Evaluation on the inhibition of pyrrol-2-yl ethanone derivatives to lactate dehydrogenase and anticancer activities

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ABSTRACT

Lactate dehydrogenase A (LDH-A) is a potentially important metabolic target for the inhibition of the highly activated glycolysis pathway in cancer cells. In order to develop bifunctional compounds as inhibitor of LDH-A and anticancer agents, two pyrrol-2-yl methanone (or ethanone) derivatives (PM1 and PM2) were synthesized and evaluated as inhibitors of LDH-A based on the enzyme assay and cell assay by spectroscopy analysis. Fluorescence and CD spectra results demonstrated that both the change of second structure of LDH-A and the affinity interaction for compounds to LDH-A gave great effect on the activity of LDH-A. In particular, low concentration of compounds (1 μ M ~ 25 μ M) could change the level of pyruvate in cancer cells. Moreover, the in vitro assay results demonstrated that pyrrol-2-yl ethanone derivatives can inhibit the proliferation of cancer cells. Therefore, pyrrol-2-yl ethanone derivatives (PM2) can be both LDH-A inhibitor and anticancer agents.

Keywords: pyrrol-2-yl ethanone; lactate dehydrogenase; inhibitor; cancer

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1. Introduction

Cancer cells referentially convert glucose into lactate through glycolysis. Glycolysis allows for continued ATP production without the need for O₂-dependent oxidative phosphorylation, and is thus an important pathway under hypoxic conditions^[1]. Target cancer supplier is a new strategy in cancer therapy^[2]. Lactate production is an essential feature of cancer cells and it is generated through LDH-A, which transforms pyruvate into lactate. Many human cancers have higher LDH-A levels than normal tissues^[3]. LDH-A is important for tumor initiation because the decreased level of LDH-A reduced cellular transformation and markedly delayed tumor formation^[4, 5]. It is also reported that the inhibition of LDH-A causes oxidative stress leading to cell death^[6]. These studies provide proof-of-concept that LDH-A is a tractable therapeutical target. Few LDH-A inhibitors are reported in the literature. Oxamate and galloflavin can increase the efficacy of cisplatin in cultured Burkitt's lymphoma (BL) cells^[7, 8]. N-hydroxy-2-carboxy-substituted indoles and N-acyl-hydrazone derivatives were reported as LDH-A-inhibitors based on enzyme assay^[9-11]. Pyruvate metabolism plays a pivotal role in cell homeostasis and energy production^[12]. Lipophilic 2,5-disubstituted pyrroles inhibit mitochondrial respiration and HIF-1 Activation^[13]. Therefore, we supposed that pyrrol-2-yl ethanone derivatives could be as inhibitors of lactate dehydrogenase in vitro and in cells. The LDH-A inhibitory activities of the compounds have been evaluated based on the conversion of L-lactate to pyruvate catalyzed by LDH-A with change in the concentration of NADH. Less report on direct interaction of compounds with LDH-A in solution was reported. Study on the interaction of compounds with LDH-A by spectroscopy methods would develop a simple and economic method for the find of potential LDH-A target anticancer compounds. Here, we report the spectroscopic analysis on the interaction of pyrrol-2-yl ethanone derivatives (Scheme 1) with LDH-A in vitro and in cells. Fluorescence titration and CD spectroscopy were firstly used in the analysis on the interaction of compounds with LDH-A.

Scheme1 The structures of PM1, PM2, PM3 and PM4.

2. Experimental

2.1 Materials and measurements

2,4-Dimethyl-1H-pyrrole, pyruvic acid, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, lactic acid, 1-methylimidazole and di(2-pyridylmethyl)amine were reagent grade. 3,5-Dimethyl-1H-pyrrole-2-carbonyl chloride (PP), 4-((bis(pyridine-2-ylmethyl)amino)methyl)phenyl)-2-(3,5-dimethyl-1H-pyrrol-2-yl)methanone (PM1) and 3-((bis(pyridine-2-ylmethyl)amino)methyl)phenyl)-2-(3,5-dimethyl-1H-pyrrol-2-yl)methanone (PM3) were synthesized as reported previously^[14, 15]. Water was purified with a Millipore Milli-Q system. The C, H and N microanalyses were performed on a Vario EL elemental analyzer. Electronic absorption spectra were recorded in the 900-190 nm region using a Varian Cary 50-BIO UV-VIS spectrophotometer. Infrared spectra were recorded on a Nicolet-470 spectrophotometer in the wavenumber range of 4000-400 cm^{-1} using KBr pellets. Fluorescence spectra were determined on a Varian Cary Eclipse fluorescence spectrophotometer. Circular dichroism was on Modle J-810 instrument, peak width 1 nm, scanning speed 50 nm/min. 0.01M PH=7.4 PBS was as solvent and samples were 6 mM (dissolving in DMSO). The electrospray mass spectra (ES-MS) were determined on a Finnigan LCQ mass spectrograph. NMR spectra were measured on a Bruker AVANCEII 400 MHz spectrometer with TMS as an internal standard and chloroform as the solvent.

2.2 Synthesis of 2-(bis(pyridin-2-ylmethyl)amino)-1-(3,5-dimethyl-1H-pyrrol-2-yl)ethanone (PM2)

3,5-Dimethyl-1H-pyrrole-2-carbonyl chloride (PP) (800 mg, 4.6 mmol) in DCM (30 ml), di(2-pyridylmethyl)amine (600 mg, 3 mmol), triethylamine (300 mg, 3 mmol) and KI (100 mg) were mixed. The resulting solution was stirred at room temperature under N_2 for 10 h. The organic solution was washed with saturated sodium chloride solution (3×20 ml), aqueous phase was extracted with DCM (3×15 ml) and dried over anhydrous Na_2SO_4 . The crude product was purified on a silica gel column eluting with ethyl acetate to obtain yellow viscous liquid PM2 (0.8 g, 75%). Anal. Calcd. for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}$: C, 71.83; H, 6.63; N, 16.75. Found (%): C, 71.89; H, 6.58; N, 16.69. IR (KBr) $/\text{cm}^{-1}$: $\nu(\text{-NH-})$ 3204 m, $\nu(=\text{CH})$ 3069 m, $\nu(\text{-CH}_2, \text{-CH}_3)$ 2850 m, $\nu(\text{C=O})$ 1617 m, $\nu(\text{C=C})$ 1590 s, $\nu(\text{C=C})$ 1481 s, $\nu(\text{C=C})$ 1432 m, $\nu(\text{C=C})$ 1364 m, $\delta(\text{CH, pyridine})$ 756 s. UV-vis (EtOH/nm) ($\epsilon \times 10^{-4}/\text{dm}^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$): 208 nm (1.55), 260 nm (1.03), 308 nm (1.62). ^1H NMR

(CDCl₃, 400MHz): δ 2.39 (s, 3H, CH₃), δ 2.47 (s, 3H, CH₃), δ 3.56 (s, 2H, CH₂), δ 3.92 (s, 4H, CH₂N), δ 5-9 (m, 9H, H-Ar), δ 12.94 (s, 1H, NH). ESI-MS (CH₃OH): m/z, 335 [PM2+H]⁺

2.2 Synthesis of 3-[3-(3,5-dimethyl-1H-pyrrole-2-carbonyl)benzyl]-1-methyl-1H-imidazol-3-ium hexafluorophosphate (PM4)

3,5-Dimethyl-1H-pyrrole-2-carbonyl-3-benzyl chloride (380 mg, 1.538 mmol) in CH₃CN (15 ml) were mixed with N-methylimidazole (131.5 mg, 1.22 mmol), then the solution were heated in microwave (400 w, 60 °C) for 5 min. Followed by addition of KPF₆ (270.7 mg, 1.5 mmol), precipitates were produced. After filtration and drying in vacuum, white solid were obtained (162 mg), yield. 64%. Anal. Calcd. for C₁₈H₂₀F₆N₃OP: C, 49.21; H, 4.59; N, 9.56. Found (%): C, 49.28; H, 4.52; N, 9.60. IR (KBr) ν /cm⁻¹: ν (-NH-) 3275 s, ν (C=O) 1570 m. UV-vis (CH₃CN/nm) ($\epsilon \times 10^{-4}/\text{dm}^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$): 206 nm (2.85), 241 nm (0.96), 314 nm (1.79). ¹H NMR (DMSO, 400 MHz): δ 11.31 (s, 1H), δ 9.24 (s, 1H), δ 7.82 (s, 1H), δ 7.72 (s, 1H), δ 7.62-7.65 (m, 2H), δ 7.55-7.57 (m, 2H), δ 5.86 (s, 1H), δ 5.49 (s, 2H), δ 3.86 (s, 3H), δ 2.21 (s, 3H), δ 1.86 (s, 3H). ESI-MS (CH₃OH): m/z, 294.24 [PM4-PF₆]⁺

2.4 Inhibition of lactate dehydrogenase

Lactate dehydrogenase (LDH-A) is considered a significant small-molecule oncology target in cancer metabolism. The oxidation of NADH is observed at 340 nm, which is a direct measure of the reduction of pyruvate to lactate in the absence of interfering reactions. The apparent Michaelis-Menten constants (K_m) of LDH-A was measured from Lineweaver-Burk plots. The reaction velocity of purified LDH-A was determined by a decrease in absorbance at 340 nm of NADH, and the value of optical density was determined at 30 s intervals for 4 min. LDH-A activity was determined at 37°C by measuring the oxidation of NADH spectrophotometrically at 340 nm as a function of time. The K_m values of LDH-A for NADH and pyruvate were determined in saturating conditions as described below. In 100 mM sodium phosphate buffer (pH 7.4), 0.5 units of LDH-A were combined with 2 mM pyruvate (pyruvate-saturated) and 15 - 170 μ M NADH, or 170 μ M NADH (NADH-saturated) and 0.42 mM - 2 mM pyruvate. These conditions were used for all assays. The reaction velocity of LDH-A was determined spectrophotometrically (UV-vis spectrophotometer) by a decrease

in absorbance at 340 nm ($\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) of NADH. Michaelis-Menten constants for substrates were determined from initial rate measurements at 37°C by nonlinear regression analysis with the Origin 8.0. Under these conditions NADH showed a K_m of 36 μM and a $V_{\max}$ of 35 ($\mu\text{mol/min}$)/mg, whereas the pyruvate showed a K_m of 607 μM and a $V_{\max}$ of 33 ($\mu\text{mol/min}$)/mg. Compounds were dissolved in stock solutions of DMSO (concentrations of DMSO during the initial rate measurements did not exceed 0.5%). Using 170 μM NADH and 2 mM pyruvate, we initially evaluated the percent inhibition of the compounds. We evaluated the apparent K'_m values in the presence of inhibitors. From the values of K'_m so obtained, K_i values for each single inhibitor were determined using double-reciprocal plots.

2.5 *Measuring of pyruvate content in cancer cells*

SMMC 7721 cells were seeded at a density of 4×10^4 cells mL^{-1} into sterile 96-well plates. Compounds were dissolved in DMSO and diluted with culture media. After cells were cultured 24 h, compounds were added into the cultured SMMC 7721 cells for 8 h. The level of pyruvate in cells was determined by dinitrophenylhydrazine colorimetric method. Cells were treated with trifluoroacetic acid (8%), then centrifugal sedimentation was removed and the supernatant was used.

2.6 *Cytotoxicity assay*

The cytotoxicity assay was in one kind of SMMC 7721 cells. Cells were cultured in RPMI 1640 medium containing 4.8 g/L of Hepes, 2.2 g/L NaHCO_3 and supplemented with penicillin/streptomycin (1000 units/ml), and 10% calf serum. SMMC 7721 cells were cultured in DMEM medium containing 10% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere in the presence of 5% CO_2 . SMMC 7721 cells were seeded at a density of 4×10^4 cells/mL into sterile 96-well plates and grown in 5% CO_2 at 37°C. Test compounds were dissolved in DMSO and diluted with culture media. After 24 h, compounds were added, treated for 48 h. Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay by measuring the absorbance at 570 nm with ELISA reader. IC_{50} data are calculated by the software provided by Nanjing University. All experiments were repeated at least three times.

3. Results and discussion

3.1 Evaluation of LDH-A activity in the presence of compounds by spectroscopic titration

Two new pyrrol-2-yl ethanone (or methanone) derivatives (PM2 and PM4) were synthesized and characterized by IR, UV, NMR and ES-MS spectra (Fig. S1-Fig. S4). The LDH-A inhibitory activities of the compounds were measured by standard enzyme kinetic experiments on rabbit LDH-A purified isoform. In the presence of L-lactate dehydrogenase, pyruvate was transformed to L-lactate, whereas NADH was oxidized to NAD^+ . The change in the concentration of NADH was measured spectrophotometrically at 340 nm. Experimental results show that the inhibition rate of compounds is dependent on their concentrations (Fig.1). The apparent Michaelis-Menten constants (K_m) of NADH and pyruvate for LDH-A were measured by Lineweaver-Burk plots. From the values of K'_m so obtained, K_i values for each single inhibitor were determined using double-reciprocal Lineweaver-Burk plots (Fig.2, Table 1). Results indicate that four compounds show moderate inhibition on the LDH-A catalyzed reaction. For PM2, K_i value is 60.8 μM vs NADH and 353.2 μM vs pyruvate. Therefore, we suggest that PM2 were found to be more competitive inhibitors of LDH-A with respect to NADH than pyruvate in the conversion of pyruvate to lactate catalyzed by this enzyme, whereby NADH is converted to NAD^+ . The K_i values (vs NADH) of phenyl group conjugated compounds (PM1, PM4 and PM4) are lower than that of PM1, indicating that electron density in the pyrrol-2-yl-methanone section is the main factor for the inhibition.

Fig. 1. Colorimetric measurement of inhibition (% relative to control) of the enzymatic activity of LDH-A in the presence of different concentration 30-150 μM of PM2. Values are reported as the mean $\pm$ the SD of three independent experiments, the SD < 3.

Fig.2. Lineweaver-Burk plots determined from triplicate experiments with inhibitors 60 μM , competition experiments with NADH and pyruvate.

Table 1 LDH-A inhibition data (^a K_i for LDH-A μM, % inhibition) obtained with compounds.

3.2 Study on the interaction of compounds with LDH-A by fluorescence spectroscopy

Enzyme assay results show that PM2 and PM4 have different effect on the conversion of pyruvate to lactate catalyzed by LDH-A. To investigate whether the inhibition of compounds to LDH-A is related to the binding interaction of compounds with LDH-A, the affinity of compounds (PM2 and PM4) toward LDH-A was investigated by fluorescence spectroscopy at different ratios of compounds:LDH-A. As shown in Fig.3, fluorescence intensity of LDH-A at 347 nm decreased gradually with increasing concentrations of PM2 and PM4. The fluorescence intensity of LDH-A at 347 nm decreased with the addition of compounds, indicating that two compounds could bind with LDH-A. In order to evaluate the combination capacity of compounds with LDH-A, K_A (binding constant) were obtained through Schatchard equation (1). Here F₀ and F are the relative fluorescence intensity of LDH-A before adding inhibitors solution and after; [Q] is the concentration of inhibitors. K_A value (2.10×10⁷ M⁻¹) of PM2 is larger than PM4 (1.91×10⁵ M⁻¹). The results indicated that PM2 has stronger affinity with LDH-A than PM4 (Table 2).

$$\log[(F_0-F)/F]=\log K_A + n \log[Q] \quad (1)$$

Fig. 3. The fluorescence emission of LDH-A at different concentrations of compounds (λ_{ex/em}=227/347 nm, PM2 (DMSO+H₂O) (a-i: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μM), PM4 (DMSO+H₂O) (a-k: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μM)

Table 2. Binding constants (K_A), binding site (n) of PM1 and PM4 with LDH-A at 298 K

3.3 Effect of compounds with the secondary structure of LDH-A

Circular dichroism (CD) spectrum of untreated LDH-A has characteristics of helix structure, with a negative peak at 195 nm and a positive ellipticity in the 210-240 nm region with a peak at 214 nm. CD spectra showed

that the ellipticity of the spectrum in the 210-240 nm region and the negative peak at 195 nm disappeared, besides there were two new positive peaks, indicating a loss of the native structure of the enzyme. Both PM2 and PM4 can change the secondary structure of LDH-A. The intensities of new appeared positive ellipticity in the 200-205 nm region and negative ellipticity in the 215-225 nm region are 0.58, -1.11 for PM2 and 0.05, -0.44 for PM4, respectively. The shift upward of the ellipticity was synonymous to loss of the secondary structure of the enzyme. Because PM2 shows better inhibition than PM4, thus we suggest that the change of secondary structure of LDH-A can be used as reference for the evaluation of LDH-A activity.

Fig. 4. CD spectra of LDH in 0.01 M PBS (pH 7.4) with PM2 and PM4: LDH (dash line), PM2+LDH (black line), PM4+LDH (short dot).

3.4 Cytotoxicity assay

The K_i values (vs NADH) of phenyl group conjugated compounds (PM1, PM2 and PM4) are lower than that of PM3, indicating that electron density in the pyrrol-2-yl ethanone (or methanone) section has great effect on the inhibition for compounds to LDH-A. The in vitro cytotoxicity of compounds (PM1, PM2, PM3, PM4) was evaluated using MTT assay by measuring anti-proliferation on HepG-2 cells. The IC_{50} values are shown in Table 3. Compound PM2 can inhibit the proliferation of the HepG-2 cells with IC_{50} 5.3 μ M, but it shows weak inhibition on the proliferation of normal liver cells (Fig. S5). It is also found that the IC_{50} values of PM1, PM2 and PM3 are lower than PM4, so we suggest that the di(pyridine-2ylmethyl)amine section in compounds (PM1, PM2, PM3, PM4) have great effect on their cytotoxicities.

Table 3 IC_{50} values of the complexes on HepG-2 cell lines. All IC_{50} values were expressed as mean values of the three experiments.

Fig. 5. The concentration dependence of PM2 and PM4 on HepG-2 cells. Cell proliferation was determined by MTT assay every 12 h. Each column represents the mean of the data from three independent experiments.

3.5 Measuring of pyruvate content in cancer cells

Based on the different IC_{50} values, compounds PM2 and PM4 were used to study their effect on the conversion of lactate in SMMC 7721 cell. The content of the intracellular pyruvate in SMMC 7721 cells was evaluated using dinitrophenyl hydrazine colorimetric assay [16] (Fig. 4). Results show that the level of the pyruvate was increased when PM2 was added into cells, indicating that PM2 can deactivate the LDH-A in cells. It was also found that the level of pyruvate for PM2 treated cells is higher than that of PM4 when the concentration of both compounds was in the range of $1\ \mu\text{M} \sim 50\ \mu\text{M}$. Hence, we suggest that PM2 is a better inhibitor for the conversion of pyruvate to lactate catalyzed by LDH-A than PM4 in SMMC 7721 cells. This result agrees with results from enzyme assay. It is also found that the level of pyruvates in SMMC 7721 cells keep constant when the concentration of compounds PM2 was more than $25\ \mu\text{M}$. Therefore, we deduce that PM2 in the range of the $1\ \mu\text{M} \sim 25\ \mu\text{M}$ were found to be inhibitors of LDH-A with respect to pyruvate in the conversion of pyruvate to lactate catalyzed by this enzyme.

Fig. 6. The content of pyruvate in SMMC 7721 cells with PM2, PM4 at different concentration.

4. Conclusions

Pyrrol-2-yl ethanone derivatives were evaluated as inhibitors of LDH-A based on the enzyme assay and cell assay. Fluorescence results showed that PM2 has stronger affinity with LDH-A than PM4, indicating that the electron density in pyrrol-2-yl ethanone (or methanone) derivatives is the main factor for their inhibition. In particular, we found that both the affinity of compounds with LDH-A and the change of secondary structure of LDH-A can be used as reference for the evaluation of LDH-A activity. Moreover, PM2 ($1\ \mu\text{M} \sim 25\ \mu\text{M}$) could

inhibit the conversion of pyruvate to lactate catalyzed by LDH-A in SMMC 7721 cancer cells. The IC₅₀ values of PM1, PM2 and PM3 are lower than PM4, so we suggest that the bis(pyridine-2-ylmethyl)amine section in compounds (PM1, PM2, PM3, PM4) give contribution to their cytotoxicities. Therefore, our results suggest that PM2 can be developed as both inhibitor of LDH-A and anticancer agents.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version.

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Figure captions

Scheme1. The structures of PM1, PM2, PM3 and PM4.

Fig. 1. Colorimetric measurement of inhibition (% relative to control) of the enzymatic activity of LDH-A in the presence of different concentration of PM2 (30-150 μ M). Values are reported as the mean $\pm$ the SD of three independent experiments, the SD < 3.

Fig. 2. Lineweaver-Burk plots determined from triplicate experiments with inhibitors (60 μ M), competition experiments with NADH and pyruvate.

Fig. 3. The fluorescence emission of LDH-A at different concentrations of compounds ($\lambda_{\text{ex/em}}$ =227/347 nm, PM2 (DMSO+H₂O) (a-i: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μ M), PM4 (DMSO+H₂O) (a-k: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μ M)

Fig. 4. CD spectra of LDH in 0.01 M PBS (pH 7.4) with PM2 and PM4: LDH (dash line), PM2+LDH (black line), PM4+LDH (short dot).

Fig. 5. The concentration dependence of PM2 and PM4 on HepG-2 cells. Cell proliferation was determined by MTT assay every 12 h. Each column represents the mean of the data from three independent experiments.

Fig. 6. The content of pyruvate in SMMC 7721 cells with PM2, PM4 at different concentration.

R1 (PM1)

R2 (PM2)

R3 (PM3)

R4 (PM4)

Scheme 1

Fig. 1.

Fig.2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Table captions

Table 1. LDH-A inhibition data (^a K_i for LDH-A μM, % inhibition) obtained with compounds.

Table 2. Binding constants (K_A), binding site (n) of PM1 and PM4 with LDH-A at 298 K.

Table 3. IC₅₀ values of the complexes on HepG-2 cell lines.

Table 1. LDH-A inhibition data (^a K_i for LDH-A μM, % inhibition) obtained with compounds.

compound	K _i / NADH ^b	K _i / Pyruvate ^c
PM1	114.9± 1.5	414.9 ± 1.7
PM2	60.8 ± 2.2	353.2 ± 1.5
PM3	100.4± 1.2	111.2 ± 2.2
PM4	140.3± 2.4	206.2± 1.5

^a K_i determination and percent of inhibition were performed in presence of compounds as described. ^b The concentration of pyruvate (2 mM) and competitive increasing concentrations of NADH (15-170 μM). ^c The concentration of NADH (170 μM) and competitive increasing concentrations of pyruvate (0.42-2 mM).

Table 2. Binding constants (K_A), binding site (n) of PM1 and PM4 with LDH-A at 298 K.

Compound	n	K _A (M ⁻¹)
PM2	1.58	2.10x10 ⁷
PM4	1.0	1.91x10 ⁵

Table 3. IC₅₀ values of the complexes on HepG-2 cell lines.

compound	IC ₅₀ *
PM1	21.5 ± 0.3
PM2	5.30 ± 0.3
PM3	10.8 ± 0.4
PM4	84.2 ± 0.3

*All IC₅₀ values were expressed as mean values of the three experiments.