Inhibition of LDH-A by oxamate induces G_2/M arrest, apoptosis and increases radiosensitivity in nasopharyngeal carcinoma cells

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Abstract. An elevated rate of glucose consumption and the dependency on aerobic glycolysis for ATP generation have long been observed in cancer cells, a phenomenon known as the Warburg effect. The altered energy metabolism in cancer cells provides an attractive opportunity for developing novel cancer therapeutic strategies. Lactate dehydrogenase (LDH), which catalyzes the transformation of pyruvate to lactate, plays a vital role in the process of glycolysis. It has been reported that the level of LDH-A expression is increased both in head and neck cancer cells and in the blood serum of nasopharyngeal carcinoma (NPC) patients, and is associated with poor prognosis. However, the effect of LDH-A inhibition on NPC cells remains unknown. Here, in the present study, we found that oxamate, a classical inhibitor of LDH-A, suppressed cell proliferation in a dose- and time-dependent manner both in CNE-1 and CNE-2 cells, two NPC cancer cell lines. LDH inhibition by oxamate induced G_2/M cell cycle arrest via downregulation of the CDK1/cyclin B1 pathway and promoted apoptosis through enhancement of mitochondrial ROS generation. N-acetylcysteine, a specific scavenger of ROS, significantly blocked the growth inhibition effect induced by oxamate. We also identified that oxamate increased sensitivity to ionizing radiation in the two NPC cancer cell lines. Furthermore, we verified similar results in tumor xenograft models. Collectively, these results suggest that LDH-A may serve as a promising therapeutic target for NPC treatment.

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Introduction

Nasopharyngeal carcinoma (NPC) is a squamous-cell carcinoma of the head and neck region, most common in Southern China and Southeast Asia (1). Radiotherapy and chemotherapy are typical conventional treatment for NPC.However, the 5-year survival rate of NPC is ~70% and local recurrence and distant metastases are the main causes of treatment failures, indicating that new therapeutic targets must be explored and developed (2).

An elevated rate of glucose consumption and the dependency on aerobic glycolysis for ATP generation are noticeable hallmarks of cancer cells, a phenomenon known as the Warburg effect. In recent years, it was also observed that glycolysis was promoted in NPC cells, and a higher ^{18}F-FDG uptake was associated with distant failure and poor prognosis in NPC patients (3,4). Since the metabolic alteration provides cancer cells with enough energy and biosynthetic precursors, metabolic enzymes involved in glycolysis have become therapeutic targets with great potential (5).

Lactate dehydrogenase (LDH), including two subunits LDH-A and LDH-B, is an enzyme widely existing in human cells and tissues. It catalyzes the transformation of pyruvate to lactate accompanied by conversion of NADH to NAD^+, which plays a vital role in the process of glycolysis. It has long been noted that LDH-A expression is upregulated in human neoplastic tissues (6). Recently, an increasing number of studies indicate that LDH-A plays an essential role in tumor maintenance, growth and progression (7-10). Furthermore, relevant studies have demonstrated that inhibition of LDH-A induces oxidative stress and suppresses tumor growth in a variety of cancer cell lines (9,11-13). Regarding NPC, LDH-A was reported to be highly expressed in head and neck cancer cells and was found to be associated with local relapse, worse survival and distant metastasis (14). In addition, several studies have also demonstrated that an increased LDH serum level is a poor prognostic factor in NPC patients (15,16). However, to date, there is no research on whether inhibition of LDH-A impairs the growth of NPC cancer cells.

In previous studies, oxamate, a competitive LDH-A inhibitor, has been shown to inhibit the growth of cervical, breast and liver cancer cells *in vitro* (17-19). Moreover, oxamate also significantly enhanced the sensitivity of cancer cells to several chemotherapy agents (20-22). Here, in the present study, the effect of LDH-A inhibition by oxamate on NPC cells was explored, and the involved mechanisms were evaluated. Moreover, the influence of oxamate on the radiosensitivity of NPC cells was examined since radiotherapy is the main treatment strategy for NPC. Finally, a tumor mouse model was employed to test the inhibitive effect of oxamate *in vivo*.

Materials and methods

Reagents and cell lines. Oxamate sodium was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). CNE-1 and CNE-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal calf serum (FCS), 5 mmol/l L-glutamine, 5 mmol/l non-essential amino acids, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C under 5% CO_2.

MTT assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed in 96-well plates to test cell viability. Cells were seeded at 10^4/well and treated with different concentrations of oxamate sodium (0-100 mmol/l) for 24, 48 and 72 h, respectively. Then 20 μl of MTT solution (5 mg/l) was added to each well, and the plates were incubated at 37˚C for another 4 h. The supernatant was discarded, and 150 μl dimethyl sulfoxide was added for 10 min. The absorbance was measured using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 570 nm. The cell viability is expressed as the percentage of untreated control cells.

Colony formation assay. Cells pretreated with 20 mmol/l oxamate sodium for 24 h were irradiated at 0-9 Gy with 6 MV X-rays from a linear accelerator (Siemens, Munich, Germany), with a dose rate of 200 cGy/min. The cells were then seeded at different densities according to the IR dose. After 14 days of incubation at 37˚C, the cells were fixed, stained with Giemsa and counted. Colonies were scored only when containing ≥50 cells. Experiments were conducted in triplicate. Survival fractions were fitted into a linear quadratic model using GraphPad Prism software (version 5.0).

Detection of LDH activity. Intracellular LDH activity was determined using an LDH activity assay kit (BioVision, Tucson, AZ, USA). The assay is based on an enzymatic coupling reaction in which LDH reduces NAD to NADH, which then reacts with a specific probe to generate a color (λ_{max} = 450 nm). Cells treated with different concentrations of oxamate were harvested and lysed for protein to measure the LDH activity. Results were normalized to the protein concentrations of the cell lysate, and the protein concentrations were determined using a BCA protein assay kit (Beyotime, Haimen, China).

Measurement of intracellular ATP. Intracellular ATP was measured using a firefly luciferase-based ATP assay kit (Beyotime) following the manufacturer's instructions. Briefly, cells were cultured in DMEM with different concentrations of oxamate sodium (0, 20, 50 and 100 mmol/l). After 24 h, cells were lysed and centrifuged at 12,000 x g for 5 min. The supernatant (100 μl) was transferred to a 24-well plate mixed with 100 μl ATP detection working dilution. Luminescence was measured by a microplate reader (Bio-Tek Instruments, Inc.). The protein concentration of each group was also determined using a BCA protein assay kit. The relative ATP level is expressed as ATP value/protein value.

Analysis of cell cycle distribution. Cells were collected at 24 h after treatment with different concentrations of oxamate sodium, then fixed with 70% ethanol and stored overnight at 4˚C. After centrifugation and washing with 1X PBS twice, the fixed cells were stained with 500 μl of propidium iodine (PI) (10 μg/ml; Sigma-Aldrich) for 30 min in the dark. Cell cycle distribution was analyzed using 10,000 cells by flow cytometry with the FACSCalibur system (Becton-Dickinson, San Jose, CA, USA).

Analysis of apoptosis. Apoptosis was tested using the Annexin V-FITC apoptosis kit (BD Biosciences, San Jose, CA, USA). Cells treated with different concentrations of oxamate sodium for 48 h were harvested and stained with Annexin V/PI for 30 min. The apoptotic fraction was detected by a flow cytometer with the FACSCalibur system.

Assay of reactive oxygen species (ROS). ROS content was tested using an ROS detection kit (Beyotime), based on the intracellular peroxide-dependent oxidation of DCFH-DA to form the fluorescent compound 2',7'-dichlorofluorescein (DCF). Cells treated with 0, 20, 50 and 100 mmol/l oxamate sodium were collected and incubated with 1 ml DCFH-DA (20 μM) for 30 min. Fluorescence intensity was detected by flow cytometry.

Western blot analysis. Cells were harvested and suspended in IP lysing buffer (Beyotime) containing protease inhibitor (25 mg/ml; Roche, Mannheim, Germany). The protein concentration was measured using the BCA protein assay kit. Then 50 μg of total protein was separated on SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in PBS with 5% non-fat dry milk for 45 min, the membranes were incubated with relevant antibodies for 1 h. Signals were detected using an ECL western blotting kit (Beyotime). The following antibodies were utilized: anti-cyclin B1 mouse antibody (1:500; Santa Cruz Biotechnology), anti-CDK1 mouse antibody (1:500; Santa Cruz Biotechnology), anti-Bcl-2 mouse antibody (1:500; Santa Cruz Biotechnology, anti-Bax mouse antibody (1:500; Santa Cruz Biotechnology), anti-caspase-3 rabbit antibody (1:1,000; Cell Signaling Technology), anti-cleaved-caspase-3 rabbit antibody (1:1,000; Cell Signaling Technology), anti-β-actin antibody (1:2,000; Santa Cruz Biotechnology), anti-mouse antibody (1:1,000; Santa Cruz Biotechnology) and anti-rabbit antibody (1:1,000; Santa Cruz Biotechnology).

Animal experiments. Female Balb/c nude mice weighing 18-22 g at 4-6 weeks of age were purchased from the Institute

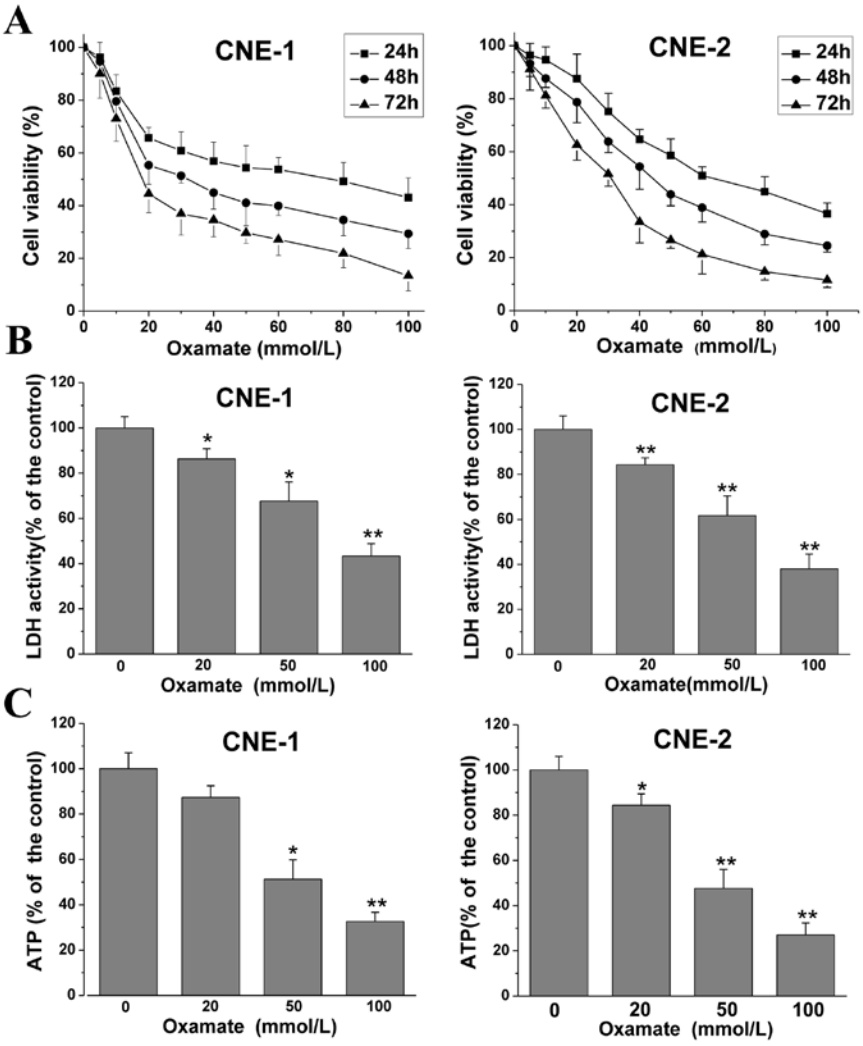

Figure 1. Oxamate inhibits cell growth, LDH enzyme activity and decreases ATP levels in CNE-1 and CNE-2 cells. (A) Cells were exposed to different concentrations of oxamate for 24, 48 and 72 h, in 96-well plates, and the effects on cell viability were tested by MTT assay. Cell viability was calculated as a percentage of untreated cells (100%); values are represented as means ± SD; n=6. (B) Cells were treated with 0, 20, 50 and 100 mM oxamate for 24 h, then intracellular LDH enzyme activity was determined using a commercially available kit. Values are represented as means ± SD; n=3. *P<0.05, **P<0.01 vs. the untreated control. (C) Cells were treated with 0, 20, 50 and 100 mM for 24 h, and intracellular ATP levels were assayed by a firefly luciferase-based ATP assay kit. Values are represented as means ± SD; n=3. *P<0.05, **P<0.01 vs. the untreated control. Each experiment was repeated three times and performed in triplicate. LDH, lactate dehydrogenase.

of Laboratory Animal Sciences (Shanghai, China) and were raised under specific pathogen-free environments. CNE-1 cells ($1x10^6$) suspended in 100 μl of 1:1 DMEM culture and Matrigel (BD Biosciences) were injected subcutaneously into the flanks of the nude mice. When tumor sizes reached ~100 mm^3, 24 mice were randomly divided into 4 groups (n=6 mice/group) and treated with PBS, oxamate, irradiation, or oxamate combined with irradiation, respectively. Oxamate was intraperitoneally injected at 750 mg/kg daily for 3 weeks, and mice were irradiated with 3.3 Gy X-rays for a consecutive 3 days (total dose of 9.9 Gy) 2 h after injection of oxamate from the second day of drug administration using a 6-MV linear accelerator (Siemens). Tumor sizes and mouse body weights were measured every 3 days for 5 weeks. Tumor volumes were calculated using the formula: Volume = length x $width^2/2$.

Statistical analysis. Each experiment was repeated three times and performed in triplicate. Data are expressed as the means ± standard deviation (SD). Differences between two groups were assessed by the two-tailed Student's t-test. All of the statistical analyses were performed using SPSS version 17.0. A P-value <0.05 was considered to indicate a statistically significant result.

Results

Inhibition of LDH by oxamate suppresses cell viability and energy metabolism in NPC cells. First, to explore the effect of oxamate on cell proliferation in NPC cells, MTT assays were conducted. CNE-1 and CNE-2 cancer cells were treated with different doses of oxamate for 24-72 h. As shown in Fig. 1A, treatment with increasing concentrations of oxamate inhibited cell proliferation in a dose- and time-dependent manner in both NPC cancer cell lines. The IC_{50} values were 74.6, 32.4 and 17.8 mmol/l and 62.3, 44.5, 31.6 mmol/l at 24, 48 and 72 h in the CNE-1 and CNE-2 cancer cells, respectively. Then, to

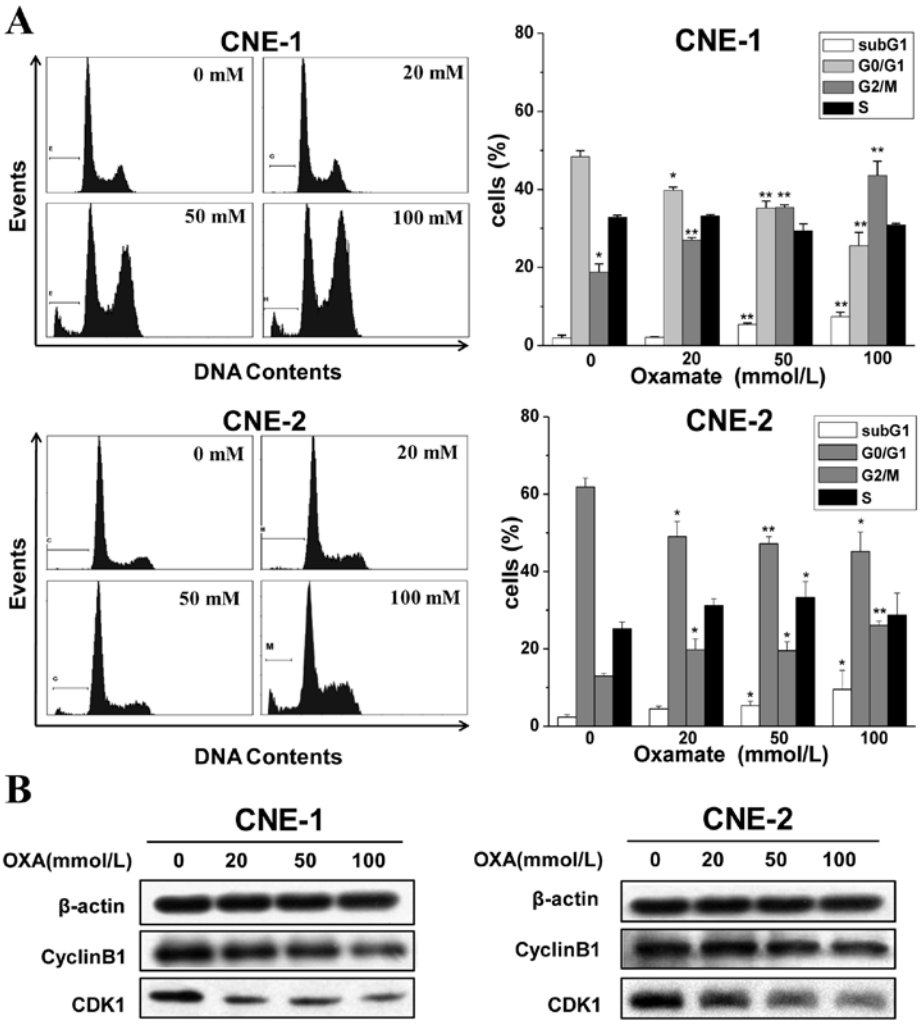

Figure 2. Inhibition of LDH by oxamate induces cell cycle arrest in the G_2/M phase in CNE-1 and CNE-2 cells. (A) Cells were exposed to 0, 20, 50 and 100 mM oxamate for 24 h, and then cells were stained with propidium iodide and analyzed using flow cytometry. Values are represented as means ± SD; n=3. *P<0.05, **P<0.01 vs. the untreated control. Each experiment was repeated three times and performed in triplicate. (B) Western blot analysis was used to detect the expression of cyclin B1 and CDK1, proteins related to G_2/M cell cycle arrest. β-actin was used as the loading control. LDH, lactate dehydrogenase; OXA, oxamate.

confirm the LDH inhibition effect of oxamate as previously reported (18,19), a commercially available kit was used to determine the intracellular LDH enzyme activity after treatment with different doses of oxamate. The results showed that oxamate significantly decreased LDH activity (Fig. 1B), which indicated that oxamate was an inhibitor of human LDH enzyme and provided evidence of the LDH inhibitory effect by oxamate for the subsequent experiments. Fig. 1C shows that oxamate markedly reduced the intracellular ATP levels. Relative ATP levels in the 20, 50 and 100 mmol/l oxamate-treated groups were 87.3±5.2, 51.3±8.5 and 32.7±4.1%, respectively, when compared to the untreated control group (100±7.0%) (P=0.21, 0.025 and 0.007) in the CNE-1 cell line, and 84.3±5.0, 47.6±8.3 and 27±5.3%, when compared to the untreated control group (100±6.2%) (P=0.002, 0.001 and <0.001) in the CNE-2 cell line. The results demonstrated that LDH inhibition by oxamate disturbed energy metabolism and decreased ATP production in the NPC cells.

Oxamate induces G_2/M arrest via downregulation of CDK1/cyclin B1 in NPC cells. Since a great majority of anticancer agents act on the cell cycle and its checkpoint (23), alterations in cell cycle distribution after oxamate treatment were investigated in NPC cancer cells. Cells were exposed to 0, 20, 50 and 100 mmol/l oxamate for 24 h, and flow cytometry was used to analyze the cell cycle changes after PI staining. As shown in Fig. 2A, there was a dose-dependent increase in the numbers of CNE-1 and CNE-2 cells in the G2/M phase after a 24-h treatment with oxamate at concentrations of 0, 20, 50 and 100 mmol/l. The percentages of cells in the G2/M phase were 18.8±2.1, 26.9±1.6, 35.4±2.3, 43.6±3.6, respectively, in the CNE-1 cells and 13.0±3.2, 19.7±2.7, 21.5±2.3 and 26.1±1.1%, in the CNE-2 cells, respectively. Meanwhile, the proportions of cells in the G_0/G_1 phase were decreased. However, the S phase fractions did not change significantly in both cell lines. In addition, it was noteworthy that there was a significant increase in the percentage of cells in the sub-G_1 phase after treatment with oxamate.

To further certify the G_2/M arrest induced by oxamate and to understand the mechanisms, western blot analysis was used to examine the changes in expression of proteins related to G_2/M transition. As shown in Fig. 2B, the expression levels

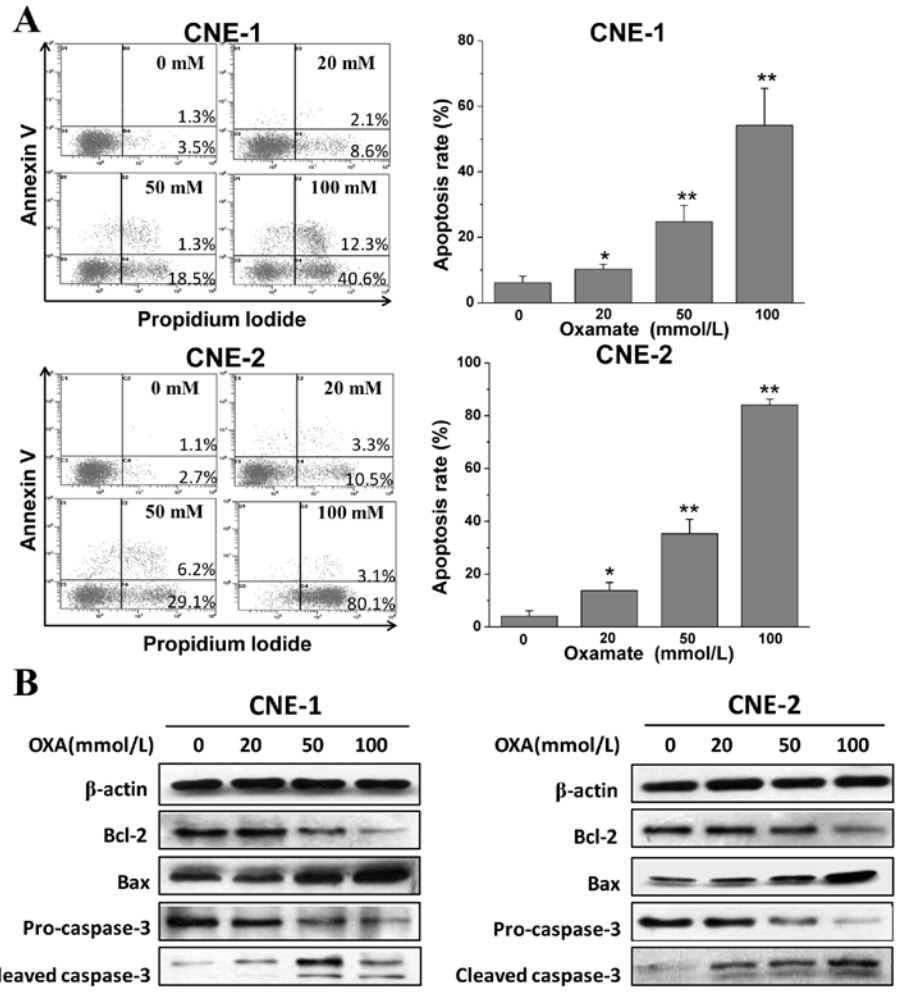

Figure 3. LDH inhibition by oxamate induces apoptosis in CNE-1 and CNE-2 cells. (A) Cells were treated with 0, 20, 50 and 100 mM oxamate for 48 h, and then cells were stained with Annexin V-FITC and propidium iodide and analyzed using flow cytometry. Values are represented as means ± SD; n=3. *P<0.05 **P<0.01 vs. the untreated control. Each experiment was repeated three times and performed in triplicate. (B) Western blot analysis was employed to detect the expression of Bax, Bcl-2, pro-caspase-3 and cleaved caspase-3, proteins related to apoptosis through the mitochondrial pathway. β-actin was used as the loading control. LDH, lactate dehydrogenase; OXA, oxamate.

of cyclin B1 and CDK1 significantly decreased in both the CNE-1 and CNE-2 cells after treatment with oxamate, which suggests that oxamate induces G_2/M arrest by modulating the expression of cyclin B1 and CDK1.

Oxamate induces apoptosis via the mitochondrial pathway. We demonstrated that LDH inhibition by oxamate impaired cell growth and increased the sub-G_1 fraction in both cell lines. To confirm that oxmate treatment induces apoptosis, cells were exposed to different concentrations of oxamate for the prolonged time of 48 h, and then Annexin V/PI staining and flow cytometric analysis were performed. After the 48-h treatment with oxamate, the percentages of early and late apoptotic cells were increased in a dose-dependent manner (Fig. 3A). Next, western blot analysis was utilized to determine the changes in expression of apoptosis-related proteins. As shown in Fig. 3B, the expression of pro-apoptotic Bax and cleaved-caspase-3 was significantly enhanced, while the anti-apoptotic signals of Bcl-2 and pro-caspase-3 were reduced notably after treatment with different concentrations of oxamate for 48 h. The results indicate that oxamate induces apoptosis via caspase-3 activation and the mitochondrial pathway in NPC cells.

Oxamate increases ROS levels in NPC cells. To further explore the mechanisms involving the inhibitory effect induced by oxamate in NPC cells, we determined the changes in ROS levels after oxamate treatment, which play an important role in the mitochondrial apoptotic pathway. As shown in Fig. 4A, oxamate dose-dependently enhanced ROS levels in both NPC cell lines. ROS levels were increased to 1.3-, 2.4- and 3.1-fold (P<0.01) after treatment with 20, 50 and 100 mmol/l oxamate for 24 h, when compared to the untreated control group. Similarly, there was an 1.5- to 3.3-fold increase (P<0.01) in the CNE-2 cells. To examine the role of ROS generation in the oxamate-induced growth inhibitory effect, oxamate-treated cells were incubated simultaneously with 10 mM N-acetylcysteine, a specific scavenger of ROS. As shown in Fig. 4B, pre-treatment with NAC significantly blocked the growth inhibitory effect in both CNE-1 and CNE-2 cell lines, indicating that ROS generation contributes partially to the anti-proliferative effect induced by oxamate.

Oxamate increases sensitivity to ionizing radiation in NPC cells. Radiotherapy is the main treatment for NPC patients at present. Thus, we examined whether LDH inhibition by

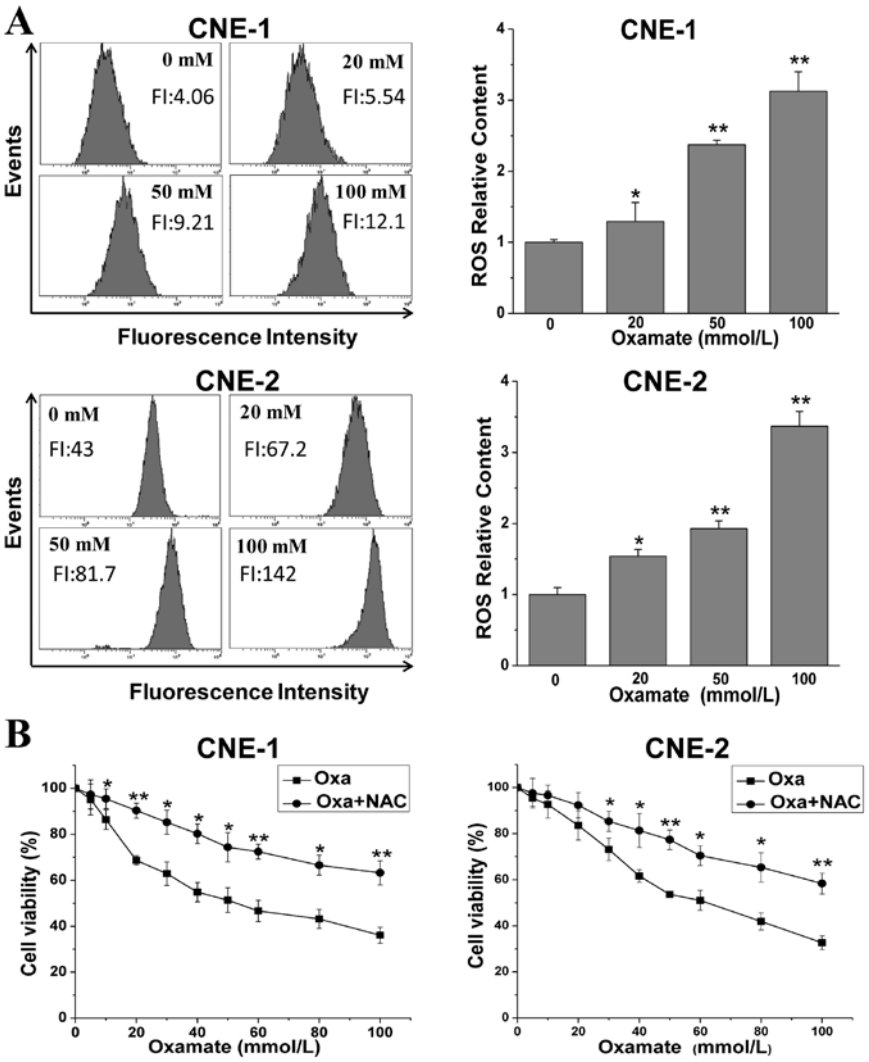

Figure 4. Induction of ROS levels by oxamate and its role in cell survival. (A) LDH inhibition by oxamate increased ROS production. Cells were treated with 0, 20, 50 and 100 mM oxamate for 24 h, and intracellular ROS levels were determined by DCFH-DA fluorescence as analyzed using flow cytometry. Values are represented as means ± SD; n=3. *P<0.05, **P<0.01 vs. the untreated control. Each experiment was repeated three times and performed in triplicate. (B) Growth inhibitory effect of oxamate was recovered by NAC. Cells were treated with different concentrations of oxamate combined with 10 mM NAC or without for 24 h in 96-well plates. Cell viability was then determined by MTT assay. Values are represented as means ± SD; n=6. *P<0.05, **P<0.01 vs. oxamate alone. ROS, reactive oxygen species; LDH, lactate dehydrogenase; OXA, oxamate.

oxamate influences the sensitivity of NPC cells to ionizing radiation. Firstly, Annexin-V/PI staining was conducted to observe the effect of oxamate on the apoptosis induced by irradiation within a short period of time, and then colony formation assays were performed to evaluate the long-term effects of the combined treatment. As shown in Fig. 5A the combination of irradiation and oxamate synergistically enhanced the apoptosis rates in both NPC cancer cells, when compared to either treatment alone. Furthermore, oxamate enhanced the IR-induced inhibition of clonogenic survival in NPC cells (Fig. 5B). The sensitivity enhancement ratios (SERs) were 1.26 and 1.35 in CNE-1 and CNE-2 cells, respectively, as determined by analyzing the linear quadratic models. These results revealed that oxamate increased the radiosensitivity in NPC cells *in vitro*, and the effects were similar in both CNE-1 and CNE-2 cells.

Oxamate improves the efficacy of irradiation in vivo. Finally, we examined whether oxamate inhibits tumor growth *in vivo* and its effects when combined with irradiation treatment. As shown in Fig. 6A, oxamate effectively delayed tumor growth *in vivo*. Moreover, combined treatment with oxamate and irradiation significantly improved the growth inhibitory effect when compared to either oxamate alone or irradiation alone. As shown in Fig. 6B that inhibition by oxamate had little influence on the body weights of the mice. A small decrease in body weights of the mice was noted following treatment with oxamate and irradiation. However, the decrease had no statistical significance when compared to the mice treated with oxamate alone.

Discussion

In the present study, we investigated the role of LDH inhibition by oxamate and its effect on radiosensitivity in two human NPC cell lines. We found that oxamate induced G_2/M cell cycle arrest via downregulation of the CDK1/cyclin B1 pathway and promoted apoptosis through enhancing mitochondrial ROS

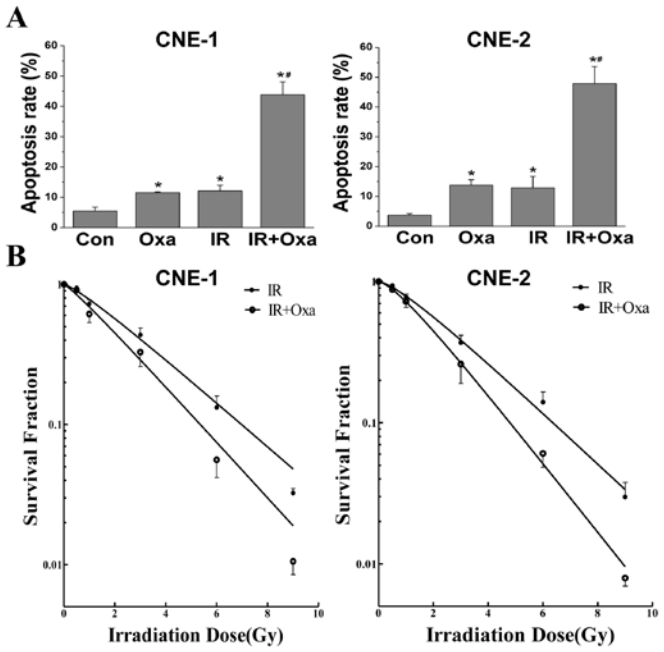

Figure 5. Oxamate increases the radiosensitivity of both CNE-1 and CNE-2 cell lines to X-rays. (A) Oxamate increases IR-induced apoptosis. Cells were pretreated with 20 mM oxamate for 24 h and exposed to 6 Gy X-ray irradiation. After incubation for another 24 h, cells were stained with Annexin V and propidium iodide and analyzed using flow cytometry. Values are represented as the means ± SD; n=3. *P<0.05 vs. the untreated control; $^{\#}$P<0.05, vs. IR alone. Each experiment was repeated three times and performed in triplicate. (B) Effects of oxamate on radiation survival curves. Cells were pretreated with 20 mM oxamate for 24 h and exposed to 0-9 Gy X-ray irradiation. Cells were incubated for another 2 weeks before fixation, staining and assessment of colony formation. Survival curves were constructed according to the linear quadratic model. Values are represented as means ± SD; n=3. Each experiment was repeated three times and performed in triplicate. Con, control; Oxa, oxamate; IR, irradiation.

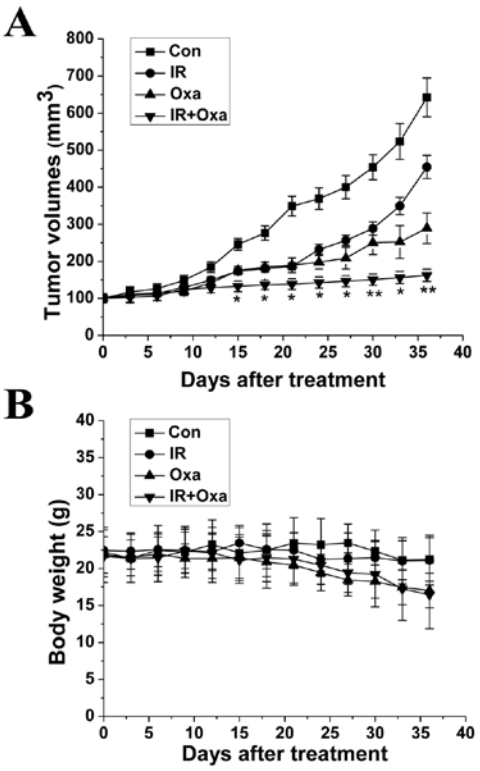

Figure 6. Combined treatment with oxamate and irradiation effectively inhibits tumor growth *in vivo*. (A) Mice bearing CNE-1 tumors received oxamate (750 mg/kg) i.p. for 3 weeks with or without irradiation of tumors (3.3 Gy x 3 fractions) for 3 consecutive days from the second day of drug administration. The control group received equal volumes of PBS (i.p.). Values are represented as means ± SD; n=6. *P<0.05, **P<0.01 vs. irradiation alone. (B) Body weight changes in mice in each group. Values are represented as means ± SD; n=6. Con, control; Oxa, oxamate; IR, irradiation.

generation. We also determined that oxamate increased sensitivity to irradiation. Furthermore, we verified similar results in the tumor xenograft model. These results indicate that LHA may serve as an attractive therapeutic target for NPC treatment.

Although the exact molecular mechanism involved in the Warburg effect remains to be explored, the altered energy metabolism in cancer cells provides an opportunity for developing novel cancer therapeutic strategies by targeting the glycolytic pathway (5). Previous studies revealed that LDH-A expression is elevated both in squamous cell head and neck cancer cells and the blood serum of NPC patients and is associated with poor prognosis (4,15,16). As anticipated, LDH inhibition by oxamate was found to impair cell growth and increase ROS production in NPC cells. Reactive oxygen species (ROS) are highly reactive molecules and free radicals containing oxygen. ROS play an important role in mitochondrial-mediated apoptosis. Moreover, mitochondria can be both a source and a target of ROS (24). As previously described, LDH is a crucial enzyme transforming pyruvate to lactate in the anaerobic glycolysis pathway. More pyruvates are forced to enter into the tricarboxylic acid (TCA) cycle when LDH is inhibited by oxamate. However, mitochondrial dysfunction and impaired oxidative phosphorylation are quite common in cancer cells, as demonstrated in many studies (25-27). In this case, the OXPHOS chains are abnormally activated and more ROS are generated, resulting in mitochondrial membrane destruction and subsequent apoptosis. In the present study, caspase-3 activation was observed by western blot analysis, and the growth inhibitory effect induced by oxamate was blocked by the ROS scavenger NAC. These data indicate that LDH inhibition by oxamate induces ROS-mediated intrinsic mitochondrial apoptosis in NPC cells.

The cell cycle involves a series of events driven by cyclins and subsequent cyclin-dependent kinases (CDKs), which further activate transcription factor proteins for cell cycle progression from one phase to another. The CDK1/cyclin B1 kinase complex plays a major role during G_2/M transition. A reduction in CDK1/cyclin B1 kinase activity triggers G_2/M cell cycle arrest (28). In the present study, LDH inhibition by oxamate induced G_2/M arrest and also a decrease in the protein levels of cyclin B1 and CDK1 in NPC cells. Thus, it is rational to postulate that G_2/M arrest induced by oxamate was mediated by reducing the activity of the CDK1/cyclin B1 kinase complex. In the present study, cell cycle progression was correlated closely with the status of energy metabolism, although upstream signals regulating the G_2/M+related proteins remain to be further investigated. Notably, dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase also induced G_2/M arrest in colorectal cancer cells in a previous study (29).

Radiotherapy plays a vitally important role in NPC treatment. Unfortunately, radiation resistance always results in subsequent recurrence and metastasis of cancer, which leads to treatment failure. It has been reported that inhibition of LDH sensitizes cancer cells to chemotherapy (18-20). However, the relationship between LDH inhibition and radiosensitivity is still unclear. In the present study, we found that oxamate significantly increased sensitivity to X-ray irradiation in NPC cells both *in vitro* and *in vivo*. As known, the 'oxygen effect' is an important phenomenon in radiation biology, which refers to the enhanced killing effect of radiation in the presence of oxic conditions. Irradiation exposure can cause DNA damage as well as mitochondrial-dependent ROS generation. In addition, ROS are also critical mediators of radiation-induced cellular toxicity (30,31). Therefore, ROS play an important role in regulating the radiosensitivity of tumor cells (32). The results in the present study showed that LDH inhibition by oxamate induced increased ROS production and mitochondrial apoptosis in NPC cells. Thus, it is reasonable to assume that the increased ROS levels induced by oxamate synergistically enhanced the DNA damage effect and toxicity of irradiation in NPC cells. Other studies also found that glycolysis inhibitors increased radiosensitivity in cancer cells (33-35). Sharma *et al* (34) reported that non-coordinated expression of antioxidant enzymes was another essential factor that led to selective radiosensitization in malignant cells, in addition to redox status.

Oxamate is a conventional competitive inhibitor of LDH-A at high concentrations, which limits its therapeutic potential in clinical practice (18,36,37). However, the present study indicates that targeting LDH-A may be a feasible therapeutic strategy for the treatment of NPC. New effective small molecules specifically targeting LDH-A are being developed, including several active compounds from Chinese traditional herbal medicine. Some have shown promising clinical utility (38-40).

In conclusion, the present study provides evidence for LDH-A inhibition in the treatment of NPC alone or combined with irradiation both *in vitro* and *in vivo*. Targeting glycolysis may be an effective strategy for NPC therapy. Further studies are required to explore whether inhibition of other key enzymes in the glycolytic pathway has similar effects as the inhibition of LDH-A. Further efforts are needed to develop highly effective novel inhibitors of the LDH-A enzyme.

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References

1. Wei WI and Sham JS: Nasopharyngeal carcinoma. Lancet 365: 2041-2054, 2005.
2. Guigay J: Advances in nasopharyngeal carcinoma. Curr Opin Oncol 20: 264-269, 2008.
3. Xie P, Yue JB, Fu Z, Feng R and Yu JM: Prognostic value of ^{18}F-FDG PET/CT before and after radiotherapy for locally advanced nasopharyngeal carcinoma. Ann Oncol 21: 1078-1082, 2010.
4. Chan SC, Chang JT, Wang HM, *et al*: Prediction for distant failure in patients with stage M0 nasopharyngeal carcinoma: the role of standardized uptake value. Oral Oncol 45: 52-58, 2009.
5. Pelicano H, Martin DS, Xu RH and Huang P: Glycolysis inhibition for anticancer treatment. Oncogene 25: 4633-4646, 2006.
6. Goldman RD, Kaplan NO and Hall TC: Lactic dehydrogenase in human neoplastic tissues. Cancer Res 24: 389-399, 1964.
7. Fantin VR, St-Pierre J and Leder P: Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9: 425-434, 2006.
8. Zhao YH, Zhou M, Liu H, *et al*: Upregulation of lactate dehydrogenase A by ErbB2 through heat shock factor 1 promotes breast cancer cell glycolysis and growth. Oncogene 28: 3689-3701, 2009.
9. Le A, Cooper CR, Gouw AM, *et al*: Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc Natl Acad Sci USA 107: 2037-2042, 2010.
10. Serganova I, Rizwan A, Ni XH, *et al*: Metabolic imaging: a link between lactate dehydrogenase A, lactate, and tumor phenotype. Clin Cancer Res 17: 6250-6261, 2011.
11. Wang ZY, Loo TY, Shen JG, *et al*: LDH-A silencing suppresses breast cancer tumorigenicity through induction of oxidative stress mediated mitochondrial pathway apoptosis. Breast Cancer Res Treat 131: 791-800, 2012.
12. Xie H, Valera VA, Merino MJ, *et al*: LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer. Mol Cancer Ther 8: 626-635, 2009.
13. Sheng SL, Liu JJ, Dai YH, Sun XG, Xiong XP and Huang G: Knockdown of lactate dehydrogenase A suppresses tumor growth and metastasis of human hepatocellular carcinoma. FEBS J 279: 3898-3910, 2012.
14. Koukourakis MI, Giatromanolaki A, Winter S, Leek R, Sivridis E and Harris AL: Lactate dehydrogenase 5 expression in squamous cell head and neck cancer relates to prognosis following radical or postoperative radiotherapy. Oncology 77: 285-292, 2009.
15. Zhou GQ, Tang LL, Mao YP, *et al*: Baseline serum lactate dehydrogenase levels for patients treated with intensity-modulated radiotherapy for nasopharyngeal carcinoma: a predictor of poor prognosis and subsequent liver metastasis. Int J Radiat Oncol Biol Phys 82: e359-e365, 2012.
16. Wan XB, Wei L, Li H, *et al*: High pretreatment serum lactate dehydrogenase level correlates with disease relapse and predicts an inferior outcome in locally advanced nasopharyngeal carcinoma. Eur J Cancer 9: 2356-2364, 2013.
17. Papaconstantinou J and Colowick SP: The role of glycolysis in the growth of tumor cells. II. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J Biol Chem 236: 285-288, 1961.
18. Thornburg JM, Nelson KK, Clem BF, *et al*: Targeting aspartate aminotransferase in breast cancer. Breast Cancer Res 10: R84, 2008.
19. Fiume L, Manerba M, Vettraino M and Di Stefano G: Impairment of aerobic glycolysis by inhibitors of lactic dehydrogenase hinders the growth of human hepatocellular carcinoma cell lines. Pharmacology 86: 157-162, 2010.
20. Zhou M, Zhao YH, Ding Y, *et al*: Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes Taxol-resistant cancer cells to Taxol. Mol Cancer 9: 33, 2010.
21. Fiume L, Vettraino M, Manerba M and Di Stefano G: Inhibition of lactic dehydrogenase as a way to increase the anti-proliferative effect of multi-targeted kinase inhibitors. Pharmacol Res 63: 328-334, 2011.
22. Zhao YH, Liu H, Liu ZX, *et al*: Overcoming trastuzumab resistance in breast cancer by targeting dysregulated glucose metabolism. Cancer Res 71: 4585-4597, 2011.
23. Shapiro GI and Harper JW: Anticancer drug targets: cell cycle and checkpoint control. J Clin Invest 104: 1645-1653, 1999.
24. Simon HU, Haj-Yehia A and Levi-Schaffer F: Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5: 415-418, 2000.
25. Modica-Napolitano JS and Singh KK: Mitochondrial dysfunction in cancer. Mitochondrion 4: 755-762, 2004.
26. Pelicano H, Lu W, Zhou Y, *et al*: Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism. Cancer Res 69: 2375-2383, 2009.

27. Hung WY, Huang KH, Wu CW, *et al*: Mitochondrial dysfunction promotes cell migration via reactive oxygen species-enhanced β5-integrin expression in human gastric cancer SC-M1 cells. Biochim Biophys Acta 1820: 1102-1110, 2012.
28. Lindqvist A, van Zon W, Karlsson Rosenthal C and Wolthuis RM: Cyclin B1-Cdk1 activation continues after centrosome separation to control mitotic progression. PLoS Biol 5: e123, 2007.
29. Madhok BM, Yeluri S, Perry SL, Hughes TA and Jayne DG: Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells. Br J Cancer 102: 1746-1752, 2010.
30. Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R and Mikkelsen RB: Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. Cancer Res 61: 3894-3901, 2001.
31. Tominaga H, Kodama S, Matsuda N, Suzuki K and Watanabe M: Involvement of reactive oxygen species (ROS) in the induction of genetic instability by radiation. J Radiat Res 45: 181-188, 2004.
32. Diehn M, Cho RW, Lobo NA, *et al*: Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature 458: 780-783, 2009.
33. Cao WG, Yacoub S, Shiverick KT, *et al*: Dichloroacetate (DCA) sensitizes both wild-type and overexpressing Bcl-2 prostate cancer cells in vitro to radiation. Prostate 68: 1223-1231, 2008.
34. Sharma PK, Bhardwaj R, Dwarakanath BS and Varshney R: Metabolic oxidative stress induced by a combination of 2-DG and 6-AN enhances radiation damage selectively in malignant cells via non-coordinated expression of antioxidant enzymes. Cancer Lett 295: 154-166, 2010.
35. Sandulache VC, Skinner HD, Wang Y, *et al*: Glycolytic inhibition alters anaplastic thyroid carcinoma tumor metabolism and improves response to conventional chemotherapy and radiation. Mol Cancer Ther 11: 1373-1380, 2012.
36. Goldberg EB and Colowick SP: The role of glycolysis in the growth of tumor cells. 3. Lactic dehydrogenase as the site of action of oxamate on the growth of cultured cells. J Biol Chem 240: 2786-2790, 1965.
37. Elwood JC: Effect of oxamate on glycolysis and respiration in sarcoma 37 ascites cells. Cancer Res 28: 2056-2060, 1968.
38. Granchi C, Bertini S, Macchia M and Minutolo F: Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials. Curr Med Chem 17: 672-697, 2010.
39. Granchi C, Roy S, Giacomelli C, *et al*: Discovery of N-hydroxyindole-based inhibitors of human lactate dehydrogenase isoform A (LDH-A) as starvation agents against cancer cells. J Med Chem 54: 1599-1612, 2011.
40. Wang Z, Wang N, Chen J and Shen J: Emerging glycolysis targeting and drug discovery from Chinese medicine in cancer therapy. Evid Based Complement Alternat Med 2012: 873175, 2012.