The effect of lactate dehydrogenase inhibitors on proliferation, motility and invasion of breast cancer cells *in vitro* highlights a new role for lactate

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Abstract. Lactate dehydrogenase (LDH) is being increasingly recognized as a major factor in the progression of breast cancer. It was previously shown that short interfering RNA-mediated knockdown of either LDH-A or -B isoform resulted in inhibition of cell motility due to reduced lactate levels in the extracellular environment. The aim of the present study was to determine the use of pharmacological LDH inhibitors to reduce aggressive behavior of breast cancer cells. The effect of LDH inhibitors was investigated in both estrogen receptor $(ER)^+$ and ER^- breast cancer cell lines and in normal breast epithelial cells. Cell proliferation, motility and invasion were measured using MTT, wound healing and cultrex assays, respectively. Changes in several key mediators of mitogenic signaling important in breast cancer cells were determined using western blotting. Treatment with various inhibitors reported to block LDH activity resulted in significant reduction in extracellular lactate level, cell proliferation, motility and invasion. This was associated with changes in the levels of vimentin, E-cadherin, p38 MAPK, ERK1/2 and AKT. A couple of these inhibitors such as quercetin and lonidamine showed preferential inhibition of cancer cell proliferation compared with normal epithelial cell inhibition. These data extend initial findings, further underlining the importance of lactate as a major factor in breast cancer progression and indicate the practical use of various commercially available LDH inhibitors as promising therapeutic agents to oppose the processes leading to cancer progression.

Introduction

Lactate dehydrogenase (LDH) activity has been reported to be associated with the pathogenesis of various forms of cancers including that of the breast, and enhanced enzymatic activity is associated with advanced disease state (1). Furthermore, enhanced activity of both of the major isoforms of LDH, LDH-A (2) and LDH-B (3) was found to be associated with aggressive forms of breast cancer. Using short interfering (si) RNA-targeting, enhanced LDH activity and lactate levels in the more aggressive estrogen receptor (ER)-downregulated breast cancer cell lines was recently demonstrated compared with the less invasive parental ER^+ line and normal breast epithelial cells (4). Knockdown of LDH-A (4,5) and LDH-B (4,6) also results in reduced breast cancer cell proliferation, migration and invasion. It is suggested that this is due to reduced levels of lactate available in the extracellular environment surrounding breast cancer cells. In fact, several reports have demonstrated that lactate may be involved in enhanced calcium signaling (7), angiogenesis (8-10), modulation of cell death (11) and suppression of anticancer immune responses (12). It has also been shown that direct addition of lactate to breast cancer cells enhances their motile behavior and is associated with enhanced ERK1/2 phosphorylation (4).

Whilst short interfering (si)RNA is an effective means of inhibiting gene expression *in vitro* to elucidate biological effects, it may be more problematic for *in vivo* clinical application, which may arguably be better achieved with pharmacological agents. Therefore, having shown the benefit of blocking lactate production/secretion, it would be of value to determine whether various pharmacological inhibitors of LDH may have potential for clinical use. These include both LDH enzyme inhibitors such as oxamate and GNE-140, as well as lactate transport inhibitors (LTIs) such as quercetin and lonidamine, often described as LDH inhibitors. Oxamate has been described in the literature as a competitive LDH inhibitor, and a possible pyruvate analog which ultimately reduces lactate production through inhibition of the conversion of pyruvate to lactate (13,14). GNE-140 is another potent LDH inhibitor which is a racemate mixture of (R)-GNE-140 and (S)-GNE-140 (15-17). Quercetin is a potent inhibitor of various molecules including lactate transport machinery such as monocaboxylate transporter 1 and LDH (18). It is a plant flavonol from the flavonoid group of polyphenols, which is found in numerous fruits and vegetables (18). Lonidamine is a derivative of indazole-3-carboxylic acid, which was shown to inhibit aerobic glycolysis in cancer cells, in part through targeting the lactate transport machinery and LDH

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activity (19-21). LTIs have been shown to have therapeutic benefits in cancer treatment (22-25). Several studies have demonstrated their ability to inhibit lactate efflux from cancer cells (26-28), lactate production, cell migration and invasion (29). Reduced lactate production and inhibition of cell proliferation was observed following treatment with different LDH inhibitors in gastric and breast cancer cell lines (30-32).

The aim of the current study was to determine the effectiveness of the LDH/LTIs oxamate, quercetin, lonidamine and GNE-140 in modulating LDH activity and lactate levels, and how this would impact cell proliferation, motility and invasion using the relatively non-invasive ER^+ cell line YS1.2 compared with the highly invasive ER^+ pII line that was previously established (33,34). In addition, the relative effect of the aforementioned LDH/LTIs on the normal breast epithelial cell line MCF10A was also investigated. To address the issue of how lactate may affect cell behavior, the effect of these inhibitors on the expression/activity of various signaling molecules including p38 MAPK, ERK1/2, AKT, Src and NF-κB involved in mitogenic processes was also examined.

Materials and methods

Cell lines. The MCF10A normal breast epithelial cells were obtained from Dr E. Saunderson and Dr J. Gomm of St Bartholomews Hospital (London, UK). The ER^- pII cell line was established in our laboratory (Profs. Luqmani and Khajah, College of Pharmacy, Kuwait City, Kuwait) by transfection of MCF7 cells originally obtained from ATCC with ER-directed short hairpin (sh)RNA plasmid as previously described (33,34). YS1.2 was also derived from MCF7 cells transfected with the shRNA plasmid but failed to downregulate ER, therefore this cell line was used as a transfected control for pII which behaves similarly to the parental MCF7 cells. For routine culture, all cancer cell lines were maintained as monolayers in advanced Dulbecco's minimum essential medium (DMEM) containing phenol red and supplemented with 5% fetal bovine serum (FBS), 600 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 6 ml/500 ml 100 x non-essential amino acids (all from Invitrogen; Thermo Fisher Scientific, Inc.), and were grown at 37˚C in an incubator gassed with an atmosphere of 5% CO_2 and maintained at 95% humidity. MCF10A cells were cultured in DMEM F12 (cat. no. SH30023.01; Cytiva) supplemented with 5% horse serum, 1x pen/strep, 20 ng/ml mouse epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin and 10 μg/ml insulin (all from Invitrogen; Thermo Fisher Scientific, Inc.) (4).

Western blotting. Cells were cultured in 6-well plates to 80-90% confluency. The medium was subsequently aspirated off, and cell monolayers were harvested by scraping and re-suspended into 300 μl of lysis buffer containing 50 mM HEPES, 50 mM NaCl, 5 mM EDTA 1% Triton X, 100 μg/ml phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin stored at -80˚C. Protein concentration was determined by the Bradford assay using BSA as standard. A total of 8 μg protein lysate was mixed with an equal volume of 2x SDS and heated at 90˚C for 10 min. Samples were loaded onto a 10% SDS-polyacrylamide gel and electrophoresed at 150 V for 1 h. Proteins were transferred to a nitrocellulose membrane and blocked with 2% BSA at 4˚C for 1 h before being incubated overnight (16 h) at 4˚C with primary antibodies (prepared in 2% BSA) against β-actin (loading control; 1:1,000 dilution; cat. no. 4970; Cell Signaling Technology, Inc.) and phospho- to total- p38 MAPK, AKT, ERK1/2, Src, NFκB, E-cadherin and vimentin (1:1,000 dilution; cat. nos. 4695, 9101, 9212, 9211, 9272, 9271, 14243, 8242, 3195 and 5741, respectively; Cell Signaling Technology, Inc.). The membrane was then washed (with 0.1% v/v Tween 20) and incubated with anti-HRP-conjugated secondary antibody (1:500 dilution; cat. no. 7074; Cell Signaling Technology, Inc.) at 4˚C for 1 h, developed with Super Signal ECL (Thermo Fisher Scientific, Inc.) and visualized with a Cell bio-imager (ChemiDoc MP System; Bio-Rad Laboratories, Inc.) (4).

Lactate assay. Cells were cultured to a density of ~10^6 cells in 6-well plates. The culture medium was removed, and protein concentration was estimated using the Bradford assay. The extracellular lactate was measured in aliquots using the EnzyChrom L-Lactate Assay Kit ECLC-100 (BioAssay Systems), following the manufacturer's protocol. Standards were prepared by dilution of a stock solution of 100 mM L-lactate in serum-free media, and 20 μl samples or standards were transferred into 96-well plates. Two reactions were performed for each sample: One with both enzymes A and B, and another without enzyme A (control). The working reagent was prepared freshly by mixing 60 μl Assay Buffer, 1 μl enzyme A, 1 μl enzyme B, 10 μl NAD and 14 μl MTT. For control, enzyme A was omitted from the reagent mix, and 80 μl working reagent was added to each sample well and mixed by pipetting up and down. The background optical density at 650 nm was measured using a plate reader at 'zero' time (OD_0) and after 20 min incubation (OD_{20}) at room temperature and subtracted from that at 565 nm. For the standard curve, the corrected OD_0 was subtracted from OD_{20}. For samples with no enzyme A control, the $\Delta OD_{no\ enzA}$ value was subtracted from ΔOD_{sample}. The $\Delta\Delta OD$ values were used to determine sample L-lactate concentration from the standard curve.

Intra- and extra-cellular lactate levels were measured. Intracellular lactate was estimated by measuring the amount released into 300 μl lysis buffer volume. However, it is noteworthy that this does not represent the actual concentration inside the cells, which will be substantially higher since the intracellular volume will be low. Since the intracellular volume could not be determined, and since the absolute concentration is not a necessary parameter for the present study, the data for the treated samples were presented either as a percentage of the control or as one cell line compared with another, providing a relative comparison (4).

LDH assay. Quantichrom LDH Kit (cat. no. DLDH-100; BioAssay Systems) was used following the manufacturer's protocol for the same samples that were prepared for the lactate assay. Freshly prepared assay reagent, containing 14 μl MTT solution, 8 μl NAD solution and 170 μl substrate buffer, were aliquoted into a 96-well plate at room temperature, and 10 μl sample was added to start the reaction. Control wells contained 200 μl H_2O (for OD_{H2O}) and 200 μl calibrator (for OD_{Cal}). The absorbance of the solution at 565nm was determined using a plate reader spectrophotometer at OD_0 and again after 25 min (OD_{25}). LDH activity was calculated according to the equations

provided in the protocol. As was explained for lactate, the intracellular LDH activity was expressed as a percentage of the control or one cell line versus another rather than in absolute units (4). In a separate experimental setup, LDH activity was measured on cell lysate rather than on live cells.

Wound healing assay. Cells were cultured in 6-well plates with complete DMEM to 80-90% confluency (on both sides of the scratch). A scratch was created in the cell monolayer using a sterile p1000 pipette tip and an image of the scratched area was immediately taken (0 h). The plates were then placed at 37˚C and 5% CO_2. After overnight (16 h) incubation, another image was taken of the same scratched area using light microscopy (magnification, 10x). The width of the scratch at 24 h was calculated as a percentage of the width at 0 h; a minimum of three areas along the scratch were measured (4). The following drugs were used: Quercetin (range, 100-500 μM; cat. no. Q4951; Sigma-Aldrich; Merch KGaA), oxamate (range, 100 μM-60 mM; cat. no. O2751; Sigma-Aldrich; Merch KGaA), lonidamine (range, 1-100 μM; cat. no. L4900; Sigma-Aldrich; Merch KGaA) and GNE-140 (range, 1-300 μM; cat. no. BCP37998; Shanghai Biochempartner Co., Ltd).

MTT assay. Cells were routinely seeded into 24-well culture plates and allowed to grow to 30-35% confluency. Cell density was determined either immediately (day 0) or after 1 and 4 days of cultivation. For the measurement, medium was removed and replaced with 500 μl MTT reagent (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) at 37˚C for 2 h. The MTT solution was then removed, and 200 μl acidic isopropanol was added to dissolve the blue formazan crystals that had formed. Plates were scanned at 595 and 650 nm (for background subtraction) using a Multiscan spectrum (Molecular Devices, LLC) spectrophotometer, and absorbance was compared between samples as a measure of proliferation (4).

Cultrex basement membrane extract cell invasion assay. Cell invasion was also assessed using Cultrex® 24-well BME cell invasion assay (Trevigen, Inc.; Bio-Techne) according to the manufacturer's instructions. All reagents were provided with the kit. In brief, the invasion chamber was coated with 100 μl 1x BME solution and incubated overnight (16 h) at 37˚C. A total of $1x10^6$ pII cells that had been serum-starved overnight (16 h) at 37˚C/5% CO_2, were re-suspended to a total of 10^6 cells/ml in DMEM (control) or DMEM containing various doses of quercetin (500 μM), oxamate (60 mM), GNE-140 (200-300 μM) or lonidamine (1-10 μM), and 100 μl suspension was loaded into the upper chamber. The lower chamber was loaded with 500 μl DMEM supplemented with 10% FBS as a chemoattractant. Cells were incubated at 37˚C/5% CO_2 and allowed to invade from the top chamber to the bottom. After 48 h, liquid from both top and bottom chambers was removed by aspiration, and chambers were gently washed with 1x cell wash buffer, provided by the supplier. Calcein-acetomethylester (AM)/cell dissociation solution complex was added to the bottom chamber and left for 1 h at 37˚C/5% CO_2. Cells internalize calcein-AM and intracellular esterases cleave the AM moiety generating free fluorescent calcein. Invading cells were determined by recording the fluorescence emission using a microplate reader with a filter set of excitation/emission at 485/535 nm (35).

Statistical analysis. The means of the experimental groups were compared with those of controls using an unpairedStudent's t-test or one-way ANOVA followed by the Bonferroni post hoc test. GraphPad Instat software (version 6; Dotmatics) was used. GraphPad Prism (version 6; Dotmatics) was used to plot graphs. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of inhibitors on LDH activity, lactate levels, cell proliferation, motility and invasion. Breast cancer cells were treated with various doses of LDH/LTIs, and their effect on intracellular LDH activity and extracellular lactate levels, as well as on cell proliferation, motility and invasion was measured. There is no evidence to suggest that any of the inhibitors studied have any direct effect on the gene expression of LDH; they were purely used as inhibitors of LDH activity/transport.

As shown in Fig. 1, treatment of pII cells with quercetin (range, 200-500 μM) reduced intracellular LDH activity (Fig. 1A) and extracellular lactate levels (Fig. 1B) in a dose-dependent manner, with significant effects observed. Treatment of pII cells with oxamate also reduced intracellular LDH activity (Fig. 1C) and extracellular lactate levels (Fig. 1D) in a dose-dependent manner but a higher dose was required (10-60 mM) compared with that of quercetin. To further confirm the LDH inhibitory effects of quercetin and oxamate, pII lysates (not live cells; Fig. 1A-D) were treated with these drugs, and it was shown that they inhibited LDH activity (range, 10-1,000 μM; Fig. 1E). Previous studies (18,22,23) have considered quercetin as an inhibitor of lactate transport. The intra and extracellular lactate levels were measured in pII cells treated with either quercetin (range, 100-500 μM) or oxamate (range, 100 μM-60 mM), and it was shown that these two agents act by directly inhibiting LDH activity rather than affecting the lactate transport machinery since there was no significant increase in the intracellular lactate level observed upon treatment with either quercetin or oxamate (Fig. 1F). Next, the effect of quercetin and oxamate on pII cell proliferation was measured using the MTT assay. Treatment with quercetin (500 μM) and oxamate (range, 40-60 mM) significantly inhibited pII cell viability on day 4 (Fig. 1G) but not on day 1 (Fig. 1H) post treatment. However, a 24-h treatment with quercetin or oxamate significantly inhibited both pII cell motility (Fig. 1A and B) and invasion (Fig. 1C). Thus, the latter effects were not due to inhibition in cell proliferation which occurred later. The effect of these inhibitors was also tested the on the ER^+ YS1.2 cells. As shown in Fig. 2, quercetin treatment inhibited YS1.2 cell proliferation in a dose-dependent manner on day 4 (Fig. 2D), but not on day 1 (Fig. 2E). Oxamate inhibited YS 1.2 cell proliferation but at a lesser degree compared with its effect on pII cells (Fig. 2D). Both inhibitors also reduced YS1.2 motility in a dose-dependent manner (Fig. 2F and G). Since it was previously shown that ER^+ breast cancer cells are motile but not invasive compared with pII cells (35), invasion assays were not performed on YS1.2 cells. Treatment with quercetin did not inhibit the proliferation of the normal breast epithelial cell line MCF10A at the same dose range

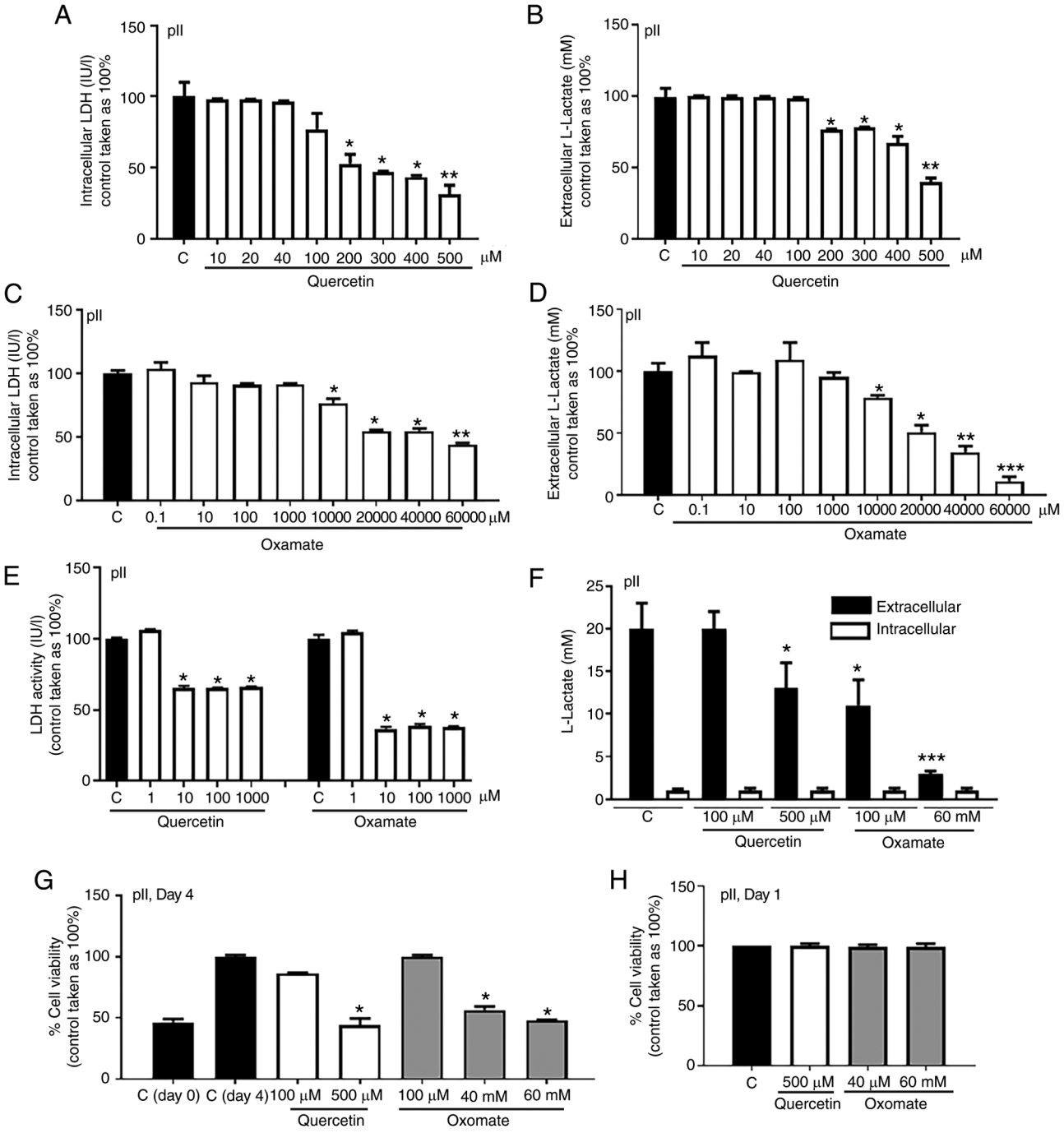

Figure 1. Effect of quercetin and oxamate treatment on breast cancer cells. (A, C) Intracellular LDH activity for (A) quercetin and (C). Extracellular lactate levels in pII cells treated with increasing concentrations of either (B) quercetin or (D) oxamate, *P<0.05 and **P<0.001. (E) LDH activity in pII cell lysate, either untreated (control) or exogenously treated with different concentrations of quercetin or oxamate, *P<0.05. (F) Intracellular and extracellular lactate levels in pII cells, either control or treated with different concentrations of quercetin or oxamate, *P<0.05 and ***P<0.0001. pII cell proliferation (using MTT assay) on (G) day 4 and on (H) day 1 post-treatment with different concentrations of quercetin or oxamate, *P<0.05. *Indicates significant difference from c; (untreated cells). C, control; LDH, lactate dehydrogenase.

effective against cancer cells (Fig. 2H), suggesting a preferential growth inhibitory effect. On the other hand, oxamate treatment did inhibit MCF10A as well as pII and YS1.2 cell viability at similar dose ranges (Fig. 2H). A motility assay for MCF10A cells was performed and it was shown that these cells are not motile (data not shown), and therefore these inhibitors were not used in the motility assay for MCF10A cells. The effect of a combination regimen of quercetin (range, 100-500 μM) with oxamate (40 mM) was also tested the on the proliferation and motility of pII cells. As shown in Fig. 2I-K, combination treatment significantly inhibited cell proliferation and motility on days 1 and 4 compared with treatment with either inhibitor.

As shown in Fig. 3, treatment of pII cells with the other LDH inhibitor, lonidamine, inhibited intracellular LDH activity (Fig. 3A) and extracellular lactate levels (Fig. 3B) in a dose dependent manner with a significant effect observed at concentration range of 1-100 μM. Treatment with lonidamine

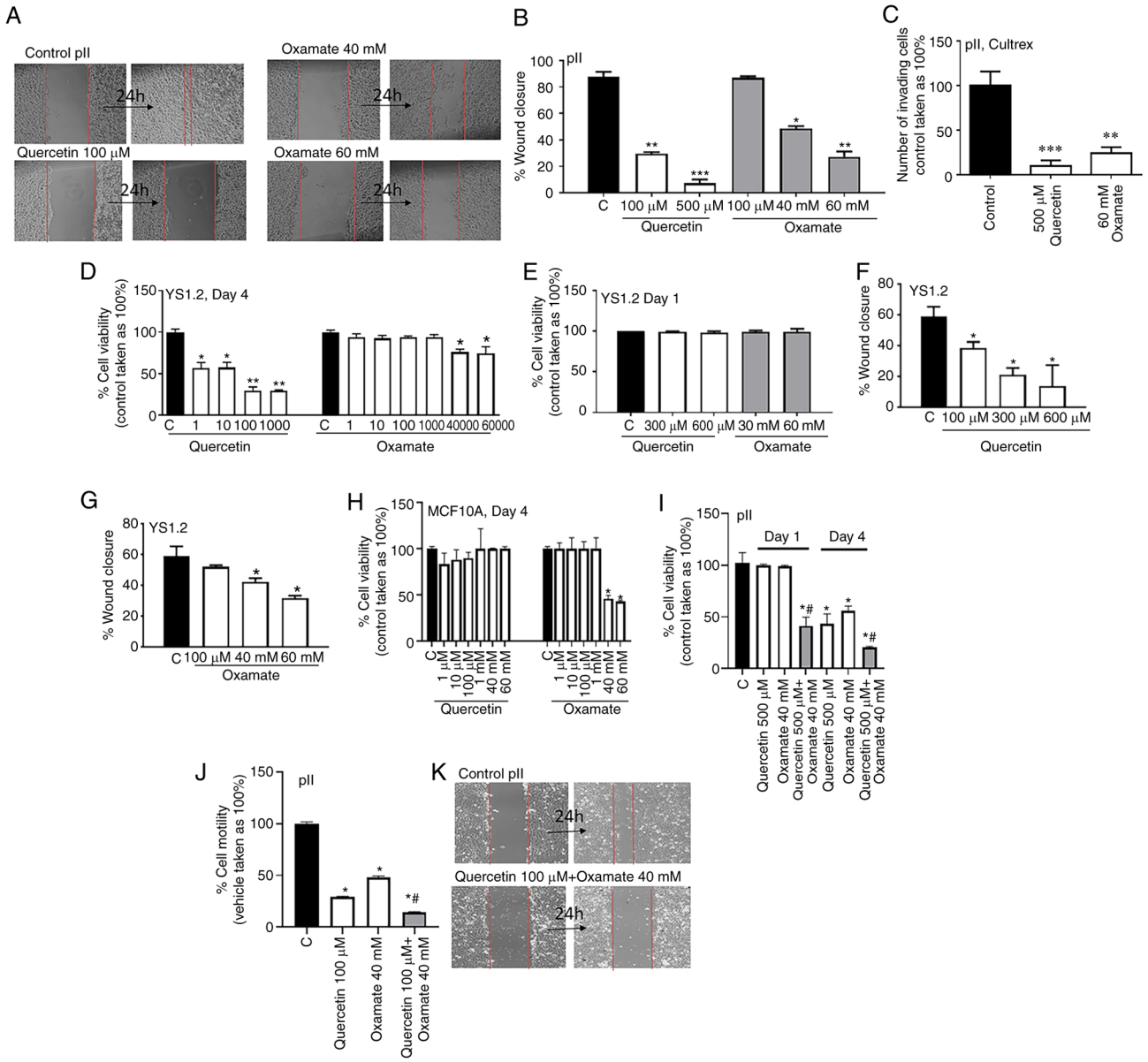

Figure 2. Effect of quercetin and oxamate treatment on breast cancer and normal epithelial cells. (A,B) pII cell motility (wound healing assay), either control or treated with different concentrations of quercetin or oxamate, *P<0.05, **P<0.001, ***P<0.0001. (C) pII cell invasion (cultrex assay), **P<0.001 and ***P<0.0001. YS1.2 cell proliferation on (D) day 4 and on (E) day 1 post-treatment with different concentrations of either quercetin or oxamate, *P<0.05 and **P<0.001. YS1.2 cell motility, either control or treated with different concentrations of either (F) quercetin or (G) oxamate, *P<0.05. (H) MCF10A cell proliferation, either control or treated with different concentrations of either quercetin or oxamate, *P<0.05 (n=3 per group). (I,J) pII cell proliferation (MTT assay) on day 1 and 4 post-treatment with either quercetin, oxamate or a combination of both drugs, *P<0.05. $^{\#}$indicates significant difference from treatment with either quercetin or oxamate alone, P<0.05. (K) pII cell motility, either control or treated with either quercetin, oxamate or combination, *P<0.05, $^{\#}$indicates significant difference from treatment with either quercetin or oxamate alone, P<0.05 (n=3 per group). *Indicates significant difference from c; (untreated cells). LDH, lactate dehydrogenase; c, control.

(starting concentration 1 μM) also reduced pII cell proliferation after 4 days of treatment (Fig. 3C), but not on day 1 (Fig. 3D). pII cell motility and invasion were also inhibited following lonidamine treatment (Fig. 3E-G). Furthermore, treatment of the ER^{+} YS1.2 breast cancer cells with lonidamine inhibited their viability (Fig. 3H) and migrative ability (Fig. 3J and K). Lonidamine did not inhibit proliferation of the normal MCF10A cells (Fig. 3L), which indicates preferential effect on breast cancer cells.

As shown in Fig. 4, in pII cells, the LDH-A inhibitor GNE-140 inhibited LDH activity (range, 200-300 μM; Fig. 4A), reduced extracellular lactate levels (Fig. 4B) and inhibited cell proliferation on day 4 but not on day 1 (Fig. 4C and D), migration (Fig. 4E and F) and invasion (Fig. 4G). GNE-140 also inhibited YS 1.2 cell proliferation on day 4 but not on day 1 (range, 100-300 μM; Fig. 4H and I) and motility (Fig. 4J). It also inhibited MCF10-A proliferation (Fig. 4K).

Effect of LDH inhibitors on the expression/activity of downstream signaling molecules in pII cells. To investigate the possible mechanisms used by LDH inhibitors to exert their actions, their effect on the level of several key intermediates of the intracellular mitogenic signaling pathway activated by EGF were studied, previously shown to be a potent stimuli of breast

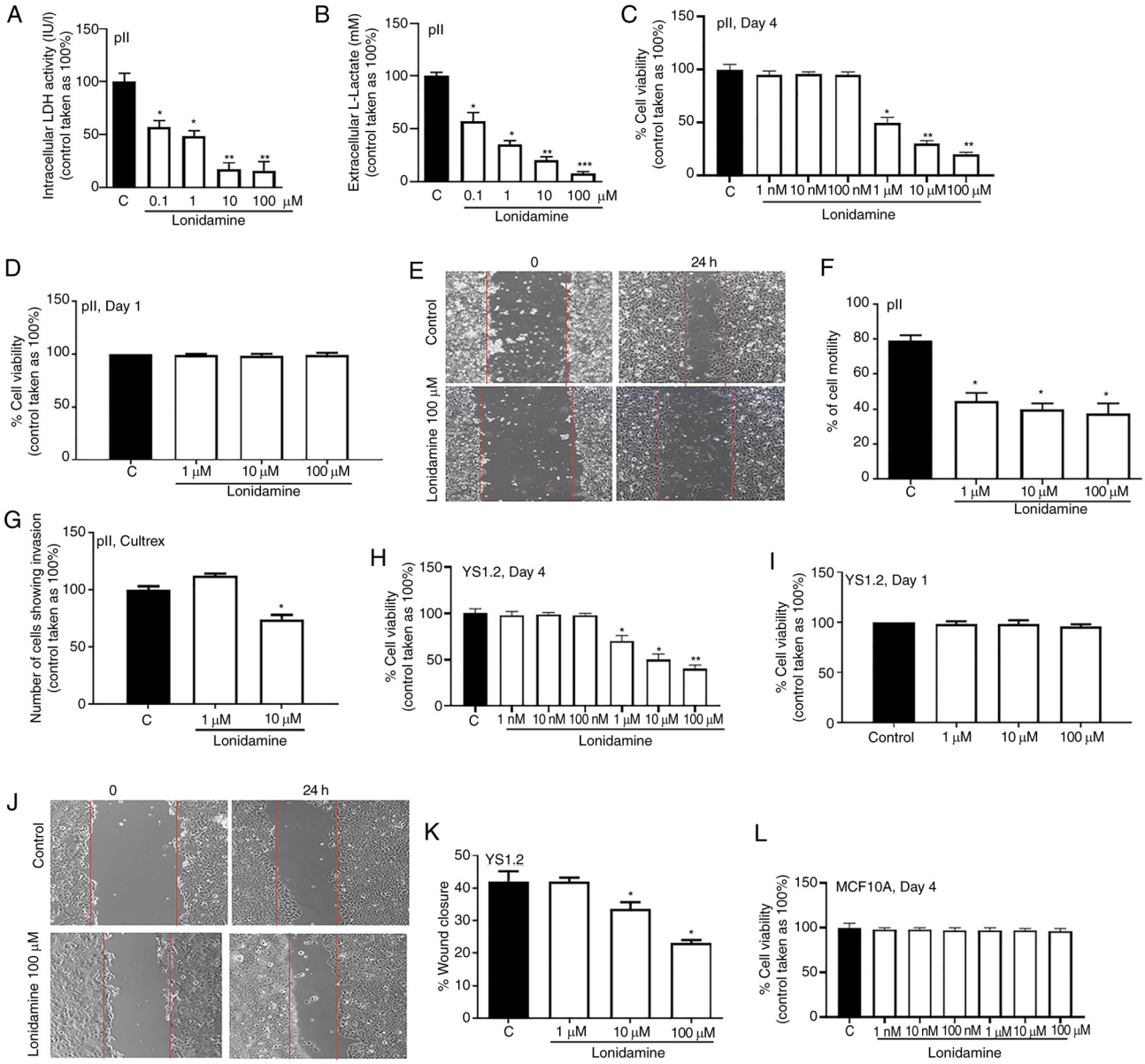

Figure 3. Effect of lonidamine treatment on breast cancer and normal epithelial cells. (A) Intracellular LDH activity for lonidamine. (B) Extracellular lactate levels for lonidamine in pII cells, *P<0.05, **P<0.001 and ***P<0.0001. pII cell proliferation on day (C) 4 and (D) 1 post-treatment with different concentrations of lonidamine, *P<0.05 and **P<0.001. (E,F) pII cell motility, either control or treated with different concentrations of lonidamine, *P<0.05. (G) pII cell invasion, *P<0.05. YS1.2 cell proliferation on (H) day 4 and on (I) day 1 post-treatment with different concentrations of lonidamine, *P<0.05 and **P<0.001. (J,K) YS1.2 cell motility, either control or treated with different concentrations of lonidamine, *P<0.05. (L) MCF10A cell proliferation, either control or treated with different concentrations of lonidamine (n=3 per group). *Indicates significant difference from c; (untreated cells). LDH, lactate dehydrogenase; c, control.

cancer cell proliferation, motility and invasion (35). Fig. 5A shows a time course of ERK1/2 phosphorylation after addition of EGF (100 ng/ml) to pII cells to determine the optimum stimulation conditions, which was subsequently used to determine the effect of the four inhibitors. As shown in Fig. 5B, pretreatment with quercetin but not oxamate or lonidamine inhibited EGF-induced p38 MAPK phosphorylation without affecting the expression of total p38 MAPK. GNE-140 reduced both p38 MAPK phosphorylation as well as expression of total p38 MAPK. Oxamate and quercetin but not lonidamine and GNE-140 inhibited EGF-induced ERK1/2 phosphorylation. Pretreatment with either oxamate, lonidamine or GNE-140 inhibited EGF-induced AKT phosphorylation. None of the LDH inhibitors affected the EGF-induced phosphorylation level of Src or NF-κB.

It was also tested if treatment with LDH inhibitors modulate the expression profile of epithelial and mesenchymal markers in pII cells. As shown in Fig. 5C, pII cells which have a mesenchymal phenotype express the epithelial marker E-cadherin at low levels and the mesenchymal marker vimentin at high levels. Treatment with either quercetin or oxamate for 24 h increased the expression of E-cadherin and reduced the expression of vimentin compared with those in the control (untreated) cells. These data suggest that reduced pII cell motility and invasion in response to treatment with LDH inhibitors is in part due to modulation in the expression of epithelial and mesenchymal markers, and the possible reversibility of the epithelial-mesenchymal transition (EMT) process.

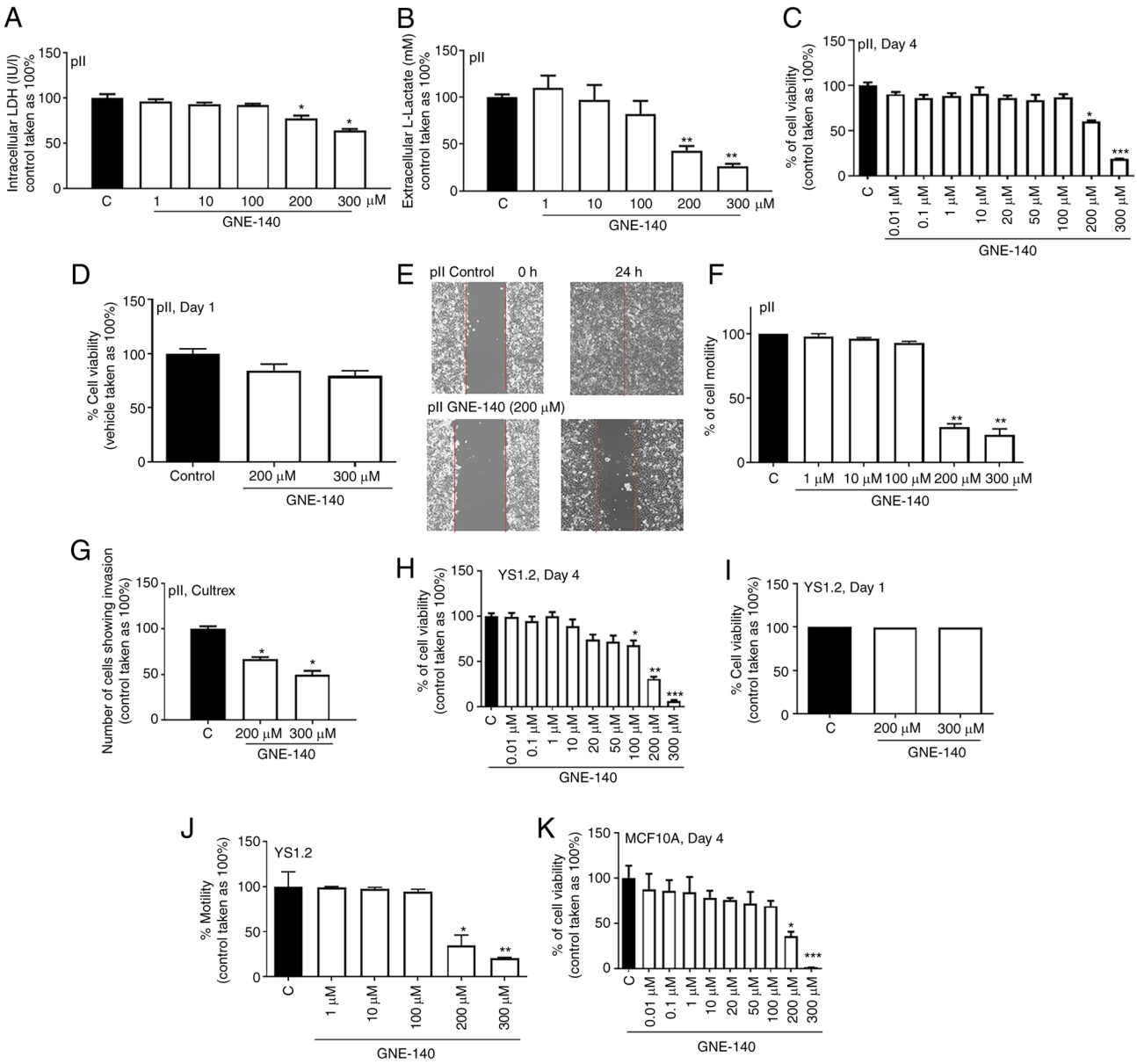

Figure 4. Effect of GNE-140 treatment on breast cancer and normal epithelial cells. (A) Intracellular LDH activity for GNE-140. (B) Extracellular lactate levels for GNE-140 in pII cells, *P<0.05 and **P<0.001. pII cell proliferation on (C) day 4 and on (D) day 1 post-treatment with different concentrations of GNE-140, *P<0.05 and ***P<0.0001. (E,F) pII cell motility, either control or treated with different concentrations of GNE-140, **P<0.001. (G) pII cell invasion, *P<0.05. YS1.2 cell proliferation on (H) day 4 and on (I) day 1 post-treatment with different concentrations of GNE-140, *P<0.05, **P<0.001 and ***P<0.0001. (J) YS1.2 cell motility, either control or treated with different concentrations of GNE-140, *P<0.05 and **P<0.001. (K) MCF10A cell proliferation, either control or treated with different concentrations of GNE-140, *P<0.05 and ***P<0.0001 (n=3 per group). *Indicates significant difference from c; (untreated cells). LDH, lactate dehydrogenase; c, control.

Discussion

In summary, the present study showed the efficacy of several drugs in reducing LDH activity and lactate levels which resulted in reduced breast cancer cell proliferation, motility and invasion in part through modulation of the expression/activity of E-cadherin, vimentin, p38 MAPK, ERK1/2 and AKT.

In the pursuit of a better understanding the transition of breast tumour cells from a non-invasive to a migratory form capable of metastatic spread, an increasing number of mainly *in vitro* but also *in vivo* studies have demonstrated that this involves a morphological change from an epithelial to a motile mesenchymal-like phenotype (36,37). Several factors can induce this transformation including loss of ER expression in breast cancer cells which were previously shown to result in acquisition of a new aggressive phenotype (36,37). These ER^{-} cells are also characterized by enhanced LDH activity resulting in excessive lactate production as compared to the parental ER^{+} cells (4). siRNA/shRNA-mediated knockdown of either LDH-A or -B isoforms reduced their motile behavior, with reduced mitogenic activity reflected by ERK1/2 phosphorylation (4). In the present study, the effect of four commercially available drugs that have been reported as LDH/LTIs was investigated on the motile and invasive behavior of ER^{+} and ER^{-} breast cancer cells to determine the potential clinical use of targeting this enzyme in advanced

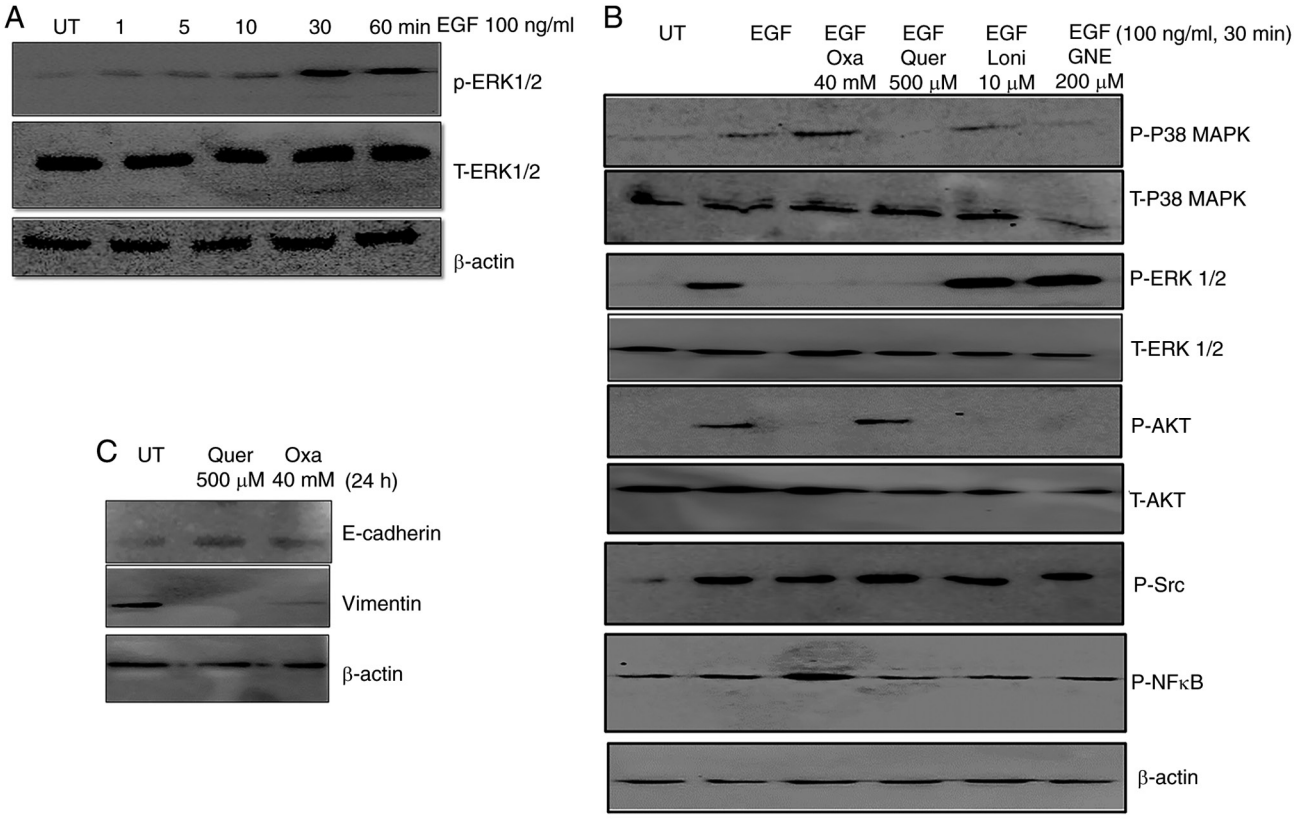

Figure 5. Effect of LDH inhibitors on the expression/activity of downstream signaling molecules in pII cells. (A) Western blotting P- and T-ERK1/2, and β-actin in pII cells exposed to EGF (100 ng/ml) for 1-60 min or left UT. (B) Western blotting P- and T-p38 MAPK, ERK1/2, AKT, Src, NF-κB and β-actin in pII cells, either UT, treated with EGF alone, or treated with EGF plus oxamate, quercetin, lonidamine or GNE-140. Inhibitors added to the cells 30 min prior to addition of EGF. (C) Western blotting of E-cadherin, vimentin and β-actin in pII cells either UT or treated with either quercetin (500 μM) or oxamate (400 mM) for 24 h. UT, untreated; EGF, epidermal growth factor; LDH, lactate dehydrogenase; P, phosphorylated; T, total; quer, quercetin; oxa, oxamate; loni, lonidamine; GNE, GNE-140.

breast cancer treatment. There is a notable discrepancy in the literature regarding the description of these inhibitors and their mode of action. For example, oxamate has been variously described as a general LDH inhibitor (38), a specific LDH-A inhibitor (13,39), and a specific LDH-C inhibitor (32) with the absence of testing its specificity of the various isoforms in these studies. Also, other studies (18,22-25) have grouped these inhibitors into two different categories: LDH enzyme inhibitors (oxamate and GNE-140) and LTIs (quercetin and lonidamine). In the current study, experimental evidence was provided suggesting that all of these inhibitors have direct inhibitory effects on intracellular LDH enzymatic activities without necessarily affecting the lactate transport machinery in ER^- and ER^+ breast cancer cell lines. In all cases, whether they act as LDH or LTIs, the net effect is to reduce the amount of lactate available in the extracellular environment, which was the case in the current study, by using the four aforementioned inhibitors.

It has been shown that oxamate treatment (range, 20-120 nM) inhibited LDH activity, lactate production and proliferation of various gastric (38) and nasopharyngeal carcinoma cell lines (13). Higher concentrations of oxamate (range, 25-75 mM) were required to inhibit these processes in medulloblastoma cells (39). Another report used oxamate (50 nM) as a specific LDH-C inhibitor without demonstrating its specificity in inhibiting this isoform in the ER^- breast cancer cell line MDA-MB-23, which resulted in reduction in cell motility without affecting cell viability (32). In the present study it was shown that oxamate treatment (range, 40-60 mM) significantly inhibited LDH activity, extracellular lactate levels, proliferation, motility and invasion in ER^- breast cancer cells.

Another way to reduce extracellular lactate levels is through targeting lactate transporters or monocarboxylate transporters (MCTs), which have been investigated as potential therapeutic targets for cancer treatment (22-25). Various MCT inhibitors have been shown to inhibit lactate efflux from cancer cells. In a previous study, the membranous expression of MCT1 in MDA-MB-468, Hs578T and BT20 cells with low levels in MCF-7 breast cancer cells was measured by immunohistochemistry (29). MCT4 was only expressed in MDA-MB-231 and SkBr3 cells, while MCT2 expression was not observed in any of the tested breast cancer cell lines. Treatment with both quercetin and lonidamine was shown to reduce lactate levels, cell migration and invasion, suggesting their use in reducing cancer cell migration (29). In the present study, it was shown that quercetin did not act by targeting the lactate transport machinery as no modulation of intracellular versus extracellular lactate levels was observed in response to quercetin addition to pII cells (Fig. 1F). It was also shown that quercetin treatment (range, 200-500 μM) significantly inhibited LDH activity, lactate levels, proliferation, motility and invasion in ER^+ and ER^- breast cancer cells (Fig. 1).

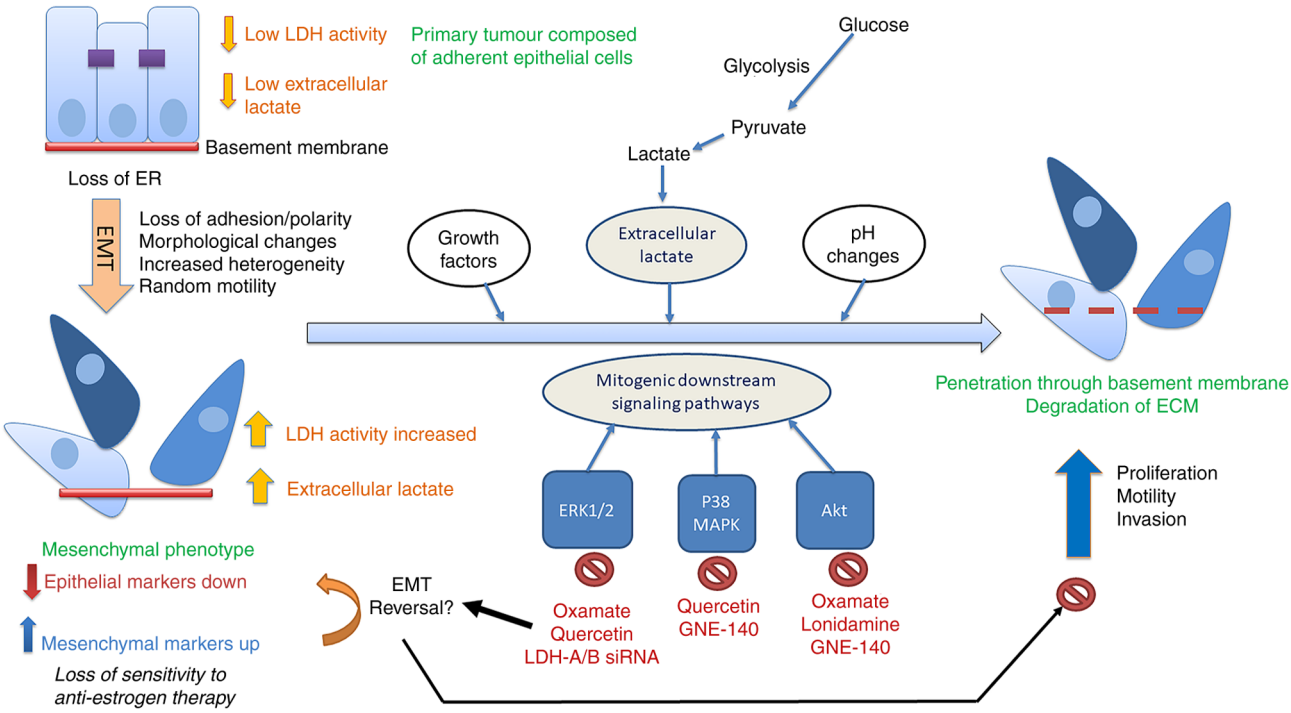

Figure 6. Model system. shRNA-mediated silencing of ER in MCF7 breast cancer cells leads to endocrine insensitivity (37). This is accompanied by transformation from an epithelial to a mesenchymal-like cell as evidenced both by their modified gene expression profile and their more fibroblast-like morphological appearance. Preliminary data suggesting EMT induction through lactate supplementation, but this requires further investigation, which will be presented in a future study. During the EMT process described *in vivo*, epithelial cells undergo unique changes which are accompanied by diminished intracellular adhesion, resulting in enhanced motility and invasion, paralleled with poor clinical outcome. Numerous factors are directly associated with these enhanced behaviors of breast cancer cells including various growth factors, downstream signaling molecules and excessive activity of LDH leading to enhanced lactate levels in the extracellular environment of cancer cells. It was previously showed that LDH activity and extracellular lactate levels are notably enhanced in the invasive ER^- breast cancer cells compared to less invasive ER^+ breast cancer cells or he normal breast epithelial cells (4). It is hypothesized that enhanced lactate levels in the ER^- cells along with their mesenchymal characteristics play a key role in their aggressive behaviors including motility and invasion. It has also been shown that targeting LDH through siRNA-mediated knockdown on LDH-A or -B isoform, or through pharmacological inhibitors (quercetin, lonidamine, oxamate and GNE-140) lead to reduced LDH activity and extracellular lactate levels. This resulted in reduced breast cancer cell proliferation, motility and invasion. Preliminary data have been provided suggesting modulation of the EMT markers upon treatment with LDH inhibitors, which requires further investigation. These pharmacological inhibitors also act on several downstream mitogenic signaling molecules involved in cancer pathogenesis, suggesting their clinical utility in breast cancer treatment. There is a consensus in the literature regarding the important role of the extracellular acidic pH in enhancing cancer pathogenesis. It was shown that this is not usually the case, since culturing ER^+ and$^-$ breast cancer cells in acidic pH condition did not enhance their proliferative, motile, or invasive capabilities (57). On the other hand, culturing ER^- breast cancer cells in alkaline pH conditions induced actin-mediated blebs which lead to enhanced cell invasion, in part through enhanced MMP levels (57-59). This raises a question regarding the role of acidic pH or the excessive lactate (which makes the pH acidic) in the extracellular environment promoting cancer pathogenesis. In the present study it was shown that this is due to lactate rather than acidic pH environment responsible for enhanced breast cancer proliferation, motility and invasion leading to poor outcomes, and targeting this machinery is promising therapeutic approach for breast cancer treatment. LDH, lactate dehydrogenase; ER, estrogen receptor; shRNA, short hairpin RNA; EMT, epithelial-mesenchymal transition.

Furthermore, lonidamine treatment (range, 0.1-100 μM) significantly inhibited LDH activity, lactate levels, proliferation, motility and invasion in ER^+ and ER^- breast cancer cells but did not affect the proliferation of the normal epithelial cell line MCF10A (Fig. 2). This indicates preferential inhibitory activity towards breast cancer but not normal cells which is of clinical importance. Indeed, normal cells produce low amounts of lactate as the pyruvate produced from glycolysis enters the Krebs cycle in the more efficient energy-producing process of oxidative phosphorylation (4). LDH activity is already low so inhibiting it would not have a notable effect. Lactate production is elevated in cancer cells which divert the pyruvate to lactate, and even more elevated in the more aggressive variant used in the present study, pII, which has transited from MCF7 to a mesenchymal-like migratory cell type that had undergone EMT.

GNE-140 has been reported as a specific LDH-A inhibitor (40,41). Boudreau *et al* (40) showed that GNE-140 treatment (10 μM) resulted in growth arrest in the glycolytic pancreatic cancer cell line MiaPaca2. In another study, GNE-140 treatment (10 μM) suppressed glycolysis in human colon adenocarcinoma and murine melanoma cells (41). In MDA-MB-231 cells, GNE-140 (range, 30-120 μM) inhibited glucose use and lactic acid production and led to growth arrest (16). In the present study, it was shown that GNE-140 treatment (range, 200-300 μM) inhibited the proliferation of both ER^+ and ER^- breast cancer cells, and the normal breast epithelial cell line (Fig. 3). It also inhibited LDH activity, lactate production and the motility and invasion of breast cancer cells.

Some LDH inhibitors have been used clinically in patients with breast cancer. Oral administration of lonidamine (range, 150-600 mg/day) either as monotherapy or add-on therapy with other anticancer agents was tolerated in patients with ovarian (42), lung (43) and breast cancer with metastatic disease (44-46), but its efficacy requires additional investigation. Furthermore, quercetin was also used clinically in

various diseases such as polycystic ovary syndrome (500 mg orally) and was shown to improve in oocyte and embryo grade through reducing luteinizing hormone, tumor necrosis factor α and interleukin 6 levels, and the pregnancy rate (47). Quercetin was also tested at higher doses (range, 800-1000 mg) in hospitalized patients with COVID-19 (48) and prostate cancer (49). To our knowledge, there is currently no clinical data regarding the use of the other LDH inhibitors oxamate and GNE-140 in patients.

To determine whether extracellular lactate may be influencing mitogenic/migratory behavior by either directly or indirectly affecting intracellular events, the levels of a few selected molecules including p38 MAPK, ERK1/2, AKT, Src and NF-κB that are known to be involved in the cell signaling cascades that control such processes were investigated. Involvement of ERK1/2 was already shown in a previous study by its reduced phosphorylation induced by siRNA-mediated LDH downregulation (4). In the present study, the effect of LDH inhibitors on the EGF-induced cell signaling pathway was investigated. Results indicated that despite all being classed as LDH inhibitors, they did not have a uniform effect. Oxamate reduces ERK1/2 and Akt phosphorylation, quercetin downregulates p38 MAPK as well as ERK1/2, lonidamine only blocks Akt phosphorylation and GNE-140 blocks p38 MAPK and Akt activation. Whilst these variations do not appear at first sight to support our hypothesis that lactate is the prime stimulus, it should be considered that, as most pharmacological agents, one or more of these inhibitors may have other actions aside from blocking lactate production. Clinically, in the context of inhibiting cancer progression, that can be seen as an added advantage. It was shown that combining two of these inhibitors had a synergistic effect which could have had beneficial implications in terms of dose reduction when considering the use of these drugs as potential therapeutic agents *in vivo*.

Lowering lactate by either oxamate or quercetin resulted in increased expression of E-cadherin and decreased expression of vimentin, an indication of a partial reversal of EMT, the process which increased their aggressiveness in the first place, consistent with previous data (36).

Several studies have suggested a role of lactate in modulating various signaling pathways. For example, lactate produced through LDH-A in activated T-cells enhances interferon-γ expression through maintaining high levels of acetyl-CoA to increase histone acetylation and transcription of the *IFNG* gene (50). Excessive lactate production in the local tissue in chronic inflammatory diseases upregulates the sodium-coupled lactate transporter SLC5A12, as well as IL-17 production by $CD4^+$ T cells through phosphorylation of signal transducer and activator of transcription 3 by PKM2 and fatty acid synthesis (51). Lactate was also shown to promote wound healing through enhancing the production of several mediators such as vascular endothelial growth factor, IL-1 and TGF-β (52). Lactate plays a vital role in NF-κB stabilization and activation which is important in resistance to targeted therapies in cancer cells (53,54). Lactate can also activate the G-protein-coupled receptor GPR81 which is followed by cyclic AMP downregulation and inhibition of protein kinase A which results in lipolysis inhibition, enhancing tumor proliferation and immune escape by cancer cells (55,56). Funding has already been obtained to study LDH-A, -B, or -AB knockdown, and lactate supplementation on the expression profile of various oncogenic molecules at both genetic and proteomic level. The data to be generated from that study will provide a better understanding of the mechanistic insights of either activating or inhibiting this important machinery in cancer progression.

A summary model for breast tumour progression based on the data from the present and previous studies (4,33-35,57-59) is shown in Fig. 6. The corollary to this is that it is an extrapolation from *in vitro* experiments requiring further *in vivo* experimentation. In this scheme, adherent epithelial cells composing the primary tumour bound by a basement membrane undergo change through a series of molecular and morphological transformations into more motile mesenchymal-like cells, in this instance through loss of ERs, but other mechanisms have also been documented (33-35). These cells proliferate under the influence of enhanced mitogenic signals, then become invasive and penetrate into the extracellular space under the influence of lactate by a mechanism yet to be determined, and pH changes previously described (57-59), with consequent penetration into the vascular circulation. LDH inhibitors lower lactate production and decrease extracellular lactate indirectly lowering the production of signaling molecules involved in proliferation and modulating the expression of epithelial and mesenchymal markers, reversing the EMT previously induced by ER loss.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the Corresponding author on reasonable request.

Authors' contributions

MAK and YAL conceived and designed the experiments and wrote the manuscript. SK and MAK performed the experiments and analyzed the data. MAK contributed reagents/materials/analysis tools. MAK and YAL confirm the authenticity of all of the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Schwartz MK: Enzymes as prognostic markers and therapeutic indicators in patients with cancer. Clin Chim Acta 206: 77-82, 1992.
2. Wang ZY, Loo TY, Shen JG, Wang N, Wang DM, Yang DP, Mo SL, Guan XY and Chen JP: LDH-A silencing suppresses breast cancer tumorigenicity through induction of oxidative stress mediated mitochondrial pathway apoptosis. Breast Cancer Res Treat 131: 791-800, 2012.
3. Bonuccelli G, Whitaker-Menezes D, Castello-Cros R, Pavlides S, Pestell RG, Fatatis A, Witkiewicz AK, Vander Heiden MG, Migneco G, Chiavarina B, *et al*: The reverse warburg effect: Glycolysis inhibitors prevent the tumor promoting effects of caveolin-1 deficient cancer associated fibroblasts. Cell cycle 9: 1960-1971, 2010.
4. Khajah MA, Khushaish S and Luqmani YA: Lactate dehydrogenase A or B knockdown reduces lactate production and inhibits breast cancer cell motility in vitro. Front Pharmacol 12: 747001, 2021.
5. Rizwan A, Serganova I, Khanin R, Karabeber H, Ni X, Thakur S, Zakian KL, Blasberg R and Koutcher JA: Relationships between LDH-A, lactate, and metastases in 4T1 breast tumors. Clin Cancer Res 19: 5158-5169, 2013.
6. Zha X, Wang F, Wang Y, He S, Jing Y, Wu X and Zhang H: Lactate dehydrogenase B is critical for hyperactive mTOR-mediated tumorigenesis. Cancer Res 71: 13-18, 2011.
7. Huang WC, Swietach P, Vaughan-Jones RD, Ansorge O and Glitsch MD: Extracellular acidification elicits spatially and temporally distinct Ca2+ signals. Curr Biol 18: 781-785, 2008.
8. Fukumura D, Xu L, Chen Y, Gohongi T, Seed B and Jain RK: Hypoxia and acidosis independently up-regulate vascular endothelial growth factor transcription in brain tumors in vivo. Cancer Res 61: 6020-6024, 2001.
9. Shi Q, Le X, Wang B, Abbruzzese JL, Xiong Q, He Y and Xie K: Regulation of vascular endothelial growth factor expression by acidosis in human cancer cells. Oncogene 20: 3751-3756, 2001.
10. Xu L, Fukumura D and Jain RK: Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway: Mechanism of low pH-induced VEGF. J Biol Chemistry 277: 11368-11374, 2002.
11. Graham RM, Frazier DP, Thompson JW, Haliko S, Li H, Wasserlauf BJ, Spiga MG, Bishopric NH and Webster KA: A unique pathway of cardiac myocyte death caused by hypoxia-acidosis. J Exp Biol 207: 3189-3200, 2004.
12. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, Gottfried E, Schwarz S, Rothe G, Hoves S, *et al*: Inhibitory effect of tumor cell-derived lactic acid on human T cells. Blood 109: 3812-3819, 2007.
13. Zhai X, Yang Y, Wan J, Zhu R and Wu Y: Inhibition of LDH-A by oxamate induces G2/M arrest, apoptosis and increases radiosensitivity in nasopharyngeal carcinoma cells. Oncol Rep 30: 2983-2991, 2013.
14. Ye W, Zheng Y, Zhang S, Yan L, Cheng H and Wu M: Oxamate improves glycemic control and insulin sensitivity via inhibition of tissue lactate production in db/db mice. PLoS One 11: e0150303, 2016.
15. Liu H, Zeng L, Yang Y, Huang Z, Guo C, Huang L, Niu X, Zhang C and Wang H: Bcl-3 regulates the function of Th17 cells through raptor mediated glycolysis metabolism. Front Immunol 13: 929785, 2022.
16. Mazzio E, Mack N, Badisa RB and Soliman KFA: Triple isozyme lactic acid dehydrogenase inhibition in fully viable MDA-MB-231 cells induces cytostatic effects that are not reversed by exogenous lactic acid. Biomolecules 11: 1751, 2021.
17. Zhai M, Cui S, Li L, Cheng C, Zhang Z, Liu J and Wei F: Mechanical force modulates alveolar bone marrow mesenchymal cells characteristics for bone remodeling during orthodontic tooth movement through lactate production. Cells 11: 3724, 2022.
18. Zhao P, Wang S, Jiang J, Gao Y, Wang Y, Zhao Y, Zhang J, Zhang M and Huang Y: Targeting lactate metabolism and immune interaction in breast tumor via protease-triggered delivery. J Control Release 358: 706-717, 2023.
19. Lacerda Q, Rochani A, Oeffinger B, Liu JB, Wessner CE, Tahmasebi A, Falatah H, Lee P, Leeper DB, Forsberg F, *et al*: Tumoral oxygenation and biodistribution of Lonidamine oxygen microbubbles following localized ultrasound-triggered delivery. Int J Pharm 625: 122072, 2022.
20. Tian LR, Lin MZ, Zhong HH, Cai YJ, Li B, Xiao ZC and Shuai XT: Nanodrug regulates lactic acid metabolism to reprogram the immunosuppressive tumor microenvironment for enhanced cancer immunotherapy. Biomater Sci 10: 3892-3900, 2022.
21. Zhang R, Li R, Zhang L, Chen G, Mo L, Jiang R, Xu X, Wang X, Zhao Y, Zhang L, *et al*: A dual-mechanism based nutrient partitioning nanoregulator for enhanced immunotherapy against anti-PD-1 resistant tumors. ACS Nano 17: 13461-13473, 2023.
22. Mathupala SP, Parajuli P and Sloan AE: Silencing of monocarboxylate transporters via small interfering ribonucleic acid inhibits glycolysis and induces cell death in malignant glioma: An in vitro study. Neurosurgery 55: 1410-1419; discussion 1419, 2004.
23. Fang J, Quinones QJ, Holman TL, Morowitz MJ, Wang Q, Zhao H, Sivo F, Maris JM and Wahl ML: The H+-linked monocarboxylate transporter (MCT1/SLC16A1): A potential therapeutic target for high-risk neuroblastoma. Mol Pharmacol 70: 2108-2115, 2006.
24. Sonveaux P, Végran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, De Saedeleer CJ, Kennedy KM, Diepart C, Jordan BF, *et al*: Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J Clin Invest 118, 3930-3942, 2008.
25. Colen CB, Shen Y, Ghoddoussi F, Yu P, Francis TB, Koch BJ, Monterey MD, Galloway MP, Sloan AE and Mathupala SP: Metabolic targeting of lactate efflux by malignant glioma inhibits invasiveness and induces necrosis: An in vivo study. Neoplasia 13: 620-632, 2011.
26. Belt JA, Thomas JA, Buchsbaum RN and Racker E: Inhibition of lactate transport and glycolysis in Ehrlich ascites tumor cells by bioflavonoids. Biochemistry 18: 3506-3511, 1979.
27. Deuticke B: Monocarboxylate transport in erythrocytes. J Membr Biol 70: 89-103, 1982.
28. Wang Q and Morris ME: Flavonoids modulate monocarboxylate transporter-1-mediated transport of gamma-hydroxybutyrate in vitro and in vivo. Drug Metab Dispos 35: 201-208, 2007.
29. Morais-Santos F, Miranda-Gonçalves V, Pinheiro S, Vieira AF, Paredes J, Schmitt FC, Baltazar F and Pinheiro C: Differential sensitivities to lactate transport inhibitors of breast cancer cell lines. Endocr Relat Cancer 21: 27-38, 2013.
30. Thornburg JM, Nelson KK, Clem BF, Lane AN, Arumugam S, Simmons A, Eaton JW, Telang S and Chesney J: Targeting aspartate aminotransferase in breast cancer. Breast Cancer Res 10: R84, 2008.
31. Eyassu F and Angione C: Modelling pyruvate dehydrogenase under hypoxia and its role in cancer metabolism. R Soc Open Sci 4: 170360, 2017.
32. Kong L, Du W, Cui Z, Wang L, Yang Z, Zhang H and Lin D: Expression of lactate dehydrogenase C in MDA-MB-231 cells and its role in tumor invasion and migration. Mol Med Rep 13: 3533-3538, 2016.
33. Al Saleh S, Al Mulla F and Luqmani YA: Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. PLoS One 6: e20610, 2011.
34. Luqmani YA, Al Azmi A, Al Bader M, Abraham G and El Zawahri M: Modification of gene expression induced by siRNA targeting of estrogen receptor alpha in MCF7 human breast cancer cells. Int J Oncol 34: 231-242, 2009.
35. Khajah MA, Al Saleh S, Mathew PM and Luqmani YA: Differential effect of growth factors on invasion and proliferation of endocrine resistant breast cancer cells. PLoS One 7: e41847, 2012.
36. Luqmani YA and Alam-Eldin N: Overcoming resistance to endocrine therapy in breast cancer: New approaches to a nagging problem. Med Princ Pract 25 (Suppl 2): 28-40, 2016.
37. Al Saleh S, Sharaf LH and Luqmani YA: Signalling pathways involved in endocrine resistance in breast cancer and associations with epithelial to mesenchymal transition (review). Int J Oncol 38: 1197-1217, 2011.
38. Zhao Z, Han F, Yang S, Wu J and Zhan W: Oxamate-mediated inhibition of lactate dehydrogenase induces protective autophagy in gastric cancer cells: Involvement of the Akt-mTOR signaling pathway. Cancer Lett 358: 17-26, 2015.
39. Valvona CJ and Fillmore HL: Oxamate, but not selective targeting of LDH-A, inhibits medulloblastoma cell glycolysis, growth and motility. Brain Sci 8: 56, 2018.
40. Boudreau A, Purkey HE, Hitz A, Robarge K, Peterson D, Labadie S, Kwong M, Hong R, Gao M, Del Nagro C, *et al*: Metabolic plasticity underpins innate and acquired resistance to LDHA inhibition. Nat Chem Biol 12: 779-786, 2016.
41. Ždralević M, Brand A, Di Ianni L, Dettmer K, Reinders J, Singer K, Peter K, Schnell A, Bruss C, Decking SM, *et al*: Double genetic disruption of lactate dehydrogenases A and B is required to ablate the 'Warburg effect' restricting tumor growth to oxidative metabolism. J Biol Chem 293: 15947-15961, 2018.

42. De Lena M, Lorusso V, Latorre A, Fanizza G, Gargano G, Caporusso L, Guida M, Catino A, Crucitta E, Sambiasi D and Mazzei A: Paclitaxel, cisplatin and lonidamine in advanced ovarian cancer. A phase II study. Eur J Cancer 37: 364-368, 2001.
43. Portalone L, Lombardi A, Antilli A, Cruciani AR, Magliacani V, Mugnaini L, Nunziati F, Perrone N, Signora M and Salvati F: Treatment of inoperable non-small cell lung carcinoma stage IIIb and IV with cisplatin, epidoxorubicin, vindesine and lonidamine: A phase II study. Tumori 85: 239-242, 1999.
44. Papaldo P, Lopez M, Cortesi E, Cammilluzzi E, Antimi M, Terzoli E, Lepidini G, Vici P, Barone C, Ferretti G, *et al*: Addition of either lonidamine or granulocyte colony-stimulating factor does not improve survival in early breast cancer patients treated with high-dose epirubicin and cyclophosphamide. J Clin Oncol 21: 3462-3468, 2003.
45. Berruti A, Bitossi R, Gorzegno G, Bottini A, Alquati P, De Matteis A, Nuzzo F, Giardina G, Danese S, De Lena M, *et al*: Time to progression in metastatic breast cancer patients treated with epirubicin is not improved by the addition of either cisplatin or lonidamine: Final results of a phase III study with a factorial design. J Clin Oncol 20: 4150-4159, 2002.
46. Pacini P, Rinaldini M, Algeri R, Guarneri A, Tucci E, Barsanti G, Neri B, Bastiani P, Marzano S and Fallai C: FEC (5-fluorouracil, epidoxorubicin and cyclophosphamide) versus EM (epidoxorubicin and mitomycin-C) with or without lonidamine as first-line treatment for advanced breast cancer. A multicentric randomised study. Final results. Eur J Cancer 36: 966-975, 2000.
47. Vaez S, Parivr K, Amidi F, Rudbari NH, Moini A and Amini N: Quercetin and polycystic ovary syndrome; inflammation, hormonal parameters and pregnancy outcome: A randomized clinical trial. Am J Reprod Immunol 89: e13644, 2023.
48. Shohan M, Nashibi R, Mahmoudian-Sani MR, Abolnezhadian F, Ghafourian M, Alavi SM, Sharhani A and Khodadadi A: The therapeutic efficacy of quercetin in combination with antiviral drugs in hospitalized COVID-19 patients: A randomized controlled trial. Eur J Pharmacol 914: 174615, 2022.
49. Henning SM, Wang P, Lee RP, Trang A, Husari G, Yang J, Grojean EM, Ly A, Hsu M, Heber D, *et al*: Prospective randomized trial evaluating blood and prostate tissue concentrations of green tea polyphenols and quercetin in men with prostate cancer. Food Funct 11: 4114-4122, 2020.
50. Peng M, Yin N, Chhangawala S, Xu K, Leslie CS and Li MO: Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. Science 354: 481-484, 2016.
51. Pucino V, Bombardieri M, Pitzalis C and Mauro C: Lactate at the crossroads of metabolism, inflammation, and autoimmunity. Eur J Immunol 47: 14-21, 2017.
52. Constant JS, Feng JJ, Zabel DD, Yuan H, Suh DY, Scheuenstuhl H, Hunt TK and Hussain MZ: Lactate elicits vascular endothelial growth factor from macrophages: A possible alternative to hypoxia. Wound Repair Regen 8: 353-360, 2000.
53. Végran F, Boidot R, Michiels C, Sonveaux P and Feron O: Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-κB/IL-8 pathway that drives tumor angiogenesis. Cancer Res 71: 2550-2560, 2011.
54. Apicella M, Giannoni E, Fiore S, Ferrari KJ, Fernández-Pérez D, Isella C, Granchi C, Minutolo F, Sottile A, Comoglio PM, *et al*: Increased lactate secretion by cancer cells sustains non-cell-autonomous adaptive resistance to MET and EGFR targeted therapies. Cell Metab 28: 848-865.e6, 2018.
55. Liu C, Wu J, Zhu J, Kuei C, Yu J, Shelton J, Sutton SW, Li X, Yun SJ, Mirzadegan T, *et al*: Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81. J Biol Chem 284: 2811-2822, 2009.
56. Roland CL, Arumugam T, Deng D, Liu SH, Philip B, Gomez S, Burns WR, Ramachandran V, Wang H, Cruz-Monserrate Z and Logsdon CD: Cell surface lactate receptor GPR81 is crucial for cancer cell survival. Cancer Res 74: 5301-5310, 2014.
57. Khajah MA, Almohri I, Mathew PM and Luqmani YA: Extracellular alkaline pH leads to increased metastatic potential of estrogen receptor silenced endocrine resistant breast cancer cells. PLoS One 8: e76327, 2013.
58. Khajah MA and Luqmani YA: Involvement of membrane blebbing in immunological disorders and cancer. Med Princ Pract 25 Suppl 2 (Suppl 2): S18-S27, 2016.
59. Khajah MA, Mathew PM, Alam-Eldin NS and Luqmani YA: Bleb formation is induced by alkaline but not acidic pH in estrogen receptor silenced breast cancer cells. Int J Oncol 46: 1685-1698, 2015.