Role of malate dehydrogenase in facilitating lactate dehydrogenase to support the glycolysis pathway in tumors

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Abstract. High aerobic glycolysis, as one of the hallmarks of cancer cells, requires nicotinamide adenine dinucleotide (NAD^+) as a vital co-factor, to guarantee the flow of glycolysis. Malate dehydrogenase (MDH), as an important enzyme in cancer metabolism, is a source of NAD^+ additional to lactate dehydrogenase (LDH). The current study aimed to elucidate the kinetic parameters of MDH in human breast cancer and evaluate its supportive role in the glycolysis pathway. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of MDH were determined in the crude extracts of human breast tumors and healthy tissue samples, which were obtained directly from the operating theatre. To assess the potential role of MDH in supporting glycolysis, the MDH activity was measured when the LDH activity was inhibited by different concentrations of oxamate, an inhibitor of LDH in breast cancer cell lines. The K_m of cancerous MDH (C-MDH) was the same as the healthy MDH, although the V_{max} of C-MDH was higher relative to the healthy MDH. Notably, the MDH activity was increased in the MDA-MB-231 cell line, which was treated with the LDH inhibitor (oxamate), but not in the MCF-7 cell line ($P<0.05$). The higher tendency of C-MDH for NAD^+ and malate generation in cancer cells is an effective approach for supporting glycolysis. Increasing MDH activity in the absence of LDH demonstrates the supportive role of MDH in glycolysis. Therefore, decreasing MDH activity and expression in a forward reaction may present as a valid molecular target to abolish its potential effect on tumor metabolism.

Introduction

High aerobic glycolysis is a predominant feature of cancer cells, which is termed the Warburg effect (1,2). This bioenergetic and metabolic characteristic permits cancer cells to survive under adverse tumor conditions (such as hypoxia) and enables them to proliferate and invade, subsequently leading to distant metastasis (3). The control point of glycolysis is glyceraldehyde 3-phosphate dehydrogenase that requires nicotinamide adenine dinucleotide (NAD^+) in the glycolytic direction. Cancer cells provide the required NAD^+ via lactate dehydrogenase (LDH), which converts pyruvate to lactate with concomitant regeneration of NAD^+. The gene expression and activity of LDH is increased in various types of tumor when compared with healthy tissue samples (4-6). However, there is another approach that supplies the required NAD^+ for glycolysis, which is cytosolic malate dehydrogenase (MDH) (7,8). MDH, as a part of the malate-aspartate shuttle, catalyzes the reversible reaction of oxaloacetate (OAA) to malate in the presence of NADH. Typically, the enzyme has two distinct forms, mitochondrial and cytosolic. The cytosolic isoform is involved in the oxidation of NADH to NAD^+, which is then used for continuing the progression of glycolysis (9).

Environmental parameters, such as pH, oxygen and nutrient availability, influence the enzyme kinetics via different approaches (10). The tumor microenvironment is defined as a heterogeneous milieu in which the oxygen pressure, pH and nutrient availability are completely different from healthy tissues (11). However, the nature and importance of the tumor environment on enzyme kinetics has been masked in certain enzyme studies, owing to the use of cell culture conditions in which pH is in the normal range, without any fluctuation, and where oxygen and nutrients are constantly accessible (12). Alteration of the enzyme characteristics

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in the tumor microenvironment, which alters the enzyme kinetics, has received little attention. Given the potential role of MDH to supply NAD^+ and the effect of stressful tumor microenvironment on enzyme kinetics, the aim of the current study was to compare the kinetic parameters of MDH between breast cancer tissue samples and cell lines (MCF-7 and MDA-MB-231) and healthy mammary tissue samples. Furthermore, the potential role of MDH for sustaining the glycolysis pathway in breast cancer cells was investigated.

Materials and methods

Clinical sample collection. Ten human breast tumor samples were obtained from Apadana Hospital (Ahvaz, Iran) during the mastectomy procedure from February 2012 to September 2013. Healthy tissue samples away from the tumor were included as controls. Two independent expert pathologists from the pathology laboratory of Apadana Hospital performed the pathological tumor tissue examination. Samples were immediately preserved in liquid nitrogen, transported to the laboratory and stored at -80°C. The study was approved by the ethics committee from Jundishapour Medical University of Ahvaz (Ahvaz, Iran; associated with Apadana Hospital) and conducted according to the Guide for Human study by the National Academy of Sciences (National Institutes of Health), and informed consent was obtained from all patients involved in the study.

Sample preparation. Frozen tumor and healthy tissue samples were homogenized (1:5; w:v) in ice cold homogenization buffer (20 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 10% v:v glycerol, 2 mM EDTA, 2 mM EGTA and 20 mM β-glycerophosphate) and a few crystals of phenylmethylsulphonyl fluoride were added at the time of homogenization. Samples were homogenized using a Miccra homogenizer (MICCRA GmbH, Müllheim, Germany), centrifuged for 10 min at 10,000 x g at 4°C to remove tissue debris. The supernatant was then centrifuged for 30 min at 25,000 x g at 4°C to obtain high speed supernatant that contained the cytoplasmic enzymes (13). Finally, the supernatant was decanted and held on ice until use. Low molecular weight metabolites and ions were removed from the supernatant by Sigma-Aldrich Sephadex G-25 columns (1x5 cm; Merck KGaA, Darmstadt, Germany) equilibrated in the homogenizing buffer. The samples were then pooled and held at 4°C until use for subsequent enzyme kinetic characterization.

Cell culture, cell suspension, cell homogenate and cytosolic fraction preparation. MCF-7 and MDA-MB-231 cells were obtained from the Pasteur Institute Collection of Cell Cultures (Tehran, Iran). MCF-7 and MDA-MB-231 cells were maintained at 37°C in the presence of 5% CO2 in RPMI-1640 supplemented with 10% inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Prior to each experiment, the culture medium was removed and the plated MCF-7 and MDA-MB-231 cells were washed with phosphate-buffered saline (PBS) medium containing 138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.4, and collected by trypsinization into 1 ml PBS medium. Cells (~$30x10^5$, grown to 80% confluence) were suspended in

3 ml cold isolation buffer (0.32 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.5). Cytosolic fractions were obtained according to a previous study (14) with certain modifications. Briefly, cells were homogenized at 4°C with a Miccra homogenizer and centrifuged at 25,000 x g for 30 min at 4°C to obtain supernatant, which contained the cytoplasmic enzymes.

Effect of oxamate on LDH and MDH activity. To evaluate the effect of oxamate (Appliechem GmbH, Darmstadt, Germany) on cell proliferation and select the appropriate oxamate concentration, cells were plated into 96-well, flat-bottomed plates at 2-4x10^3 cells/100 μl per well. After the overnight incubation at 37°C, triplicate wells were treated with varying concentrations of oxamate, ranging from 5 to 80 mM for 3 days. The relative percentage of metabolically active cells relative to the untreated controls was then determined on the basis of the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazine. The quantity of 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide that is converted to formazine indicates the number of viable cells. The results were assessed in a 96-well format plate reader by measuring the absorbance at a wavelength of 540 nM (*A*540 nm). The percentage of metabolically active cells was compared with the percentage of control cells growing in the absence of oxamate in the same culture plate. The half maximal inhibitory concentrations (IC_{50}) were determined by nonlinear regression analysis using the equation for a sigmoid plot. The MCF-7 and MDA-MB-231 cells were seeded in 25-cm^2 flasks in duplicate. MCF-7 cells were treated (based on the IC_{50} results) with 30 and 60 mM oxamate, and MDA-MB-231 cells were treated with 10 and 20 mM oxamate for 72 h, with untreated cells serving as controls. Oxamate was administered at a cell confluency of 70% for each cell line. After 72 h, cells were harvested and processed for cell lysate preparation according to the previous method for cell culture preparation. LDH activity was determined by monitoring the rate of conversion from NADH to NAD^+ in the present of 1.5 mM pyruvate and 0.25 mM NADH. MDH activity was measured using the same method as for LDH, with 1 mM OAA used instead of pyruvate as a substrate. The results were normalized to the LDH and MDH activity per protein content. The protein concentrations were quantified using the Bradford protein assay (15), and experiments were performed in triplicate.

Enzyme assay and kinetic parameters. MDH activity was measured in the presence of OAA with NADH as substrates for the forward reaction and malate with NAD^+ as substrates for the reverse reaction. The lowest concentration of each substrate, which demonstrated maximum velocity, constant rate of product formation and linear regressions of activities for serial dilutions of enzyme, was assigned as the optimum substrate concentration.

Reactions were initiated by adding 10 μl of crude enzyme to a 200-μl total reaction volume by using 20 mM Tris-Hcl buffer (pH 8) in the microplate well. Activity was monitored at 340 nM (*A*340 nm) for assessing the conversion of NADH to NAD^+ (or vice versa) by using a BioTek PowerWave X2 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) and Gen5 version 2.0 software (BioTek Instruments, Inc.; kinetic mode, reading interval=39 sec).

Data were analyzed using microplate analysis and kinetics programs 3.51 (16,17). Kinetics 3.51 computer program fitted data through a nonlinear least squares regression for determination of the Michaelis-Menten constant (K_m; the substrate concentration resulting in half-maximal activity) and maximum velocity (V_{max}) values.

The K_m and V_{max} were calculated from the mean of three separate series of determinations. Total protein content was measured using the Bradford method and bovine serum albumin (Sigma-Aldrich; Merck KGaA) as standard. Due to the potential existence of endogenous NADH to NAD$^+$ interconversion (i.e., NADH oxidation by complex I activity) in the crude extract, the NADH to NAD$^+$ interconversion was surveyed in each sample to eliminate the possible existence of its effect. This interconversion was determined by adding the NADH (0.5-1 mM) or NAD$^+$ (3-5 mM) into the samples and monitoring the absorbance change at 340 nm.

Statistical analysis. Data were expressed as the mean ± SEM, from independent determinations on separate preparations of enzymes. Data were analyzed using Student's t-test and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Kinetic properties of MDH and LDH. Optimum assay conditions for MDH in the forward reaction were 1 mM OAA and 0.25 mM NADH in tumor and healthy tissue samples, and 1.5 mM pyruvate and 0.25 mM for LDH. It should be noted that no NADH to NAD$^+$ interconversion activity (or vice versa) was observed in the crude extract samples.

The maximal activity of MDH in tumor samples for malate formation (6,978±9.1 mU/g) was significantly greater than the values in the healthy tissue samples (5,651±12.7 mU/g; Table I) ($P<0.05$). The K_m OAA of MDH were not identified to be significantly different between the tumor (0.023±0.004 mM) and healthy (0.02±0.006 mM) samples ($P>0.05$). The maximal activity of LDH in the tumor samples for lactate formation (8,476±7.2 mU/g) was significantly higher than that of the healthy tissue samples (5,330±9.2 mU/g) ($P<0.05$; Table I).

Effect of oxamate on LDH and MDH activity. The effect of oxamate on LDH and MDH activity in the MCF-7 cell lines was evaluated at two concentrations, 30 and 60 mM. Compared with the control (9.087±0.07 mU/mg), LDH activity was not significantly decreased by 30 mM oxamate after 72 h (8.8±0.20 mU/mg); however, 60 mM oxamate significantly decreased LDH activity (6.73±0.09 mU/mg). Following treatment with 60 mM oxamate ($P<0.05$), MDH activity remained stable and was not significantly changed in the MCF-7 cell line (Fig. 1; $P>0.05$).

LDH and MDH activity of MDA-MB-231 were measured following treatment with 10 and 20 mM oxamate for 72 h. Compared with the control (10.763±0.17 mU/mg), LDH activity of MDA-MB-231 was significantly decreased by treatment with 10 mM (9.53±0.06 mU/mg) and 20 mM (7.87±0.1 mU/mg) oxamate ($P<0.05$). Notably, MDH activity was increased by ~3-fold following treatment with 10 (2.183±0.20 mU/mg) and 20 mM (2.153±0.29 mU/mg) oxamate compared with the control (0.785±0.06 mU/mg; Fig. 2), and no significant

Table I. Kinetic parameters of malate dehydrogenase and lactate dehydrogenase from breast tumor and healthy tissue samples.

Kinetic parameter	Tumor	Healthy
K_m OAA (mM)	0.023±0.004	0.02±0.006
V_{max} OAA (mU/g)	6,978±9.1[a]	5,651±12.7
V_{max} pyruvate (mU/g)	8,476±7.2[a]	5,330±9.2

Assays were conducted at 25°C and data are presented as the mean ± standard error of the mean, (n=3). Independent determinations were performed on each of the 10 tumor and normal samples. [a]$P<0.05$ vs. healthy. K_m, Michaelis-Menten constant; V_{max}, maximum velocity; OAA, oxaloacetate.

difference was identified between the increase of MDH activity following 10 and 20 mM oxamate treatment ($P>0.05$).

Discussion

High aerobic glycolysis is one of the main hallmarks of cancer cells, which is driven by multiple enzymes. The enzyme rate is regulated via two different processes as follows: i) Change in the total quantity of the enzyme present (V_{max}); ii) change in one or more kinetic constants (K_m or V_{max}). These processes and the enzyme stability are strongly influenced by the composition of the intracellular milieu in which the enzyme operates (10). Given the different microenvironmental conditions of the tumor compared with the healthy tissue, the enzyme function may differ between healthy tissue and tumor samples. The data from the current study supported the hypothesis and indicated that the kinetics of MDH, as one of the important enzymes in metabolism, has a distinctive feature in tumors and may be an alternative approach for supporting the glycolysis pathway.

The maximum activity measured was assumed to be primarily due to the cytosolic fraction, as the highest concentration of OAA used in the assay method (1 mM) was considerably greater than that (by 0.04 mM), which was known to inhibit the activity of the mitochondrial fraction (14). Furthermore, to demonstrate that the samples were free of the mitochondrial enzyme form, the supernatants, made from specimens, were assessed for NADH oxidase activity, as the mitochondrial marker and the interrupter of dehydrogenase. The results clarified that there was no NADH oxidase activity; therefore, the supernatant was free of mitochondrial particles.

The present data indicated that the maximum activity of the cancerous MDH (C-MDH) for producing NAD$^+$ and malate is higher than the normal MDH (N-MDH). This result is consistent with the current hypothesis regarding the role of MDH in supporting NAD$^+$ pool. In addition, a greater V_{max} of MDH causes a higher level of NAD$^+$, which may be used as a precursor for sustaining a high rate of aerobic glycolysis in tumors. According to the attained results, the required NAD$^+$ for the continuous flow of glycolysis may be supplied by cytoplasmic MDH in addition to eminent LDH in tumors. In the current study, the LDH kinetics in tumor and healthy tissue samples were evaluated. The V_{max} of C-LDH for the generation
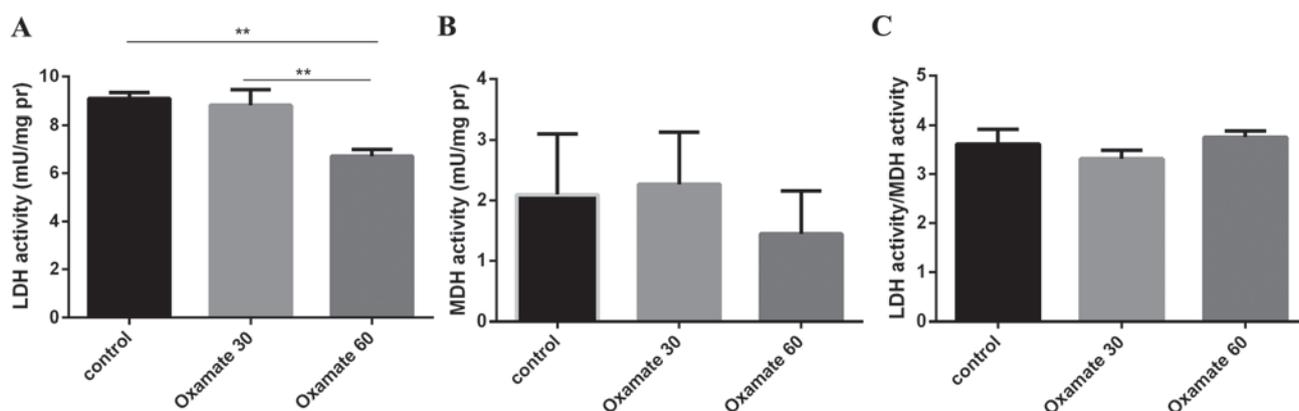
Figure 1. LDH and MDH activity patterns in the MCF-7 cell line with and without (control) LDH inhibitor, oxamate (30 and 60 mM). (A) LDH activity was significantly decreased by 60 mM oxamate, although not with 30 mM oxamate. (B) MDH activity was not affected as a result of LDH inhibition. (C) LDH/MDH activity ratio was unchanged following LDH inhibition. LDH, lactate dehydrogenase; MDH, malate dehydrogenase; pr, protein. $^{**}P<0.01$.
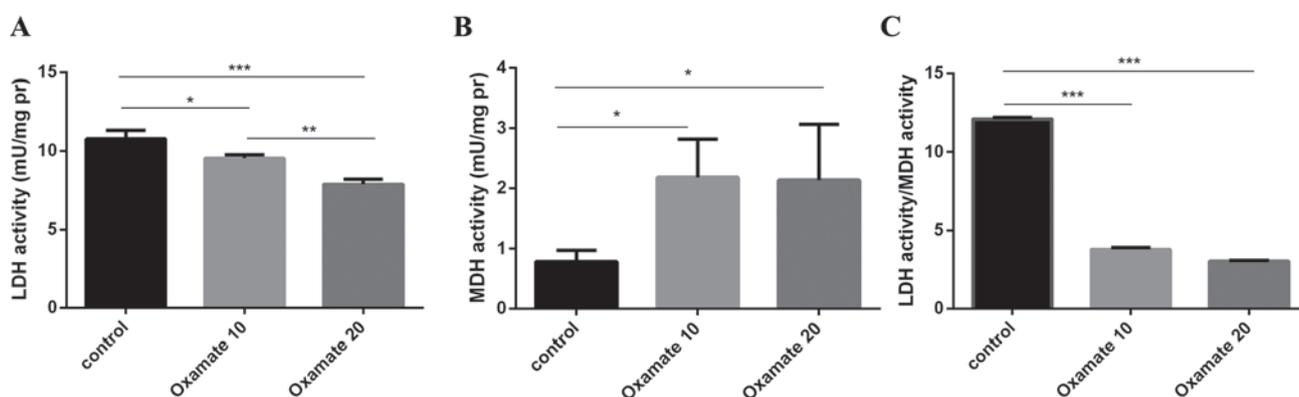
Figure 2. LDH and MDH activity patterns in the MDA-MB231 cell line with and without (control) LDH inhibitor, oxamate (10 and 20 mM). (A) Compared with the control, LDH activity was significantly decreased with 10 and 20 mM oxamate. The difference between 10 and 20 mM oxamate was also significant. (B) MDH activity significantly increased following LDH inhibition with 10 and 20 mM oxamate compared with the control. (C) LDH/MDH activity ratio was decreased compared with the control, following LDH inhibition with 10 and 20 mM oxamate. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

of NAD^+ and pyruvate was ~2-fold higher than N-LDH. This is consistent with previous studies, which demonstrated markedly higher LDH activity and gene expression in tumors when compared to the relevant healthy samples (5,18,19). In the current study, while LDH and MDH had the same maximum activity in the healthy samples, in the tumor samples, the two were increased and LDH had a greater V_{max} than MDH, demonstrating the more significant role of LDH in tumorigenesis. Thus, increasing the activity of LDH and MDH may be a strategy of tumorigenesis and proliferation for adapting to the stressful tumor environment.

Malic enzyme (ME) produces more pyruvate from the abundant quantity of malate generated by C-MDH. Pyruvate is used as an LDH substrate and supports high aerobic glycolysis (20). Therefore, MDH, through producing a high level of malate, sustains a high glycolysis rate in an indirect model. Thus, MDH supports high aerobic glycolysis in tumors by generating two metabolites: NAD^+ and malate. In order to verify the role of MDH in supporting glycolysis, the MDH activity was measured in breast cancer cell lines, treated by oxamate (the inhibitor of LDH). Notably, the reduction of LDH activity by oxamate was concomitant with the increase of the MDH activity in MDA-MB-231 cells, although the MDH activity in the MCF-7

cells was stable following oxamate treatment. Furthermore, the ratio of LDH to MDH demonstrated the pattern of the MDH activity increment subsequent to oxamate treatment. The ratio of LDH to MDH decreased in the MDA-MB-231 cells following oxamate treatment; however, no change was observed in the MCF-7 cells (Figs. 1 and 2). Increasing the MDH activity in MDA-MB-231 cells, compared with the MCF-7 cells, is associated with the glycolysis patterns in the two cancer cell types. MDA-MB-231 cells have a higher rate of glycolysis than MCF-7 cells and are more dependent on glycolysis as the main energy source (21). Thus, MDA-MB-231 cells were more susceptible to glycolysis and LDH inhibition. It is conceivable that cancer cells increase the MDH activity to compensate for the LDH deficiency and produce NAD^+ for sustaining a high rate of aerobic glycolysis, even though the production rate of NAD^+ by MDH in comparison with LDH is negligible. This finding can also be considered in LDH-associated cancer therapy, where LDH is targeted to disrupt the glycolysis pathway as the mainstream energy pathway in cancer cells. In recent years, efforts have been made to eliminate LDH activity and gene expression (22,23). Evaluation of alternative metabolism pathways is required, where LDH activity is inhibited to halt the glycolysis pathway, as the cancer cells employ various approaches (such as MDH) to

repair their deficiency in order to support the glycolysis pathway. Given the role of MDH in supporting glycolysis, inhibition of MDH activity and gene expression, concomitant with LDH, may assist with halting the glycolysis pathway and enhancing LDH removal efficiency. Furthermore, the obtained results regarding increasing MDH activity in the presence of oxamate are consistent with a previous report (21), which indicated the more malignant MDA-MB-231 cells are more dependent on glycolysis and LDH than the benign MCF-7 cells.

The current data are not consistent with the findings of Balinsky *et al* (24) where they demonstrated that cytosolic MDH in tumor and healthy breast tissues exerts the same activity, whereas the current results demonstrate that MDH has higher maximal activity in tumor tissue samples when compared with healthy tissue samples. The difference between these two studies may be associated with the various methods applied, for example Balinsky *et al* (24) used an electrophoretic method for enzyme activity detection whereas a spectrophotometric assay of LDH and MDH activities was used in the current study. The results regarding the K_m of the forward reaction are in contrast to the results of the study by Grisham *et al* (13), which is one of the initial studies on the kinetics of MDH in tumors. The current results indicated that the affinity of MDH in forward reaction is the same in tumor and healthy tissue samples. By contrast, Grisham *et al* (13) expressed that the K_m of OAA in tumor samples was higher than that in healthy tissue samples. Therefore, it can be concluded that the difference between these two studies is justified by considering the fact that Grisham *et al* (13) used 0.2 and 2 mM of OAA as the highest concentration of OAA in healthy and tumor tissue samples, respectively, whereas in the current study, the highest OAA concentration in the two tissue types was 1 mM. The present data demonstrated that the concentrations >1 mM exerted an inhibitory effect on MDH activity and that 1 mM was the optimum concentration in the two types of tissue.

The kinetic differences indicated that MDH from healthy and tumor tissue samples may exist in distinct structural states, which may be associated with the various tumor microenvironmental conditions. The condition of the milieu, where the enzyme operates, may affect the enzyme kinetics. It is important to note that the kinetic diversity of MDH may be due to post-translational modification during tumorigenesis. Further investigations are required to detect the post-translational modification of MDH in cancer tissue samples and recognize the effect on the MDH structure and the kinetic parameters.

In conclusion, the hypothesis of the present study, which addressed the kinetics and role of MDH in supplying NAD$^+$, remains unanswered as the enzyme were not purified and fully characterized. However, the results obtained in the current study are, to the best of our knowledge, the first step in this field. The results highlight another approach to support glycolysis in MDA-MB-231 and propose that cancer cells adapt to a situation, where the energy generation pathway is targeted through LDH inhibition.

Acknowledgements

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