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Discovery of potent pyruvate dehydrogenase kinase inhibitors and evaluation of their anti-lung cancer activity under hypoxia†

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Targeting pyruvate dehydrogenase kinases (PDKs) reverses the Warburg effect, which could be a potential therapeutic target for anti-cancer drug discovery. In this paper, we identified 12 potential PDK inhibitors by virtual ligand screening of a chemical library, and then further verified them by an enzymatic assay, in which **6**, **7**, and **11** strongly inhibited the function of PDKs, with IC₅₀ values of 1.26, 0.62, and 0.41 μM against PDK1, respectively, and showed a similar inhibitory effect on PDK2, PDK3, and PDK4. However, we failed to correlate the observed inhibitory activity against PDKs with cellular activity under normal conditions. In contrast, **7** and **11** inhibited NCI-H1975 cell proliferation under hypoxia, with EC₅₀ values of 4.66 and 3.88 μM, respectively, suggesting that **7** and **11** could be promising leads for further development of PDK inhibitors in cancer treatment.

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1. Introduction

In most tumor cells, pyruvate is converted into lactic acid in the cytosol through aerobic glycolysis rather than oxidative phosphorylation (OXPHOS) in the mitochondrion, regardless of the oxygen level.^{1,2} The switch to aerobic glycolysis is known as the Warburg effect, which is considered as a hallmark of dysregulated metabolism in many types of cancer.³ Thus, targeting this alteration represents therapeutic opportunities in anticancer treatment.^{4,5}

To facilitate the aerobic glycolysis pathway, PDKs are over-expressed in many tumor cells, which could regulate the activity of the pyruvate dehydrogenase complex (PDC) for converting pyruvate into acetyl-CoA for OXPHOS.⁶ Four PDK isoforms (PDK1, PDK2, PDK3, and PDK4) have been isolated and identified,⁷ of which, PDK1 was found to be most closely associated with tumor development and metastasis.⁸ In particular, PDK1 has been shown to be significantly over-expressed in different kinds of human tumors as compared with their stromal tissues,^{9–11} which was often associated with poor prognosis.¹² Moreover, the overexpression of PDK2 contributed to HIF1 accumulation and mitochondrial mutations, which both resulted in cancer cell development and de-

terioration.¹³ In addition, evidence suggested that the expression of PDK3 contributed to drug resistance, which was positively correlated with the severity of colon cancer and negatively related to disease-free survival.¹⁴ Similarly, siRNA knockdown of PDK4 inhibited bladder cancer cell proliferation, while up-regulation of PDK4 increased bladder cancer cell growth.¹⁵ Collectively, these results indicated that PDKs, especially PDK1, could be a potential target for cancer therapy.

To date, a number of PDK inhibitors with different mechanisms have been disclosed in the open literature and/or patents. Dichloroacetate (DCA, Fig. 1) was reported as a PDK inhibitor for the treatment of diabetes and lactic acidosis, and recently it has been used in an anticancer study.¹⁶ However, the high effective dosage hampered its routine application in clinical practice. AZD7545 bound to the lipoamide-binding site of PDK2 with a very high binding affinity but was not used in anticancer studies.¹⁷ 4,5-Diarylisoxazole¹⁸ was reported as an ATP-competitive inhibitor, which bound to the ATP pocket of PDK1 (IC₅₀ = 0.60 μM) and displayed a moderate anticancer activity (EC₅₀ = 9.21 μM) under normal conditions. Recently, Moore J. D. *et al.*¹⁹ reported a novel pan-isoform PDK inhibitor named VER-246608. Analysis of the 2.6 Å X-ray co-crystal of PDK2-VER-246608 (PDB code: 4V25) suggested that VER-246608 bound to the ATP binding pocket and formed a series of direct hydrogen bonds with various residues of PDK2, including Asp282 and Thr346, which are conserved amino acids in PDKs. Based on the PDK2-VER-246608 complex, herein we report a computer-aided high-throughput virtual screening approach and multiple bioassays to identify cellular active PDK inhibitors under hypoxia.

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Fig. 1 Representative samples of the reported PDK inhibitors.

2. Results and discussion

2.1 Inhibitor selection from high throughput virtual screening of a chemical library

Firstly, the co-crystal of the PDK2-VER-246608 complex was downloaded from the Protein Data Bank and prepared using Sybyl-X 2.0 software. As shown in Fig. 2, a chemical library containing 45 000 compounds was filtered to exclude compounds that do not fit the drug-likeness properties, then the remaining 12 854 compounds were optimized by the ligand structure preparation procedure in the Sybyl software. The optimized compounds were docked into the ATP binding pocket of PDK2 (PDB code: 4V25). Because the PDK1-ATP complex is not available in the Protein Data Bank, we selected the PDK2-VER-246608 complex as the template for docking. PDK1 (PDB code: 2Q8F) and PDK2 (PDB code: 4V25)

share a very high similarity in their structures, with an alignment root mean square (RMS) less than 1.1 (Å). In addition, all critical amino acid residues involved in the ATP binding pocket between PDK1 and PDK2 are conserved, which both justify the use of the PDK2-VER-246608 structure as a template in our docking studies to find PDKs, especially PDK1 inhibitors.

The top-ranked 200 compounds with higher total binding scores were selected for further consideration. Potential compounds were selected if they formed direct hydrogen bond interactions with the amino acids Asp282 and Thr346 in PDK2. Following the flow chart of the multistep virtual screening strategy, 12 candidates were chosen, and their chemical structures are shown in Fig. 3. All of the 12 candidates displayed similar binding modes to VER-246608 in PDK2 (Fig. 4), forming a series of direct hydrogen bond interactions with Asp282 and Thr346, which are conserved amino acids in the ATP binding pocket of PDKs. Then, we docked the 12 candidates into the ATP binding pockets of PDK1, PDK3, and PDK4, respectively. All of the compounds formed direct hydrogen bonds with Thr383 and Asp318 for PDK1, Thr352 and Asp287 for PDK3, Thr358 and Asp293 for PDK4 (see ESI† Fig. S1–S3). Finally, the 12 candidates were purchased from commercial sources, which were then evaluated for their biological roles in PDKs.

Fig. 2 Multi-step virtual screening strategy for the discovery of hit compounds.

2.2 Inhibitors blocked the PDK function

We tested the inhibitory potencies of 2–13 against PDK1 by using a biochemical assay, and VER-246608 was used as the positive control. As shown in Fig. 5A, some of the compounds displayed promising inhibitory efficacies against PDK1 at 10 μ M, except 3, 9, 10, 12, and 13, which showed little inhibitory effect on PDK1. However, when the test concentration was lowered to 1 μ M, only 6, 7 and 11 were observed to display satisfactory inhibitory activities. So, we further evaluated the full-dose inhibitory effect of 6, 7 and 11 on the enzyme. As shown in Fig. 5B–D, the IC_{50} values of 6, 7 and 11 were 1.26, 0.62, and 0.41 μ M, respectively, indicating weak potencies as compared to VER-246608 (IC_{50} = 95.7 nM), but still a good starting point as lead compounds for developing active PDK1 inhibitors. Since PDK1 shares a very high similarity in structure with PDK2, PDK3 and PDK4, compounds inhibiting PDK1 may also have an inhibitory effect on the other isoforms. Thus, 6, 7 and 11 were further tested for their

Fig. 3 Chemical structures of the identified inhibitors from the screening library.

Fig. 4 Proposed binding model of the selected compounds **2–13** in the ATP pocket of PDK2. A–L: Compounds **2–13** in the ATP binding pocket of PDK2. All the amino acid residues involved in the hydrogen bond interactions were labeled and shown as stick models, the formed hydrogen bonds were shown as yellow dashed lines, and the graphics of 3D views were drawn using PyMOL.

Fig. 5 The compounds that inhibited the PDK1 protein function. **A:** Compounds 2–13 and VER-246608 at 1 and 10 μM reduced the PDK1 activity. **B–D:** IC_{50} values of 6, 7, and 11 against PDK1. Values were reported as the mean $\pm$ SD of three independent experimental determinations. * $P < 0.05$, versus VER-246608 at 1 μM.

inhibitory effect on the isoforms. As expected, all the three compounds inhibited PDK2, PDK3 and PDK4. In particular, for 11, the IC_{50} values were 1.5, 3.9, and 6.8 μM for PDK2, PDK3, and PDK4, respectively (Table 1). This observation was in accordance with our molecular docking studies, another proof supporting our virtual screening results. To further verify this result, we titrated 11 with PDK1 buffer solution, and a K_d value of 0.95 μM was generated, suggesting that 11 bound to PDK1 (see the ESI†).

2.3 Inhibitors inhibited NCI-H1975 cell proliferation under hypoxia

With the compounds exhibiting satisfactory biochemical activities, next we turned our attention to addressing whether they reduce the proliferation of cancer cells. Therefore, the anticancer activities of compounds 6, 7, 11, VER-246608 and 2,2-dichloroacetophenone (DAP, a PDK inhibitor with anticancer activity)¹⁶ against NCI-H1975 cells were measured by the

MTT assay. As shown in Fig. 6A, all of the identified compounds and VER-246608 showed weak potencies against NCI-H1975 cells under normal conditions. However, after NCI-H1975 cells were treated with whole media containing cobalt dichloride (100 μM) to induce hypoxia for 72 h, the anti-proliferative activity of the compounds were investigated. As shown in Fig. 6B, 7 and 11 strongly inhibited cancer cell growth at 10 μM, with EC_{50} values of 4.28 and 3.59 μM (Fig. 6C and D), respectively. Meanwhile under the same experimental conditions, VER-246608 still weakly reduced NCI-H1975 proliferation at both concentrations, indicating that 7 and 11 outperformed VER-246608 in inhibiting NCI-H1975 proliferation under hypoxia.

2.4 Inhibitors reduced PDC phosphorylation and lactate formation in NCI-H1975 cells

Compounds 7 and 11 exhibited the most potent activities at both biochemical and cellular levels under hypoxia, so next we would like to know whether the two compounds would lead to the reduction of the PDC phosphorylation level in NCI-H1975 cells. NCI-H1975 cells were incubated with 7, 11, DAP and VER-246608 for 12 h, then the PDC phosphorylation level was analyzed using the western blotting experiment. As shown in Fig. 7A, 7 and 11 at 2 μM dramatically reduced PDC phosphorylation; DAP (10 μM) and VER-246608 (2 μM) also showed similar activities to 7 and 11. Targeting PDK1 should result in a switch of pyruvate metabolism from lactate

Table 1 Compounds that inhibited PDKs

Compds.	IC_{50} (μM)		
	PDK2	PDK3	PDK4
6	2.5 ± 0.45	7.8 ± 1.6	12.3 ± 2.2
7	2.6 ± 0.45	4.8 ± 1.1	8.7 ± 1.4
11	1.5 ± 0.21	3.9 ± 0.59	6.8 ± 1.3
VER-246608	0.13 ± 0.03	0.20 ± 0.01	0.24 ± 0.02

Fig. 6 Compounds that reduced NCI-H1975 cell proliferation under hypoxia. A/B: Compounds 6, 7, 11, DAP and VER-246608 at 1 and 10 μM reduced NCI-H1975 cell proliferation under normal conditions/under hypoxia. C/D: EC_{50} values of 7 and 11 against NCI-H1975 cell proliferation. Values were reported as the mean $\pm$ SD of three independent experimental determinations. * $P < 0.05$, versus the control group.

Fig. 7 Compounds 7 and 11 affected the PDK1 function. A: 7 and 11 at 2 μM reduced PDC phosphorylation in NCI-H1975 cells under hypoxia; DAP (10 μM) and VER-246608 (2 μM) were used as the positive control. B: Compounds 7, 11, DAP and VER-246608 at 5 μM reduced lactate formation in NCI-H1975 cells under hypoxia. C/D: Compounds 7 and 11 were docked into the PDK binding pocket and formed direct hydrogen bonding interactions with the amino acids in PDK. * $P < 0.05$, versus the control group.

production to OXPHOS. To verify this hypothesis, NCI-H1975 cells were treated with 7 and 11 at 5 μM for 4 h, then the

changes in lactate formation were measured. As shown in Fig. 7B, lactate formation was decreased by treatment with 7

and **11**, which is consistent with our hypothesis. Finally, we docked **7** and **11** into the ATP binding pocket of PDK2 (Fig. 7C and D), and direct hydrogen bonds formed between the compounds and amino acids Asp282, Thr346, and Gly286, all of which contributed to the PDK function.

3. Conclusions

In summary, we have reported herein the discovery of two potent PDK inhibitors, namely, **7** and **11**, by high throughput virtual screening and multiple bioassays. Compounds **7** and **11** were evaluated by a biochemical assay, which suggested that both compounds inhibited PDK activity. However, it is a challenge to correlate the observed PDK inhibition with cellular activity under normal conditions. In contrast, **7** and **11** reduced NCI-H1975 cell proliferation with EC₅₀ values of 4.28 and 3.59 μM , respectively, under hypoxia. Moreover, **7** and **11** attenuated lactate formation in NCI-H1975 cells, suggesting that the two compounds could serve as potential modulators to reprogram the glucose metabolic pathways in cancer cells. Collectively, these data demonstrated that **7** and **11** could be promising leads for the development of potent inhibitors of PDKs, especially PDK1. Efforts are ongoing to further explore the biological roles of **7** and **11** in cancer cells and improve their potency *via* structural modifications and optimization based on PDK crystal structures, and the results will be reported in due course.

4. Experimental section

4.1 Materials and cell culture

VER-246608 was synthesized as previously described.¹⁶ DAP was purchased from Sigma. All of the identified compounds were purchased from a local agent, with purities of not less than 95%. The primary antibodies against α -tubulin and the pyruvate dehydrogenase E1 α subunit were purchased from Invitrogen. NCI-H1975 was purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640. The culture medium was supplemented with 10% fetal bovine serum. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C in incubators, which were used at the exponentially growing stage for all biological experiments.

4.2 PDK activity evaluation

The PDK1 kinase activity was measured following a previously reported method with a little modification.⁴ 25 μL of double-distilled water and 5 μL assay buffer (10 $\times$, 250 mM Tris-HCl, 10 mM EDTA, 5 mM EGTA, 10 mM DTT, 50 mM MgCl₂) were added to 96-well plates. Then, 5 μL of compound (100 or 10 μM) were added to each well, except for the two control wells that should be added with 5 μL of 1% DMSO PBS buffer. Then, 5 μL ATP (10 μM) and 5 μL substrate (PDC E1, 0.2 μg per well) were added to all the wells. Finally, 5 μL PDK1, PDK2 and PDK3 (10 μM) or 5 μL PDK4 (20 μM) in protein buffer was added to all the wells to initialize phosphorylation except for the control well. The plate was mixed and in-

cubated at 37 °C for 30 min. Then, 50 μL of the appropriate Kinase-Glo reagent was added to each well. The plate was shaken and incubated for 10 min at room temperature. The luminescence was recorded on a microplate reader. The dose-response relationships of **6**, **7**, and **11** which inhibit PDK1 activity were studied by using the above-mentioned experimental protocol.

4.3 Cell viability

Cancer cell viability was measured by using the MTT assay. A suspension of NCI-H1975 cancer cells (3000 per well) was seeded in 96-well plates and cultured in a medium for 24 h with/without cobalt dichloride (100 μM), which was used to induce hypoxia conditions. Then, working compound solutions, namely, **10** and 1 μM , were prepared in a culturing medium and added to the corresponding plates (100 μL per well), in which the cells were incubated for 72 h. Then, the culturing medium was removed and 100 μL fresh medium containing 0.5 mg mL⁻¹ MTT was added. After 4 h of incubation, the supernatant was removed and 100 μL DMSO was added to each well. The absorbance of each well was measured at 490 nm using a microplate reader. Once the potential compounds (**7** and **11**) were identified, dose-response evaluation was conducted to determine the exact EC₅₀ values of these compounds. Eight different concentrations of **7** and **11** (40, 20, 10, 5, 1, 0.5, 0.1 and 0.01 μM) were used on NCI-H1975 cells to determine the EC₅₀ values.

4.4 Western blot analysis

NCI-H1975 cells were seeded in 6-well plates and treated with VER-246608, DAP, **7**, and **11** for 12 h. Then, the treated cells were incubated in cell lysate buffer with gentle shaking for 2 min and centrifuged at 10 000 rpm at 4 °C for an extra 5 min. Then, proteins were extracted with RIPA buffer containing a protease inhibitor cocktail. Equal amounts of total proteins per well were loaded for separation. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, blocked with TBST containing 3% BSA for 1 h at room temperature; then the primary antibody (anti-p-PDC E1 (1:500 dilution)) in the primary antibody diluent was added, and the mixture was incubated for 2 h at room temperature. After washing, the secondary antibody was added and incubated for 1 h at room temperature. Membranes were scanned using the ChemiDoc MP Imaging System (Bio-Rad) after 5 min of incubation in the Clarity Western ECL substrate.

4.5 Lactate measurement

A suspension of NCI-H1975 cells (2×10^5 per well) was seeded in 24-well plates and cultured overnight. Then, DAP, VER-246608, **7** and **11** at 5 μM were added into the plate, which were then incubated at 37 °C for 4 h. The medium was transferred into Eppendorf tubes, which were centrifuged for 10 min (10 000 rpm). Finally, 1 mL medium was collected, and lactate production was evaluated using a Nova BioProfile Flex analyzer.

4.6 Molecular docking

Molecular docking was conducted using the Surflex-Dock program. The crystal structure of PDK was obtained from the Protein Data Bank, which was prepared with all H addition and charge addition by the AMBER7 FF99 method. The structures of small molecules were subjected to polar H addition and energy optimized with a Tripos force field and charge optimized with the Gasteiger–Hückel method.

4.7 Statistical analysis

Data are reported as mean $\pm$ SD. Statistical analysis was performed using GraphPad Prism version 5.0 for Windows. * $P < 0.05$ is considered to be statistically significant.

Conflicts of interest

The authors declare no conflicts of interest.

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