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Discovery of potent human lactate dehydrogenase A (LDHA) inhibitors with antiproliferative activity against lung cancer cells: virtual screening and biological evaluation†

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Human lactate dehydrogenase A (LDHA) plays a crucial role in the promotion of glycolysis in invasive tumor cells, and recently, it has been considered as a vital therapeutic target in the field of oncology. Herein, by combining docking-based virtual screening with *in vitro* biochemical evaluation, we report the discovery of a potent LDHA inhibitor with antiproliferative activity. The enzymatic assay suggested that the identified compound **7** has a good inhibitory activity (IC_{50} = 0.36 μM) against LDHA. The *in vitro* cytotoxicity assay demonstrated that compound **7** reduces the growth of A549 and NCI-H1975 lung cancer cells, with EC_{50} values of 5.5 and 3.0 μM, respectively. These findings indicate that compound **7** could be employed as a useful probe to explore the pharmacology of LDHA.

1. Introduction

Unlike normal cells, most cancer cells have been found to exhibit a higher uptake of glucose and an increased rate of glycolysis such that it tends to convert glucose into lactate in the cytoplasm, even under aerobic conditions.[1] Despite the lower efficiency in energy production by glycolysis, the increased rate of glycolysis in cancer cells is believed to ensure a rapid energy supply and biomass required for cellular proliferation.[2,3] This metabolic alteration is recognized as an emerging hallmark of cancer and thus inhibition of the altered metabolic processes has been considered as a promising approach in the search for novel anticancer therapies.[4]

Among the enzymes and transporters involved in glycolysis, lactate dehydrogenase enzyme (LDH) plays a central role in tumor glycolysis. Structurally, LDH is a tetrameric enzyme, generally composed of two major subunits: LDHA and LDHB.[5] LDHA uses NADH as a cofactor to transfer a hydride to the pyruvate ketone moiety, converting pyruvate into lactate in cytosol, whereas LDHB preferentially catalyzes the reverse reaction in which lactate is converted to pyruvate.[6] It has been reported that LDHA is overexpressed in human tumor cells[7,8] and is correlated with tumor size and poor prognosis.[9,10] Moreover, elevated expression of LDHA causes higher lactate production, which further triggers extracellular acidosis, resulting in a low pH, thus facilitating tumor invasion and metastasis.[11] Silencing LDHA expression by shRNA in tumor cells induces a reduction in the cell proliferation, a markedly delayed tumor migration, and *in vivo* tumorigenesis.[12,13] Collectively, these observations indicate that LDHA can be an attractive target for inhibiting the proliferation of tumor cells.

To date, several examples of human LDHA inhibitors have been described in the open scientific and patent literature.[14] As shown in Fig. 1, gossypol is a natural polyphenol dialdehyde that could inhibit the proliferation of lung cancer cells (EC_{50} = 20 μM); moreover, it also showed an ability to inhibit LDHA (K_i = 1.9 μM). However, its toxicity towards normal cells limits its further clinical application.[15] FX11 is an

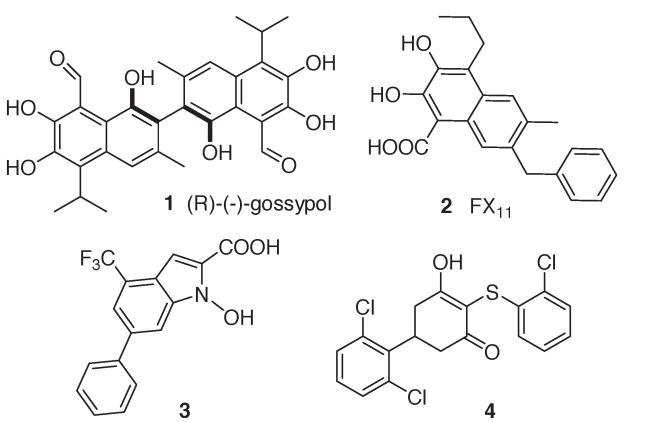

Fig. 1 Chemical structures of the representative LDHA inhibitors.

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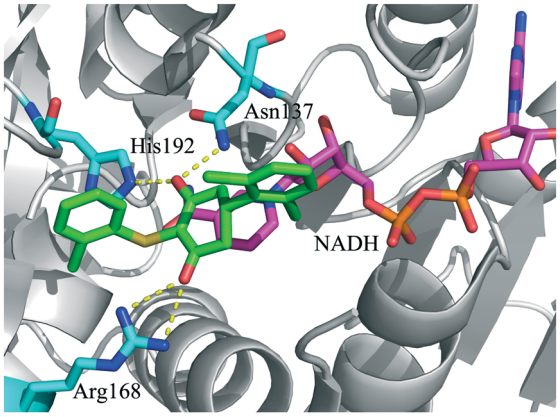

Fig. 2 Binding modes of compound 4 in LDHA. Ligand, cofactor NADH, and the important amino acid residues are shown as stick models (PDB: 4QO8) and H-bonds are shown as yellow dashed lines. The graphic of 3D view was drawn using PyMOL.

Table 1 The identified compounds were tested on purified human LDHA for their inhibitory potency and on A549 and NCI-H1975 lung cancer cells for their growth inhibition rates (%)

Comp.	LDHA inhibition IC_{50} (μM)[a]	Growth inhibition (%)[b]	
		A549	NCI-H1975
5	7.5 ± 0. 82	<10.0	<10.0
6	1.24 ± 0.21	47.2 ± 7.8	74.8 ± 7.2
7	0.36 ± 0.08	67.4 ± 5.9	92.5 ± 7.8
8	2.21 ± 0.32	46.8 ± 5.7	72.5 ± 6.7
9	1.96 ± 0.24	39.8 ± 5.3	62.5 ± 7.5
10	0.96 ± 0.14	63.4 ± 6.7	79.5 ± 6.5
4	2.45 ± 0.33	No inhibition	No inhibition

[a] LDHA inhibition activities were reported as the arithmetic mean of three separate measurements. [b] Antiproliferative inhibitions (%) against A549 and NCI-H1975 cells were determined from cytotoxicity experiments in which cells were treated with the identified compounds at 10 μM.

efficient LDHA inhibitor, with a K_i value of 8 μM, which has been proven to inhibit tumor progression *in vitro* and *in vivo*, whereas the reactive catechol portion in FX11 makes it unsuitable as a drug candidate for further development. Moreover, the binding behaviors of FX11 with LDHA were undetectable in an NMR biochemical assay.[13] *N*-Hydroxyindole 3 was evaluated as a promising LDHA selective inhibitor, which also showed inhibitory activities in a wide range of tumor cells.[16] Another known LDHA inhibitor is compound 4, with an IC_{50} value of 0.87 μM against LDHA *in vitro*. Unfortunately, compound 4 was inactive in the cell-based assay when the tested concentration reached 50 μM.[17] Recently, Purkey *et al.* reported the optimization of a trisubstituted hydroxylactam (analog of compound 4), from which a cell-active molecule (MiaPaca2 EC_{50} = 0.67 μM) with potent LDHA inhibitory activity (IC_{50} = 3 nM) was discovered.[18]

Although a number of LDHA inhibitors have been reported by academic and industrial groups, some of these display limited cellular activities. Therefore, it is very important to discover novel LDHA inhibitors with antiproliferative activity. Herein, we report a virtual screening approach that allowed to identify new LDHA inhibitors with interesting inhibitory potencies *in vitro* enzymatic and antiproliferative assays.

2. Results and discussion

The crystal structure of the LDHA-compound 4 complex (PDB entry 4QO8) was obtained from a protein data bank as the template for molecular docking. The complex was optimized by hydrogen addition and energy minimization using the Sybyl-X 2.0 software. Then, a library with 16 000 compounds of diverse chemical structure was downloaded from the ZINC database (http://zinc.docking.org). The compounds were filtered to discard some compounds with unfavorable physicochemical properties that did not meet the drug-like criteria ($350 < MW < 500$, $-2 < \mathrm{clog}P < 5$, $0 <$ rotational bonds < 10, $0 <$ hydrogen bond donors < 5, $0 <$ hydrogen bond acceptors < 10, $20 <$ polar surface area < 140). The remaining 6217 compounds were prepared by the ligand structure preparation procedure in the software. Finally, the optimized compounds were docked into the binding pocket of

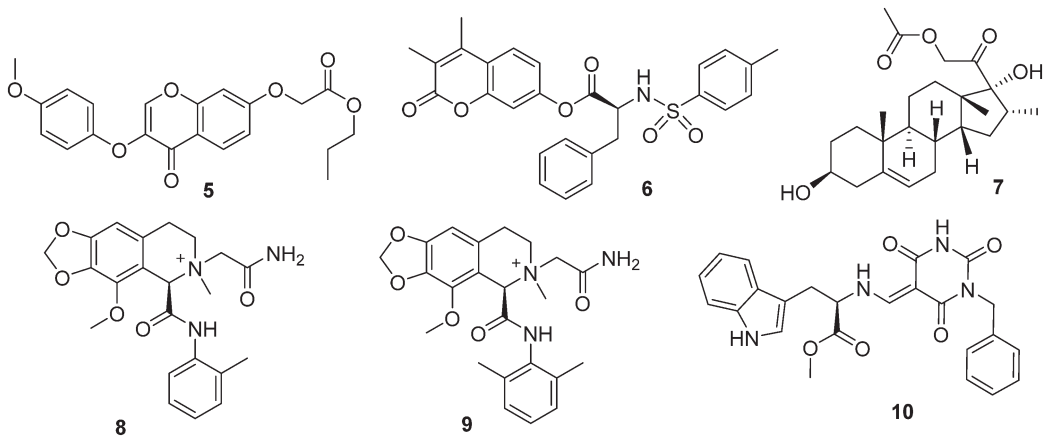

Fig. 3 Chemical structures of the identified compounds.

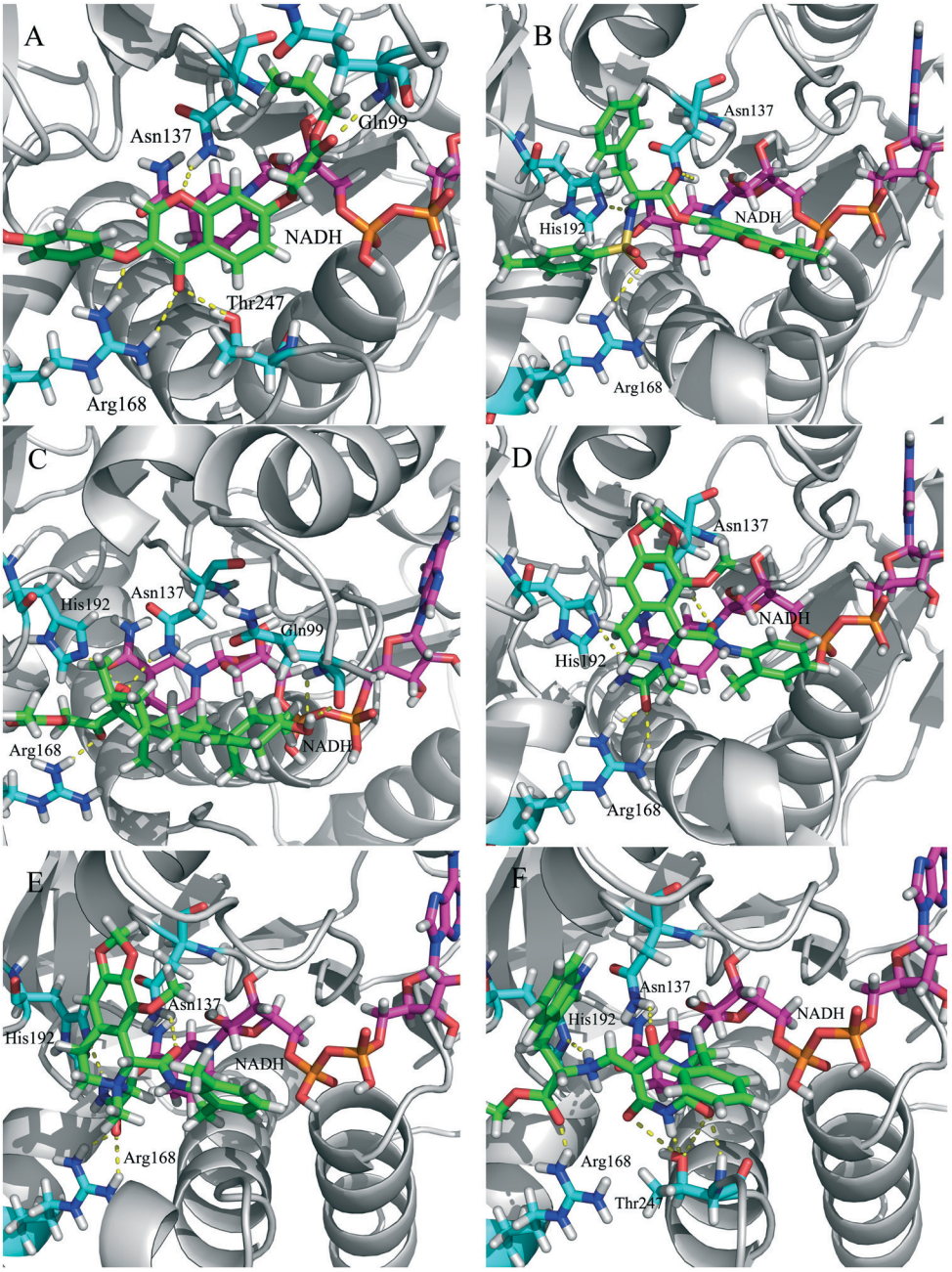

Fig. 4 Predicted binding modes of the identified inhibitors in the active pocket of LDHA. (A) Compound **5** was docked into the active pocket of LDHA and it formed hydrogen bonds with the Gln 99, Asn 137, Arg 168, and Thr 247 residues of LDHA. (B) Compound **6** was docked into the active site of LDHA and it formed hydrogen bonds with the Asn 137, Arg 168, and His 192 residues of LDHA. (C) Compound **7** was docked into the active site of LDHA and it formed hydrogen bonds with the Gln 99, Asn 137, Arg 168, and His 192 residues of LDHA. (D) Compound **8** was docked into the active site of LDHA and it formed hydrogen bonds with the Asn 137, Arg 168, and His 192 residues of LDHA. (E) Compound **9** was docked into the active site of LDHA, and it formed hydrogen bonds with the Asn 137, Arg 168, and His 192 residues of LDHA. (F) Compound **10** was docked into the active site of LDHA and it formed hydrogen bonds with the Asn 137, Arg 168, His 192, and Thr 247 residues of LDHA. All the amino acid residues, inhibitors, and cofactor NADH are shown as stick models, and H-bonds are shown as yellow dashed lines. The graphics of 3D views were drawn using PyMOL.

compound 4 in LDHA using the Surflex-Dock software. Similar to compound 4 (Fig. 2), the potential compounds that form hydrogen bonds with the residues Arg 168, Asn 137, and His 192 of LDHA were selected for further analyses. Following this procedure, six compounds (Fig. 3) were identified and purchased from a local supplier.

The identified candidates and compound 4 were tested for their inhibitory activity towards human LDHA by monitoring the disappearance of NADH during the conversion of pyruvate into lactate. As shown in Table 1, all the identified compounds exhibited an interesting inhibitory potency, with IC_{50} values ranging from 0.36 to 7.5 μM (Table 1). Compounds 7

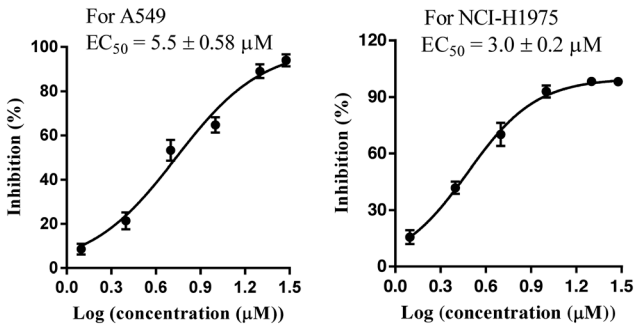

Fig. 5 Dose–response curves of compound 7 against A549 (left) and NCI-H1975 (right) lung cancer cells.

and **10** displayed the most potency against LDHA, with IC_{50} values of 0.36 and 0.96 μM, respectively, which were better than that of the reference compound **4** (IC_{50} = 2.45 μM). The elevated inhibitory activity against LDHA could be partially explained by the evidence obtained from the docking experiment. As shown in Fig. 4, the identified compounds 7 and **10** displayed the same hydrogen bonds interactions as compound **4**. Additionally, they exhibited hydrogen bond interactions with the residues Gln 99 and Thr 247 of LDHA, which may have enhanced the inhibitory activity towards LDHA. In the docking experiment, we observed that the total binding score was 9.00 for compound 7 when bound to LDHA in the presence of the NADH cofactor, whereas the total binding score dropped to 5.04 in the absence of NADH, suggesting that the binding of compound 7 to LDHA required prior association of the NADH cofactor. To verify this observation, an LDHA solution was titrated with compound 7 in the presence of NADH in an isothermal titration calorimetry (ITC) experiment, and a decent K_d (1.1 μM) was derived, indicating that compound 7 directly interacts with LDHA, whereas the K_d value was undetectable in the absence of NADH under the same experimental conditions.

Next, we wanted to determine whether the identified compounds displaying LDHA inhibition could inhibit the growth of A549 and NCI-H1975 lung cancer cells. For this purpose, we tested the anticancer activity of the identified compounds against two cancer cell lines using an MTT assay. As shown in Table 1, most of the compounds, especially **6**, 7, **8**, and **10**, displayed good anticancer activities, with the inhibition rates of 74.8%, 92.5%, 72.5%, and 79.5%, respectively, and strongly inhibited the proliferation of NCI-1975 cells at 10 μM. Note that compound 7 showed the most potent

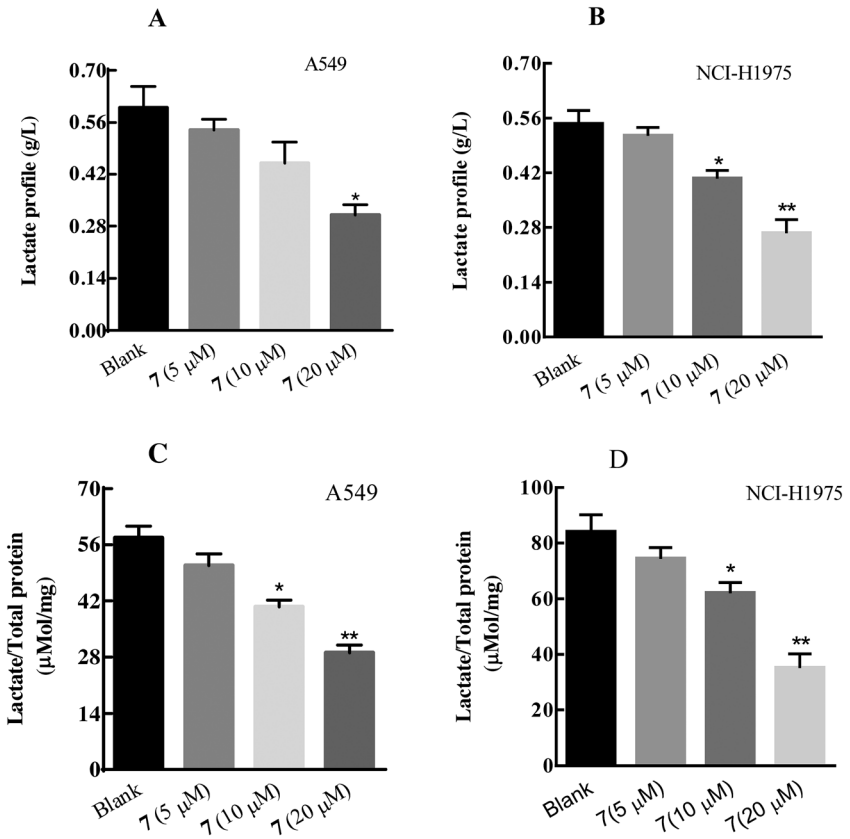

Fig. 6 Effect of compound 7 on the lactate production in A549 and NCI-H1975 cancer cell lines. A: A549 cells were treated with compound 7 at 5, 10, and 20 μM for 4 h, and then the lactate produced was measured by *Nova Bioprofile Flex* analyzer; B: NCI-H1975 cells were treated with compound 7 at 5, 10, and 20 μM for 4 h, and then the lactate produced was measured by *Nova Bioprofile Flex* analyzer; C: A549 cells were treated with compound 7 at 5, 10, and 20 μM for 12 h, and then the lactate produced was measured by lactate assay kit; D: NCI-H1975 cells were treated with compound 7 at 5, 10, and 20 μM for 12 h, and then the lactate produced was measured by lactate assay kit. *$P < 0.05$, *versus* blank group.

antiproliferative activity, with an EC_{50} value as low as 5.5 μM against A549 and 3.0 μM against NCI-1975 (Fig. 5).

Since compound 7 displayed a promising inhibitory activity towards LDHA, it also showed the highest antiproliferative activity against lung cancer cells; thus we decided to further study this compound. Inhibiting the activity of LDHA could lead to the switch of pyruvate consumption from lactate production to oxidative phosphorylation in the mitochondria, and this conversion would decrease the lactate formation in the cytoplasm. Therefore, lactate production in A549 and NCI-H1975 cancer cells was measured using the *Nova Bioprofile Flex* analyzer. As shown in Fig. 6A and B, the lactate formation was significantly decreased in A549 and NCI-1975 cancer cells after treatment with compound 7 at 5, 10, and 20 μM for 4 h. To confirm this finding, we examined lactate formation using a lactate assay kit. As shown in Fig. 6C and D, lactate production displayed a similar trend as measured by the *Nova Bioprofile Flex* analyzer in A549 and NCI-1975 cancer cells.

3. Conclusions

In summary, we discovered a potent LDHA inhibitor *via* a virtual screening approach and biochemical assays. The identified inhibitor 7 was tested by *in vitro* enzymatic and antiproliferative assays, with both assays suggesting that compound 7 targets LDHA and exhibits cellular activity. The lactate production assays revealed that compound 7 reduced the formation of lactate in A549 and NCI-H1975 cancer cells. These findings indicate that compound 7 could be employed as a useful probe to explore the pharmacology of LDHA.

4. Experimental protocols

4.1. Materials and cell culture

The identified compounds **5–10** (purity >95%) used in the experiments were purchased from local suppliers. Stock solutions were prepared by dissolving the compounds in DMSO at a concentration of 40 mM and the solutions were then stored at −80 °C. Compound **4** was synthetized following the procedure reported by P. S. Dragovich *et al.*[17] Purified human LDHA was obtained from Lee Biosolutions (St Louis, Missouri, USA). A549 and NCI-1975 lung cancer cells were purchased from American Type Culture Collection (ATCC). A549 was cultured in DMEM/F12 (1 : 1, Gibco), whereas NCI-1975 was maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in 5% CO_2 at 37 °C.

4.2. Antiproliferative assay

The antiproliferative activity of the identified compounds was measured by the MTT assay. A suspension of A549 and NCI-1975 cancer cells (5000 per well in a 90 μL medium) was seeded in 96-well plates and cultured for 24 h. Then, stock solutions of the identified compounds (100 μM) were prepared by diluting the stock solution in a PBS buffer. Compounds (10 μL) were added to the 96-well plates (90 μL medium with cells), which were incubated for another 72 h. Then, 10 μL of MTT solution (5 mg mL^{-1}) was added to each well. After 4 h of incubation, solvent was removed and 100 μL DMSO was added to the 96-well plates. The absorbance of each well (measured as OD values) was observed at a wavelength of 570 nm. The growth inhibition rates (%) of the compounds at 10 μM were calculated by the following equation:

$$\text{Growth inhibition (\%)} = 100 - \frac{\text{Mean OD values of individual test group}}{\text{Mean OD values of control group}} \times 100$$

After the most potent compound 7 was identified, dose–response studies were carried out to determine the exact EC_{50} values of compound 7. Six different concentrations of compound 7 ranging from 300 to 12.5 μM (300, 200, 100, 50, 25, 12.5 μM) were added to the 96-well plates (in 90 μL medium). The experiment was conducted following the above mentioned protocol. The OD values were read using a Microplate Reader, and the results were expressed as EC_{50} values, which were the mean value obtained from three independent experiments.

4.3. Lactate production measured by the Nova Bioprofile Flex analyzer

A suspension of A549 and NCI-1975 lung cancer cells (6×10^5 per well) was seeded in 6-well plates and cultured overnight. Then, compound 7 at 5, 10, and 20 μM concentrations was added to the corresponding wells and the plates were incubated at 37 °C for 4 h. Then, the medium was transferred to microcentrifuge tubes, which were centrifuged for 5 min (8000 r min^{-1}). Finally, 1 mL of medium was collected and the lactate production was determined by the Nova Bioprofile Flex analyzer (Nova Biomedical).

4.4. Lactate production measured using a lactate assay kit

A549 and NCI-1975 lung cancer cells were treated with compound 7 at 5, 10, and 20 μM concentrations for 12 h in a serum-free medium, and the lactate production was measured using a lactate assay kit (Biovision, CA, USA). Moreover, total proteins from cell lysates were collected and quantified for the normalization of lactate release.

4.5. LDHA enzymatic assay

The assay described herein has already been reported in the literature.[17] The experiment was performed in a 96-well plate, with the following enzyme and buffer: 2 nM LDHA, 60 μM NADH, 50 mM Hepes (pH 7.2), 0.01% (v/v) Triton X-100, 0.01% (0.1 mg mL^{-1}) bovine gamma-globulin, 2 mM DTT, and 60 μM pyruvate. For the enzymatic reaction, the identified compounds were added to a mixture of enzyme and NADH. The assay plates were then incubated at room temperature for 10 minutes and a baseline reading was

conducted using a microplate reader at an excitation wavelength of 340 nm and an emission wavelength of 480 nm to identify any compounds that interfere with NADH fluorescence. Then, pyruvate was added to the assay plates and these plates were read at an excitation wavelength of 340 nm and at an emission wavelength of 480 nm for 10 minutes every 2.5 seconds. A suitable linear timeframe was selected (150–400 s) to calculate the slope of each concentration tested herein. The bottom of the curve was set to the background rate and the top of the curve was set to no inhibitor (DMSO only) control wells rate.

4.6. ITC analysis

The LDHA solution was diluted to 10 μM in a buffer solution with pH 7.2 containing 50 mM Hepes, 100 mM NaCl, 2 mM DTT, and 1% DMSO. Working solutions of compound 7 (100 μM in 1% DMSO buffer solution) were prepared by diluting the stock solution of compound 7. NADH was dissolved in double-distilled water. ITC experiments were conducted using a Microcal iTC200 microcalorimeter (GE Healthcare). The reaction cell contained 300 μL of LDHA. Titrations were performed with every injections of 2.5 μL of titrant(s) in the reaction cell, which was maintained at 25 °C. All the ITC data were initially analyzed by the Origin 7, and then followed by curve-fitting to one-site model to obtain the binding parameter.

4.7. Molecular docking

Molecular docking was performed using a Surflex Dock program of the Sybyl-X 2.0 package. The crystal structure of LDHA was obtained from the Protein Data Bank (PDB ID: 4QO8). The crystal structure of LDHA was determined with all polar H and charge added (AMBER7 FF99). The structures of a small molecule library were downloaded from the zinc database (http://zinc.docking.org/). First, the commercially available small molecule database was filtered following the drug-like rules, and the remaining compounds were subjected to the polar H addition, were energy optimized with a tripos force field, and charged optimized *via* the Gasteiger-Huckel method. The protocol was generated in a ligand mode with the threshold was kept at 0.50 and the bloat was 0. Ring flexibility was considered and other parameters during the docking program were determined *via* a number of attempts.

4.8. Statistical analysis

Data are reported as mean ± SD. Statistical analysis was performed using GraphPad Prism version 5.0 for Windows. $^{*}p < 0.05$ is considered as statistically significant.

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