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BRIEF ARTICLE

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Identification of Pyrido[1,2-α]pyrimidine-4-ones as New Molecules Improving the Transcriptional Functions of Estrogen-Related Receptor α

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Supporting Information

ABSTRACT: The nuclear estrogen-related receptor α (ERRα) plays a central role in the regulation of expression of the genes involved in mitochondrial biogenesis and oxidative metabolism. We have successfully identified a series of pyrido[1,2-*a*]pyrimidin-4-ones as new agonists enhancing the transcriptional functions of ERRα. The compounds potently elevated the mRNA levels and the protein levels of ERRα downstream targets. Consequently, the compounds improved the glucose and fatty acid uptake in C2C12 muscle cells.

The estrogen-related receptors (ERRs) belong to an orphan nuclear receptor subfamily.[1] Three isoforms of ERRs (ERRα, -β, and -γ)[2] have been identified with distinct expression profiles and diverse biofunctions.[3] ERRα is predominately expressed in metabolically active tissues such as muscle and adipose and plays a central role in the regulation of metabolism and energy homeostasis.[3] ERRβ is associated with early development,[4] and its postnatal expression is highly restricted. It could be detected only at low level in liver, stomach, skeletal muscle, kidney, etc. Although the exact biological functions of ERRγ still remain elusive, it is widely expressed in a number of adult human and mouse tissues, including spinal cord and central nervous system.[5]

Genetic and functional analysis have demonstrated that ERRα is a critical regulator of mitochondrial biogenesis and plays a central role in the regulation of expression of oxidative metabolism genes[6] by interacting with transcriptional cofactor PGC-1 (peroxisome proliferator-activated receptor γ coactivators)[7] and RIP-140 (nuclear receptor-interacting protein 140),[8] etc. The downstream target genes of ERRα include MACD (medium-chain acyl-coenzyme A dehydrogenase),[9] PDK4 (pyruvate dehydrogenase kinase 4),[10,11] ATP5b (ATP synthase 5b),[12] and other key components of oxidative metabolism. Therefore, ERRα has been considered a novel potential drug target and selective ERRα agonists might be developed as new therapeutic agents for the treatment of type II diabetes and other metabolic disorders.[13] However, recent results from Patch et al. revealed that a diaryl ether-based inverse agonist of ERRα potently normalized the serum triglyceride and insulin levels and improved glucose tolerance both in diet-induced murine models and in an overt diabetic rat model.[14] These disagreements indicate the complexity of ERRα regulatory pathways. It is highly desired to identify new ERRα agonists and inverse agonists as chemical probes to further validate ERRα as a new drug target for metabolic diseases.

X-ray crystallographic studies revealed that ERRα possesses constitutively active conformations to interact with coactivator PGC-1α or RIP-140, etc.[15,20] The ligand binding domains of ERRα is highly occupied by side chains, making it difficult to accommodate synthetic ligands without disrupting its constitutively active conformation. Although several classes of ERRα inverse agonists have been identified by high-throughput screening,[14−18] small molecular agonists of ERRα are lacking[19] likely because of the tiny binding pocket of the ERRα ligand binding domain (LBD)[20,21] (Figure 1, **1**−**5**). The failure to identify good ERRα agonists has disappointed many scientists, suggesting that "ERRα is an intractable molecular target"[21] for designing direct small-molecule ERRα agonists.

Structural feature analysis on the reported ERR modulators clearly revealed that almost all of the known ERR modulators possessed a carbonyl group or its isosteric moieties (Figure 1). An X-ray crystallographic study had also demonstrated that the carbonyl group contributed greatly to the binding of GSK4716 (Figure 1, **6**) with the ERRγ LBD domain by interacting with Arg-316 in ERRγ.[22,23] Therefore, a carbonyl focused library with diverse scaffolds was constructed for identifying new ERRα agonists. In this paper, we report the identification of pyrido-[1,2-*a*]pyrimidin-4-ones as new molecules improving the transcriptional functions of ERRα by screening our self-constructed carbonyl focused library.

CHEMISTRY

The simply substituted pyrido[1,2-*a*]pyrimidin-4-ones were synthesized by direct condensation/cyclization of 2-aminopyridines with substituted β-ketone esters in PPA (polyphosphorus acid) at 100 °C. For the 7-phenyl, 8-phenyl, 7-cyclopropyl, or 8-cyclopropyl analogues, they were prepared by a palladium catalyzed Suzuki coupling reaction of 7- or 8-bromopyrido[1,2-*a*]pyrimidin-4-one with phenylboronic acid (**11**) or cyclopropyl acid (**10**) (Scheme 1).

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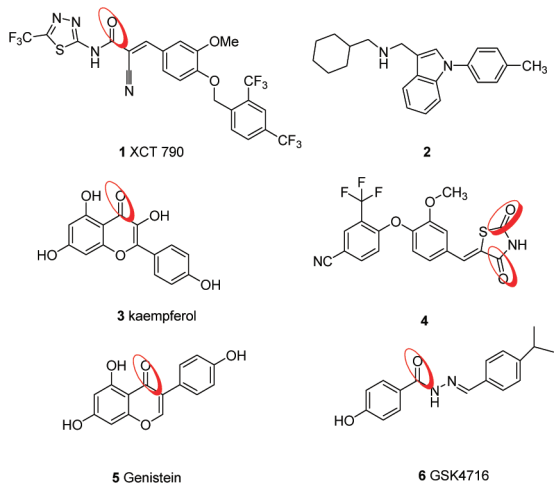
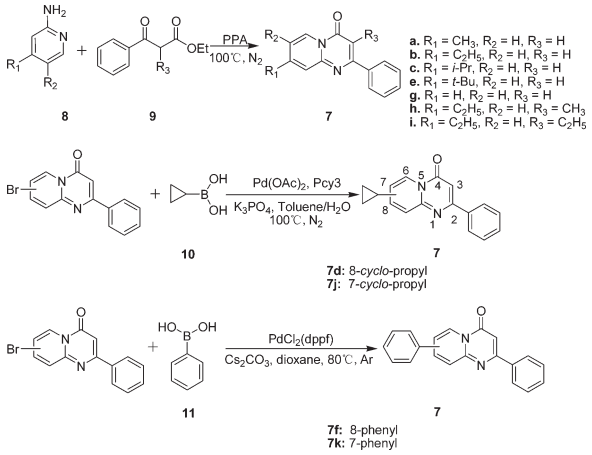


Figure 1. Chemical structures of reported small molecular modulators of ERRs: **1**−**4**, ERRα inverse agonists; **5**, ERRα agonist; **6**, ERRγ agonist.

Scheme 1. Synthesis of Pyrido[1,2-*a*]pyrimidin-4-one Derivatives



RESULTS AND DISCUSSION

The compounds were primarily screened by using a well established cell-based reporter gene assay to monitor their regulatory functions on the transcriptional activity of ERRα.[14−19,22] The heterologous reporter assay was performed in 293FT cells by transient transfection with a vector expressing LBD domain of human ERRα fused to Gal4 DNA binding domain and a reporter plasmid containing firefly luciferase gene. A well characterized ERRα inverse agonist kaempferol[18] was used as a reference compound to validate the screening conditions.

Among all of the compounds tested, 8-methyl-2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (**7a**, Figure 2) effectively enhanced the ERRα-driven luciferase activity at 10.0 μM after a 24 h incubation, indicating that **7a** was an agonist that up-regulated the transcriptional functions of ERRα (Figure 2A). By use of **7a** as a lead compound, new pyrido[1,2-*a*]pyrimidin-4-one derivatives were designed, synthesized, and evaluated (Scheme 1). The results suggested that the 8-methyl group could be replaced by a slightly larger hydrophobic group to achieve a better agonizing effect on ERRα. For instance, when the 8-methyl group was

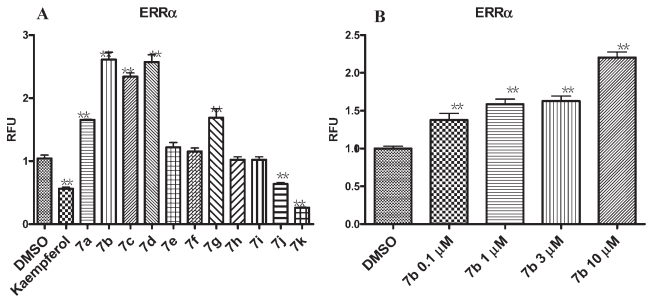


Figure 2. Pyrido[1,2-*a*]pyrimidin-4-ones potently agonize the transcriptional function of ERRα. (A) **7a**−**g** agonize the transcriptional function of ERRα at 10 μM, while **7j** and **7k** are inverse agonists of ERRα. (B) **7b** elevates the transcriptional function of ERRα in a dose dependent manner. Results are the means of at least three independent experiments (**, $p < 0.01$).

replaced by an ethyl, isopropyl or cyclopropyl group, the bioactivity was obviously improved, which elevated the transcriptional function of ERRα by 2.7-, 2.5-, and 2.6-fold at 10.0 μM, respectively (Figure 2A, **7b**−**d**). However, when the 8-position was substituted by a *tert*-butyl (**7e**) or a phenyl (**7f**), the potency was decreased. The removal of the 8-methyl group in **7a** did not lead to obvious change of the bioactivity (**7g**). The results also demonstrated that a substituent at the 3-position might be detrimental to its agonistic function on ERRα; the 3-methyl (**7h**) and 3-ethyl (**7i**) compounds were obviously less potent than the corresponding **7b**. Interestingly, when the hydrophobic group was merged to the 7-position from the original 8-position, the resulting compounds (**7j** and **7k**) became reverse agonists of ERRα, which inhibited the transcriptional function of ERRα (Figure 2A). Further investigation demonstrated that **7b** improved the transcriptional function of ERRα in a dose-dependent manner (Figure 2B). The investigation also demonstrated that **7b** effectively elevated the ERRα-driven luciferase activity in kaempferol pretreated 293FT cells, in which the basal constitutive activity of ERRα was reduced (Supporting Information). These results further suggested the agonizing effect of **7b** on ERRα.

The selectivity of the new ERRα agonists over other nuclear receptors (i.e., ERRβ, ERRγ, ERα, and ERβ (estrogen receptor α and estrogen receptor β)) was also investigated. Not surprisingly, **7b** also moderately elevated the transcriptional functions of ERRγ. However, the compound did not obviously affect the function of ERRβ in a similar gene reporter assay. Different from the fact that kaempferol strongly agonized the functions of ERα and ERβ,[18] **7b** did not show effects on ERα or ERβ at 20.0 μM (Supporting Information).

Taking **7b** as an example, the agonizing effect of pyrido[1,2-*a*]-pyrimidin-4-ones on ERRα was further validated by investigating the expression changes of ERRα targeted genes. MCAD, PDK4, and ATP5b are key components of oxidative metabolism, and the transcription of these genes are regulated by transcriptional factor ERRα. Highly consistent with our primary reporter screening results (Figure 2), **7b** obviously increased the mRNA levels of MCAD, PDK4, and ATP5b in a dose dependent manner monitored in a quantitative real-time PCR assay (parts A, B, and C of Figure 3). Not surprisingly, the protein levels of PDK4 and ATP5b were also elevated as determined by a Western blot analysis (Figure 3D) after a 24 h treatment.

ERRα plays a central role in mitochondrial biogenesis and oxidative metabolism. Theoretically, a cell permeable small molecule

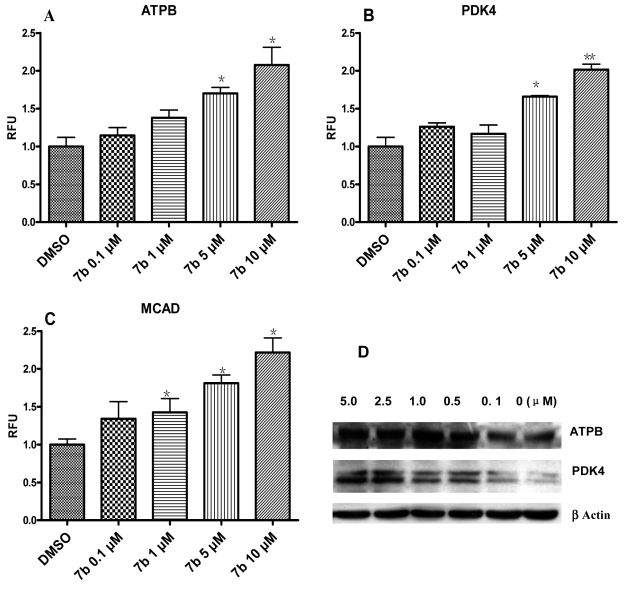


Figure 3. Compound **7b** potently up-regulates the transcription of ERRα genes. (A−C) **7b** dose-dependently increased the mRNA levels of MCAD, PDK4, and ATP5b in a quantitative real-time PCR assay. (D) **7b** elevates the protein levels of PDK4 and ATP5b in dose dependent manner determined by a Western blot analysis. Results are representative and the mean of at least three independent experiments (*, $p < 0.05$; **, $p < 0.01$).

ERRα agonist would enhance the lipid metabolism and improve the glucose and fatty acid uptake in muscle cells.[24−26] Therefore, the influence of **7b** on glucose and fatty acid uptake was also investigated in well differentiated C2C12 mouse muscle cells. Indeed, **7b** moderately improved the glucose and fatty acid uptake in C2C12 muscle cells in a dose-dependent manner (Figure 4A,B).

In summary, pyrido[1,2-*a*]pyrimidin-4-ones were successfully identified as new small molecular agonists of ERRα receptor. Although the precise mechanism remains elusive, our study demonstrated that pyrido[1,2-*a*]pyrimidin-4-ones could enhance the transcription of ERRα downstream target genes and improve the glucose and fatty acid uptake in C2C12 muscle cells. Our results may provide an interesting basis for further validation of ERRα as a new drug target for metabolic diseases.

EXPERIMENTAL SECTION

General Procedure for Synthesis of Substituted Pyrido-[1,2-*a*]pyrimidin-4-ones 7a−c,e,g−i. A mixture of 2-aminopyridine **8** (1.00 mmol) and the substituted β-keto ester **9** (1.50 mmol) in polyphosphorus acid (2.00 g) was heated at 100 °C with vigorously stirring. After 1.0 h, the mixture was cooled in an ice bath and neutralized with 5% aqueous sodium hydroxide. The solid precipitate was collected by filtration, washed with water. The crude products were purified by recrystallization from ethanol.

8-Methyl-2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (7a). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (d, J = 7.2 Hz, 1 H), 8.06−8.03 (m, 2 H), 7.49−7.43 (m, 4 H), 6.90 (dd, J = 1.6, 7.2 Hz, 1 H), 6.80 (s, 1 H), 2.43 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.1, 158.5, 151.0, 148.3, 137.4, 130.5, 128.7, 127.4, 126.5, 124.7, 117.8, 99.1, 21.3. HRMS (ESI): exact mass calcd for $C_{15}H_{12}N_2O$ $[M + H]^+$, 237.1022, found 237.1024. Anal. ($C_{15}H_{12}N_2O$) C, H, N.

Procedure for Synthesis of 7f and 7k. A mixture of 7-bromo or 8-bromo 2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (0.5 mmol), phenylboronic acid (0.55 mmol), Cs_2CO_3 (1.0 mmol), and $PdCl_2$-(dppf) (0.05 mmol) in dioxane (2.0 mL) was stirred at 80 °C under an argon atmosphere. The mixture was cooled to room temperature after complete consumption of starting material as monitored by TLC. Water (10 mL) and EtOAc (10 mL) were added to the reaction mixture. The organic phase was separated, and the aqueous phase was further extracted with EtOAc (10 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash chromatography to provide the desired product.

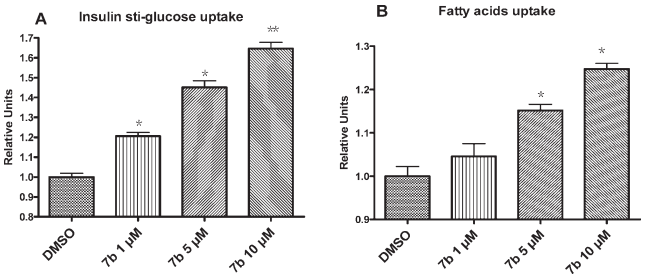


Figure 4. Compound **7b** improves the insulin-stimulated glucose uptake and fatty acid uptake in well differentiated C2C12 mouse muscle cells. (A) **7b** dose-dependently improves the insulin-stimulated glucose uptake. (B) **7b** improves the fatty acid uptake in dose dependent manner. Results are the mean of at least three independent experiments (*, $p < 0.05$; **, $p < 0.01$).

2,8-Diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (7f). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.00 (d, J = 7.6 Hz, 1 H), 8.26−8.21 (m, 2 H), 8.07 (d, J = 2.0 Hz, 1 H), 8.02−8.00 (m, 2 H), 7.77 (dd, J = 2.0, 7.6 Hz, 1 H), 7.62−7.53 (m, 6 H), 6.98 (s, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.7, 157.5, 151.0, 147.7, 136.7, 135.2, 130.7, 130.3, 129.4, 128.7, 127.4, 127.2, 127.2, 121.7, 114.7, 98.4. HRMS (ESI): exact mass calcd for $C_{20}H_{14}N_2O$ $[M + H]^+$, 299.1179, found 299.1182. Anal. ($C_{20}H_{14}$-N_2O) C, H, N.

Procedure for Synthesis of 7d and 7j. To a solution of 7-bromo or 8-bromo 2-phenyl-4*H*-pyrido[1,2-a]pyrimidin-4-one (0.5 mmol), cyclopropylboronic acid (0.6 mmol), potassium phosphate (1.75 mmol), and tricyclohexylphosphine (0.05 mmol) in toluene (2.0 mL) and water (100 uL) under a nitrogen atmosphere was added palladium acetate (0.025 mmol). The mixture was heated to 100 °C for 3 h and then cooled to room temperature. Water (5 mL) was added and the aqueous portion extracted with EtOAc (2 × 10 mL). The combined organics were washed with brine (10 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography to afford the desired compounds.

8-Cyclopropyl-2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (7d). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.83 (d, J = 7.2 Hz, 1 H), 8.20−8.15 (m, 2 H), 7.53−7.50 (m, 4 H), 7.04 (dd, J = 2.0, 7.6 Hz, 1 H), 6.86 (s, 1 H), 2.21−2.15 (m, 1 H), 1.22−1.17 (m, 2 H), 1.03−0.99 (m, 2 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.7, 157.6, 155.9, 150.5, 136.8, 130.5, 128.6, 127.1, 126.6, 120.3, 114.2, 97.3, 15.1, 11.1. HRMS (ESI): exact mass calcd for $C_{17}H_{14}N_2O$ $[M + H]^+$, 263.1179, found 263.1176. Anal. ($C_{17}H_{14}N_2O$) C, H, N.

Cell Lines and Cell Culture. See Supporting Information.

Transient Transfection and Dual Luciferase Reporter Assay. 293FT cells were seeded in 96-well plate at a density of 10 000 cells/well in DMEM containing 10% FBS. At 18−24 h after plating, transit transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Total DNA for transfections included Gal4-DBD-ERRα-LBD vector (0.5 ng), pcDNA-PGC-1α vector (0.5 ng), pFRlaczeo vector (25 ng), and internal control vector pRLTK renilla (3 ng). After cells were transfected for 6 h, compounds were then added for an additional 24 h. Luciferase activity was

measured by dual luciferase reporter assay system (Promega) according to the manufacturer's instruction on a Veritas microplate luminometer. Relative luciferase units were the ratio of the absolute activity of firefly luciferase to that of renilla luciferase. The experiment was done in triplicate, and results are representatives of at least three independent experiments.

Western Blot Analysis. For myotube formation, C2C12 myoblasts at ~100% confluence was changed to DMEM supplemented with 2% donor equine serum and the cells were maintained for an additional 5−7 days. During myotube formation, the medium was changed every day. Then cells were treated with **7b** of indicated concentration or vehicle control. After an indicated period of time, Western blot was carried out according to the protocol provided by Cell Signaling Technology Ltd.

Real-Time PCR Analysis. RNA isolation and real-time analysis were performed according to a previous report.[27] Briefly, total RNA was isolated from C2C12 myocytes pretreated with **7b** for 20 h using Trizol reagent (Invitrogen), and isolated total RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen). For real-time PCR analysis, cDNA samples were used in quantitative PCR reaction in the presence of fluorescent dye Cybergreen (Bio-Rad, Benicia, CA, U.S.). The following PCR conditions were applied: 5 min, 95 °C; 40 × (10 s, 95 °C; 20 s, 60 °C; 1 s, 70 °C). After each elongation step, the reaction was quantified in a reading step and the product quality tested by melting curve analysis. Relative abundance of mRNA was calculated after normalization to Gapdh mRNA. Sequences for the primers used in this study are shown in the table in Supporting Information.

Analysis of Cellular Glucose and Fatty Acid Uptake. After a 24 h treatment of **7b**, C2C12 myocytes were glucose-starved for 10 min in Krebs Ringer Hepes (KRH) buffer (pH 7.5) containing 120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM Hepes, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.1% BSA. Fifteen minutes after incubation with insulin (100 nM), tracer [1,2-^{3}H]-2-deoxy-D-glucose (0.93 GBq/mmol, Amersham Pharmacia, Buckinghamshire, U.K.) was then added for 15 min and glucose uptake was assayed in triplicate for each condition. Fatty acid uptake assays were initiated by incubating cells for 20 min in KRH buffer containing 5.4 mM glucose and [1-^{14}C]oleic acid (2.04 GBq/mmol, Amersham Pharmacia, Buckinghamshire, U.K.) bound to fatty acid-free BSA. The cells were washed and cellular incorporated [^{3}H] and [^{14}C] radioactivity was determined by liquid scintillation counting. The abundance of radioactivity was normalized to protein content.

■ ASSOCIATED CONTENT

Supporting Information. Chemical data for **7b,c,e,g−k**, general information for chemistry and biology, and procedure for Western blot analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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■ ABBREVIATIONS USED

PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; DNA, deoxyribobucleic acid; cDNA, complementary DNA; RNA, ribobucleic acid; mRNA, messenger RNA; BSA, bovine serum albumin

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