^+/CD206^+$) [20, 21].

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

Analysis of LLC/R9 growth and metastasis showed that the combined usage of DCA at a total dose of 0.75 g/kg and MTF at a total dose of 0.3 g/kg was ineffective. As can be seen from Fig. 1, the tumor growth kinetics in mice administered with MTF in combination with DCA was not significantly different from that in controls and animals treated with MTF alone.

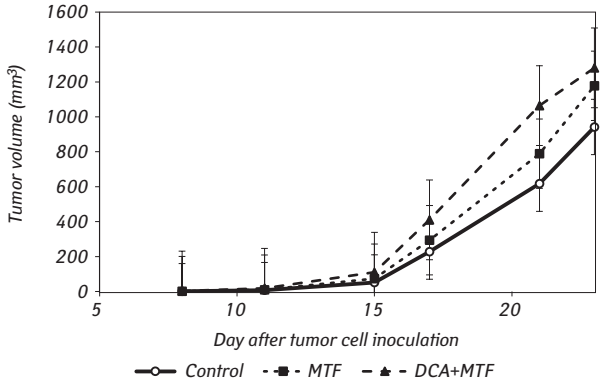


**Fig. 1.** The effect of DCA in combination with MTF upon LLC/R9 primary tumor growth kinetics *in vivo*. *$p < 0.05$ as compared to control

**Table.** The effect of DCA in combination with MTF upon LLC/R9 growth and metastasis values

| Group | The number of mice | The tumor volume ($mm^3$) | The number of metastases | The volume of metastases ($mm^3$) |
|---|---|---|---|---|
| Control | n = 10 | 941.9 ± 169.9 | 19.7 ± 5.5 | 33.8 ± 14.6 |
| MTF | n = 9 | 1178.1 ± 142.6 | 18.3 ± 3.5 | 21.9 ± 5.2 |
| DCA+MTF | n = 13 | 1280.3 ± 101.5 | 19.6 ± 3.3 | 26.6 ± 6.6 |

In the group of animals treated with MTF or MTF in combination with DCA, the volume of primary tumors, the number and volume of lung metastases on day 23 after tumor transplantation were also not significantly different from those in control (Table).

Similarly, in animals treated with MTF or MTF in combination with DCA, glucose and lactate levels in the blood plasma did not differ significantly from corresponding indices in the control. The obtained data are presented in Fig. 2.

Analysis of the EPR spectra of tumor tissue samples showed that DCA in combination with MTF had no significant effect on ETC Mt in tumor cells. This was evidenced by virtually identical levels of nitrosylation of non-heme (DNICs) and heme proteins, as well as levels of iron-sulfur centers in Mt of tumor cells in mice treated with DCA in combination with MTF and the control (Fig. 3).

It should be noted also that in tumors of mice treated with MTF alone, a high level of nitrosylation of DNICs in ETC Mt of tumor cells was observed, which exceeded that of control and animals treated with MTF together with DCA, up to 107% ($p < 0.05$) and 82% ($p < 0.01$), respectively. The detected peak may be related to the ability of MTF to enhance NO production and the subsequent formation of paramagnetic complexes, in particular, DNICs, which can be detected by EPR [22].

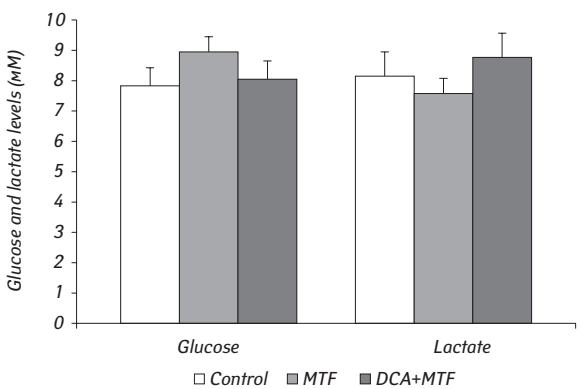


**Fig. 2.** The effect of DCA in combination with MTF on glucose and lactate concentrations in the blood plasma of LLC/R9 bearing mice

Phenotyping of tumor cells with antibodies against CD68 and CD206 revealed that in mice treated with both MTF alone or in combination with DCA, the total amount of TAMs ($CD68^+$ cells) in the tumor was almost by 27% ($p < 0.05$) and by 43% ($p < 0.05$) lower, respectively, than the corresponding index in the control (Fig. 4, 5). Instead, we did not observe significant macrophage redistribution due to the action of the test drugs on M1/M2 subpopulations known to reflect their antitumor (in the case of M1-phenotype — $CD68^+$/$CD206^-$ cells) or stimulating (in the case of M2-phenotype — $CD68^+$/$CD206^+$ cells) activity [23, 24].

Thus, there was no effect of DCA in combination with MTF on LLC/R9 tumor growth and metastasis *in vivo,* at least in administered doses. The complete absence of an inhibitory antitumor effect of DCA in combination with MTF was associated with the absence of significant changes in the functional state of ETC Mt in tumor cells and circulating levels of glucose and lactate as well as with the decrease of the total number of TAMs in a tumor.

According to literature data one of the mechanisms of antitumor action of both DCA and MTF is their influence on the functional state of immune cells, in particular, TAMs and their M1/M2 polarization [25, 26]. So, it was shown that DCA via the suppression of lactic acid production by tumor cells decreases ARG1 expression and arginase activity in tumor-infiltrating immune cells [25]. MTF is known to mediate its antitumor and anti-angiogenic activities via its effect on macrophage polarization [26]. Moreover, MTF-mediated TAM polarization from M2 to M1 phenotype is dose-dependent.

In our work, MTF both alone and in combination with DCA caused a significant decrease in the total number of TAMs in the tumor. But such a decrease in the total number of TAMs was not sufficient to realize the antitumor effect of the drugs, although high TAMs count in malignant tumors is usually associated with

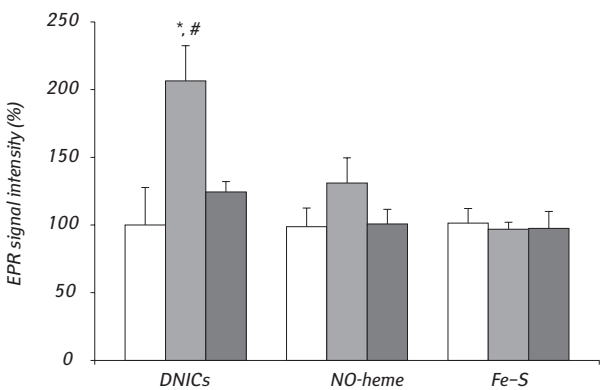


**Fig. 3.** The effect of DCA in combination with MTF on the nitrosylation of heme and non-heme proteins, and Fe–S center level in Mt of LLC/R9 cells *in vivo*. *, #$p < 0.05$ as compared to control and mice, treated by DCA in combination with MTF, correspondently.

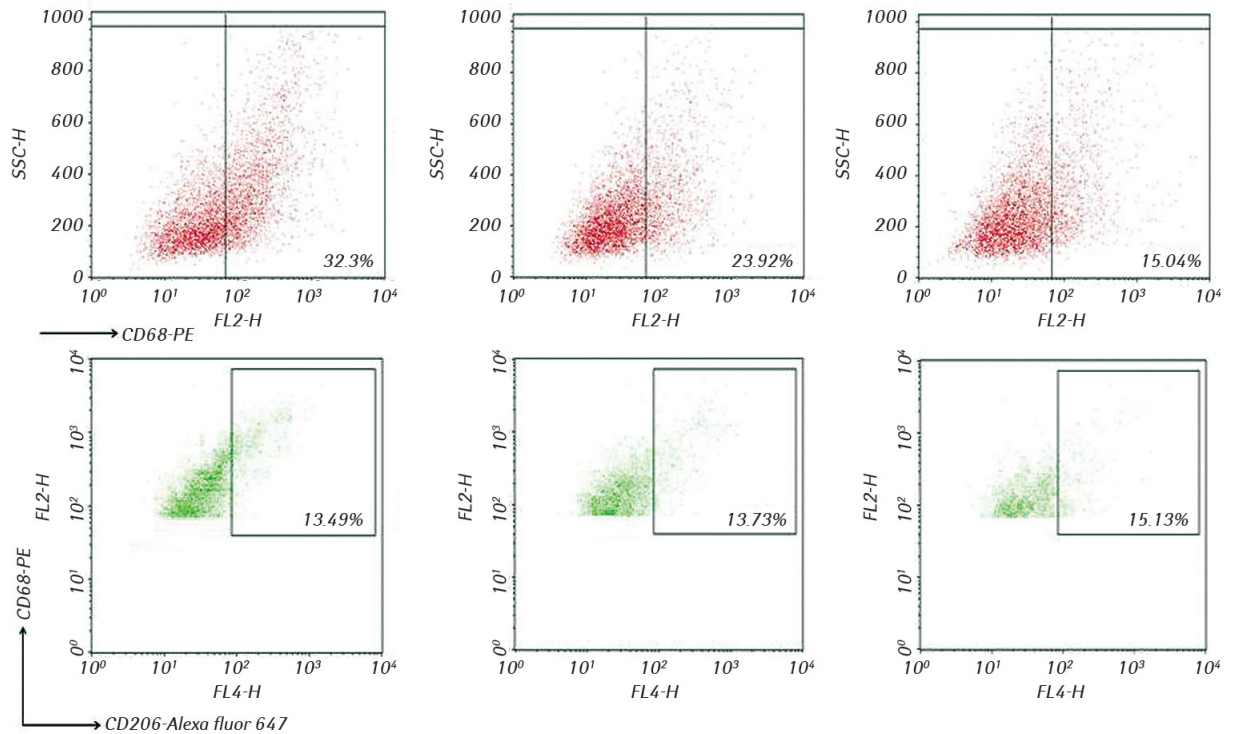


**Fig. 4.** Flow cytometry images of CD68$^+$ TAMs (upper row) and CD68$^+$/CD206$^+$ TAMs subset (bottom row) in LLC/R9 tumors from mice treated with water (control), MTF and MTF in combination with DCA

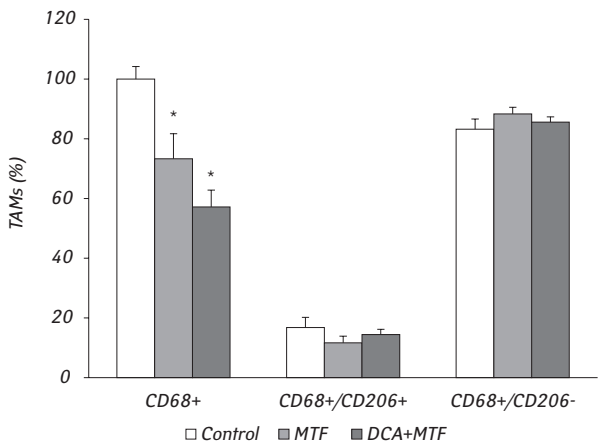


**Fig. 5.** The effect of DCA in combination with MTF on the total amount of TAMs (CD68$^+$) and their M1 (CD68$^+$/CD206$^-$) and M2 (CD68$^+$/CD206$^+$) subsets in the primary tumor of LLC/R9 bearing mice. *$p < 0.05$ as compared to control.

a negative prognosis [27]. Besides, the drug-induced changes in the total number of TAMs in the tumor were not accompanied by a shift of TAM polarization from M2 to M1 phenotype, which is known to reflect their antitumor activity [26].

The complete absence of an inhibitory antitumor effect of DCA in combination with MTF was surprising taking into account our previous data that MTF, even at a relatively low noncytotoxic concentration, was capable of potentiating the cytotoxic/cytostatic action of DCA against LLC/R9 cells *in vitro* [16]. Moreover, previously, we have shown that DCA in combination with MTF exerted extremely high efficacy against C6 glioma *in vivo*, resulting in a 50% prolongation of the average lifespan of rats with intracranial transplanted tumors [14].

Analysis of obtained data revealed that the actions of tested drugs against glioma C6 and LLC/R9 cells were similar *in vitro* and distinct *in vivo*. According to *in vitro* data, MTF in a similar manner suppressed DCA-induced oxidative phosphorylation and reactivated glycolysis in both C6 glioma and LLC/R9 cells. Also, in both cases, MTF in combination with DCA did not lead to the expected increase in apoptosis, and in the case of glioma cells even caused a significant decrease in the apoptosis level as compared to DCA-induced one [14].

*In vivo* data revealed that the ability of MTF to enhance the antitumor efficacy of DCA against C6 glioma was associated with their effects on glucose metabolism not only in tumor cells but also in normal cells. At the systemic level, this was realized, in particular, by reducing the glucose and lactate levels in the blood of tumor-bearing rats [14, 15]. In contrast, the absence of an antitumor effect of DCA in combination with MTF against LLC/R9 was simultaneously associated with the absence of significant changes in metabolism of both tumor cells and tumor-bearing host.

Therefore, the antitumor efficacy of the combined usage of DCA and MTF can significantly depend on the type of a tumor and both their local effects within a tumor and their multiple systemic impacts.

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