

Research Article

Potential of Paclitaxel Anti-tumor Activity by Galloflavin or Oxamate as Lactate Dehydrogenase Inhibitors

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*Alaa E. Elsisy, Samia S. Sokar, Sara R. El-Mahrouk, Sally E. Abu-Risha**

Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tanta University, Tanta, Egypt

ABSTRACT

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Increased aerobic glycolysis in cancer, a phenomenon known as the Warburg effect, has been observed in various tumors and represents a major biochemical alteration associated with malignant transformation. Several cancers display an elevated expression of lactate dehydrogenase-A (LDH-A), which is involved in tumor initiation, maintenance, and progression. Significantly, inhibition of LDH-A has been reported to have an antiproliferative effect on breast cancer and inhibited tumor progression. Accordingly, several LDH-A inhibitors are being tested for their anticancer activity, such as oxamate and galloflavin. In the current study, the anti-tumor activity of oxamate and galloflavin was tested *in vitro* using MCF7 and OVCAR-3 human carcinoma cell lines. Furthermore, both drugs were examined in combination with paclitaxel (Taxol). Additionally, the potential anti-tumor effect of oxamate in the solid Ehrlich carcinoma (SEC) mouse model was examined alone and in combination with paclitaxel. Oxamate and galloflavin significantly reduced cell survival of MCF7 and OVCAR3 cell lines. They also caused significant reductions in LDH enzyme activity and ATP cellular content in addition to a significant increase in MDA content. Both oxamate and galloflavin potentiated the anticancer effect of paclitaxel both *in vivo* and *in vitro*. Moreover, potentiation of apoptosis and the anti-angiogenic effect of paclitaxel by oxamate were found *in vivo*. In conclusion, LDH inhibitor may represent a promising agent that enhances the anti-tumor activity of paclitaxel chemotherapy.

Keywords: Apoptosis, Galloflavin, Oxamate, Paclitaxel, Warburg effect.

INTRODUCTION

In contrast to normal cells, which rely primarily on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, most cancer cells rely on aerobic glycolysis, a phenomenon named as "the Warburg effect".¹ LDH catalyzes the interconversion of pyruvate and reduced nicotinamide adenine dinucleotide (NADH) generated by glycolysis to lactate and NAD⁺. LDH-A promotes the reduction of pyruvate to lactate to regenerate

NAD⁺ from NADH, whereas LDH-B favors the reverse reaction.^{2,3} LDH-A is a key enzyme involved in the Warburg effect and in sustaining cancer's glycolytic phenotype through regenerating the NAD⁺ required to drive glycolysis in cancer cells.⁴ In addition, LDH-A plays a key role in the growth and progression of breast cancer cells. Thus, several human cancers display elevated expression of LDH-A. Significantly, the inhibition of LDH-A has been reported to have an antiproliferative effect on breast carcinoma and inhibited tumor progression.⁵⁻⁹ Furthermore, complete deficiency of LDH-A does not induce any symptoms in humans under normal conditions, which indicates that selective LDH-A inhibitors could only present minimal side effects.¹⁰

*Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tanta University, Tanta, Egypt, 31527.
E-mail address: sally.abouresha@pharm.tanta.edu.eg

Therefore, LDH-A is considered an attractive target for the development of novel anti-tumor agents.

It has been demonstrated that oxamate, a metabolic inhibitor of LDH, has shown to be a promising anticancer agent. However, the detailed mechanism remains largely unclear.¹¹ In previous studies, oxamate has been shown to inhibit the growth and metastasis of cervical, breast, and liver cancer cells *in vitro*.¹²⁻¹⁴ Furthermore, oxamate also significantly enhanced the sensitivity of cancer cells to several chemotherapeutic agents.¹⁵⁻¹⁷ Galloflavin (GF) is another LDH inhibitor that inhibits both isoforms of LDH.¹⁸ By serving as a competitive inhibitor with NADH for LDH, GF has been shown to disrupt aerobic glycolysis and decrease cell viability effectively across many cancer cell types.¹⁹⁻²¹ It was found that GF inhibited lactate production and ATP synthesis of human hepatocellular carcinoma cells in addition to apoptosis induction.²²

Taxol is an important chemotherapeutic agent in the treatment of human breast cancer as well as other tumor malignancies.^{23,24} Taxol primarily targets the microtubules of cancer cells. It stabilizes the microtubule structure by disrupting the dynamic equilibrium between soluble tubulin dimers and their polymerized form.²⁵ Taxol resistance is frequently emerged, and therefore, the identification of chemosensitizers for cancer chemotherapy is an area of intensive investigation.²⁶ Oxamate may also be useful in treating paclitaxel-resistant cancers. Paclitaxel/oxamate combinational treatment revealed a synergistic inhibitory effect in paclitaxel-resistant breast cancer cells by promoting apoptosis.¹⁵

The present study aimed to investigate the potential anticancer effect of oxamate and galloflavin as LDH inhibitors in breast and ovarian cancer cell lines *in vitro*. Also, the potential anticancer effect of oxamate in solid Ehrlich carcinoma (SEC) in mice was examined, and the molecular mechanisms involved in any potential therapeutic effects were clarified.

1. MATERIALS AND METHOD

2.1. Cell cultures

2.1.1. Cell lines

MCF7 (breast carcinoma line), OVCAR3 (ovarian carcinoma line) were used. They were obtained frozen in liquid nitrogen from the American Type Culture Collection (ATCC). These cell lines were maintained in the VACSERA cell culture unit by serial sub-culturing. Dulbecco's Modified Eagle's Medium (DMEM) outgrowth medium was used for culture. The cell lines were maintained in a complete medium, which consists of Eagle's minimum essential medium (MEM), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 50 µg / ml gentamicin, 2 mmol / L L-glutamine, 1 mmol / L sodium pyruvate and 10 % heat-inactivated fetal calf serum at 37°C

in a humidified atmosphere of 95 % air and 5 % CO₂. Tumor cells were harvested by overlaying the monolayer with a solution of 0.05 % trypsin and 0.5 mmol / L EDTA and then resuspended in a complete medium. The cells were plated in 24-well plates at a concentration of 106 cells per well and grown for 24 hours.

2.1.2. Compounds and reagents

Paclitaxel, Bristol-Myers Squibb (New York, USA). Oxamate, Sigma-Aldrich (St Louis, Missouri, USA), was freshly dissolved in sterile saline. Galloflavin, Sigma-Aldrich (St Louis, Missouri, USA), was freshly dissolved in a DMSO vehicle, which is composed of 10% DMSO and 90% saline.

2.1.3. Cell Proliferation assay

The CellTiter 96® aqueous one solution cell proliferation assay (Promega Corporation, USA) was used. After the cells were plated in 24-well plates at a concentration of 106 cells/ well and grown for 24 hours, cells were incubated with the following drugs: Oxamate (40mM),²⁷ Galloflavin (250µM),¹⁹ Paclitaxel (10µM),²⁸ combination of oxamate (40mM) and paclitaxel (10 µM), combination of Galloflavin (250 µM) and paclitaxel (10 µM), and then incubated for 48 hours. Control wells were treated with vehicles alone (DMSO, cremophore). Negative control well was prepared with no addition of vehicles or drugs. After 48 hours, 20µl of CellTiter 96 Aqueous one solution reagent was added into each well of the 24-well assay plate containing the sample in 100µl of culture medium. And the plate was incubated for an additional 4 hours at 37°C in a humidified 5% CO₂ atmosphere. Finally, the absorbance was measured at 490nm using ELISA reader. Calculations: The percentage of surviving cells was calculated as a percentage of untreated control cells²⁹: % Survival = (A treated cells / A control cells) X 100

2.1.4. Preparation of cell culture supernatant

Cell culture medium was centrifuged at 3000 rpm for 20 minutes, then supernatant removed and the cell suspension diluted with PBS (PH 7.4) to 106 cells/ ml, repeated freeze-thaw cycles, then cells were damaged and intracellular components released, centrifugation was done, and the supernatant taken for further assessment of additional parameters.

2.1.5. Determination of LDH enzyme activity, ATP cellular content in human cancer cell lines

LDH enzyme activity was determined using ELISA kit (SunRed, PELOBIOTECH GmbH, Germany); ATP cellular content was determined using ELISA kit (SunRed, PELOBIOTECH GmbH, Germany). Standard curves were prepared using kit-provided standards.

2.1.6. Determination of Lipid Peroxides Level (measured as MDA) in Human Cancer Cell Lines

Malondialdehyde (MDA), the product of lipid peroxidation, is used as an indirect measure of lipid peroxidation according to Yoshioka et al.,³⁰ after phase separation, the butanol layer was isolated, and the absorbance of the pink color product was measured at 535 nm using a double beam spectrophotometer.

2.2. Animal study

2.2.1. Animals

Adult female Swiss albino mice (18–20 g, aged 8 weeks) were purchased from the National Cancer Institute (NCI) (Cairo, Egypt) and maintained under standard laboratory conditions (25°C, 5% relative humidity, 12-h light-dark cycle). All mice had ad libitum access to standard mouse chow and water and were acclimated for at least 1 week prior to experiment initiation. All procedures reported here were approved by the Ethical and Animal Care Committees of Faculty of Pharmacy, Tanta University.

2.2.2. Experimental protocol

Ehrlich ascites carcinoma (EAC) cells were kindly supplied from the Pharmacology and Experimental Oncology Unit of NCI. EAC cells were then maintained via serial IP passages into female Swiss albino mice. After 10 days, the host mice were euthanized by cervical dislocation, and the cells recovered by collection with a syringe. After recovery from the host, the cells were washed in normal saline and then enumerated (via hemocytometer) for use in passing or in the experiments below. For induction of the SEC, mice were injected subcutaneously into the right thigh of the lower limb with 0.2 ml of viable EAC cells (i.e., at 2×10^6 /ml). Mice were then randomly allocated into one of the six experimental groups (each containing eight mice) that then received: (A) saline (control); (B) cremophore (control); (C) paclitaxel (20 mg/kg, I.P.);³¹ (D) oxamate (300 mg/kg, I.P.);²⁷ (E) a combination of paclitaxel (20 mg/kg) and oxamate (300 mg/kg). Daily treatment with saline or oxamate was initiated on day 7 post-EAC implantation and continued for a total of 21 days. Paclitaxel treatments occurred on days 7, 11, 15, and 19 after EAC injection.

2.2.3. Sample collection

At the end of the protocol (i.e., day 22 post-EAC injection), the mice were killed by cervical dislocation, and the solid tumor was removed. Portions of the solid tumor were fixed in 10% neutral-buffered formalin (pH 7.4) for subsequent histopathological examination. Other portions were washed with normal saline and placed at -80°C for later measurement of MDA content, IL-17, caspase-3 activity, vascular endothelial growth factor activity.

2.2.4. Assessment of SEC malondialdehyde (MDA) content

SEC tissue MDA, the product of lipid peroxidation, is used as an indirect measure of lipid peroxidation, according to Yoshioka et al.³⁰. After phase separation, the butanol layer was isolated, and the absorbance of the pink color product was measured at 535 nm using a double beam spectrophotometer.

2.2.5. Assessment of IL-17a content, caspase-3, and VEGF activity in SEC

100 mg of SEC tissue was homogenized in 10 volumes of ice-cold phosphate-buffered saline (PBS, pH 7) containing protease inhibitor cocktail and 0.05% (v/v) tween 20. Centrifugation at 3000 rpm for 10 min was made for samples, and the supernatant was collected and used for determination of IL-17a content using mouse IL-17a ELISA kit (SunRed, PELOBIOTECH GmbH, Germany); determination of caspase-3 activity using mouse caspase-3 ELISA kit (SunRed, PELOBIOTECH GmbH, Germany); determination of VEGF activity using mouse VEGF ELISA kit (SunRed, PELOBIOTECH GmbH, Germany). Standard curves were constructed using kit-provided standards.

2.2.6. Histopathological examination of SEC sections

Samples of SEC tissue were dehydrated in alcohol series and then embedded in paraffin wax. Then, staining with hematoxylin and eosin (H and E) was made to paraffin sections, each was nearly 5µm thickness. Photomicroscope (Olympus BX 51, Olympus America, Melville, NY) was used for the examination of these sections with 100X and 400X magnified images. The H&E stained sections were evaluated for histological changes, such as fibrous stroma, tumor giant cell formation, vacolated nucleus, increased mitotic division, and necrosis of neoplastic masses.^{32, 33}

2.2.7. Statistical analysis

Graphpad prism 5.0 Demo (Graphpad software, San Diego, CA) was used for statistical analysis of different groups. Comparison of data groups was carried out using one-way analysis of variance (ANOVA) followed by Tukey, multiple comparison tests. The significant difference was accepted when $P < 0.05$.

3. RESULTS

3.1. Cytotoxicity of oxamate, galloflavin, alone and/or paclitaxel on MCF7 human breast cancer cell lines

The addition of oxamate (40 mM) or galloflavin (250 µM) to the culture medium of MCF7 cell line caused a significant ($P > 0.05$) reduction in the % cell survival by 40.4%, 42%, respectively compared to that of the control vehicles. Similarly, paclitaxel (10 µM) caused a significant ($P > 0.05$) reduction by 43.1% compared to that of the control cremophore medium. The addition of a combination of either

oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to the culture medium of MCF7 cell line caused a significant ($P>0.05$) reduction in the % cell survival by 61.5%, 66.6%, respectively compared to that of the control medium. It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to the culture medium of MCF7 cell line caused a significant ($P>0.05$) reduction in the % cell survival by 32.4%, 41.2%, respectively compared to the paclitaxel-treated medium (**Figure 1**).

3.2. Cytotoxicity of oxamate, galloflavin, alone and/or paclitaxel on OVCAR3 human ovarian cancer cell line

The addition of oxamate (40 mM) or galloflavin (250 μ M) to the culture medium of OVCAR3 cell line caused a significant ($P>0.05$) reduction in the % cell survival by 34.2%, 31.5%, respectively compared to that of the control vehicles. Similarly, the addition of paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in the % cell survival by 37.9% compared to that of the control cremophore medium. The addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in the % cell survival by 60.5%, 67.7%, respectively compared to that of the control medium. It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in the % cell survival by 36.3%, 47.9%, respectively compared to the paclitaxel-treated medium (**Figure 2**).

3.3. Effects of oxamate, galloflavin, alone and/or paclitaxel on LDH enzyme activity in MCF7 human breast cancer cell line

The addition of oxamate (40 mM) or galloflavin (250 μ M) to the MCF7 culture medium caused a significant ($P>0.05$) reduction in LDH enzyme activity by 69.3%, 67.4%, respectively, compared to that of the control vehicles. On the other hand, the addition of paclitaxel (10 μ M) to MCF7 culture medium caused a significant ($P>0.05$) elevation in LDH enzyme activity by 35.6% compared to that of the control cremophore medium. The addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to the MCF7 culture medium caused a significant ($P>0.05$) reduction in LDH enzyme activity by 70.3%, 68.7%, respectively compared to that of the control medium. It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to MCF7 culture medium caused a significant ($P>0.05$) reduction in LDH enzyme activity by 78.1%, 76.9%, respectively compared to the paclitaxel-treated medium (**Figure 3**).

3.4. Effects of oxamate, galloflavin, alone and/or paclitaxel on LDH enzyme activity in OVCAR3 human ovarian cancer cell line

The addition of oxamate (40 mM) or galloflavin (250 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in LDH enzyme activity by 53.4%, 61.3%, respectively, compared to that of the control vehicles. On the other hand, the addition of paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) elevation in LDH enzyme activity by (35.04%) compared to that of the control cremophore medium. The addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in LDH enzyme activity by 58.1%, 64.2%, respectively compared to that of the control medium. It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in LDH enzyme activity by 69%, 73.5%, respectively compared to the paclitaxel-treated medium (**Figure 4**).

3.5. Effects of oxamate, galloflavin, alone and/or paclitaxel on ATP cellular content in MCF7 human breast cancer cell line

The addition of oxamate (40 mM) or galloflavin (250 μ M) to MCF7 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 40.8%, 43.7%, respectively compared to that of the control vehicles. Similarly, the addition of paclitaxel (10 μ M) to MCF7 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 33.5% compared to that of the control cremophore medium. The addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to MCF7 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 54.5%, 58.7%, respectively compared to that of the control medium. It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to MCF7 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 31.6%, 37.9%, respectively compared to the paclitaxel-treated medium (**Figure 5**).

3.6. Effects of oxamate, galloflavin, alone and/or paclitaxel on ATP cellular content in OVCAR3 human ovarian cancer cell line

The addition of oxamate (40 mM) or galloflavin (250 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 39.8%, 38.3%, respectively, compared to that of the control vehicles. Similarly, the addition of paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 35% compared to that of the control cremophore medium. The addition of a combination of either

oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 59.6%, 61.2%, respectively compared to that of the control medium. It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) reduction in ATP cellular content by 37.9%, 40.4%, respectively compared to the paclitaxel-treated medium (**Figure 6**).

3.7. Effects of oxamate, galloflavin, alone and/or paclitaxel on lipid peroxides level (measured as MDA) in MCF7 human breast cancer cell line

The addition of oxamate (40 mM) or galloflavin (250 μ M) to MCF7 culture medium caused a significant ($P>0.05$) elevation in MDA cellular content by 222.9%, 267.4%, respectively compared to that of the control vehicles. Similarly, the addition of paclitaxel (10 μ M) to MCF7 culture medium caused significant ($P>0.05$) elevation in MDA cellular content by 241.3% compared to that of the control cremophore medium. The addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to MCF7 culture medium caused a significant ($P>0.05$) elevation in MDA cellular content by 314%, 351.3%, respectively compared to that of the control medium. It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to MCF7 culture medium caused a significant ($P>0.05$) elevation in MDA cellular content by 21.3%, 32.2%, respectively compared to the paclitaxel-treated medium (**Figure7**).

3.8. Effects of oxamate, galloflavin, alone and/or paclitaxel on lipid peroxides level (measured as MDA) in OVCAR3 human ovarian cancer cell line

The addition of oxamate (40 mM) or galloflavin (250 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) elevation in MDA cellular content by 237.2%, 267%, respectively compared to that of the control vehicles. Similarly, the addition of paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) elevation in MDA cellular content by 255.4% compared to that of the control cremophore medium. The addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) elevation in MDA cellular content by 318.9%, 354.6%, respectively compared to that of the control medium.

It was found that addition of a combination of either oxamate (40mM)/paclitaxel (10 μ M) or galloflavin (250 μ M)/paclitaxel (10 μ M) to OVCAR3 culture medium caused a significant ($P>0.05$) elevation in MDA cellular content by

17.9%, 27.9%, respectively compared to the paclitaxel-treated medium (**Figure 8**).

3.9. Effects of oxamate alone and/or paclitaxel on lipid peroxides level (measured as MDA) in SEC-bearing mice

Mice-bearing SEC showed a significant ($P>0.05$) increase in MDA content by 367.4% compared to the normal control group. Similarly, oxamate treatment (300 mg/kg) resulted in a significant ($P>0.05$) increase in MDA content by 39.6% compared to the SEC control saline group. Similarly, treatment of mice-bearing SEC with paclitaxel (20mg/kg) resulted in a significant ($P>0.05$) increase in MDA content by 39.1% compared to the SEC control cremophore group. Mice-bearing SEC that received a combination of oxamate/paclitaxel showed a significant ($P>0.05$) increase in MDA content by 64.9% compared to the SEC control cremophore group. It was found that treatment of mice-bearing SEC with oxamate/paclitaxel combination caused a significant ($P>0.05$) increase in MDA content by 18.5% compared to the paclitaxel-treated group (**Figure 9**).

3.10. Effects of oxamate alone and/or paclitaxel on IL-17a content in SEC-bearing mice

Mice-bearing SEC showed a significant increase (58.7%) in IL-17a cellular content compared to the normal control group. On the other hand, treatment of mice with oxamate (300 mg/kg) resulted in a significant reduction (30.6% of control SEC values) in IL-17a cellular content. However, treatment of mice with paclitaxel (20mg/kg) resulted in a significant increase (40.9% of control normal values) in IL-17a cellular content. Mice received a combination of oxamate and paclitaxel showed a significant reduction (31.9% of control SEC values) in IL-17a cellular content. It was found that treatment of mice with oxamate or oxamate and paclitaxel combination caused significant reductions (22.4%, and 23.3%, respectively) in IL-17a cellular content compared to the paclitaxel group (**Figure 10**).

3.11. Effects of oxamate alone and/or paclitaxel on caspase-3 activity in SEC-bearing mice

Mice-bearing SEC showed a significant increase (299%) in caspase-3 activity compared to the normal control group. On the other hand, treatment of mice with oxamate (300 mg/kg) resulted in a significant increase (125% of control SEC values) in caspase-3 activity. However, treatment of mice with paclitaxel (20mg/kg) resulted in a significant increase (74.9% of control normal values) in caspase-3 activity. Mice that received a combination of oxamate and paclitaxel showed a significant increase (166.5% of control SEC values) in caspase-3 activity. It was found that treatment of mice with oxamate and paclitaxel combination caused a significant increase (52.4%) in caspase-3 activity compared to paclitaxel group (**Figure 11**).

3.12. Effects of oxamate alone and/or paclitaxel on VEGF activity in SEC-bearing mice

Mice-bearing SEC showed a significant increase (1014%) in VEGF activity compared to the normal control group. On the other hand, treatment of mice with oxamate (300 mg/kg) resulted in a significant decrease (55.5% of control SEC values) in VEGF activity. Similarly, treatment of mice with paclitaxel (20mg/kg) resulted in a significant decrease (54.1% of control normal values) in VEGF activity. Mice that received a combination of oxamate and paclitaxel showed a significant decrease (75.3% of control SEC values) in VEGF activity. It was found that treatment of mice with oxamate and paclitaxel combination caused a significant decrease (46.3%) in VEGF activity compared to paclitaxel group (Figure 12).

3.13. Correlation between IL-17a and VEGF activity

As demonstrated in (table 1), it was found that there is a strong positive correlation between IL-17a and VEGF activity in groups received oxamate ($r=0.934$, $P> 0.00$) or oxamate/paclitaxel combination ($r=0.841$, $P> 0.05$).

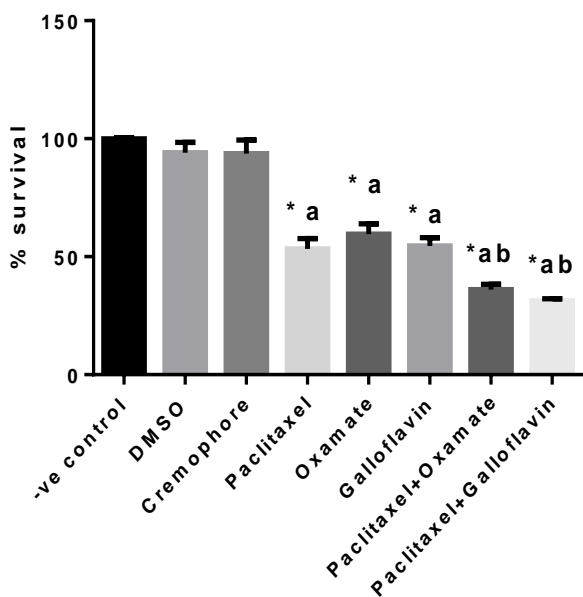


Figure 1: Cytotoxicity of oxamate, galloflavin, alone, and/or paclitaxel on MCF7 human breast cancer cell line. Effects of control vehicles (DMSO, cremophore), oxamate (40mM), galloflavin (250µM), paclitaxel (10µM), the combination of paclitaxel (10µM) with either oxamate (40mM) or galloflavin (250 µM) on the % cell survival of MCF7 cell line. Data are presented as the mean±SD of % survival of MCF7 cell line. n=5. *Significant difference from negative control medium at $p> 0.05$. ^aSignificant difference from control vehicles medium at $p> 0.05$. ^bSignificant difference from paclitaxel medium at $p> 0.05$.

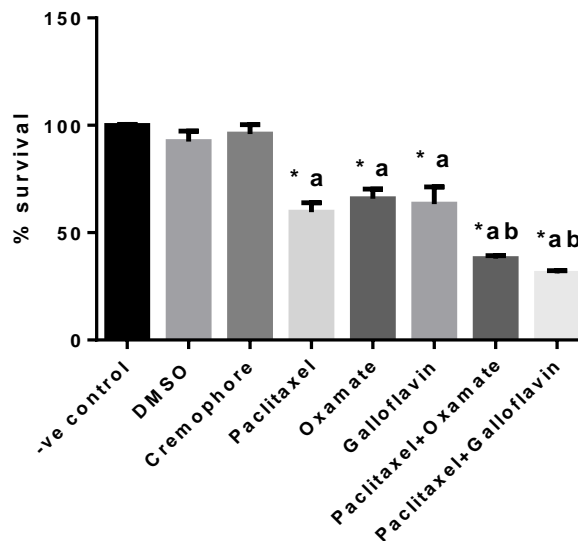


Figure 2: Cytotoxicity of oxamate, galloflavin, alone, and/or paclitaxel on OVCAR3 human ovarian cancer cell line. Effects of control vehicles (DMSO, cremophore), oxamate (40mM), galloflavin (250µM), paclitaxel (10µM), combination of paclitaxel (10µM) with either oxamate (40mM) or galloflavin (250 µM) on the % cell survival of OVCAR3 cell line. Data are presented as the mean±SD of % survival of OVCAR3 cell line. n=5. *Significant difference from negative control medium at $p> 0.05$. ^aSignificant difference from control vehicles medium at $p> 0.05$. ^bSignificant difference from paclitaxel medium at $p> 0.05$.

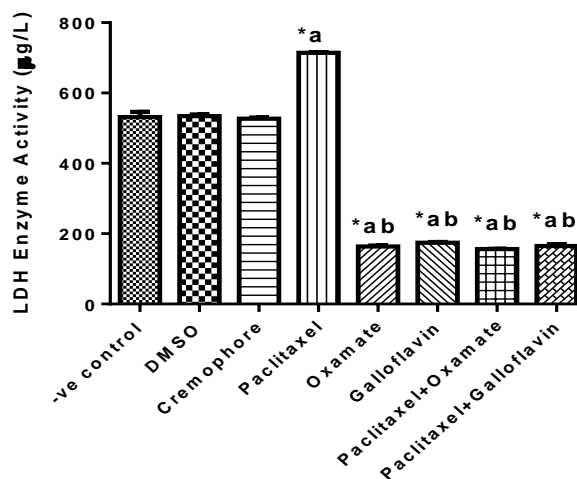


Figure 4: Effects of oxamate, galloflavin, alone, and/or paclitaxel on LDH enzyme activity in OVCAR3 human ovarian cancer cell line. Effects of control vehicles (DMSO, cremophore), oxamate (40mM), galloflavin (250µM), paclitaxel (10µM), a combination of paclitaxel (10µM) with either oxamate (40mM) or galloflavin (250 µM) on LDH enzyme activity of OVCAR3 cell line. Data are presented as the mean±SD of LDH enzyme activity of OVCAR3 cell line. n=5. *Significant difference from negative control medium at $p> 0.05$. ^aSignificant difference from control vehicles medium at $p> 0.05$. ^bSignificant difference from paclitaxel medium at $p> 0.05$.

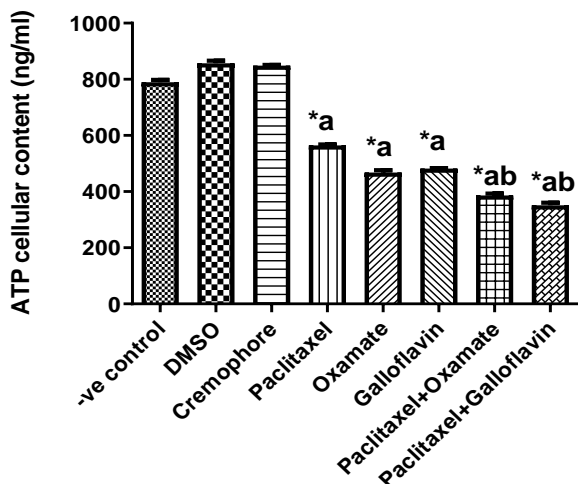


Figure 5: Effects of oxamate, galloflavin, alone, and/or paclitaxel on ATP cellular content in MCF7 human breast cancer cell line. Effects of control vehicles (DMSO, cremophore), oxamate (40mM), galloflavin (250µM), paclitaxel (10µM), a combination of paclitaxel (10µM) with either oxamate (40mM) or galloflavin (250 µM) on ATP cellular content of MCF7 cell line. Data are presented as the mean±SD of ATP cellular content of MCF7 cell line. n=5. *Significant difference from negative control medium at p> 0.05. ^aSignificant difference from control vehicles medium at p> 0.05. ^bSignificant difference from paclitaxel medium at p> 0.05.

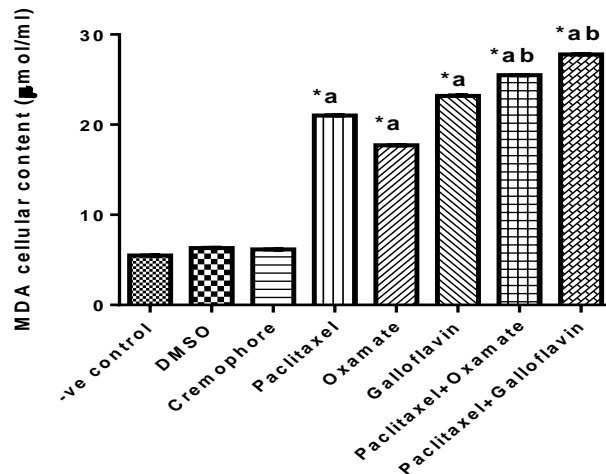


Figure 7: Effects of oxamate, galloflavin, alone, and/or paclitaxel on lipid peroxides level (measured as MDA) in MCF7 human breast cancer cell line. Effects of control vehicles (DMSO, cremophore), oxamate (40mM), galloflavin (250µM), paclitaxel (10µM), a combination of paclitaxel (10µM) with either oxamate (40mM) or galloflavin (250 µM) on MDA cellular content of MCF7 cell line. Data are presented as the mean±SD of MDA cellular content of MCF7 cell line. n=5. *Significant difference from negative control medium at p> 0.05. ^aSignificant difference from control vehicles medium at p> 0.05. ^bSignificant difference from paclitaxel medium at p> 0.05.

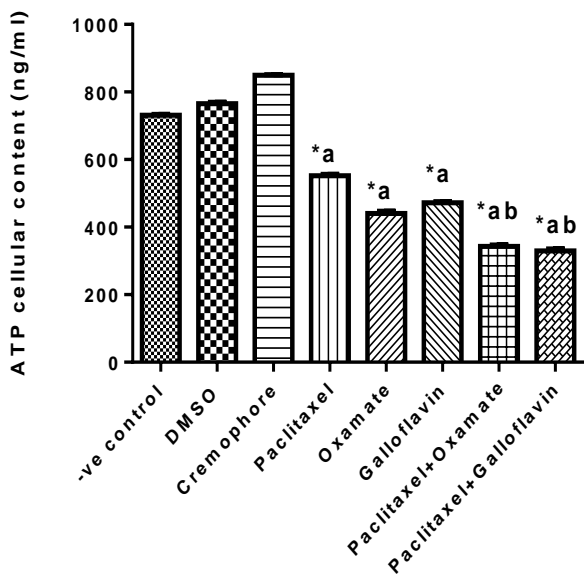


Figure 6: Effects of oxamate, galloflavin, alone, and/or paclitaxel on ATP cellular content in OVCAR3 human ovarian cancer cell line. Effects of control vehicles (DMSO, cremophore), oxamate (40mM), galloflavin (250µM), paclitaxel (10µM), a combination of paclitaxel (10µM) with either oxamate (40mM) or galloflavin (250 µM) on ATP cellular content of OVCAR3 cell line. Data are presented as the mean±SD of ATP cellular content of OVCAR3 cell line. n=5. *Significant difference from negative control medium at p> 0.05. ^aSignificant difference from control vehicles medium at p> 0.05. ^bSignificant difference from paclitaxel medium at p> 0.05.

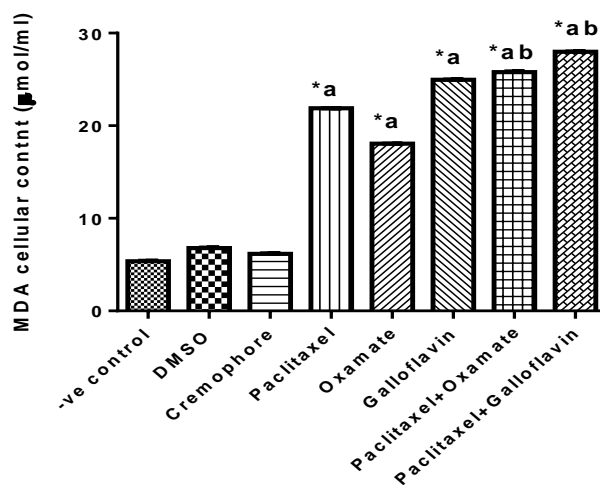


Figure 8: Effects of oxamate, galloflavin, alone, and/or paclitaxel on lipid peroxides level (measured as MDA) in OVCAR3 human ovarian cancer cell line. Effects of control vehicles (DMSO, cremophore), oxamate (40mM), galloflavin (250µM), paclitaxel (10µM), a combination of paclitaxel (10µM) with either oxamate (40mM) or galloflavin (250 µM) on MDA cellular content of OVCAR3 cell line. Data are presented as the mean±SD of MDA cellular content of OVCAR3 cell line. n=5. *Significant difference from negative control medium at p> 0.05. ^aSignificant difference from control vehicles medium at p> 0.05. ^bSignificant difference from paclitaxel medium at p> 0.05.

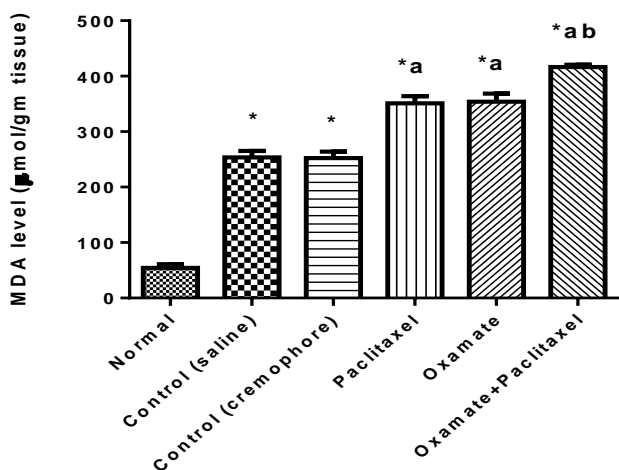


Figure 9: Effects of oxamate alone and/or paclitaxel on lipid peroxides level (measured as MDA) in SEC-bearing mice. Mice-bearing SEC were treated with control vehicles (saline or cremophore), oxamate (300mg/kg, IP), paclitaxel (20mg/kg, IP), or a combination of oxamate and paclitaxel. MDA cellular level was determined at the end of the treatment period. Data are presented as the mean±SD of MDA cellular level (nmol/ gm tissue), n=8. *Significant difference from the normal control group at p> 0.05. ^aSignificant difference from SEC control group at p> 0.05. ^bSignificant difference from paclitaxel group at p> 0.05.

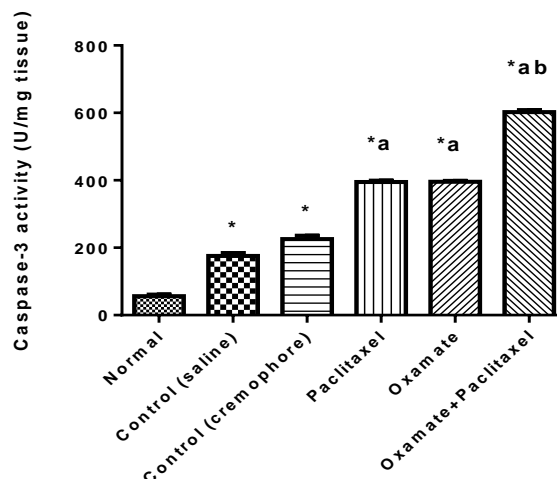


Figure 11: Effects of oxamate alone and/or paclitaxel on caspase-3 activity in SEC-bearing mice. Mice-bearing SEC were treated with control vehicles (saline or cremophore), oxamate (300 mg/kg, IP), paclitaxel (20 mg/kg, IP), or a combination of oxamate and paclitaxel. Caspase-3 activity was determined at the end of the treatment period. Data are presented as the mean±SD of caspase-3 activity (U/ mg tissue), n=8. *Significant difference from the normal control group at p> 0.05. ^aSignificant difference from SEC control group at p> 0.05. ^bSignificant difference from paclitaxel group at p> 0.05.

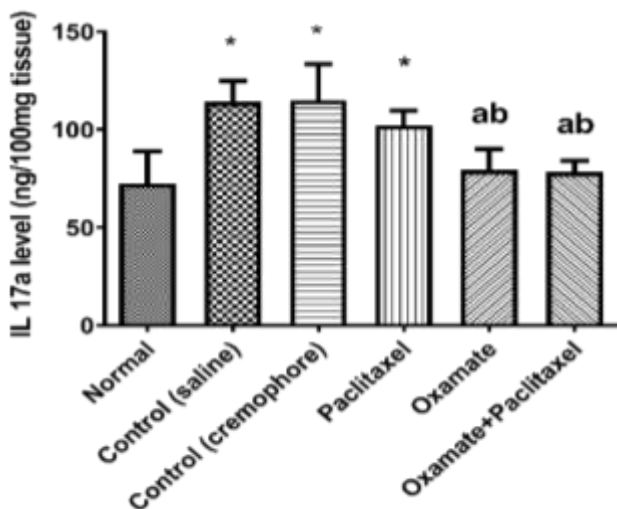


Figure 10: Effects of oxamate alone and/or paclitaxel on IL-17a cellular content in SEC-bearing mice. Mice-bearing SEC were treated with control vehicles (saline or cremophore), oxamate (300 mg/kg, IP), paclitaxel (20 mg/kg, IP), or a combination of oxamate and paclitaxel. IL-17 level was determined at the end of the treatment period. Data are presented as the mean±SD of IL-17 level (ng/ gm tissue), n=8. *Significant difference from the normal control group at p> 0.05. ^aSignificant difference from SEC control group at p> 0.05. ^bSignificant difference from paclitaxel group at p> 0.05.

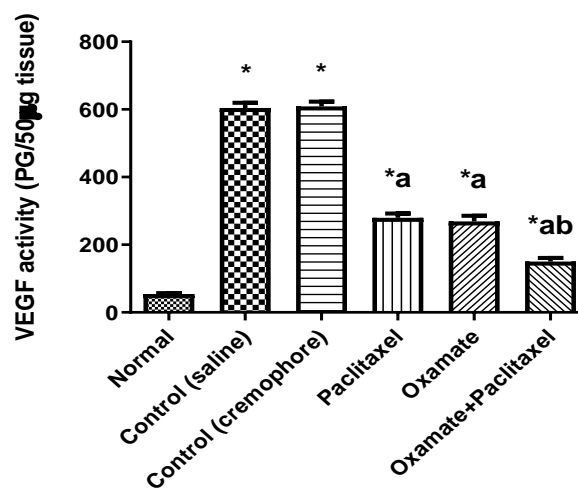


Figure 12: Effects of oxamate alone and/or paclitaxel on VEGF activity in SEC-bearing mice. Mice-bearing SEC were treated with control vehicles (saline or cremophore), oxamate (300 mg/kg, IP), paclitaxel (20 mg/kg, IP), or a combination of oxamate and paclitaxel. VEGF activity was determined at the end of the treatment period. Data are presented as the mean±SD of VEGF activity (PG/ 50 µg tissue), n=8. *Significant difference from normal control group at p> 0.05. ^aSignificant difference from SEC control group at p> 0.05. ^bSignificant difference from paclitaxel group at p> 0.05.

Table 1: Correlation between Il-17a and VEGF activity in SEC-bearing mice; r: correlation coefficient, n=8

Group	R	p-value
Normal	0.935	0.006*
SEC Control saline	.437	0.337
SEC Control cremophore	-0.717	0.109
Paclitaxel (20 mg/kg)	0.979	0.001**
Oxamate (300 mg/kg)	0.934	0.000**
Paclitaxel / Oxamate	0.841	0.036*

3.14. Effects of oxamate and/or paclitaxel on histopathological examination of SEC sections

Histopathological examination of SEC sections from mice received control vehicles (saline) revealed neoplastic masses from pleomorphic densely basophilic neoplastic cells, little fibrous stroma, and tumor giant cell formation (**Figure 13a**), SEC sections from mice received control vehicles (cremophore) revealed neoplastic masses from pleomorphic densely basophilic neoplastic cells, vacolated nucleus and increased mitotic division (**Figure 13b**). SEC sections from mice treated with oxamate (300 mg/kg) showed neoplastic masses from pleomorphic densely basophilic vacuolated nucleus neoplastic cells, with moderate necrosis of neoplastic masses (Fig. 13. c). SEC sections from mice treated with paclitaxel (20 mg/kg) revealed neoplastic masses showing moderate necrosis of neoplastic cells with nests pleomorphic densely basophilic neoplastic cells (Fig. 13. d). SEC sections from mice received a combination of oxamate and paclitaxel showed neoplastic masses showing severe necrosis of neoplastic cells (**Figure 13e**).

4. Discussion

There is a fundamental difference between tumor and normal cells regarding the metabolism and source of energy. While normal cells obtain the majority of required energy via mitochondrial oxidative phosphorylation and only utilize LDH-A when oxygen is sparse, tumor cells obtain their energy mainly via aerobic glycolysis.³⁴ This process of utilizing glycolysis as a source of energy for cell growth and proliferation even in the presence of oxygen is called "The Warburg effect." It is implicated in the inhibition of tumor suppressors and activation of oncogenes⁴. However, it provides a wide range of potential targets for therapy.³⁵⁻³⁷

This study investigated the effects of oxamate and galloflavin on the growth of MCF7 and OVCAR3 cell lines in order to confirm their cytotoxicity. It was found that addition of oxamate, galloflavin, or paclitaxel to the culture medium of MCF7 or OVCAR3 cell lines significantly decreased the cell survival compared to that of the control vehicles. Yang et al.

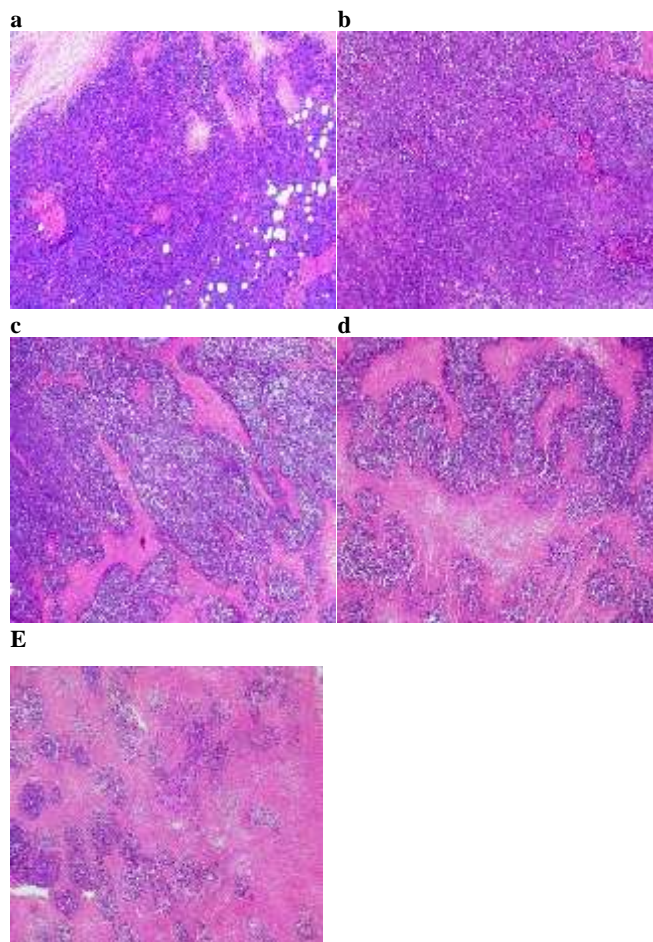


Figure 13: Effects of oxamate and/or paclitaxel on histopathological examination of SEC sections (photos a-e). (a) SEC sections from mice received control vehicles (saline) revealed neoplastic masses from pleomorphic densely basophilic neoplastic cells, little fibrous stroma and tumor giant cell formation (HE, 100x); (b) SEC sections from mice received control vehicles (cremophore) revealed neoplastic masses from pleomorphic densely basophilic neoplastic cells, vacolated nucleus and increased mitotic division (HE, 100x); (c) SEC sections from mice treated with oxamate (300 mg/kg) showed neoplastic masses from pleomorphic densely basophilic vacuolated nucleus neoplastic cells, with moderate necrosis of neoplastic masses (HE, 100x); (d) SEC sections from mice treated with paclitaxel (20 mg/kg) revealed neoplastic masses showing moderate necrosis of neoplastic cells with nests pleomorphic densely basophilic neoplastic cells (HE, 100x); (e) SEC sections from mice received a combination of oxamate and paclitaxel showed neoplastic masses showing severe necrosis of neoplastic cells(HE, 100x)

found that oxamate exerted a dose- and time-dependent impairment of the non-small cell lung cancer cells viability³⁸. Similarly, Zhai et al. and Li et al. reported that inhibition of LDH by oxamate suppressed cell viability and energy metabolism in nasopharyngeal carcinoma cells^{34, 39}. Similar results were reported in pituitary adenoma cells⁴⁰. Furthermore, Farabegoli et al. demonstrated that galloflavin not only inhibited cell growth and proliferation but also

increased the expression of proteins involved in apoptosis signals¹⁹.

In the present study, the activity of LDH enzyme was determined in MCF7 and OVCAR3 cell lines *in vitro*. It was found that the addition of oxamate or galloflavin to the culture medium of MCF7 or OVCAR3 cell lines caused a significant reduction in LDH enzyme activity compared to that of the control vehicles. These results are in agreement with Lu et al.,⁴¹ who showed that treatment of human pancreatic adenocarcinoma cells with oxamate reduced LDH-A activity through direct inhibition of LDH-A. It has been reported that oxamate decreased lactate production and increased pH, which in turn inhibited LDH.²⁷ Furthermore, Thornburg et al.¹³ reported that oxamate is a competitive inhibitor of human LDH-A. Li et al. confirmed that incubation of NPC cell lines with oxamate resulted in a time-dependent inhibition of LDH.³⁹

Galloflavin results are in agreement with the results of Farabegoli et al., and Manerba et al.^{19,22}; also, targeted inhibition of LDH by GF showed promising anti-tumor activity in endometrial cancer cell lines and primary cultures of endometrial cancer cells.⁴² Moreover, Fiume et al. found that GF strongly hindered the interaction of the enzyme with ssDNA in SW620 cells derived from human colorectal cancer.²⁰

Zhou et al. demonstrated that taxol treatment enhanced LDH-A mRNA expression in cancer cells¹⁵. Since LDH-A enhances glycolysis and hinders mitochondrial oxidative phosphorylation, it plays an important role in taxol resistance, and potentially its downregulation might overcome the taxol resistance in patients with breast cancer. It is also reported that oxamate treatment decreased the LDH activity and impaired the cell viability in a dose-dependent manner in both MDA-435 (breast cancer cells) and 435TR1 cells (taxol-resistant clones). However, the taxol-resistant 435TR1 cells were more sensitive to oxamate than MDA-435 cells. Although the efficiency of ATP generation via glycolysis is lower than that of oxidative phosphorylation, the ATP production rate of glycolysis is nearly 100 times faster than that of oxidative phosphorylation. In tumors, more ATP is required for cell growth and survival, thus elevated aerobic glycolysis differentiates malignant tumors from benign tumors and normal cells, a phenomenon that was identified by Otto Warburg⁴³ and has been repeatedly confirmed.⁴⁴

The present study investigated the effect of glycolytic inhibitors oxamate and galloflavin on ATP cellular content *in vitro*. It was detected that the addition of galloflavin to the culture medium of MCF7 cell line caused a significant reduction in ATP cellular content compared to that of the control vehicles. This result is in agreement with Manerba et al.,²² that owed the decreased cell viability observed with GF used to the impairment of aerobic glycolysis and the decrease in ATP synthesis, which were manifested by the reduced lactate production. In addition, Farabegoli et al. confirmed that one of the GF mechanisms of action was via ATP

depletion which resulted in down-regulation of the signaling pathways needed for cell survival ending with cell death.¹⁹

Malondialdehyde acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes.⁴⁵ It was reported to be higher in carcinomatous than normal tissue,⁴⁶ and their levels were linked with advanced clinical stages.⁴⁷ Therefore, MDA contents were found to be elevated in breast and ovarian cancer cell lines and in mice-bearing SEC. In the present study, LDH inhibitors significantly increased MDA content both *in vivo* and *in vitro*.

These results are in agreement with Le et al., who demonstrated that reduction of LDH-A expression by siRNA or inhibition of LDH-A by FX11 in P493 B-lymphoid cells resulted in an increase in oxygen consumption.⁸ Enhanced oxygen consumption was expected to increase the production of mitochondrial reactive oxygen species (ROS), particularly because glycolysis, which diverts pyruvate to lactate, diminishes cellular oxidative stress.⁴⁸ Le et al.,⁸ also found that reduction of LDH-A expression with siRNA markedly increased necrosis or late cell death.

In the present study, paclitaxel significantly increased MDA content both *in vivo* and *in vitro*. Paclitaxel induces cytotoxic effects by increased production of ROS and reactive nitrogen species.⁴⁹ These results are consistent with former studies, indicating that PTX can increase the production of ROS.⁵⁰⁻⁵⁴ Interleukin-17A was claimed to favor the tumor progression via promoting cancer cell proliferation, invasion, dissemination, and angiogenesis as well as resistance to chemotherapy.⁵⁵⁻⁵⁷ Moreover, Cochaud et al. reported that IL-17A to be a poor prognostic factor in breast cancer.⁵⁸ In the present study, oxamate resulted in a significant reduction in IL-17a cellular content. As a result of IL-17 reduction, oxamate caused a significant reduction in VEGF level.

Shime et al.⁵⁹ demonstrated that inhibition of LDH activity significantly reduced the concentration of lactic acid in the conditioned medium, and this was matched with a decreased ability of the conditioned medium to enhance toll-like receptor (TLR) ligand-stimulated IL-23p19 promoter activity. IL-23 then drives peptide-activated T cells to produce IL-17. Some reports stated that the enhanced production of pyruvate from lactate oxidation activates NF- κ B and HIF-1, lead to overexpression of some growth factors required for angiogenesis such as VEGF, basic fibroblast growth factor (bFGF), and stromal cell-derived factor-1 (SDF-1).^{60,61} In addition, Végran et al.⁶² demonstrated that lactate-stimulated NF- κ B activation in ECs was associated with IL-8-mediated autocrine angiogenesis and this pathway promoted EC migration and tube formation *in vitro*, as well as lactate-triggered tumor angiogenesis *in vivo*. Ghani et al.⁶³ demonstrated that VEGF release followed the lactate-induced reduction of the NAD⁺ /NADH via reduction of ADP-ribosylations. With or without oxygen, lactate-induced VEGF release from macrophages.⁶⁴ Accordingly, oxamate decreased VEGF content in SEC-bearing mice in the present study.

The present study reported that paclitaxel resulted in a significant increase in IL-17a cellular content compared to the normal control group. Vicari et al.⁶⁵ demonstrated that paclitaxel increased the efficacy of a toll-like receptor 9 (TLR 9) agonist in a model of renal cell carcinoma by reducing the number and inhibiting the activity of regulatory T cells in a TLR 4-independent manner. This synergism was associated with decreased IL-10 expression and increased IL-17 secreting CD4+ T cells.

Our results revealed a strong positive correlation between IL-17a and VEGF content in groups that received oxamate, paclitaxel, and oxamate/paclitaxel combination. Apoptosis is a process of programmed cell death regulated by many intracellular pathways such as Bax, Bcl-2, caspases, and p53 gene.⁶⁶ It is a predominant mechanism by which cancer chemotherapeutic agents kill cells.⁶⁷ Thornburg et al.,¹³ Zhou et al.¹⁵ reported that oxamate is able to induce apoptotic cell death. Sheng et al. demonstrated that LDH-A inhibition induced the production of ROS and cytosolic Ca²⁺, which decreased the inner mitochondrial membrane potential. That led to activation of caspase-9 and caspase-3 which ended in apoptosis.⁶⁸

In our study, oxamate/ paclitaxel combination showed significant elevation in caspase 3 compared to monotherapy indicating increased apoptosis. Similarly, a significant increase of the levels of cleaved PARP after treatment with the combination of taxol and oxamate compared to treatment with a single agent.¹⁵

5. CONCLUSION

In conclusion, oxamate or galloflavin induced a significant decrease in survival of MCF7 and OVCAR3 cell lines, caused a significant increase in MDA content, significant decrease in LDH and ATP cellular content. Moreover, treatment of SEC with LDH inhibitors increased MDA content, reduced IL-17A. Oxamate also prompted apoptosis in SEC tissue and suppressed angiogenesis. Furthermore, LDH inhibitor potentiated the anticancer effect of taxol through inhibition of LDH enzyme, which was upregulated by taxol, also through reduction of inflammatory mediators and ATP, as well as apoptosis and anti-angiogenesis. Therefore, LDH inhibitors may represent excellent adjuvants to paclitaxel for the treatment of certain types of cancers.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution:

Study design: Alaa E. El-Sisi, Samia S. Sokar, Sara R. El-Mahrouk, Sally E. Abu-Risha. Practical experiments and data analysis: Sara R. El-Mahrouk, Sally E. Abu-Risha. Writing manuscript: Alaa E. El-Sisi, Samia S. Sokar, Sara R. El-Mahrouk, Sally E. Abu-Risha

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