IMPACT OF LACTIC ACIDOSIS ON THE SURVIVAL OF LEWIS LUNG CARCINOMA CELLS

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Aim: To investigate the effect of lactic acidosis on the survival of Lewis lung carcinoma cells under glucose-deprived conditions. *Materials and Methods*: LLC/R9 variant of Lewis lung carcinoma cells was cultured in glucose deficit or complete culture medium. Conditions of lactic acidosis, lactosis, and acidosis were generated in glucose deficit medium. Cell survival, cell cycle, apoptosis, autophagy, and the content of glucose, lactate, vascular endothelial growth factor in the culture medium were determined. Light and fluorescent microscopy, flow cytometry, spectrophotometry, and ELISA were used. *Results:* It has been found that 24 h incubation of tumor cells under lactic acidosis caused (i) the reduction of the number of living cells by 33% ($p < 0.05$) and 56% ($p < 0.05$); (ii) the inhibition of apoptosis by 4.3-fold ($p < 0.05$) and 3.3-fold ($p < 0.05$); (iii) the reduction of the rate of glucose consumption by 2-fold ($p < 0.05$) and 2.5-fold ($p < 0.05$); (iv) an increase of lactate production more than twice ($p < 0.05$) and 1.6-fold ($p < 0.05$) compared with these indexes under conditions of glucose deficiency or complete glucose-containing medium, respectively. However, on the second day of culture under lactic acidosis, the number of viable cells reached a maximum, in contrast to culture in the complete medium. The number of live cells on the seventh day of culture under lactic acidosis exceeded almost $2-3$ times ($p < 0.05$) that in the culture under conditions of the glucose deprivation or in complete medium. On the third day under lactic acidosis the autophagolysosomes count was 54% ($p < 0.05$) lower that that under glucose deficit. *Conclusions*: Lactic acidosis promoted the survival and proliferation of Lewis lung carcinoma cells by energy system reprogramming directed on inhibition of apoptosis and autophagy, a significant decrease in the rate of glucose utilization and activation of glutaminolysis and, consequently, increase of the lactate production rate. Inhibition of lactate production by tumor cells may be considered as a promising approach for more efficient antiangiogenic treatment of cancer.
Key Words: Lewis lung carcinoma, lactic acidosis, lactosis, glucose deficiency.

It is known that unlike normal tissue, microenvironment of malignant cells in a tumor characterized by a shortage of energy and the accumulation of plastic substrates and their metabolic products, gradually increasing with the distance from the vessel. This microenvironment occurs, in part, as a result of disability of tumor angiogenesis, and causes the metabolic stress in a tumor cell — the dominance of energy consumption *vs* its production. Tumor cells cannot effectively adapt to this metabolic stress because the predominant mechanism of adenosine triphosphate (ATP) generation in tumor cells is inefficient glycolysis. This offers the prospect of inducing the death of cancer cells, creating or increasing imbalance between the requirement of cells in energy and low level of its production (creating a "metabolic catastrophe") [1, 2].

Modern antiangiogenic cancer therapy is aimed at inducing a "metabolic catastrophe" in tumor cells by inhibiting tumor angiogenesis [3]. The efficiency of antiangiogenic therapy depends on the simultaneous fulfillment of two conditions: effective inhibition of tumor angiogenesis and tumor cell death induced by nutrient deprivation, which is caused by a significant reduction in tumor vascularity. Meanwhile, in many cases, despite the effective inhibition of the growth of blood vessels, expected antitumor action doesn't occur [4–7]. The reason for this lack of anti-

tumor effect is the tolerance of tumor cells to metabolic stress. So, understanding the mechanisms that ensure the survival of tumor cells under metabolic stress is important for improving the efficiency of current antiangiogenic cancer therapy and development of new anticancer drugs.

The aim of the work was to investigate the effect of lactic acidosis on the survival of Lewis lung carcinoma cells under conditions of metabolic stress induced by glucose deprivation.

MATERIALS AND METHODS

Cell culture. In the work, LLC/R9 variant of Lewis lung carcinoma cells was used [8]. Tumor cells are maintained *in vitro* in complete RPMI 1640 medium (Sigma, USA) supplemented with 10% fetal calf serum (fetal bovine serum (FBS), Sigma, USA) and 40 µg/ml gentamicin at 37 °C in humidified atmosphere of 5% CO_2.

To assess the survival of tumor cells under lactic acidosis LLC/R9 cells at exponential growth phase were seeded in 24-well plates (1.5 · 10^5 cells/well) in complete RPMI 1640 medium and incubated overnight. Then this medium was replaced with fresh media, which differed by the content of glucose, lactate, and pH (Table 1).

Table 1. Glucose and lactate content and pH of the culture medium

Culture conditions	Glucose, mM	Lactate, mM	pH
Lactic acidosis	3.0 ± 0.1	14.0 ± 0.7	6.70 ± 0.01
Lactosis	3.0 ± 0.1	14.0 ± 0.7	7.40 ± 0.01
Acidosis	3.0 ± 0.1	1.6 ± 0.7	6.70 ± 0.01
Glucose deficit	3.0 ± 0.1	1.6 ± 0.1	7.40 ± 0.01
Complete medium	9.0 ± 0.5	1.6 ± 0.1	7.40 ± 0.01

For this, we used as a base glucose-free RPMI 1640 medium (Sigma, USA), which, after adding 10%

FBS, was contained glucose and lactate in a final concentration of 3.0 mM and 1.6 mM, respectively, pH 7.4. Lactic acidosis was generated by adding lactic acid (Sigma, USA) to the culture medium to a final concentration of 14.0 mM and pH 6.7; lactosis was generated by adding sodium lactate (Sigma, USA) to a final concentration of 14.0 mM and pH 7.4; acidosis was generated by adding HCl to a final pH of 6.7. Complete culture medium after adding 10% of FBS contained 9.0 mM glucose, 1.6 mM lactate, pH 7.4. After replacing the medium the cells were additionally incubated under standard conditions for 7 days and then following indexes were assessed: the number of living cells and their cell cycle distribution, apoptosis rate, the level of vascular endothelial growth factor (VEGF) production, autophagy induction, glucose and lactate content in the culture medium.

The number of cells and their viability was evaluated by the routine method by direct counting using a 0.4% solution of trypan blue.

Cell cycle distribution was assessed by flow cytometry according to [9]. Briefly, the cells were resuspended in hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100), which contained 5 mg/ml propidium iodide, and DNA content was analyzed by flow cytometry at a wavelength of 488 nm.

The number of apoptotic cells was determined by staining with Hoechst 33258 dye (Sigma, USA) using a fluorescent microscope. To do this, the cells were stained with Hoechst 33258 at a final concentration of 1 mg/ml at 37 °C for 20 min, washed with saline and then the changes in nuclear morphology were evaluated, by counting at least 500 cells/sample. Apoptotic cells were determined by the presence or fragmented nuclei or condensed chromatin, apoptosis rate was expressed as a percentage of total cells.

The VEGF level in cell culture medium was detected by ELISA using mouse VEGF kit (Invitrogen, USA) according to the manufacturer's protocol. The rate of VEGF production by tumor cells was calculated based on changes in VEGF concentration in the culture medium and in the number of living cells in the time course.

The level of autophagy in tumor cells was determined using a fluorescent marker monodansylcadaverine (MDC) (Sigma, USA) [10]. Shortly, the cells were incubated with MDC at a final concentration of 0.05 mM at 37 °C for 20 min, washed with phosphate buffered saline (PBS) and lysed using 10 mM Tris-HCl solution with the addition of 1% Triton X-100. Intracellular MDC levels were determined using fluorometer (excitation filter 360/40 nm, emission filter 528/20 nm). Fluorescence intensity was normalized to the number of living cells.

Glucose level was measured in the medium samples deproteinized with zinc hydroxide by the enzymatic glucose-oxidative method using a set for determining glucose in biological fluids (Sigma, USA) according to the manufacturer's protocol.

The level of lactate was measured in medium samples deproteinized with perchloric acid by the enzymatic spectrophotometric method using lactate dehydrogenase (Sigma, USA) [11].

In all cases, the studied medium samples were collected and stored at −20 °C prior to measurement.

The rate of glucose consumption and lactate production in tumor cells was calculated based on the concentration of substances in the culture medium and changes in the number of living cells in the time course.

Statistical analysis. All measurements were done in triplicate and data are presented as mean ± s.e. Statistical analysis was performed by descriptive methods and Student's *t*-test using Microsoft Excel software and Microcal Origin (MicroSoft Inc., USA). *p* values of less than 0.05 were considered as significant.

RESULTS

As it has been found lactic acidosis with glucose deprivation significantly promoted survival of LLC/R9 cells. Despite the slowing proliferation of the studied cells on the 1st day of culture under lactic acidosis, already on the 2nd day, their proliferation rates increased dramatically (Fig. 1). On the 1st day of culture under lactic acidosis, the total number of living cells was almost by 33% ($p < 0.05$), 41% ($p < 0.05$), and 56% ($p < 0.05$) lower than that under conditions of the deficit of glucose, lactose, and complete medium, respectively.

At the same time the maximal number of living cells, which was achieved under lactic acidosis on the 2nd day, almost 1.5-fold ($p < 0.05$) exceeded that under conditions of the glucose deprivation, lactosis and acidosis. In addition, the maximal number of tumor cells achieved under lactic acidosis with glucose depletion did not differ significantly from the corresponding value under conditions of cell incubation in complete medium.

In the remote terms, the number of cells that survived on the 7th day of incubation (without replacement of the media) under conditions of lactic acidosis and acidosis (in both cases at pH 6.7) was almost by 2–3-fold ($p < 0.05$) higher than that in conditions of glucose deprivation or complete medium (in both cases, at pH 7.4).
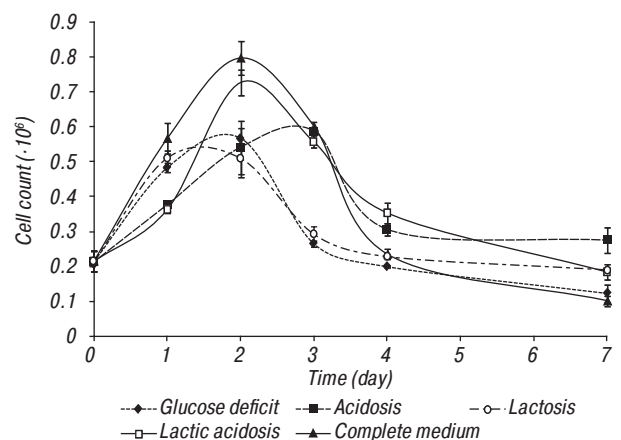

Fig. 1. Growth kinetics of LLC/R9 cells *in vitro* under different culture conditions

On the 1st day of cell culture, lactic acidosis caused the reduction of glucose consumption rate by cells, which clearly correlated with a decrease in prolifera-

tion rate at this time. In the conditions of lactic acidosis this index was almost twice lower ($p < 0.05$) compared to that under glucose and lactose deficiency and 2.5-fold lower ($p < 0.05$) than that in the complete medium (Table 2). Acidosis, as well as lactic acidosis, led to a significant reduction of the mentioned index to 0.200 ± 0.003 μmol/(10^6 cells·h) ($p < 0.05$), which was associated with the lowest proliferation rate of tumor cells and the presence of glucose in culture medium even on the 4th day, unlike all other conditions of incubation, and the highest cell survival even on the 7th day.

Interestingly, along with the low rate of glucose consumption by cells under lactic acidosis their lactate production rate was the highest ($p < 0.05$) compared to all other culture conditions (see Table 2). Moreover, the ratio between lactate production and glucose consumption significantly exceeded an expected value in the case of an effective glycolysis.

Table 2. Glucose consumption and lactate production rates by LLC/R9 cells under different culture conditions

Culture conditions	Glucose consumption rate, μmol/(10^6 cells·h)	Lactate production rate, μmol/(10^6 cells·h)
Glucose deficit	0.328 ± 0.001	0.646 ± 0.003
Acidosis	0.197 ± 0.003	0.278 ± 0.022
Lactosis	0.319 ± 0.003	0.746 ± 0.204
Lactic acidosis	0.164 ± 0.012	1.328 ± 0.048
Complete medium	0.417 ± 0.030	0.807 ± 0.055

Lactic acidosis significantly reduced the percentage of apoptotic cells (Fig. 2). So, if at the 1st day apoptosis rate was almost the same in all cases, starting from the 2nd day the percentage of apoptotic cells under lactic acidosis was 4.3-fold ($p < 0.05$), 5-fold ($p < 0.05$) and 3.3-fold ($p < 0.05$) lower compared to that under conditions of glucose deprivation, lactosis and complete medium, respectively.
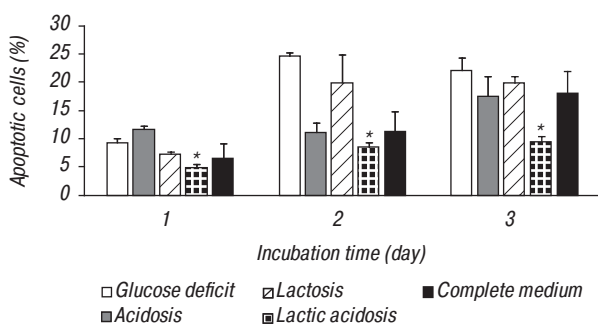

Fig. 2. Apoptosis level under different culture conditions. *$p < 0.05$ as compared to that in the conditions of glucose deficiency

Significant changes in the cell cycle distribution related to the culture conditions were not observed (Table 3). Hence, inhibition of cell proliferation, found in the conditions of lactic acidosis on the 1st day and in conditions of acidosis during 1–3 days apparently occurred due to blocking cell proliferation in all phases of the cell cycle.

There have been no significant effect of lactic acidosis at the background of glucose deficiency on VEGF production in LLC/R9 cells (Fig. 3). At least, during the 1st day, VEGF production rate in cells LLC/R9 in conditions of lactic acidosis did not differ significantly from that of all other culture conditions.

Table 3. LLC/R9 cell cycle distribution under different culture conditions

| Culture conditions	Phase	Incubation time, day			
		1	2	4	7
Lactic acidosis	G0/G1	53.5 ± 1.0	61.6 ± 1.2	60.9 ± 0.5	44.8 ± 0.3
	S	36.4 ± 0.1	27.0 ± 1.1	33.7 ± 1.2	48.8 ± 0.6
	G2/M	10.1 ± 1.0	11.4 ± 0.1	5.5 ± 0.7	6.5 ± 0.8
Acidosis	G0/G1	58.4 ± 0.2	62.3 ± 2.2	55.5 ± 0.8	41.8 ± 0
	S	31.1 ± 0.6	25.5 ± 1.5	37.4 ± 0.7	45.2 ± 1.0
	G2/M	10.5 ± 0.4	12.2 ± 0.7	7.2 ± 0.1	13.0 ± 1.0
Glucose deficit	G0/G1	53.9 ± 0.7	59.8 ± 0.8	58.0 ± 0.7	47.6 ± 0.2
	S	35.4 ± 1.0	23.6 ± 0.2	34.2 ± 0.9	45.9 ± 1.2
	G2/M	10.7 ± 0.2	16.6 ± 1.0	7.8 ± 0.2	6.5 ± 1.1
Lactosis	G0/G1	55.1 ± 1.5	61.5 ± 0.4	65.2 ± 0.6	52.6 ± 1.6
	S	34.6 ± 1.8	23.1 ± 0.6	27.1 ± 0.2	39.1 ± 0.7
	G2/M	10.3 ± 0.2	15.4 ± 0.1	7.7 ± 0.3	8.4 ± 1.0
Complete medium	G0/G1	59.0 ± 0.9	57.8 ± 0.7	66.1 ± 1.3	56.5 ± 3.3
	S	30.7 ± 0.3	26.0 ± 0.1	28.8 ± 1.5	36.2 ± 1.7
	G2/M	10.3 ± 0.5	16.2 ± 0.8	4.7 ± 0.3	7.3 ± 1.6
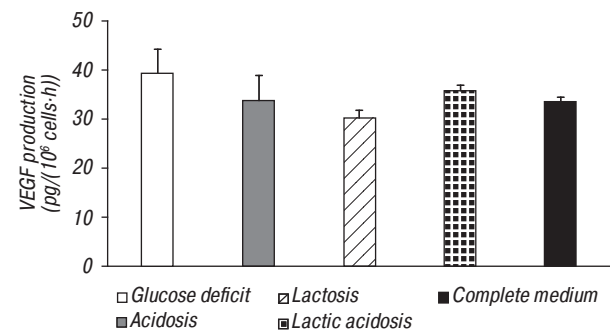
Fig. 3. VEGF production by LLC/R9 cells under different culture conditions

Induction of autophagy in tumor cells was tested using MDC, which is considered as an autophagolysosomal marker due to its ability to accumulate in cytoplasmic vacuoles. The data on the number of lysosomes in LLC/R9 cells are shown in Fig. 4. On the 3rd day of culture, maximal induction of tumor cell autophagy under glucose depletion was observed in the conditions of the glucose deficiency and lactosis. Instead, autophagy induction under acidosis and lactic acidosis was almost 49% ($p < 0.05$) and 54% ($p < 0.05$) lower than that in the conditions of glucose deprivation.
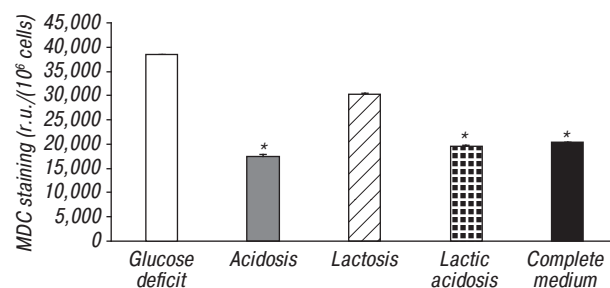

Fig. 4. The number of lysosomes in LLC/R9 cells under different culture conditions. *$p < 0.05$ as compared to that in the conditions of deficiency of glucose and lactosis

DISCUSSION

It is known that the microenvironment of tumor cells is usually characterized by the deficiency of nutrient substrates and accumulation of metabolic products, including excessive lactate and consequently low extracellular pH [12]. Despite metabolic stress generated by malignant cells and their high dependence on energy and nutrient substrates, they show tolerance

to adverse conditions and provide not only survival but also highly proliferative and metastatic activity.

One of the mechanisms of malignant cells survival in adverse conditions, including nutrient deficiency, is limiting cell proliferation and/or transition into dormancy [13]. The mechanisms of survival of tumor cells also include a process of autophagy, i.e. lysosomal degradation of intracellular components providing malignant cells with an alternative source of nutrients and energy substrates [13–16]. Growth factors, including VEGF, induce tumor angiogenesis that results in the enhancement of supplying tumor with oxygen and nutrients [17]. The resistance of tumor cells to metabolic stress can also be associated with impaired mechanisms of apoptotic death [18]. Reprogramming of energy metabolism, for example, increased glucose uptake and activation of glycolysis enzymes on the background of hypoxia, is one of the key mechanisms of survival of malignant cells, as they almost always exist in unfavorable metabolic microenvironment [19].

In this work lactic acidosis with glucose deprivation significantly promoted cell survival of metastatic Lewis lung carcinoma LLC/R9 cells. However, to survive in such conditions, tumor cells at the initial terms of their incubation significantly inhibited the processes requiring major energy consumption, such as proliferation, apoptosis, and autophagy.

Thus, when the cells just began to adapt to the conditions of lactic acidosis the significant slowing of proliferation has been observed. In the same period lactic acidosis caused a significant inhibition of apoptosis and autophagy, whose level under these conditions in the LLC/R9 cells was the lowest among all other culture conditions. Obviously, in terms of further enhancement of nutrient substrates deficiency on the background of lactic acidosis one could expect induction of autophagy processes for cell survival. At least in the later periods of cell incubation under conditions of unfed culture, in these cells there was observed activation of autophagy [20].

Slowing of the proliferation of LLC/R9 cells, observed in the early stages of incubation under lactic acidosis, was accompanied by a significant decrease in the rate of glucose utilization by these cells. If in the complete culture medium the rate of glucose consumption was the highest, then in the conditions of lactic acidosis it was the lowest. This index under lactic acidosis was almost twice lower than that in the conditions of glucose deprivation and lactosis.

Low consumption of glucose by tumor cells led to the fact that glucose was determined in the culture medium even on the 3rd day under lactic acidosis, while in the conditions of glucose deficiency and lactosis the media were completely depleted by glucose at the 2nd day. Interestingly, the lowest rate of glucose consumption in conditions of lactic acidosis was accompanied with the highest rate of lactate production by tumor cells. Moreover, the level of lactate in the culture medium of these cells is much higher than this, expected in the case of complete metabolism of glucose through glycolysis.

These changes evidenced on a significant adjustment in the regulation of energy metabolism of LLC/R9 cells,

caused by lactic acidosis. These changes can be explained by activation of oxidative phosphorylation and glutaminolysis under conditions of lactic acidosis. The latter was confirmed by the high speed of lactate production in LLC/R9 cells and the high levels of lactate in the culture medium under lactic acidosis. One should note that lactosis, unlike lactic acidosis, did not lead to such a pronounced increase in lactate production rate in LLC/R9 cells and consecutive activation of glutaminolysis.

As it is known, glutaminolysis is a major (after glycolysis) way to provide rapidly tumor cells with energy. Activation of glutaminolysis in tumor cells by extracellular lactate was shown in the work [21], in which signaling cascades that are activated with lactate were thoroughly investigated. It was found that in oxygenated malignant human SiHa and HeLa cells lactate enters the cell through monocarboxylate transporter 1 (MCT1), which stabilizes HIF-2α, which transactivates c-Myc, which in turn enhances the absorption of glutamine by cells and activates oxidative phosphorylation.

The important role of lactate in the survival of tumor cells is also confirmed by its ability to induce tumor angiogenesis through the activation of HIF-1α [22] as well as the ability of malignant cells and normal cells co-opted in the tumor process, to use it as an energy substrate [23, 24]. We also know that lactate can cause degradation of the extracellular matrix, increase tumor cell migration activity, which is known to correlate with their metastatic potential [25, 26].

Therefore, the main adaptive reactions aimed at the conservation of mentioned ATP-consuming processes in LLC/R9 cells and related to their subsequent survival under lactic acidosis occurred only during the 1st day of culture. These reactions included dramatic inhibition of proliferation, apoptosis, and autophagy, decreased glucose consumption rate and activation of glutaminolysis. Starting from the 2nd day the active proliferation of these cells resumed: the maximal number of live cells under lactic acidosis was as high as that in complete medium. The levels of dead and apoptotic cells under lactic acidosis were the lowest not only in terms of the initial incubation, but also for the whole culture duration.

Interestingly, acidosis, as well as lactic acidosis contributed to increased survival of LLC/R9 cells. Obviously, under acidosis, these cells used similar mechanisms for their survival. However, slowing of the proliferation of these cells under acidosis was much more pronounced than that in conditions of lactic acidosis, and continued for up to 3 days of culture. In addition, lactate production in LLC/R9 cells under acidosis was significantly lower than that in the conditions of lactic acidosis. However, exactly acidosis provided the maximal survival of LLC/R9 cells on the 7th day in all the studied culture conditions. Thus, the increased survival of tumor cells under acidosis was associated with a slowing of proliferation and inhibition of glycolysis.

The ability of acidosis to promote survival of LLC/R9 cells was also confirmed by the data on their survival in extreme conditions of incubation, namely in PBS supplemented with 10% FBS. As shown

in Table 4, survival of LLC/R9 cells after 3-day incubation in PBS, pH 6.7 was twice higher ($p < 0.05$), than that in the conditions of incubation in PBS, pH 7.4.

Table 4. Survival of LLC/R9 cells under incubation in PBS with different pH

Culture condition	Viable cell count (10^6 cells)	
	0 day	3rd day
Control (PBS + 10% FBS, pH 7.4)	0.15 ± 0.02	0.0319 ± 0.003
Acidosis (PBS + 10% FBS, pH 6.7)	0.15 ± 0.02	0.0763 ± 0.009*

Note: *$p < 0.05$ as compared to control.

The role of acidification of the microenvironment of tumor cells, but rather an important contribution to the excess of hydrogen ions in the extracellular environment to their survival is discussed in several studies [27–29]. This feature of the tumor microenvironment could be related, in particular, to a predominance of cells with glycolytic phenotype and is actively considered as a promising target for anticancer therapy.

Thus, our findings suggested that acidosis promoted survival of tumor cells, but unlike lactic acidosis provided lower proliferative potential. Lactic acidosis not only contributed to the survival of tumor cells but also allow malignant cells actively proliferate under glucose deprivation. Activation of proliferation of LLC/R9 cell was preceded by a short period of adaptation to metabolic stress, which includes reprogramming of its energy system, aimed at energy conservation by inhibiting apoptosis and autophagy, a significant decrease in the rate of glucose utilization and activation of glutaminolysis and as a result, increased lactate production rate. Inhibition of lactate production by tumor cells may be viewed as a promising approach for improving the efficiency of antiangiogenic cancer therapy.

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