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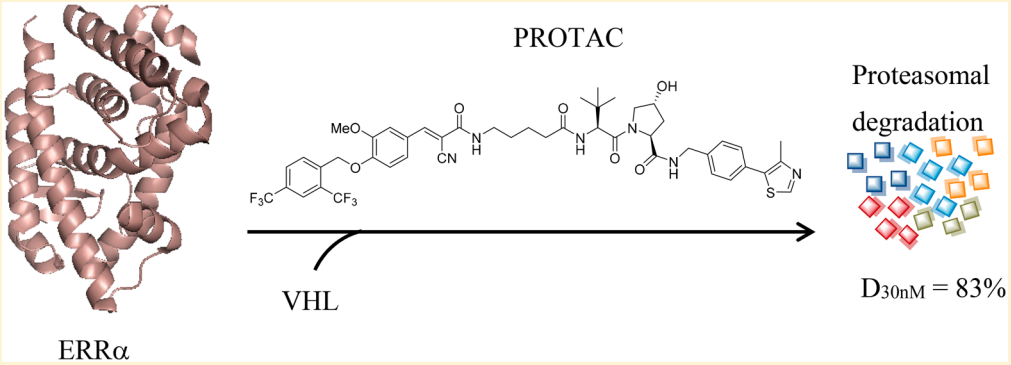
Identification of New Small-Molecule Inducers of Estrogen-related Receptor α (ERRα) Degradation

Lijie Peng,*[,†,#] Zhensheng Zhang,[†,#] Chong Lei,[†] Shan Li,[†] Zhang Zhang,[†] Xiaomei Ren,[†] Yu Chang,[†] Yan Zhang,[‡] Yong Xu,*[‡] and Ke Ding*[,†]

[†]International Cooperative Laboratory of Traditional Chinese Medicine Modernization and Innovative Drug Development, Ministry of Education (MOE) of China, Guangzhou City Key Laboratory of Precision Chemical Drug Development, School of Pharmacy, Jinan University, 601 Huangpu Avenue West, Guangzhou 510632, China

[‡]Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, 190 Kaiyuan Avenue, Guangzhou 510530, China

Supporting Information



ABSTRACT: A series of (*E*)-3-(4-((2,4-bis(trifluoromethyl)benzyl)oxy)-3-methoxyphenyl)-2-cyanoacrylamide derivatives were designed and synthesized as new estrogen-related receptor α (ERRα) degraders based on the proteolysis targeting chimera (PROTAC) concept. One of the representative compounds **6c** is capable of specifically degrading ERRα protein by >80% at a relatively low concentration of 30 nM, becoming one of the most potent and selective ERRα degraders to date. Compound **6c** could be utilized as a new powerful research tool for further biological investigation of ERRα.

KEYWORDS: *Estrogen-related receptor α (ERRα), proteolysis targeting chimera (PROTAC), protein degradation*

Estrogen-related receptors (ERRs) belong to an orphan nuclear receptor superfamily.[1] Three isoforms of ERRs (i.e., ERRα,[2] β, and γ[3]) have been identified to date. ERRβ is associated with early development,[4] whereas ERRα and ERRγ are being considered as novel potential targets for metabolic disorders.[5,6] ERRα is the first-discovered and most-studied member of this subgroup and was named because of its relatively high homology with estrogen receptor α (ERα). In addition to the cross-talk with ERα signals,[7] ERRα also plays a critical role in regulating metabolism and energy homeostasis[8] by interacting with multiple transcriptional cofactors, such as the peroxisome proliferator-activated receptor γ coactivator 1 proteins (i.e., PGC-1α and PGC-1β),[9] receptor-interacting protein 140 corepressor (RIP-140),[10] etc.

A small molecule up-regulating function of ERRα had been suggested as a new potential therapeutic strategy for type II diabetes and other metabolic disorders, based on its crucial roles in mitochondrial oxidative phosphorylation (OXPHOS) and fatty acid oxidation processes.[6] However, whole-body ERRα knockout animals are unexpectedly lean and non-diabetic.[11] Pharmacological inhibition of ERRα by using an inverse agonist also exhibited *in vivo* antidiabetic efficacy.[12] These paradoxical results indicate complicated and diverse functions of ERRα in metabolic regulation.

ERRα/PGC-1α(β) transcriptional axis also mediates metabolic adaptations of various types of human cancer cells.[13−15] For instance, metabolic alterations driven by ERRα obviously stimulate proliferation, migration, angiogenesis, metastasis, and drug resistance of human breast cancer cells.[16−18] ERRα overexpression has also been frequently detected in Her2+ and triple-negative breast tumors and associated with poor clinical outcome of patients.[19] Pharmacological inhibition and gene knockout of ERRα were proven to mitigate breast cancer progression both *in vitro* and *in vivo*.[20,21]

To facilitate biological investigation on the target, several ERRα inverse agonists and small molecules improving its transcriptional function have been discovered.[12,22−32] Most recently, a proteolysis targeting chimera (PROTAC) compound was also designed and synthesized by Crews et al. to induce the degradation of ERRα (Figure 1).[33]

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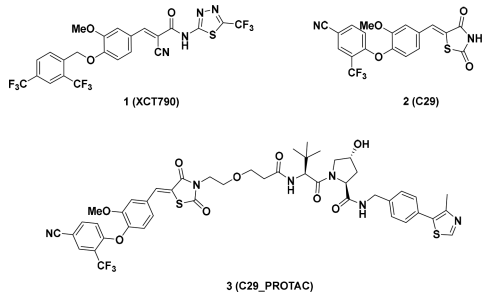


Figure 1. Chemical structures of representative ERRα inverse agonists and previously reported ERRα degrader.

PROTAC is a new "chemical-small-molecule-induced protein knockdown" technology to recruit an E2/E3 ligase to the target protein and promote polyubiquitination and subsequent protein degradation via proteasome.[34] Comparing with the classical activity inhibitors, PROTAC can mimic the natural proteasome degradation process to totally abolish functions of target protein. Additionally, obvious advantages, such as superior target specificity[35] and accessibility to "undruggable" targets,[36] make PROTAC as an attractive strategy for next generation drug discovery.[37,38]

Herein, we would like to report our effort to identify a new series of PROTAC-based ERRα degradation inducers with obviously improved potency.

Architecturally, PROTAC molecules possess two distinct ligand moieties that are able to bind with the target protein and E2/E3 ubiquitin ligase, respectively, and a covalently connecting linker.[34] Therefore, the first step for designing a degrader is to identify a novel ERRα ligand.

Compound **1** (XCT790)[23] is the first reported and widely investigated ERRα inverse agonist with an IC_{50} value of 0.37 μM in a GAL4-ERRα cell-based transfection assay. A preliminary computational study suggested that compound **1** could fit into a ligand-induced pocket of ERRα (PDB code: 3K6P, Figure 2A). The α-cyano-α, β-unsaturated carbonyl

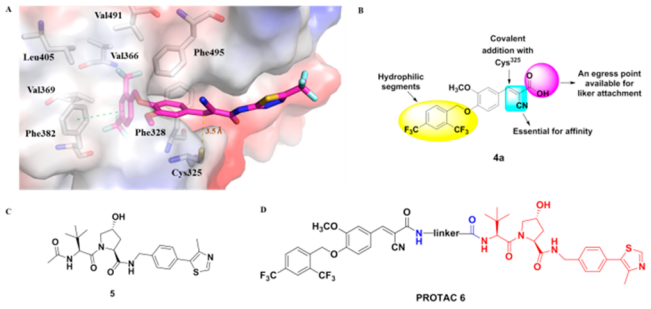
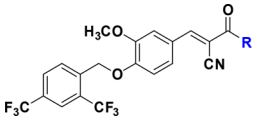


Figure 2. (A) Predicted binding mode of compound **1** with ERRα (PDB code: 3K6P). (B) Structure–activity relationship (SAR) overview for compound **4a**. (C) Chemical structure of von-Hippel-Lindau (VHL) ligand **5**. (D) Chemical structure of designed PROTACs **6**.

moiety is close to Cys325 residue with a distance of 3.5 Å, suggesting a covalent interaction could be formed. The diaryl ether core was embedded in a hydrophobic pocket formed by Phe382, Val369, Leu405, Val366, Val491, and Phe495 residues, whereas the thiadiazoloacylamide group of **1** seemed to extend toward solvent exposing surface of the protein and did not form any defined interaction with ERRα. It was hypothesized that the thiadiazoloacylamide group in compound **1** could be removed without affecting the binding with ERRα. Compound **4a** was thus designed and synthesized based on this hypothesis (Figure 2B).

Activity of compound **4a** to block the protein–protein interaction of ERRα with PGC-1α coactivator was determined by utilizing a well-established time-resolved fluorescence resonance energy transfer (TR-FRET) assay.[30] Under the experimental conditions, reference compounds **1** and **2** exhibited strong inhibitory potencies with IC_{50} values of 61.3 and 33.0 nM, respectively, which are comparable to the reported data.[12,30] Encouragingly, the structurally simplified molecule **4a** displayed a ~11-fold improved potency with an IC_{50} value of 5.67 nM. Additionally, removal of the thiadiazoloacylamide group also resulted in a significant solubility improvement of **4a** (data not shown). Highly consistent to our prediction, the terminal carboxylic acid in **4a** could be replaced by a hydrophilic group, such as an unsubstituted (**4c**) or substituted (**4d**, **4e**, and **4f**) amide without affecting the binding affinity with ERRα. However, esterification of the carboxylic acid (**4b**) caused a ~4-fold potency loss in the TR-FRET assay. These data collectively suggested that the terminal carboxylic acid group could be a feasible position to introduce a linker for further PROTAC molecule design (Table 1).

Table 1. *In Vitro* Binding Affinities to ERRα of Inverse Agonists 4a–4f[a]



Cpds	R	TR-FRET[a] IC_{50} (nM)
4a	OH	5.67 ± 0.58
4b	OCH_3	20.33 ± 1.53
4c	NH_2	4.33 ± 1.53
4d	$NHCH_2CH_2OCH_3$	4.33 ± 0.58
4e	$NHCH_2CH_2CH_2CH_2COOH$	6.00 ± 1.00
4f	$NHCH_2CH_2OCH_2CH_2COOH$	4.67 ± 0.58
2		33.0 ± 1.73
1		61.33 ± 6.11

[a]Affinity to ERRα was determined by using a TR-FRET assay (Invitrogen, USA). The data are means from at least three independent experiments.

Several small-molecule E2/E3 ligase ligands,[39] such as Mouse Double Minute 2 (MDM2) ligand Nutlin derivatives, cellular inhibitor of apoptosis protein 1 (cIAP1) inhibitor bestain, cereblon (CRBN) ligand thalidomide derivatives, and the von-Hippel-Lindau (VHL) ligand, have been incorporated into PROTAC molecules to mediate degradation of the target proteins. It is also clear that the E2/E3 ubiquitin ligase ligand choice[40] has significant impact on efficiency and target specificity of a PROTAC molecule. The VHL ligand had been previously proven to be highly efficient to induce degradation of ERRα protein.[33] Therefore, VHL ligand **5** (Figure 2C), which harbors a solvent-exposing acetamide group for conjugating a linker,[41] was first utilized for our new ERRα degrader design.

A series of PROTAC molecules were first designed and synthesized by hybridizing **4a** and VHL amino building block **13** with different linkers (Scheme 1). Carboxylic acid **4a** was coupled with amino ester **10** in the presence of HATU and

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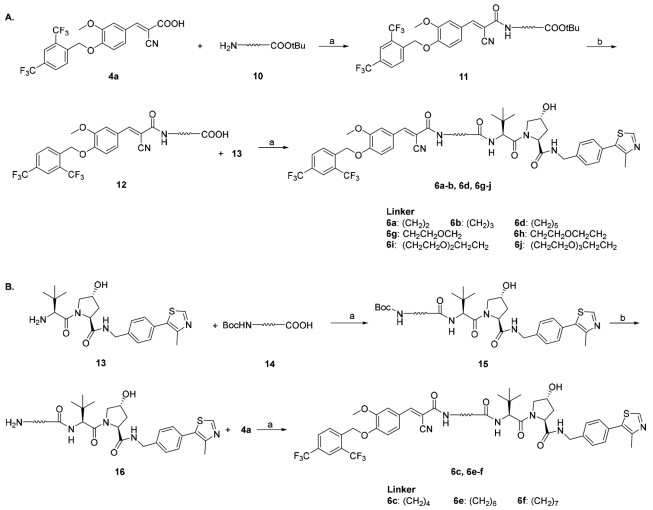
Scheme 1. Synthesis of PROTACs 6a−6j[a]



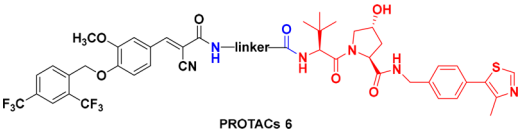
[a]Reagents and conditions: (a) 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo [4,5-*b*]-pyridinium 3-oxide hexafluorophosphate (HATU), *N*,*N*-diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), room temperature (rt), 39−71%; (b) trifluoroacetic acid (TFA), dichloromethane (DCM), rt.

DIPEA at room temperature in DMF to give the amide **11**, which was deprotected with TFA to afford the corresponding carboxylic acid intermediate **12**. Upon coupling with VHL amino building block **13** (prepared as previously described, Supporting Information), the designed PROTACs (**6a−b**, **6d**, **6g−j**) were generated. Differently, ERRα-PROTACs (**6c**, **6e−f**) and the epimeric **6c** (**6c**-Epi) were prepared by using an alternative protocol. VHL amino ligand **13** was coupled with Boc-protected amino acid **14**, and the resulting compound **15** was treated with TFA to give **16**. The final products were obtained by condensation of **16** and **4a**. Compound **6c**-Epi was synthesized by using an epi-VHL building block harboring a reversed (*S*) stereochemistry at the proline 4-position.[33]

Binding affinities of the new hybrids and their function to degrade ERRα were determined by utilizing *in vitro* TR-FRET assay and Western blotting analysis in the MDA-MB-231 cells, respectively (Table 2 and Supporting Information). The first reported ERRα PROTAC **3** (C29_PROTAC) was utilized as a positive control in both assays. Compound **3** exhibited an IC_{50} value of 8.0 nM to block the protein−protein interaction of ERRα with PGC-1α peptide and induced approximately 40% protein degradation at 100 nM ($D_{100\ nM}$) after 4.0 h treatment, which are similar to the previously reported data.[33]

It was shown that length and chemical types of the hybrid linker had minor influence on ERRα binding affinities of the PROTAC molecules. Compounds **6a−6j** exhibited strong binding with ERRα, with IC_{50} values ranging from 5.33 to 12.67 nM, which are comparable to the parental molecule **4a**. These data further supported that our initial hypothesis that the terminal carboxylic acid group in **4a** is a feasible position for E2/E3 ligase ligand linkage. However, the introduced linkers had significant impact on ERRα protein degradation capabilities of the molecules. For instance, compounds **6a** and **6b**, which harbor a 2-carbon or 3-carbon linker, exhibited almost the same ERRα inhibitory potencies with IC_{50} values of 7.3 and 6.3 nM, respectively. However, they displayed significantly varied capabilities to induce degradation of ERRα protein, with $D_{30\ nM}$ and $D_{100\ nM}$ values of 28%, 50%

Table 2. Binding Affinities and Degradation Activities to ERRα of PROTACs 6a−6j



PROTACs 6

Cpds	linker	TR-FRET[a] IC_{50} (nM)	$D_{30\ nM}$[b] (%)	$D_{100\ nM}$[b] (%)
6a	$(CH_2)_2$	7.33 ± 3.06	28	50
6b	$(CH_2)_3$	6.33 ± 1.15	58	85
6c	$(CH_2)_4$	12.67 ± 2.89	83 ($D_{10\ nM}$ = 39)	96
6d	$(CH_2)_5$	5.67 ± 1.53	58	78
6e	$(CH_2)_6$	12.33 ± 2.52	23	71
6f	$(CH_2)_7$	7.67 ± 0.58	3	9
6g	$CH_2CH_2OCH_2$	11.33 ± 0.58	79	92
6h	CH_2CH_2O CH_2CH_2	9.33 ± 4.16	63 ($D_{10\ nM}$ = 25)	89
6i	$(CH_2CH_2O)_2$ CH_2CH_2	5.33 ± 2.52	0	15 ($D_{300\ nM}$ = 19)
6j	$(CH_2CH_2O)_3$ CH_2CH_2	7.00 ± 1.00	0	4 ($D_{300\ nM}$ = 10)
6c-Epi	$(CH_2)_4$	5.33 ± 1.15	6	NT[c]
3		8.00 ± 2.65	1	40

[a]Binding affinity with ERRα was determined by using a TR-FRET assay. [b]*D* (%): % degradation is measured at indicated concentration by using Western blot assay and generated by ImageJ software analysis.[46] The data are means from at least three independent experiments. Representative Western blots can be found in the Supporting Information (Figure S2). [c]NT: not tested.

and 58%, 85%, respectively. Although a 4-carbon linker caused an approximately 2-fold ERRα binding affinity loss, the resulting compound **6c** exhibited the strongest protein degradation potency, with $D_{30\ nM}$ and $D_{100\ nM}$ values of 83% and 96%, respectively. It could even induce approximately 39% of ERRα degradation at a concentration as low as 10 nM ($D_{10\ nM}$). These results strongly support the hypothesis that each PROTAC causes a unique conformational change and that protein degradation requires certain plasticity in binding.[42] The compound with a 5-carbon linker (**6d**) also displayed strong ERRα degradation potency, whereas a further longer carbon linker (e.g., 6- or 7- carbon linker) caused an obvious protein-degrading potency loss (**6e** and **6f**). The investigation also revealed that the linker could be tolerated by an oxygen atom-containing moiety to retain the strong ERRα degrading potency. Compounds **6g** and **6h** exhibited $D_{100\ nM}$ values of 92% and 89%, respectively. It was noteworthy that compound **6h** harbors the same alkyl ether linker with that of previously reported ERRα degrader **3**,[33] but its potency is significantly stronger than that of compound **3**. For instance, the ERRα degrading potency of compound **3** was almost abolished at 30 nM (with a $D_{30\ nM}$ value of approximately 1%), but compound **6h** induced 25% and 63% ERRα degradation at concentrations of 10 and 30 nM, respectively. Similar to observation on the compounds with pure carbon linkers, longer polyethylene glycol (PEG) linkers also caused significant protein degrading potency loss of the resulting compounds (**6i** and **6j**). Not surprisingly, although **6c**-Epi exhibited strong binding with the target protein ERRα, its ERRα degrading function was totally abolished because the epi-VHL building block almost completely prevented the binding with VHL E3 ligase.[33]

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Molecules obtained by hybridizing compound **4a** with small molecular ligands of other E2/E3 ligases (e.g., MDM2, cIAP1 and CRBN) were also designed and synthesized. However, none of them exhibited superior ERRα degradation potency to that of molecule **6c** (Supporting Information).

The selectivity of compound **6c** to induce ERRα degradation was further validated in MDA-MB-231 breast cancer cells (Figures 3 and 4). It was confirmed that

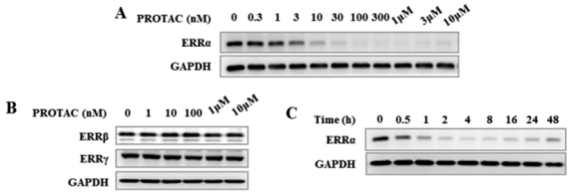

Figure 3. Degradation effects of PROTAC **6c** on ERRα, ERRβ, and ERRγ proteins in MDA-MB-231 cells by Western blot analysis. (A) PROTAC **6c** dose-dependently degrades ERRα protein. (B) PROTAC **6c** has no effect on the levels of ERRβ and ERRγ protein at indicated concentration. (C) Time-points degradation analysis of PROTAC **6c** at 30 nM.

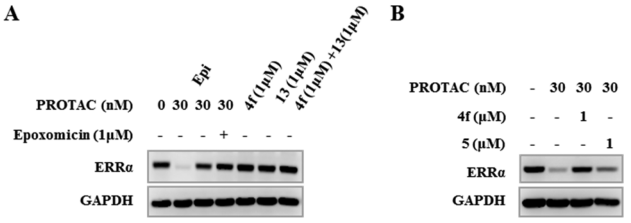

Figure 4. Mechanism study of PROTAC **6c** in MDA-MB-231 cells by Western blotting analysis. (A) PROTAC **6c**-mediated ERRα degradation is abrogated by pretreatment with proteasome inhibitor Epoxomicin. (B) PROTAC **6c**-mediated ERRα degradation is abrogated by pretreatment with ERRα ligand **4f** or VHL ligand **5**.

compound **6c** dose-dependently induced ERRα degradation with an efficacious dose as low as 3.0 nM at 4.0 h (Figure 3A), whereas **6c** did not show obvious effect on ERRβ and ERRγ homologues at relatively high concentrations (up to 10.0 μM, Figure 3B) at 4.0 h. These data are highly consistent with the specific binding of **6c** with ERRα protein (Supporting Information). A time-course investigation further revealed that compound **6c** could initiate ERRα degradation after 30 min exposure and demonstrated a maximum effect at approximately 4.0 h after treatment. However, the level of ERRα could be recovered after 48 h (Figure 3C), which might be due to resynthesis of the protein and/or other intracellular feedback response. Further investigation also showed that **6c** potently decreased protein levels of ERRα downstream target genes, e.g., ATP5B, medium-chain acyl CoA dehydrogenase (MCAD), and pyruvate dehydrogenase kinase 4 (PDK4) in the MDA-MB-231 cells after a 24 h treatment (Supporting Information).

Proteasome is importantly involved in the E2/E3 ligase mediated protein degradation.[43,44] In order to elucidate the mechanism of **6c**-induced ERRα degradation, we further investigated the contribution of proteasome in ERRα degrading process. MDA-MB-231 cells were preincubated with 1.0 M proteasome inhibitor Epoxomicin,[45] then treated with **6c** at 30 nM for 4.0 h. Western blot analysis suggested that pretreatment of proteasome inhibitor successfully rescued the ERRα degradation induced by **6c**, validating the crucial role of proteasome system in VHL-mediated ERRα degradation (Figure 4A). However, no protein degradation was observed for direct treatments of ERRα ligand **4f** and VHL amino building block **13**, either in a single agent or in a combination (Figure 4A), whereas pretreatment with ERRα ligand **4f** or VHL ligand **5** successfully abrogated the ERRα degradation mediated by **6c** (Figure 4B). These data collectively supported the necessity of ternary complex formation for ERRα degradation.

In summary, a series of (*E*)-3-(4-((2,4-bis(trifluoromethyl)-benzyl)oxy)-3-methoxyphenyl)-2-cyanoacrylamide derivatives were designed and synthesized as new ERRα degraders based on a proteolysis targeting chimera concept. Compound **6c** is capable of specifically degrading ERRα protein by >80% at a relatively low concentration of 30 nM, representing one of the most potent and selective ERRα degraders to date. Mechanism investigation further validated that **6c**-mediated ERRα degradation requires the ternary complex formation and ubiquitin proteasome participation. PROTAC **6c** could be utilized as a new powerful chemical tool for further investigating functions of ERRα in physiological and pathological states.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00025.

Synthetic procedures and compound characterization, procedures, and *in vitro* TR-FRET assay, Western blot analysis, ^{1}H NMR, ^{13}C NMR spectra of compounds **6a**–**6j** (PDF)

Recommended compound characterization checklist (XLS)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel: +86-20-85221523. Fax: +86-20-85224766. E-mail: dingke@jnu.edu.cn (K.D.).

*E-mail: elva_0916@jnu.edu.cn (L.P.).

ORCID

Shan Li: 0000-0001-5269-5631

Yong Xu: 0000-0003-3601-0246

Ke Ding: 0000-0001-9016-812X

Author Contributions

#These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ERR, estrogen-related receptor; ER, estrogen receptor; OXPHOS, oxidative phosphorylation; PROTAC, proteolysis targeting chimera; TR-FRET, time-resolved fluorescence resonance energy transfer; VHL, von-Hippel-Lindau; MDM2, Mouse Double Minute 2; cIAP1, cellular inhibitor of apoptosis protein 1; CRBN, cereblon; DIPEA, *N*,*N*-diisopropylethylamine; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluorophosphate; TFA, trifluoroacetic acid; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; rt, room temperature; IC_{50}, half maximal (50%) inhibitory concentration of a substance; PEG, polyethylene glycol; MCAD, medium-chain acyl CoA dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4.

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