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Development of Dihydroxyphenyl Sulfonylisoindoline Derivatives as Liver-targeting Pyruvate Dehydrogenase Kinase Inhibitors

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Abstract

Pyruvate dehydrogenase kinases 1–4 (PDK1–4) negatively control activity of the pyruvate dehydrogenase complex (PDC) and are up-regulated in obesity, diabetes, heart failure and cancer. We reported earlier two novel pan-PDK inhibitors PS8 [4-((5-hydroxyisoindolin-2-yl)sulfonyl)benzene-1,3-diol] (**1**) and PS10 [2-((2,4-dihydroxyphenyl)sulfonyl)isoindoline-4,6-diol] (**2**) that targeted the ATP-binding pocket in PDKs. Here, we developed a new generation of PDK inhibitors by extending the dihydroxyphenyl sulfonylisoindoline scaffold in **1** and **2** to the entrance region of the ATP-binding pocket in PDK2. The lead inhibitor (*S*)-3-amino-4-(4-((2-((2,4-dihydroxyphenyl)sulfonyl)isoindolin-5-yl)amino)piperidin-1-yl)-4-oxobutanamide (**17**) shows a ~8-fold lower IC₅₀ (58 nM) than **2** (456 nM). In the crystal structure, the asparagine moiety in **17** provides additional interactions with Glu-262 from PDK2. Treatment of diet-induced obese mice with **17** resulted in significant liver-specific augmentation of PDC activity, accompanied by improved glucose tolerance and drastically reduced hepatic steatosis. These findings support **17** as a potential glucose-lowering therapeutic targeting liver for obesity and type 2 diabetes.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX.

- Synthetic protocols and analytical data of common intermediates and **3** to **28** (PDF)
- Molecular formula strings (CSV),

INTRODUCTION

The macromolecular mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate to give rise to acetyl-CoA and is the gate-keeping enzyme linking glycolysis and the Krebs cycle. Due to its strategic location, the regulation of PDC activity is critical for glucose homeostasis and fuel preferences between glucose and fatty acids in the glucose-fatty acid cycle¹. The mammalian PDC is acutely modulated by reversible phosphorylation²³. The phosphorylation of PDC by the four isoforms of pyruvate dehydrogenase kinase (PDK 1–4) results in inactivation; and dephosphorylation by the two pyruvate dehydrogenase phosphatase (PDP) isoforms (PDPs 1–2) restores PDC activity⁴. PDKs and PDPs are anchored to the E2p/E3BP core of PDC through interactions with the inner lipoyl-bearing domain. The phosphorylation state of PDC is controlled by relative activities of PDKs and PDPs in tissues. When blood glucose is high upon the consumption of carbohydrate, PDC is largely dephosphorylated and active to promote glucose disposal and the storage of fatty acids as triglycerides. When glucose levels are low during fasting, PDC is highly phosphorylated and inactive, so as to preserve the substrates for gluconeogenesis (pyruvate, lactate and alanine) and facilitate glucose synthesis and fatty acid oxidation¹.

Different PDKs are distinctively up-regulated in obesity⁵⁶, diabetes⁷⁸, heart failure⁹, ischemia¹⁰ and cancer¹¹²¹³. Single knockout in PDK2¹⁴ or PDK4⁶ and double knockout in PDK2 and PDK4¹⁵ result in enhanced glucose tolerance and significantly attenuated hepatic steatosis in mice fed high-fat diet. Suppression of PDK activity impedes tumor growth¹⁶¹⁷¹², reduces glucose levels in diabetes¹⁸ and improves bioenergetics in heart failure⁹. Therefore, PDKs are potential therapeutic targets for these important human diseases.

At present, there are no effective PDK inhibitors for pre-clinical or clinical studies targeting the PDKs. The commonly used dichloroacetate (DCA) is a low-potency, non-specific PDK inhibitor¹⁹, and the high doses required for this compound produce neurological toxicity and promote tumor growth²⁰. The pro-drug design for DCA in the form of a triphenylphosphonium DCA²¹ or diisopropylamine²² conjugates facilitates import of these compounds into mitochondria, resulting in efficient inhibition of PDK activity in cell culture. However, both DCA conjugates were unsuitable for *in vivo* studies because the ester bond is presumably cleaved by esterases in plasma. Dihydrolipoamide mimetics including

(R)-4-(3-chloro-4-(3,3,3-trifluoro-2-hydroxy-2-methylpropanamido)phenylsulfonyl)-N,N-dimethylbenzamide (AZD7545)²³ and secondary amides of (R)-3,3,3-Trifluoro-2-hydroxy-2-methylpropionic acid²⁴ inhibit PDK2 by abrogating its interaction with the E2/E3BP core of PDC²⁵²⁶. Paradoxically, these dihydrolipoamide mimetics bind to the lipoyl-binding pocket of core-free PDK4 and strongly stimulates kinase activity; the results preclude these compounds as *bona fide* PDK inhibitors. Recently, pan-PDK inhibitors PS8 [4-((5-hydroxyisindolin-2-yl)sulfonyl)benzene-1,3-diol] (**1**) and PS10 [2-((2,4-dihydroxyphenyl)sulfonyl)isindoline-4,6-diol] (**2**) targeting the ATP-binding pocket were shown to selectively inhibit PDK activity, accompanied by marked increase in hepatic PDC activity in diet-induced obese mice²⁷. A related pan-PDK inhibitor N-(4-(2-chloro-5-methylpyrimidin-4-yl)phenyl)-N-(4-((2,2-difluoroacetamido)methyl)benzyl)-2,4-dihydroxybenzamide (Ver-246608) also targeting the ATP-binding pocket shows anti-proliferative properties to cancer cells under nutrient-depleted conditions²⁸. A covalent PDK inhibitor morpholine-4-carbothioic dithioperoxyanhydride (JX06) was proposed to suppress kinase activity by modifying a conserved cysteine-240 close to the ATP-binding pocket in PDK1; the growth of cancer cells with high dependence on glycolysis were impeded by this PDK inhibitor²⁹. However, it does not inhibit PDK4 efficiently, and the selectivity of covalent cysteine-residue modification by this compound is unknown.

We sought to develop a new generation of PDK inhibitors that can be used to improve glucose metabolism and correct metabolic dysfunction *in vivo*. The afore-mentioned PDK inhibitor **1** and **2** targeting the ATP-binding pocket displayed high selectivity for PDK²⁷ over the structurally related Hsp90³⁰. Prolonged treatment with **1** or **2** resulted in improved glucose tolerance with notably diminished hepatic steatosis in DIO mice²⁷. The current communication reports the continuation of structure-based inhibitor optimization for considerably improved potency and pharmacokinetic properties of these PS-series compounds. These second generation of PDK inhibitors have potential to become drugs for the treatment of obesity and type 2 diabetes.

RESULTS AND DISCUSSION

Baell and Holloway³¹ reported substructures that frequently appear as hits in high throughput screening assays. These structural elements are designated as pan assay interference subcompounds (PAINS). Many of the PAINS compounds reported have questionable structure function relationship (SAR) and are also known to contain reactive functional groups. The Pipeline Pilot protocol (see the Experimental section) was applied to filter PAINS elements among all representative classes of compounds presented here. None of these compounds contained a PAINS substructure.

As shown in Figure 1a, the ATP-binding pocket in PDK2 consists of four regions (adenine, ribose, triphosphate and entrance), designated according to the location in contact with distinct chemical moieties of the bound ATP. The superimposition of the PDK2-ATP structure with the PDK2- **2** structure reveals profoundly different binding modes for ATP and the PDK inhibitor. Compound **2** with a dihydroxyphenyl sulfonylisindoline scaffold binds primarily to the adenine region and its neighborhood, leaving the ribose and triphosphate regions of the ATP-binding pocket largely unoccupied. Attempts to extend **2**-

based compounds into the ribose and triphosphate regions of the ATP-binding pocket to improve the potency of these PDK inhibitors were unsuccessful; none of these new compounds show lower IC_{50} 's over **2**. On the other hand, in the PDK2- **2** structure, a bound tartrate molecule from the crystallization buffer showed good electron density proximal to the entrance region of the ATP-binding pocket (Figure 1b). We therefore adopted a different strategy to develop more potent PDK inhibitors than **2** by targeting the entrance region.

We described previously that the 6-hydroxyl group at the R_2 position of the isoindoline ring in **1** or **2** make a hydrogen bond to Glu-262 of PDK2²⁷ and confers sub-micromolar IC_{50} 's (Table 1). We attempted to identify the chemical moiety that can serve as a secondary scaffold to extend a PS-series PDK inhibitor to the entrance region of the ATP-binding pocket. SAR analysis was performed with various substitutions made on the isoindoline ring. In **3**, replacement of the hydroxyl group in **1** by an amino group causes a 2-fold higher IC_{50} . The result suggests the amino group is a poorer donor than the hydroxyl for making hydrogen bonds to Glu262. Installments of bulky ring structures in the R_2 position as in **4** and 4-((5-(piperidin-4-ylamino)isoindolin-2-yl)sulfonyl)benzene-1,3-diol (**5**) lead to significantly better IC_{50} than the parental **1**. The smaller R_2 substitution in **5** compared to **4** results in a better IC_{50} (195 nM) in **5** than **4**. This may be due to the overall higher R_2 polarity in **5** than **4** and not the size difference between these two installments. The beneficial effect of polarity is evidenced by the markedly reduced potency in **6** compared with **5**, when the more polar piperidine in **5** is replaced by a cyclohexane in **6**. This is expected because the PDK2 protein in this region is hydrophilic and negatively charged (Figure 1a). The secondary amine linked to the isoindoline in **4**, **5** or **6** proves to be also critical for maintaining the inhibitor potency, as its absence in **7** and **8** results in considerably higher IC_{50} 's compared to **4**, **5** and **6**.

The above SAR results identified **5** as a suitable intermediate compound that allows additional moieties to be incorporated into the R position (a secondary amine) of the piperidine ring (Table 2). Several substitutions for making possibly more hydrogen-bond donors or acceptors were tested. These included carboxylic acids with different alkyl chain lengths (**10–12**), a sugar (**13**), amino acids (**14–18**), a benzoic acid (**19**) and a glycol (**20**). Only those compounds with incorporated amino acids show significant improvements in IC_{50} 's over parental compound **5**. The best compound is (*S*)-3-amino-4-((2-((2,4-dihydroxyphenyl)sulfonyl)isoindolin-5-yl)amino)piperidin-1-yl)-4-oxobutanamide (**17**) with an asparagine moiety added to the piperidine ring; it shows an IC_{50} of 58 nM, which is 3.4-fold better than **5**.

The above results established **17** with an asparagine-decorated piperidine ring as the best PDK inhibitor among those tested. We then dissected the SAR of different linkers (R) between the isoindoline ring and asparagine moiety of parental **17** (Table 3). It was speculated that interactions between asparagine moiety and PDK2 may be affected by the size, angle and flexibility of the linker. Among the azacycloalkyl rings of different sizes (**21–25**) and diamino alkyl chains of various lengths (**26** and **27**) tested, the azacyclohexyl piperidine linker in **17** still shows the best IC_{50} for inhibition of PDK2 activity (Table 3).

However, similar potencies or IC_{50} 's to **17** were exhibited by **23** and **26**, despite the presence of distinctly different linkers in the latter two compounds than that in **17**.

To shed light on modes of binding responsible for improved inhibition potencies, crystal structures of PDK2 in complex with **5** and **17** were determined. To prevent possible interference of tartrate with binding of **17** to PDK2, we developed a new crystallization condition in which Na-tartrate was omitted from the previous crystallization buffer²⁷. Crystals grown in the absence of tartrate were soaked with **17** and the structure of the PDK2 in complex with **17** was solved. The PDK2 protein packed into crystals in different space groups with comparable resolutions under two crystallization conditions: $I4_122$ for the PDK2- **5** structure with tartrate and $P6_4$ for the PDK2- **17** structure without tartrate (Table 4). The entire PDK2 structures solved with these two crystals in different space groups are virtually identical with the superimposition root-mean-square deviation (RMSD) of 0.88 Å. Figure 2 shows side-by-side comparisons of binding modes between **2**, **5** and **17** with respect to their polar interactions with PDK2, and water molecules in the entrance region of the ATP-binding pocket. As expected, the nitrogen atom between the isoindoline and piperidine in **5** or **17** (Figure 2b and 2c) assumes the same position as the hydroxyl oxygen atom in **2** (Figure 2a), and forms a hydrogen bond to Glu-262 from PDK2. The piperidine ring in compound **5** or **17**, replaces the water molecule w2 in the PDK2- **2** structure, whereas w1 and w3 are displaced from their original positions also in the PDK2- **2** structure. It should also be noted that in the PDK2- **5** structure, w3 serves as a bridge by making hydrogen bonds with the piperidine nitrogen and the carbonyl oxygen on the Glu-262 main chain (Figure 2b). Unexpectedly, the structure of PDK2- **17** shows that the asparagine moiety of **17** does not fit into the putative "tartrate pocket"; instead it docks to the entrance region of that pocket and forms a bridged hydrogen bond to Glu-262 through a water molecule w7; moreover, two water molecules, w4 and w8, fill the vacancy caused by the absence of tartrate (Figure 2c). Furthermore, $F_o - F_c$ omit maps (blue mesh) contoured at 3σ indicate that in contrast to the good density associated with the entire bound **5** (Figure 2b), the amino and amide groups of the asparagine moiety in **17** lack density (Figure 2c). The relatively weak density signal on the asparagine moiety suggests that this portion of the bound **17** may not be in the most favorable conformation.

In addition to the high potency on PDK2 (IC_{50} = 81 nM and 58 nM with or without E2E3BP, respectively), **17** also shows good inhibition on three other PDK isoforms according to *in vitro* assays (Table 5). Compared with IC_{50} values of **1** and **2** on the sub-micromolar scale²⁷, **17** makes significant improvements on the inhibition of PDK2 and PDK4, but not PDK1 and PDK3. We showed previously that the anchoring of PDK3 to the E2/E3BP core, as existed in the native PDC macromolecular structure, markedly reduces the binding affinity of PDK3 to nucleotides ATP and ADP³². The results explain the improved IC_{50} of **17** for PDK3 in presence of E2/E3BP compared to its absence (Table 5), as **17** targets the ATP-binding pocket,

The binding of **1**, **2**, **5** and **17** to PDK2 were analyzed by isothermal calorimetry (ITC), producing dissociation constants (K_d) of 426 nM, 156 nM, 110 nM and 22 nM, respectively. The binding of these compounds to PDK2 is mainly enthalpy driven; however, the higher

binding affinity of **5** than **1** or **2** results from gains in the entropy term (decrease to 4.64 from 5.54 or 6.57 kcal/mol in $-T \Delta S$) (Figure 3). These favorable significant changes in entropy are likely attributed to the replacement of ordered waters by the piperidine ring in the PDK2-**5** structure (Figure 2b). Compared to **5**, the presence of the asparagine moiety in **17** is apparently responsible to the large 3.4 kcal/mol gain in enthalpy change (ΔH) by the polar contacts to PDK2 (Figure 2c), despite the 2.7 kcal/mol loss in entropy (Figure 3).

Compound **17** levels in plasma, liver, and muscle female CD-1 mice were monitored after receiving a single- of dose at 30 mg/kg by intraperitoneal injection; these results were compared to those obtained with **2** at 70 mg/kg (Table 6). Despite the comparable terminal $t_{1/2}$ in the plasma, **17** shows a markedly faster clearance (CL/F) from plasma than **2** at 0.27 g/min and 8.13 g/min, respectively. Based on the AUC_{last} term, **17** concentration persisted in muscle is >10-fold lower than **2** in the same tissue. In contrast to muscle and plasma, **17** remained in the liver (AUC_{last}) is >10-fold higher than **2**. Moreover, the unit-time clearance (CL/F) of **17** in liver is >30-fold slower than **2**. Taken together, the above pharmacokinetic data strongly suggest that compared to **2**, **17** preferentially targets liver over muscle and plasma.

The liver-specific accumulation of **17** in comparison to **2** is striking. It is possible that physiochemical properties of the two compounds may in part explain this behavior. Liver pH is predicted to be around pH 7³³. At this pH, **2** is expected to be largely negatively charged based on calculation of pKa values using the pKa calculator plugin from ChemDraw Professional Version 15.0.0.106. On the other hand, approximately 30% of **17** is predicted to be both positively and negatively charged with multiple calculated pKa values of 5.6, 6.3 and 12.4 using the same program. It is possible that one of the cationic liver influx transporters such as OCT1 or OCT3, which are highly expressed in the liver and known to transport both cations and zwitterions³⁴, are responsible for the selective accumulation of **17** in the liver.

Compound **17** was administered to diet-induced obese (DIO) mice, which had been on a high-fat diet for 18 weeks, at 40–45 mg/kg/day using osmotic mini-pumps. PDC activity in liver, heart and muscle was measured after two weeks of the treatment. PDC activity in **17**-treated liver was increased by about 6-fold compared to the vehicle control (Figure 4a). In contrast, no PDC activation by **17** was detected in heart and muscle. The liver-specific augmentation of PDC activity is consistent with the vastly preferential uptake of **17** by the liver than the muscle and plasma, as indicated by their AUC_{last} values (Table 6). The prominent increase in hepatic PDC activity correlates well with decreased phosphorylation of the E1 α subunit (pE1) in the liver homogenate (Figure 4b), indicating the direct inhibition of PDK activity by **17** in the liver.

Results from the glucose tolerance tests (Figure. 4c) show that when challenged with 1.5 g/kg of glucose, the plasma glucose level in vehicle-treated DIO mice, which was below 200 mg/dl at 0 min, peaked at 540 mg/dl at 30 min and was reduced to 300 mg/dl at 120 min. In **17**-treated DIO mice, the glucose concentration at 0 min was slightly lower than that in the vehicle-treated animals, reached 375 mg/dl at 30 min and returned to below 200 mg/dl at 120 min. The two groups of animals show significant differences ($p < 0.05$) in glucose

concentrations at 20, 30, 60, and 120 min, with lower glucose levels uniformly observed in the **17**-treated DIO mice. The data therefore suggest that the **17** treatment increases glucose tolerance over vehicle-treated mice. Finally, noticeably larger amounts of fat were present in the liver of the vehicle-treated DIO mice compared with the **17**-treated, when the liver slices were stained with Oil Red O (Figure 4d). The accumulated hepatic fat was primarily macrovesicular in vehicle-treated DIO mice and became microvesicular in the **17**-treated counterpart.

CONCLUSIONS

The present study has focused on the development of a second generation of dihydroxyphenyl sulfonylisoindoline derivatives as pan-PDK inhibitors. The significantly improved IC₅₀ of lead **17** was achieved by extending the **2** scaffold via the piperidine linker to the entrance region of the ATP-binding pocket. The presence of the R group in asparagine provides additional contact with Glu-262 so as to improve binding affinity to PDK2, as disclosed by the crystal structure of the PDK2- **17** complex.

Liver is the major organ responsible for maintaining homeostasis and continuous supply of glucose, lipids and other essential metabolites to peripheral tissues³⁵. A prominent feature of **17** is its preferential uptake and retention by the liver. This property confers significant augmentation of hepatic PDC activity converting the liver from a normally gluconeogenic organ³⁶ to a glucose oxidative machinery. The liver-specific inhibition of PDKs by **17** is also desirable. Systemic inactivation of PDKs in PDK2/PDK4 double knockout mice results in hypoglycemia and hypothermia in fasting mice due to exhausted glucose oxidation¹⁵. Moreover, targeting PDK inhibitors to the liver may prevent extra-hepatic toxicity and improve the efficacy of glucose-lowering therapeutics for the treatment of obesity and type 2 diabetes³⁷. This notion is further buttressed by the improved glucose tolerance with drastically reduced hepatic steatosis in **17**-treated DIO mice.

EXPERIMENTAL SECTION

Chemical Synthesis

All reactions were carried out under an atmosphere of nitrogen in flame-dried glassware with magnetic stirring unless otherwise indicated. Commercially obtained reagents were used as received. Solvents were dried by passage through an activated alumina column under argon. Liquids and solutions were transferred via syringe. All reactions were monitored by thin-layer chromatography with E. Merck silica gel 60 F254 pre-coated plates (0.25 mm). Structures of the target compounds in this work were assigned by use of NMR spectroscopy and MS spectrometry. The purities of all compounds were >95% as determined on Waters HPLC (Column: X Bridge C18, Eluents: 0.1% NH₄HCO₃/H₂O and CH₃CN) with 2998PDA and 3100MS detectors, using ESI as ionization. Pre-HPLC is used to separate and refine high-purity target compounds. ¹H and ¹³C NMR spectra were recorded on Varian Inova-400 or 500 spectrometers. Data for ¹H NMR spectra are reported relative to CDCl₃ (7.26 ppm), CD₃OD (3.31 ppm), or DMSO-d₆ (2.50 ppm) as an internal standard and are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, br = broad), coupling constant J (Hz), and integration.

PAINS Substructure Filters

A Pipeline PilotTM (v 9.0.2, Biovia, Inc.) protocol was developed by annotating and incorporating SMARTS with collaborative input from Jonathan Baell³¹³⁸. The protocol was employed to identify PAINS elements within each representative class of structures presented here (**5**, **12**, **13**, **17**, **19** and **20**). It provided the relevant PAINS family designation using structural information in the Mol file format.

Proteins

Recombinant human PDK2 was expressed and purified as a N-terminal His₆-tagged SUMO fusion protein with a tobacco-etch-virus protease (TEV) cleavage site in front of the N-terminal PDK2 sequence²⁶, and was used directly for the activity and binding assays. For crystallization, the protein was subjected to a TEV-protease digestion, and the untagged PDK2 protein was purified on a Superdex 200 column in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 5 mM DTT. The purified protein was concentrated to 35–40 mg/ml and stored at –80°C in small aliquots. Recombinant human PDK1, PDK3 and PDK4 were expressed and purified as described previously²⁶.

Assay for Inhibition of PDK Activity

To determine the IC₅₀ for PDK inhibitors, a mixture containing 0.05–0.2 μM PDK, 6 μM E1, with or without 0.5 μM of the PDC core E2/E3BP, and various amounts of inhibitor was incubated at 25°C for 10 min in a buffer of 10 mM MOPS (pH 7.5), 5 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 0.01% (v/v) Tween-20, and 0.05 mg/ml bovine serum albumin before the addition of ATP to 50 μM to initiate the reaction. All inhibition titrations were performed at 10 dose-points in a 3.162-fold dilution series, with each inhibitor concentration tested in duplicate. The range of titration was chosen to have the IC₅₀ roughly in the middle of the titration. The remaining steps were described previously²⁷. IC₅₀ values were obtained by the curve fitting of inhibition isotherms using SigmaPlot 12.0 (Systat Software, Inc.).

Isothermal Titration Calorimetry (ITC)

The SUMO-tagged PDK2 was dialyzed against one liter of the dialysis buffer containing 50 mM MOPS, pH 7.0, 50 mM KCl, 1 mM MgCl₂, and 0.5 mM β-mercaptoethanol. Ligand solutions (100–200 μM) were placed in the titration syringe and injected in 8-μl increments into the reaction cell containing 1.4 ml of 10–20 μM PDK2 at 15°C in a VP-ITC microcalorimeter (GE Healthcare, Piscataway, NJ). Both protein and ligand were prepared in the dialysis buffer plus 4% of DMSO. All of the ITC data were initially analyzed by the NITPIC program³⁹ to construct the baseline, followed by curve-fitting in Origin 7 to obtain binding parameters. The concentrations of SUMO-tagged PDK2 protein were determined by measuring A₂₈₀ and using calculated molar extinction coefficients (M⁻¹·cm⁻¹) of 49,530.

Crystallization of PDK2 and Inhibitor Complexes

Crystals of human PDK2 for soaking with **5** and **17** were obtained by the hanging-drop vapor-diffusion method under two different crystallization conditions. For **5**, the PDK2 protein solution was mixed with the same volume of a well solution containing 0.9 M ammonium tartrate, 0.1 M sodium acetate pH 4.6 and was kept in a 20°C incubator. For **17**,

the well solution containing 9% isopropanol, 0.1 M MgCl₂, 50 mM ammonium sulfate, and 0.1 M sodium acetate (pH 5.6) was incubated with an equal volume of the PDK2 solution at 4°C. Mature PDK2 crystals were transferred to a fresh soaking solution comprising the well solution, 5% glycerol and **5** or **17**. After overnight incubation, crystals were serially transferred to a cryo-solution containing 20% glycerol and snap frozen in liquid nitrogen.

Structure Determination and Refinements

All X-ray diffraction data for PDK2 and PDK2-inhibitor complexes were collected at beamline 19-ID at the Advanced Photon Source, Argonne National Laboratories. Diffraction data for each PDK2-inhibitor complex were collected from a single crystal. The molecular replacement, structure modeling and refinement were performed as described previously⁴⁰. The crystal structure of inhibitor-free human PDK (PDB code 2BTZ) was used as the search model.

Pharmacokinetic Studies

Six- to eight-week old female CD-1 mice (Charles River Laboratories, Wilmington, MA) were dosed IP with 70 mg/kg **2**, (0.2 ml/mouse in 5% ethanol and 95% 0.1 M sodium bicarbonate, pH 9) or 30 mg/kg **17** (0.2 ml/mouse in 10% DMSO/20% dH₂O/70% of a 25% solution of 2-hydroxypropyl)- β -cyclodextrin in water). At various times post-dose, mice were euthanized and blood and tissues collected. Plasma was isolated from whole blood, and tissues were harvested and homogenized in PBS. Compound was extracted using a two-fold excess of methanol (**17**) or acetonitrile (**2**) containing 0.1% formic acid and an internal standard, n-benzyl-benzamide (**17**) or tolbutamide (**2**). Extracted compound was evaluated in comparison to standard curves prepared in the same matrix using a Sciex 3200 QTRAP mass spectrometer coupled to a Shimadzu LC. Compound **2** was detected in negative MRM (multiple reaction monitoring) mode using the following transitions: compound **2** 322.1 to 172.8; tolbutamide 269.1 to 169.9. Compound **17** was detected in positive MRM mode using the following transitions: **17** 504.294 to 298.3; n-benzyl-benzamide 212.1 to 91.1. Chromatographic conditions were as follows for **2**: Buffer A: Water + 0.1% formic acid; Buffer B: methanol + 0.1% formic acid; flow rate 1.5 ml/min; column Agilent C18 XDB column, 5 micron packing 50 X 4.6 mm size ; 0 – 1.5 min 3% B, 1.5 – 2.2 min gradient to 100% B, 2.2 – 3.5 min 100% B, 3.5 – 3.6 min gradient to 3% B, 3.6 – 4.5 3% B. Conditions for **17** were identical except the gradient to 100% B was extended to 1.5 to 2.5 min. The hold at 100% B was still completed at 3.5 min. Pharmacokinetic parameters were calculated using noncompartmental analysis with Phoenix WinNonlin (Pharsight Corp, Cary, NC). Terminal T_{1/2}, half-life of the terminal phase; C_{max}, observed maximum plasma concentration; T_{max}, time to reach C_{max}; AUC_{last}, area under the concentration-time curve from 0 to the last measured point.

Glucose Tolerance Test

Mice were fasted for 6 hours after compound treatment. Ten hours after compound administration, 1.5 g/kg of glucose was delivered intraperitoneally to mice. Tail vein serum samples were collected immediately before and 15, 30, 60 and 120 minutes after the glucose

challenge. Plasma glucose concentrations (mg/dL) were determined using glucose strips in a BAYER glucose meter.

Treatments of Diet-induced Obese Mice with PDK Inhibitor

Six- to eight-week old C57BL/6J male mice were obtained from the local campus breeding colony at UT Southwestern Medical Center (Dallas, TX) and randomized into two groups: vehicle- and treated with **17**. Prior to the treatment, mice were fed with a 60% high-fat diet, which contained 32% saturated and 68% unsaturated fat (catalog number: D12492, Research Diet Inc. New Brunswick, NJ) for 18 weeks to produce DIO mice. Compound **17** was dissolved in 100% DMSO and then diluted to make a 10% DMSO aqueous solution containing 17.5% (w/v) (2-hydroxypropyl)- β -cyclodextrin for delivery. Animals were dosed at 40–45 mg/kg using osmotic pumps (ALZET Osmotic Pumps model 2001), which were implanted into the subcutaneous cavity on the back of animals. At the end of two-week treatment, animals were euthanized using carbon dioxide asphyxiation followed by cervical dislocation and dissection. Blood was harvested by cardiac puncture and stored on ice. Acidified citrate dextrose was used as an anticoagulant. Immediately after blood collection, heart, liver, kidneys and both hind-leg quadriceps muscles were removed and snap frozen in liquid nitrogen. Average ischemia time before organ harvest was about 2 to 3 min. PDC activities in mouse tissues were assayed as described previously²⁷. Blood was centrifuged in an Eppendorf 5415R refrigerated microcentrifuge at $9,300 \times g$ for 5 min to isolate plasma; the latter was subsequently stored at -80°C . Animal used in the above studies were housed and handled in accordance with guidelines defined by the Institutional Animal Care and Use committee at UT Southwestern Medical Center.

Western Blotting

SDS-PAGE gels were run using 15–20 μg of protein lysate per lane. Western blots were transferred to PVDF membranes for 2 hrs at 200 mV. PVDF membranes were blocked with 5% non-fat dried milk and then probed using polyclonal antibodies to pyruvate dehydrogenase/decarboxylase E1 α and to phosphorylated E1 α (pE1 α). The E1 α antibody was obtained from MitoSciences/Abcam (Cambridge, MA). Antibodies against the phosphorylated serine (pSer293) residue of the E1 α subunit were purchased from EMD Millipore/Calbiochem Biochemical (Billerica, MA). One milliliter of Luminata Forte western HRP (Millipore Corporation, Billerica, MA) substrate reagent was pipetted across the membrane for signal detection in a FluorChem E system (Cell Biosciences, Santa Clara, CA).

Blood Biochemistry

The levels of lactate, cholesterol, and triglyceride were measured by Vitros 250 blood chemistry analyzer (Johnson & Johnson Inc.) in the Metabolic Phenotyping Core in UT Southwestern Medical Center.

Histochemistry of the Liver

Histological examination of the liver was performed in the institutional Immunohistochemistry Laboratory. Liver tissue was dissected, grossly trim then fixed by

immersion for 48 hrs in 4% formalin/PBS (4% formic acid, 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.5) at 4°C. Liver samples were then transferred to 10% (w/v) sucrose in PBS and incubated at 4°C for 24 hrs. Tissues were incubated in 18% sucrose in PBS at 4°C for 24 hours. Finally, samples were transferred to a fresh 18% sucrose solution and embedded in optimal cutting temperature compounds (OCT), cryo-sectioned and stained with Oil Red O.

Statistical Analysis

Data are shown as mean $\pm$ standard deviation. Prism 6.0 (GraphPad Inc.) was used to perform the two-tailed Student t test for comparison between groups, and non-linear regression to fit inhibition curves. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ are considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

PDC	pyruvate dehydrogenase complex
E1	pyruvate dehydrogenase
E2	dihydrolipoyl transacetylase
E3	dihydrolipoamide dehydrogenase
E3BP	E3-binding protein
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase
DCA	dichloroacetate
Hsp90	heat-shock protein 90
DIO	diet-induced obese
RMSD	root-mean-square deviation
ITC	isothermal titration calorimetry
SAR	structure activity relationship
PAINS	pan assay interference subcompounds

HTS	high throughput screening
GTT	glucose tolerance test
DMSO	dimethyl sulfoxide
PBS	phosphate buffer saline

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Figure 1.

The PDK inhibitor **2** binds to the ATP binding pocket in the PDK2 structure (PDB entry 4MPN). (a) Superimposition of the ATP-binding pocket (surface representation) occupied by ATP (the stick model) with the one by **2**. Compound **2** binds to both the adenine and entrance regions, but not the phosphate region, of the pocket. The blue and red colors indicate positively and negatively charged surfaces, respectively. (b) The PDK2 structure showing both the bound **2** and tartrate. The $F_o - F_c$ omit map (blue mesh) of the bound tartrate is contoured to 3σ .

Figure 2.

The PDK2 ATP-binding pocket occupied by **2**, **5** or **17**. (a) Interactions of the bound **2** and tartrate with side chains of the amino acids juxtaposed to ATP binding pocket (4MPN). (b) The bound **5** and tartrate showing similar interactions to (a). (c) The bound **17** in the absence of tartrate. The F_o-F_c omit map (blue mesh) of bound **5** or **17** and water molecules is contoured to 3σ .

Figure 3.

Thermodynamic analysis of **1**, **2**, **5** and **17** binding to PDK2. Thermodynamic signatures of inhibitor binding were obtained by isothermo titration calorimetry using the Gibbs equation

$G = H - T S$, where G is Gibbs binding energy; H , binding enthalpy; S , binding entropy; T , absolute temperature. The derived dissociation constants (K_d) for **1**, **2**, **5** and **17** are 426 nM, 156 nM, 110 nM and 22 nM, respectively.

Figure 4.

Augmentation of PDC activity in liver with improved glucose tolerance and lessened hepatic steatosis in **17**-treated diet-induced obese mice. C57BL6J male mice fed high-fat diet for 18 weeks were treated with **17** at 40–45mg/kg/day by mini pumps for two weeks. Tissues were harvested and analyzed for (a) PDC activity and (b) protein levels of phosphorylated E1α subunit (pE1) and total E1α (E1) in liver homogenates with GAPDH as a loading control. (c) Glucose tolerance test. **17**- and vehicle-treated DIO mice were fasted for 6 h, followed by injection of 1.5 g glucose/kg by intraperitoneal injection. Blood glucose levels were monitored at 0–2 h after the glucose injection. *, $p < 0.05$. (d) Oil Red O stains of liver slices (20× magnification) from vehicle- and **17**-treated DIO mice.

Table 1

IC₅₀ for PDK2 activity of dihydroxyphenylsulfonylisoindoline compounds substituted at R₁ and R₂ position.

Compounds	R ₁	R ₂	IC ₅₀ , nM
1	H	OH	570
2	OH	OH	456
3	H	NH ₂	1177
4	H		416
5	H		195
6	H		1196
7	H		4371
8	H		1346

^aPDK2 activity was assayed in mixture containing 50 μ M ³²P- γ -ATP, 6.0 μ M E1 protein in the absence and presence of increasing concentration of the compound. The incubation was for 2 min at 22°C. PDK2 activity was determined by the incorporation of radioactivity into the E1 protein. IC₅₀ was referred to the compound concentration required to inhibit 50% of PDK2 activity.

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Table 2

IC₅₀ for PDK2 activity of compounds with the piperidine ring substituted at the R position

Compounds	R	IC ₅₀ , nM
5	H	195
10		1,657
11		1,610
12		629
13		432
14		91

Compounds	R	IC ₅₀ , nM
15		68
16		72
17		58
18		580
19		565

Compounds	R	IC ₅₀ , nM
20		154

Table 3

IC₅₀ for PDK2 activity of compounds with substitution in the linker (R position)

Compounds	R	IC ₅₀ , nM
17		58
21		156
22		221
23		65
24		767
25		1514

Compounds	R	IC_{50} , nM
26		67
27		357

Table 4

Crystallographic Data Collection and Refinement Statistics

	PDK2+ 5	PDK2+ 17
PDB ID	5J71	5J6A
Space group	I4 ₁ 22	P6 ₄
Cell dimensions		
<i>a</i> , <i>b</i> (Å)	110.75	109.39
<i>c</i> (Å)	228.58	84.42
α, β, γ (°)	α=β=γ=90	α=β=90, γ=120
Resolution (Å)	50-1.65 (1.68-1.65)	50-2.05 (2.09-2.05)
<i>R</i> _{merge}	4.9 (94.9)	6.8(91.8)
<i>I</i> / σ <i>I</i>	46.7 (2.7)	24.7(2.2)
Completeness (%)	99.8 (100.0)	99.5(99.2)
Redundancy	11.3 (11.4)	7.3(7.1)
Refinement		
Resolution (Å)	1.65	2.05
No. reflections	83039	34146
<i>R</i> _{work} / <i>R</i> _{free} (%)	0.184/0.197	0.196/0.229
No. atoms	5834	2902
Protein	5532	2758
Ligand	63	35
Water	239	109
<i>B</i> -factors		
Protein	20.4	31.1
Ligand	14.9	29.9
Water	28.5	33.7
R.m.s. deviations		
Bond lengths (Å)	0.010	0.008
Bond angles (°)	1.057	0.897

* Values in parentheses are for highest-resolution shell.

Table 5IC₅₀ Values of 17 for the Four PDK Isoforms

	IC ₅₀ of 17, μ M - E2/E3BP ^a	+E2/E3BP
PDK1	2.54 $\pm$ 0.34	2.06 $\pm$ 0.90
PDK2	0.058 $\pm$ 0.011	0.081 $\pm$ 0.026
PDK3	45.8 $\pm$ 6.4	1.71 $\pm$ 0.31
PDK4	0.284 $\pm$ 0.051	0.416 $\pm$ 0.135

^aKinase activity was assayed as described in Table 1 for the different PDK isoforms in the absence or presence of 60-meric E2/E3BP core of the PDC (n = 3). The molar ratio of E1 to the E2/E3BP core was 40 to 1.

Table 6Mouse pharmacokinetic parameters for **2** and **17**^a

	Compound 2, 70 mg/kg IP	Compound 17, 30 mg/kg IP
Plasma		
Terminal t _{1/2} ^b	339 min	288 min
C _{max}	29,000 ng/ml	5443 ng/ml
T _{max}	10 min	10 min
AUC _{last}	1,494,106 min*ng/ml	234,088 min*ng/ml
V _z /F	679 ml	1289 ml
CL/F	1.39 ml/min	3.11 ml/min
Liver		
Terminal t _{1/2}	100 min	303 min
C _{max}	5707 ng/g	18,767 ng/g
T _{max}	10 min	30 min
AUC _{last}	245,088 min*ng/g	2,682,301 min*ng/g
V _z /F	1174 g	116 g
CL/F	8.13 g/min	0.266 g/min
Muscle		
Terminal t _{1/2}	514 min	324 min
C _{max}	17,426 ng/g	2417 ng/g
T _{max}	30 min	10 min
AUC _{last}	885,986 min*ng/g	83,083 min*ng/g
V _z /F	1720 g	4043 g
CL/F	2.32 g/min	8.64 g/min

^aFemale CD-1 mice were dosed IP with 70 mg/kg **2** or 30 mg/kg **17**. Whole blood, liver and skeletal muscle (specifically the *vastus lateralis*) were collected at different time points up to 24 hours (n=3). The compound concentrations were analyzed by LC/MS/MS.

^bTerminal t_{1/2}, half-life of the terminal phase; C_{max}, observed maximum plasma concentration; T_{max}, time to reach C_{max}; AUC_{last}, area under the concentration time curve from 0 to the last measured point; V_z/F, apparent volume of distribution during terminal phase; and CL/F, volume of plasma cleared of the drug per unit time, where F is the fraction bioavailable as compared to an IV dose, which is not known.