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Oleanolic acid derivative NPLC441 potently stimulates glucose transport in 3T3-L1 adipocytes via a multi-target mechanism

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ABSTRACT

The natural product oleanolic acid (OA) has been discovered to exhibit varied pharmacological functions including anti-inflammation, anti-tumor and anti-diabetes, while appropriate synthetic oleanolic acid derivatives seem to possess more potent activities. Here we identified a new oleanolic acid derivative, 3-β-(2-carboxybenzoyloxy)-oleanolic acid (NPLC441), which functioned as a competitive PTP1B inhibitor and enhanced insulin-stimulated phosphorylation of IR and AKT in HepG2 cells. As an RXRα antagonist, it could selectively activate LXRα:RXRα heterodimer and increase the promoter activities of ABCA1 and ABCG1 genes in transient transfection assays. Quantitative RT-PCR and Western blot analyses suggested that NPLC441 could up-regulate GLUT4 expression in 3T3-L1 adipocytes, and such effect was further proved to be dependent on LXRα:RXRα activation. Moreover, 2-deoxyglucose uptake technology-based characterization demonstrated that this compound could stimulate glucose uptake in 3T3-L1 adipocytes. Finally, NPLC441 was observed to be able to suppress 11β-HSD$_1$ expression in HepG2 cells, following the discovery that activation of LXRα:RXRα could repress the expression of 11β-HSD$_1$. Compared with NPLC441, OA showed no effects on the transactivation of either LXRα:RXRα heterodimer or RXRα-LBD. Our work is thus expected to provide a new insight into the anti-diabetic application for oleanolic acid derivatives via multi-target mechanism, and NPLC441 could be used as a potential lead compound for further research.

1. Introduction

Oleanolic acid (OA) and ursolic acid are ubiquitous triterpenoids synthesized in various plants, most of which are widely used in Asian medicine for therapies of inflammatory, tumor, hepatitis and diabetes [1–3]. However, the pharmacological activities of these triterpenoids themselves are relatively weak. To improve the potential pharmacological potencies,

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Abbreviations: ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; BSA, bovine serum albumin; DEX, dexamethasone; FXR, farnesoid X receptor; FXRE, FXR response element; GLUT4, glucose transporter 4; GR, glucocorticoid receptor; IR, insulin receptor; IRS, insulin receptor substrate; LAR, leukocyte antigen-related tyrosine phosphatase; LXR, liver X receptor; LXRE, LXR response element; OA, oleanolic acid; PPAR, peroxisome proliferator-activated receptors; PPRE, PPAR response element; PTP1B, protein tyrosine phosphatase 1B; RA, rentinoic acid; RXR, retinoid X receptor; SPR, surface plasmon resonance; TC-PTP, T-cell PTP; 11β-HSD$_1$, 11β-hydroxysteroid dehydrogenase type 1.

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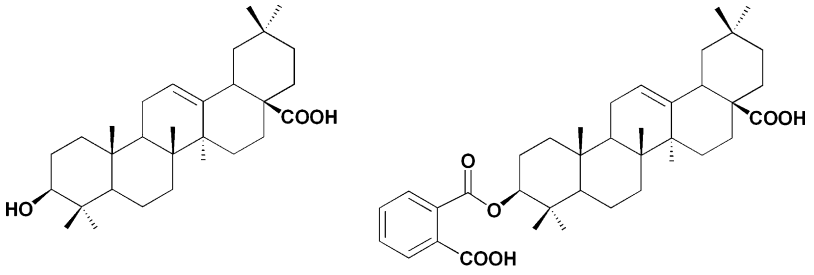

Fig. 1 – Structures of oleanolic acid and NPLC441.

varied OA derivatives have been synthesized [4,5]. For example, the synthetic OA derivative 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) exhibited potent differentiating, anti-proliferative, and anti-inflammatory activities [6]. During the past decades, relevant targets for the synthetic oleanane triterpenoids were disclosed, such as IκB kinase, transforming growth factor-β (TGF-β) and peroxisome proliferator-activated receptor-γ (PPARγ) [6,7]. Here, we identified a new OA derivative, 3-β-(2-carboxybenzoyloxy)-OA (NPLC441, Fig. 1), which functioned as both PTP1B inhibitor and RXRα antagonist that selectively activated LXRα:RXRα heterodimer.

Protein tyrosine phosphatases (PTPases), including PTP1B, PTPα, leukocyte antigen-related (LAR) tyrosine phosphatase, CD45 and T-cell PTP (TC-PTP), are negative regulators against insulin signaling pathway [8–12]. Among these, PTP1B has been identified as a promising drug target for the prevention and treatment of type 2 diabetes as investigated by the fact that the PTP1B gene knock-down mice displayed normalized plasma glucose level and improved insulin sensitivity, and overexpression of PTP1B in rat adipose cells impaired metabolic actions of insulin [13–15]. For this reason, efforts have been made to discover specific PTP1B inhibitors both from the synthetic compounds and natural products in recent years [16–20]. For example, the marine natural product *Hyrtiosal* was identified as a PTP1B inhibitor that stimulated glucose transporter 4 (GLUT4) translocation [16] and a specific PTP1B inhibitor KR61639 was reported as an anti-hyperglycemic agent [17].

The retinoid X receptors (RXRα, RXRβ, RXRγ) belong to the nuclear receptor (NR) superfamily, which plays a central role in regulating critical cellular pathways essential for mammalian physiology and development [21]. RXRs exert their functions mainly through their ability to act as obligatory heterodimer partners for many members of the NR family [22] and these RXR-heterodimers can be classified as two types: nonpermissive heterodimers that can only be activated by the partner's ligand and permissive heterodimers that can be activated either by an RXR or partner-specific ligand, including peroxisome proliferator-activated receptors (PPARs):RXR, farnesoid X receptor (FXR):RXR and liver X receptors (LXRs):RXR [23,24].

LXRs were activated by oxysterol ligands and originally discovered as important regulators of lipid and lipoprotein metabolism [25]. In macrophages and other peripheral cells, LXRs control the transcription of several genes involved in cellular cholesterol efflux, including ATP-binding cassette (ABC)A1, ABCG1, and apolipoprotein E [26]. Recent studies revealed that LXRs also play important roles in the regulation of glucose metabolism [27–29]. For example, studies on the diet-induced insulin-resistance rats revealed that activation of LXRα, but not of LXRβ, could enhance glucose tolerance and increase peripheral glucose disposal [27]. In addition, LXRα directly regulates the expression of the insulin-responsive GLUT4 in adipocytes [28] and down-regulates the expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD_1) that was suggested to be a key factor in the development of insulin resistance syndromes [29,30].

The OA derivative NPLC441 as a determined LXRα:RXRα heterodimer activator could suppress 11β-HSD_1 expression and sensitize insulin signaling in HepG2 cells. Cell-based assay indicated that NPLC441 could stimulate GLUT4 expression and glucose uptake in 3T3-L1 adipocytes. Our results are expected to have provided a new insight into the anti-diabetic application for OA derivatives via multi-target mechanisms and suggested that NPLC441 might be a promising lead compound for further research.

2. Materials and methods

2.1. *Reagents*

All cell culture reagents were purchased from GIBCO. Penicillin–streptomycin, biotin, rosiglitazone, chenodeoxycholic (CDCA), *p*-nitrophenyl phosphate (pNPP) and Ru486 were purchased from Sigma–Aldrich. Compound-2 was purchased from CalBioChem. TO901317 was obtained from Cayman. Phospho-IR, IR, phospho-AKT, AKT antibodies were purchased from Cell-signaling, GLUT4 antibody was purchased from Santa Cruze, 11β-HSD_1 antibody was obtained from Cayman chemical and GAPDH antibody was from Kangcheng Bio-tech (Shanghai, China). 2-[^{3}H]-Deoxyglucose was purchased from PerkinElmer Life Sciences. All the other solvents and reagents were purchased commercially and were used without further purifications.

2.2. *Plasmids*

The pGEX-4T-1-PTP1B (aa. 1–321) and pGEX-4T-1-TC-PTP (aa. 1–288) were constructed with BamHI–HindIII and BamHI–EcoRI sites, respectively. The pCDNA3.1-PPARγ and pSV-PPRE-Luc were described previously [31]. The pGEX-2T-LAR

(aa. 1282–1888) and pGEX-2T-CD45 (aa. 560–1256) were kindly provided by Prof. Rafael Pulido (Instituto de Investigaciones Citológicas, Spain), the pGEX-2T-PTPα (aa. 182–803) by Prof. Frank R. Jirik (Department of Biochemistry & Molecular Biology, University of Calgary, Canada), the pCMX-hFXR by Dr. Stefan K. Westin (Department of Biology, X-Ceptor Therapeutics, Inc., San Diego, CA), the pGL3-FXRE-Luc vector by Dr. Majlis Hermansson (AstraZeneca. Mölndal, Sweden), the pTK-Luc-2xLXRE-1 plasmid by Dr. Peter A. Edwards (The Medicine Institute at University of California, Los Angeles) and the pGL3-hABCA1 (−919/+224)-Luc and pGL3-hABCG1 (−1013/+123)-LXREB-Luc plasmids were kindly donated by Prof. Gerd. Schmitz (Institute for Clinical Chemistry, University of Regensburg) and Dr. Steven L. Sabol (Laboratory of Developmental Biology NHLBI Division of Intramural Research), respectively. The truncated GLUT4 promoter (−478 to +223) construct was generated from the pGL3-hGLUT4 promoter that was a gift from Dr. Hilde I. Nebb (Institute for Nutrition Research, University of Oslo, Norway) and the GLUT4 promoter-LXRE-mut was constructed as described previously [28]. The UAS-TK-Luc reporter was generously donated by Dr. Daniel P. Kelly (Washington University School of Medicine, USA) and the fusion constructs of Gal4DBD-LXRα-LBD, Gal4DBD-RXRα-LBD and Gal4DBD-GR-LBD were generated by using pCDNA3.1-LXRα, pCDNA3.1-RXRα and pCI-nGFP-C656G (kindly provided by Dr. Gordon Hager, National Cancer Institute, Laboratory of Receptor Biology and Gene Expression) as templates, respectively.

2.3. PTP1B enzymatic assay and inhibition kinetics

The enzymatic assay of PTPases, including PTP1B, CD45, TC-PTP, LAR and PTPα were carried out as described previously [32]. Briefly, the reaction was performed incubating 5 mM substrate pNPP and 5 μg enzyme in assay buffer (50 mM HEPES, pH 7.3, 100 mM NaCl, 0.1% BSA, and 1 mM DTT) at 30 °C and the real-time enzyme kinetics was monitored by measuring the OD_{410} using the microplate spectrophotometer (Bio-Rad) for at least 10 min. In the kinetic analysis, five different concentrations of pNPP (0.1, 0.5, 1, 3, 5 mM) were used as PTP1B substrate in the absence or presence of NPLC441 (9, 12 μM). The inhibition mode for the inhibitor was determined from the Lineweaver–Burk plot, while the inhibition was expressed as the K_i value from the plot of the Lineweaver–Burk slopes against the inhibitor concentration.

2.4. Cell culture and differentiation

HEK293T (human embryonic kidney) cells were cultured in Delbecco's modified eagle's medium (DMEM), HepG2 (hepatoma) cells were cultured in α-MEM. The 3T3-L1 cells were cultured in DMEM containing biotin (8 mg/L) and Ca-pantothenate (4 mg/L). All the cells were cultured at 37 °C in media supplemented with 10% FBS and in a humidified atmosphere with 5% CO_2. The differentiation procedure of 3T3-L1 preadipocytes followed the classic method [33], briefly, 2 days after 100% confluence, cells were stimulated with MDI stimulus (0.115 mg/L methylisobutylxanthine (MIX), 0.39 mg/L dexamethasone (DEX) and 1 mg/L insulin) for 3 days, and then the medium was changed to fresh medium containing 1 mg/L insulin for another 3 days.

2.5. Transfections and luciferase assays

Transient transfection was carried out in 24-well plates. Cells were transfected in opti-MEM with the transfection reagent Lipofectamine-2000 (Invitrogen, USA) according to the manufacturer's protocols. And the renilla luciferase reporter pRL-SV40 (50 ng/well) was used to normalize the transfection efficiency. After a 5-h transfection, the medium was replaced with fresh DMEM supplemented with 10% FBS, and the cells were further stimulated with vehicle (DMSO) or compound(s) for another 18-h. Finally, cells were lysed and luciferase activities were measured using Dual Luciferase Assay System kit (Promega).

2.6. Mammalian one-hybrid assay

The mammalian one-hybrid assays were used to investigate the effects of NPLC441 or OA on LXRα-LBD, RXRα-LBD and GR-LBD. HEK-293T cells were transiently co-transfected with the UAS-TK-Luc reporter and the fusion construct of Gal4DBD-LXRα-LBD, Gal4DBD-RXRα-LBD, Gal4DBD-GR-LBD, respectively. After a 5-h transfection, the cells were further treated with vehicle (DMSO) or compound(s) for another 18-h, and then cells were lysed and luciferase activities were measured.

2.7. Real-time quantitative RT-PCR analysis

Total RNA was isolated using TRIZOL® Reagent (Promega). The first strand synthesis was performed using oligo-dT primers with the Superscript first strand synthesis system for RT-PCR (Genery Biotech, Shanghai, China). The quantity of mRNA was detected by a real-time quantitative RT-PCR (qRT-PCR) approach using the ready-to-use assay SYBR green PCR core reagents kit based on the Taqman technology (Applied Biosystems, Stockholm, Sweden). The threshold cycles (C_t) for the GLUT4 and the GAPDH control signals were determined in triplicate experiments, and the relative RNA quantities were calculated using the comparative Ct method, where ΔC_t is $C_{t_{GLUT4}} - C_{t_{GAPDH}}$. The ΔC_t values were used to calculate $2^{\Delta C_t}$. PCR primer pairs for amplification of the two genes are as follows: GLUT4: 5′-CAACGTGGCTGGGTAGGCA-3′ (sense), 5′-ACACATCAGCCCAGCCGGT-3′ (antisense); GAPDH: 5′-CCACTCACGGCAAATTCAACGGCA-3′ (sense), 5′-TCCAGGCGGCACGTCAGATCCACG-3 (antisense).

2.8. Western blot

For IR and AKT phosphorylation analysis, HepG2 cells were seeded into 12-well plates and grew to 80–100% confluence, cells were incubated with varied compounds in serum-free α-MEM media, after 4-h incubation, cells were left untreated or stimulated with insulin (16.7 nM) for 5 min and the stimulation was terminated by rinsing twice the cells with ice-cold PBS. Cells were harvested and lysed with 2× SDS sample buffer. The phosphorylation levels of IR and AKT were analyzed by Western blot with phospho-IGF-I receptor beta (Tyr1135/1136) (1:1000)/insulin receptor beta (Tyr1150/1151) (19H7) (1:1000) and phospho-AKT (Ser473) (1:1000)/AKT (1:1000) antibodies, respectively. For GLUT4 protein analysis,

the 3T3-L1 adipocytes (the 8th day after MDI stimulation) were seeded to 24-well plates, adipocytes (the 10th day) were treated with compounds or vehicle (DMSO) in serum-free DMEM media for 24 h, cells were harvested and GLUT4 protein levels were investigated by Western blot with GLUT4 antibody (1:1000). For 11β-HSD_1 expression analysis, HepG2 cells were seeded into 12-well plates and stimulated with vehicle (DMSO) or compounds in α-MEM media containing 10% charcoal-treated FBS for 48 h, cells were harvested and blotted with 11β-HSD_1 antibody. In the above-mentioned Western blot experiments, GAPDH (1:10,000) was used as an internal control for protein loading.

2.9. Ligand binding assay

Binding affinity of NPLC441 or OA towards RXRα-LBD was assayed by the surface plasmon resonance (SPR)-based BIAcore 3000 instrument following the reported method [31]. Proteins to be covalently bound to the chips were diluted in 10 mM sodium acetate buffer (pH 4.5) to a final concentration of 0.10 mg/ml. Then the proteins were immobilized to CM5 chips using a standard amine-coupling procedure. Before experiments, baseline was equilibrated with a continuous flow of running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4). And then different concentrations of NPLC441 were injected into the channels at a flow rate of 20 μL/min for 60 s, followed by disassociation for 120 s. BIAevaluation software version 3.1 (Biacore) and the 1:1 Langmuir binding fitting model were used to determine the equilibrium dissociation constant (K_D) of compounds.

2.10. 2-Deoxyglucose uptake assay

3T3-L1 adipocytes were preincubated with vehicle or varied concentrations of NPLC441 in serum-free DMEM media for 2.5 h and in Krebs buffer (10 mM HEPES buffer, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1.25 mM $MgSO_4$) containing 0.5% BSA for another 30-min, and then cells were either left untreated or stimulated with 17 nM insulin for 5 min. Glucose uptake was determined by the addition of 2-[^{3}H]-deoxyglucose (final concentration, 0.5 μCi/ml). The reaction was stopped by washing three times with ice-cold PBS. Cells were lysed by 0.1% Triton. Finally, radioactivity was calculated by scintillation counter, and glucose uptake was assessed by scintillation counting. Results were normalized for protein content measured by BCA assay.

Table 1 – Inhibition of NPLC441 against a panel of protein tyrosine phosphatases

Enzyme	IC_{50} (μM)
PTP1B	11.8
CD45	32.0
TC-PTP	38.3
LAR	–
PTPα	–

–: no inhibition at 100 μM.

2.11. Statistical analysis

The data were presented as mean ± S.D. for at least three separate determinations for each group. Differences between groups were examined for statistical significance using Student's *t*-test. $p < 0.05$ was used to indicate a statistically significant difference.

3. Results

3.1. NPLC441 is a competitive PTP1B inhibitor

In the evaluation of NPLC441 inhibition activity against PTP1B, the assay system was set up as reported previously [32]. In brief, the GST-tagged PTP1B (aa. 1–321) was expressed in *E. coli*, and pNPP was used as the reaction substrate. NPLC441 was identified as a PTP1B inhibitor (Table 1, IC_{50} = 11.8 μM), whose inhibition activity is a little stronger than OA against PTP1B (IC_{50} = 23.9 μM) [34]. The inhibition mode determination indicated that NPLC441 is a competitive inhibitor of PTP1B (Fig. 2) with K_i value of 16.9 μM.

To date, a number of PTP1B inhibitors have been reported, but few of them showed good selectivity over other PTPases due to the structural similarity among these enzymes, particularly between PTP1B and TC-PTP, which share ~80% sequence identity between their catalytic domains. In the current work, we have evaluated the selectivity of NPLC441 against a panel of PTPases, including TC-PTP, LAR, PTPα, and CD45. As shown in Table 1, NPLC441 exhibited an inhibition preference toward PTP1B *versus* the other examined PTPases.

3.2. NPLC441 increases insulin-stimulated phosphorylation of IR and AKT

PTP1B negatively regulates insulin signaling pathway by dephoshorylation of IR and IRS [35]. To investigate whether NPLC441 as a PTP1B inhibitor could sensitize insulin signaling, serum-starved HepG2 cells were treated with series of

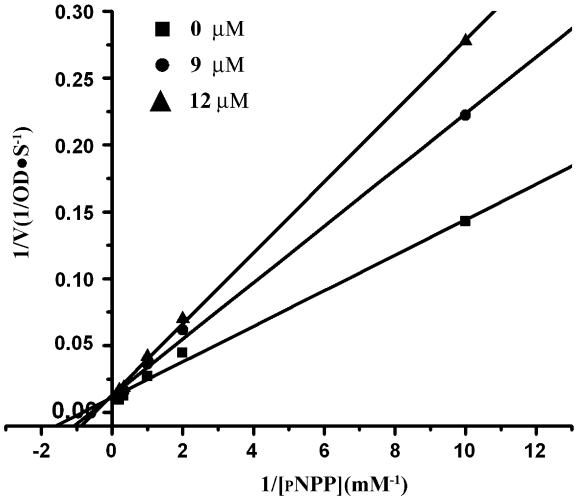

Fig. 2 – Inhibition kinetics of PTP1B by NPLC441. The Lineweaver–Burk plot of pNPP hydrolysis catalyzed by PTP1B in the absence or presence of NPLC441 (9, 12 μM).

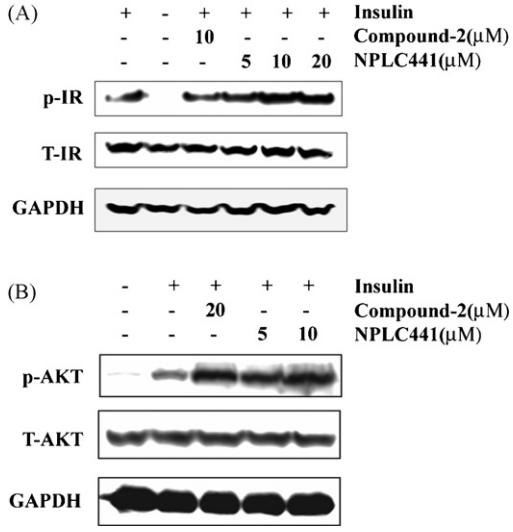

Fig. 3 – NPLC441 enhanced insulin-stimulated IR and AKT phosphorylation. HepG2 cells were serum-starved and untreated (–) or treated with compound-2 or various concentrations of NPLC441 for 4 h. Cells were stimulated with (+) or without (–) insulin (16.7 nM) for 5 min. Cell lysates were analyzed by Western blot using specific antibodies. (A) Effect of NPLC441 on insulin-stimulated IR phosphorylation. p-IR, phosphorylated insulin receptor; T-IR, total protein of insulin receptor. (B) Effect of NPLC441 on insulin-stimulated AKT phosphorylation. p-AKT, phosphorylated protein kinase B; T-AKT, total protein of protein kinase B.

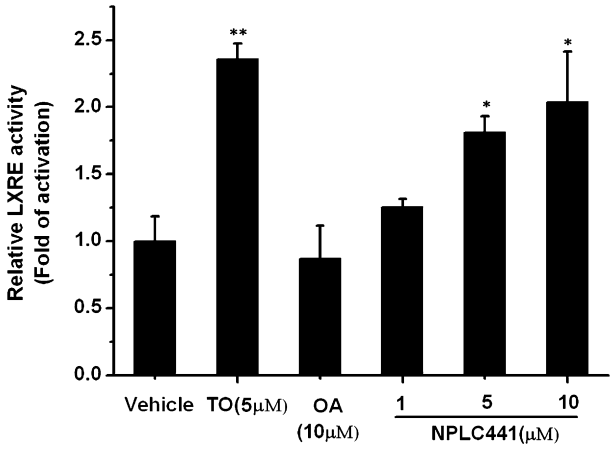

Fig. 4 – NPLC441 activated LXRα:RXRα heterodimer transactivation. HEK-293T cells were transiently transfected with LXRE-luc reporter (pTK-Luc-2xLXRE-1) in combination with human LXRα and RXRα expression vectors (pcDNA3.1-LXRα and pcDNA3.1-RXRα). The transfected cells were treated with vehicle, TO901317 (TO, 5 μM), OA (10 μM) and varied concentrations of NPLC441, respectively for 18 h. The transactivation activities of LXRα:RXRα heterodimer were evaluated by measuring the luciferase activities. Values are means ± S.E., $n = 3$. $^{**}P < 0.01$ *vs.* vehicle, $^{*}P < 0.05$ *vs.* vehicle, vehicle = 1.

concentrations of NPLC441 or compound-2 (a reported specific inhibitor of PTP1B as a positive control) [36]. After a 4-h incubation, the cells were left untreated or stimulated with 16.7 nM of insulin for 5 min. As shown in Fig. 3A and B, the insulin-stimulated phosphorylations of both IR and AKT were significantly increased with treatment of NPLC441, thereby indicating that NPLC441 could enhance the insulin-induced activation of IR and AKT in HepG2 cells.

3.3. *NPLC441 as an RXRα antagonist selectively activates LXRα:RXRα heterodimer*

The potential effects of NPLC441 on the transactivation of LXRα:RXRα heterodimer were investigated according to the cell-based assay system, in which HEK-293T cells were transiently transfected with LXR response element (LXRE)-driven luciferase reporter (pTK-Luc-2xLXRE-1) (2xLXRE sequence: ggtaTGGTCAggcaAGTTCAtgtc) [37] in combination with the expression constructs of LXRα and RXRα (pCDNA3.1-LXRα and pCDNA3.1-RXRα). The transfected cells were subsequently treated with vehicle (DMSO), OA, NPLC441 and TO901317 (a reported synthetic agonist of LXRα) [38], respectively. The transactivation activity of LXRα:RXRα heterodimer was evaluated by luciferase assay. As indicated in Fig. 4, TO901317 (5 μM) significantly increased the transactivation activity of LXRα:RXRα by 2.4-fold compared to vehicle, while NPLC441 also induced a similar enhancement in a dose-dependent manner, OA, however, showed no effect on this heterodimer.

LXRα is classified as a permissive RXR-heterodimer which can be activated by a ligand of either LXRα or RXRα [23]. To determine which receptor (LXRα or RXRα) is the target protein for NPLC441, we performed a mammalian one-hybrid assay. In the assay, HEK-293T cells were transiently co-transfected with the UAS-TK-Luc reporter and the fusion construct of Gal4DBD-LXRα-LBD or Gal4DBD-RXRα-LBD. The transactivation activities of LXRα-LBD and RXRα-LBD were evaluated by luciferase assay. As shown in Fig. 5, NPLC441 rendered no effect on LXRα-LBD (Fig. 5A), while it antagonized 9-*cis*-RA (RA) in the transactivation of RXRα-LBD (Fig. 5B), indicating that RXRα is possibly the target receptor of NPLC441 in the LXRα:RXRα heterodimer complex. In addition, we also found that OA was completely inactive in the transactivation of RXRα-LBD (Fig. 5B).

To further validate the binding of NPLC441 to RXRα, we performed a ligand binding assay by surface plasmon resonance (SPR) technology-based Biacore 3000, in which RXRα-LBD protein was immobilized on CM5 chip and NPCL441 was injected onto the sensor chip to detect the binding affinity. As shown in Fig. 5C, NPLC441 dose-dependently bound to RXRα-LBD with K_D value of 0.72 μM, inconsistent with the results from mammalian one-hybrid assay. While OA had a very weak binding affinity against RXRα-LBD (K_D = 321 μM) (Fig. 5D). All these results thereby suggested that NPLC441 is not an LXRα ligand but an RXRα antagonist, and could activate the LXRα:RXRα heterodimer.

In considering the fact that RXRα can act as an obligatory heterodimer partner for many other nuclear receptors such as

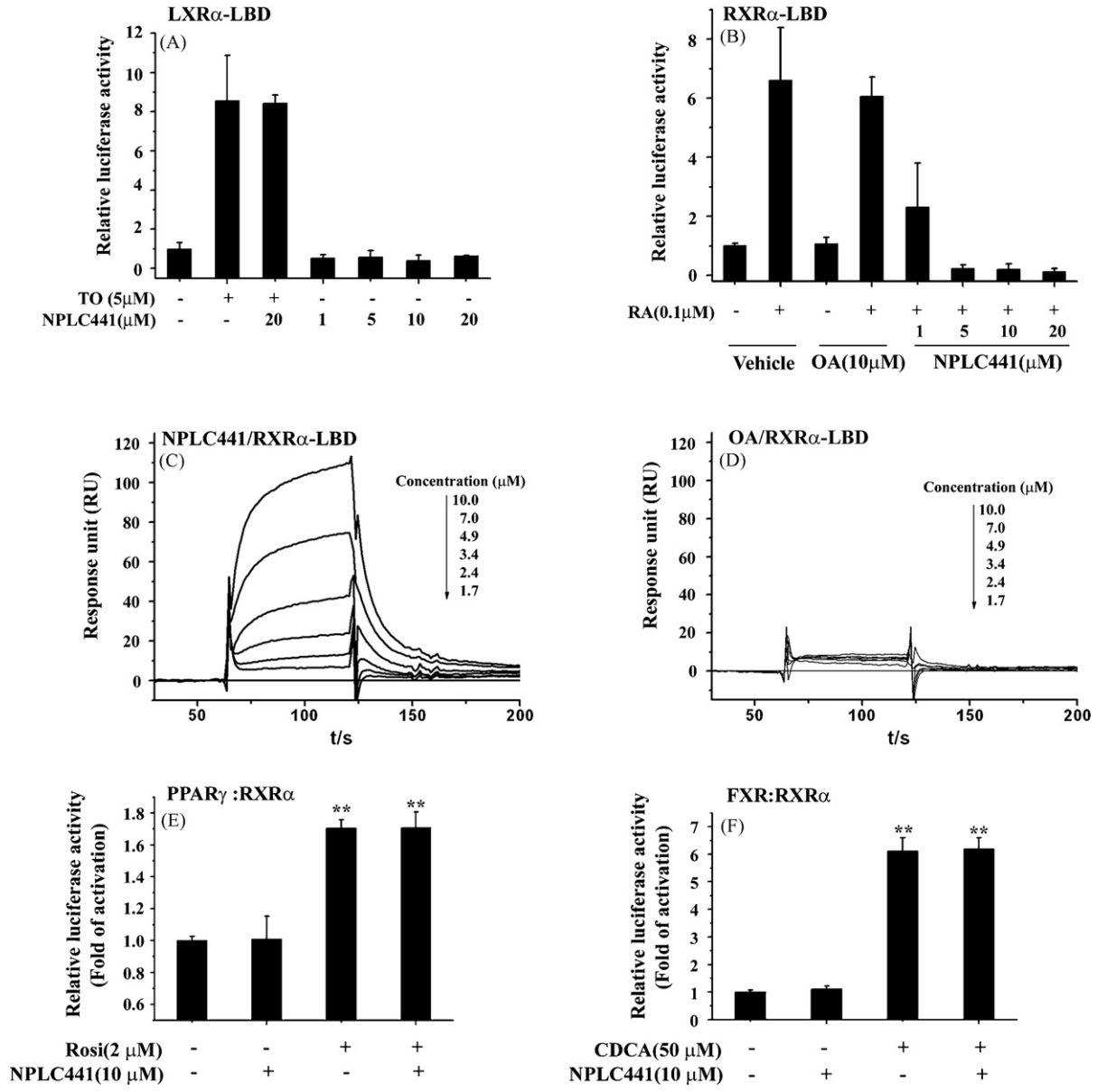

Fig. 5 – NPLC441 as an RXRα antagonist selectively activated LXRα:RXRα heterodimer. Effect of NPLC441 on LXRα-LBD and RXRα-LBD were investigated by mammalian one-hybrid assays, in which, HEK-293T cells were transiently co-transfected with UAS-TK-Luc reporter and the fusion construct of Gal4DBD-LXRα-LBD (A) or Gal4DBD-RXRα-LBD (B). The binding affinity of NPLC441 and OA to RXRα-LBD was examined by SPR technology-based BIAcore 3000 (C and D), in the assay, the RXRα-LBD protein was immobilized to CM5 chips using a standard amine-coupling procedure. The sensorgrams were obtained from injection of series of concentrations of NPLC441 or OA over the immobilized RXRα-LBD. Effects of NPLC441 on the transactivation of PPARγ:RXRα (E) or FXR:RXRα (F) heterodimers were investigated by luciferase assays. In the assays, HEK-293T cells were transiently transfected with luciferase reporters (pSV-PPRE-Luc or pGL3-FXRE-Luc) in combination with the expression constructs of corresponding receptors (pCDNA3.1-PPARγ or pCMX-hFXR) and RXRα (pCDNA3.1-RXRα), respectively. The transfected cells were then treated with compound(s) or vehicle (−) for 18 h. Rosiglitazone (Rosi, 2 μM) and chenodeoxycholic acid (CDCA, 50 μM) were used as positive agonists for PPARγ and FXR, respectively. The transactivation activities of both heterodimers were evaluated by measuring the luciferase activities. Values are means ± S.E., $n = 3$. $^{**}P < 0.01$ *vs.* vehicle, vehicle = 1.

PPARs, FXR, *etc.* [22], we thus tested the effects of NPLC441 on PPARγ:RXRα and FXR:RXRα heterodimers as two representatives by transient transfection assays. As indicated in Fig. 5E and F, NPLC441 was inactive on the two permissive RXRα-heterodimers, suggesting its good selectivity for LXRα over other examined nuclear receptors.

Next, we investigated the possible functional role of NPLC441 in the regulation of LXRα:RXRα target genes ABCA1

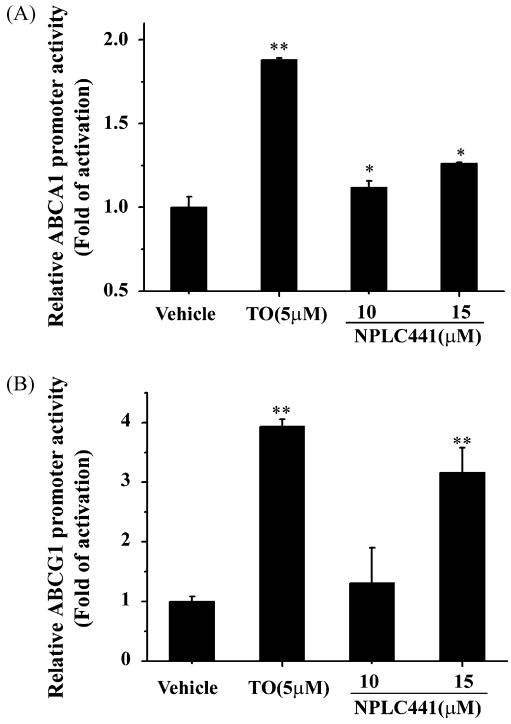

Fig. 6 – NPLC441 increased hABCA1 and hABCG1 promoter activities in transient transfection assays. HEK-293T cells were transiently co-transfected with pGL3-hABCA1 (−919/+224)-Luc (A) or pGL3-hABCG1 (−1013/+123)-LXREB-Luc (B), and the expression constructs of LXRα and RXRα (pCDNA3.1-LXRα, pCDNA3.1-RXRα). The transfected cells were stimulated with vehicle, TO901317 (TO) or NPLC441 for 18 h, and the activities of hABCA1 and hABCG1 gene promoters were evaluated by luciferase assays. Values are means ± S.E., $n = 3$. $^{*}P < 0.05$ *vs.* vehicle, $^{**}P < 0.01$ *vs.* vehicle, vehicle = 1.

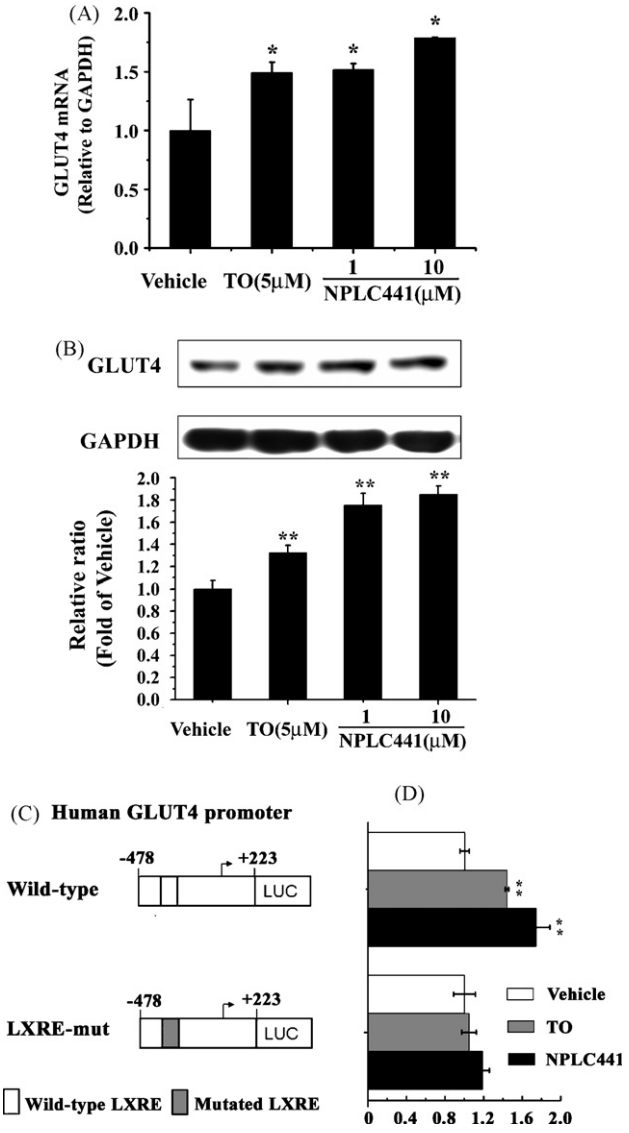

Fig. 7 – NPLC441 enhanced GLUT4 expression in 3T3-L1 adipocytes through its activation against LXRα:RXRα heterodimer. 3T3-L1 adipocytes were treated with TO901317 (TO, 5 μM) or NPLC441 (1, 10 μM) for 24 h, then the cells were harvested for qRT-PCR (A) and Western blot (B) assays. The immunoblots are representative of three separate experiments, and the values shown in the bar graph below the representative blots are means ± S.E., $n = 3$. (C) A schematic presentation of the wild-type hGLUT4 promoter (−478/+223)-Luc construct and a mutant form LXRE-mut bearing a LXRE mutation. The arrow points out the transcription start site. (D) Transient transfection of wild-type (−478/+223) or LXRE-mut hGLUT4 promoter reporters into HEK-293T cells. The transfected cells were then treated with vehicle (DMSO, *white*), TO901317 (TO, 5 μM, *light gray*) or NPLC441 (10 μM, *black*) for 18 h, and the GLUT4 promoter activities were subsequently evaluated by luciferase assays. Values are means ± S.E., $n = 3$. $^{*}P < 0.05$ *vs.* vehicle, $^{**}P < 0.01$ *vs.* vehicle, vehicle = 1.

and ABCG1 [26]. In this study, HEK-293T cells were transiently co-transfected with human ABCA1 or ABCG1 gene promoter cloned upstream of the luciferase gene (pGL3-hABCA1(−919/+224)-Luc [39] or pGL3-hABCG1 (−1013/+123)-LXREB-Luc [40]), and the expression constructs of LXRα and RXRα. The activities of hABCA1 and hABCG1 gene promoters were thus evaluated by luciferase assays. As expected, NPLC441 could both increase the promoter activities of ABCA1 and ABCG1 genes (Fig. 6).

Therefore, all the above-mentioned results have revealed that NPLC441 as an RXRα antagonist could selectively activate LXRα:RXRα heterodimer and regulate its target genes ABCA1 and ABCG1.

3.4. *NPLC441 enhances GLUT4 expression in 3T3-L1 adipocytes by activating LXRα:RXRα heterodimer*

As has been reported, activation of LXRα:RXRα heterodimer might lead to the increase of GLUT4 expression in adipose tissue [28]. We have thus examined the ability of NPLC441 in the up-regulation of GLUT4 expression. In the assay, fully differentiated 3T3-L1 adipocytes were treated with NPLC441 or

TO901317 (as a positive control) for 24 h. The transcription and expression levels of GLUT4 gene were measured by qRT-PCR and Western blot assays. As shown in Fig. 7A, NPLC441 could increase GLUT4 gene transcription in a dose-dependent manner. Similarly, Western blot results indicated that the expression levels of GLUT4 protein could be significantly increased by the presence of NPLC441 (Fig. 7B). Therefore, all these results thus suggested that NPLC441 could up-regulate GLUT4 gene transcription and protein expression in 3T3-L1 adipocytes.

Subsequently, we determined whether the above-mentioned up-regulation of GLUT4 expression induced by NPLC441 was dependent on LXRα:RXRα heterodimer activation. By considering the fact that a functional LXR response element (LXRE) is located at −463 bp to the transcriptional initiation site in human GLUT4 promoter [25], we constructed a truncated GLUT4 promoter (−478/+223) containing a mutated LXRE (LXRE-mut) following the reported methods [28] (Fig. 7C). HEK-293T cells were then transiently transfected with the wild-type or the LXRE-mut GLUT4 promoter in combination with LXRα and RXRα expression vectors. After 5-h transfection, cells were treated with TO901317 (5 μM) or NPLC441 (10 μM) for another 18 h. GLUT4 promoter activities were thus evaluated by measuring luciferase activities. As indicated in Fig. 7D, the activity of the wild-type construct was significantly increased by the treatment of either TO901317 (1.5-fold) or NPLC441 (1.7-fold), whereas such enhancements were abolished when LXRE-mutation was introduced into the GLUT4 promoter. These results thus clearly suggested that the effect of NPLC441 on GLUT4 expression was mediated by the LXRα:RXRα heterodimer.

3.5. *NPLC441 stimulates the basal glucose uptake in 3T3-L1 adipocytes*

As indicated above, NPLC441 could both enhance the insulin signaling and GLUT4 expression, we therefore investigated its potential effects on the glucose uptake. In this study, the 3T3-L1 adipocytes were preincubated with vehicle or varied concentrations of NPLC441 in serum-free DMEM media for 2.5 h and then in Krebs buffer containing 0.5% BSA for another 30-min, cells were either left untreated or stimulated with 17 nM insulin for 5 min. Glucose uptake was determined by the addition of 2-[^{3}H]-deoxyglucose. As indicated in Fig. 8, a dose-dependent increase of glucose uptake in 3T3-L1 adipocytes was observed when treated with various concentrations of NPLC441.

3.6. *NPLC441 suppresses 11β-HSD$_1$ expression in HepG2 cells*

It is reported that activation of LXR could suppress the expression of 11β-HSD$_1$ [29]. In the current study, we discovered that NPLC441 could also suppress 11β-HSD$_1$ expression in HepG2 cells, although it is an RXRα antagonist functioning as an agonist of LXRα:RXRα heterodimer. In the assay, HepG2 cells were treated with vehicle (DMSO), NPLC441, TO901317 (known LXRα agonist), Ru486 (known glucocorticoid receptor antagonist as a positive control) [41], respectively for 48 h, the protein levels of 11β-HSD$_1$ were examined by Western blot. As indicated in Fig. 9A, all the compounds, TO901317 (5 μM), Ru486 (1 μM) and NPLC441 (10 μM), could significantly suppress the expression of 11β-HSD$_1$ in HepG2 cells.

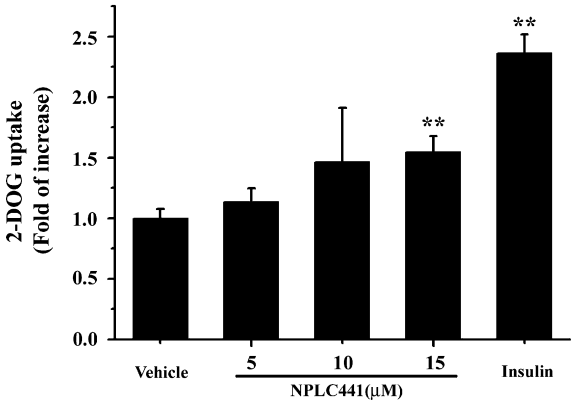

Fig. 8 – NPLC441 stimulated the basal glucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with the indicated concentrations of NPLC441 or vehicle (DMSO) in serum-free DMEM media for 2.5 h and then in Krebs buffer containing 0.5% BSA for another 30-min, thereafter, cells were left untreated or treated with 17 nM insulin for 5 min. Glucose uptake was measured after 5-min incubation of 2-[^{3}H]-deoxyglucose (2-[^{3}H]-DOG). Radioactivity was calculated by scintillation counter, and glucose uptake was assessed by scintillation counting. Results were normalized for protein content measured by BCA assay. Values are means ± S.E., $n = 3$. $^{**}P < 0.01$ *vs.* vehicle, vehicle = 1.

To rule out the possibility that NPLC441 is a ligand of glucocorticoid receptor (GR) which was suggested to directly regulate the expression of 11β-HSD$_1$ [42,43], mammalian one-hybrid assay was carried out. In the assay, HEK-293T cells were transiently co-transfected with the UAS-TK-Luc reporter and the fusion construct of Gal4BD-GR-LBD. The transfected cells were then treated with vehicle, Ru486 or NPLC441, respectively in the absence or presence of 1 nM dexamethasone (DEX, known GR agonist) [44], the luciferase activities were subsequently measured to evaluate the effect of NPLC441 on GR-LBD. As shown in Fig. 9B, Ru486 significantly antagonized DEX in activating the GR transactivation, while NPLC441 showed no effect on the GR-LBD.

All the above-mentioned results thereby suggested that NPLC441 suppressed 11β-HSD$_1$ expression in HepG2 cells as an LXRα:RXRα heterodimer agonist.

4. Discussion

Oleanolic acid (OA) and its derivatives exhibited multiple biological and pharmacological functions, including anti-inflammatory, anti-tumorigenic and anti-hepatitis effects [1,2]. Recently, the anti-diabetic effects of these triterpenoids have been also studied [45–47]. For example, it is reported that OA glycosides that are purified from plants, but not OA itself,

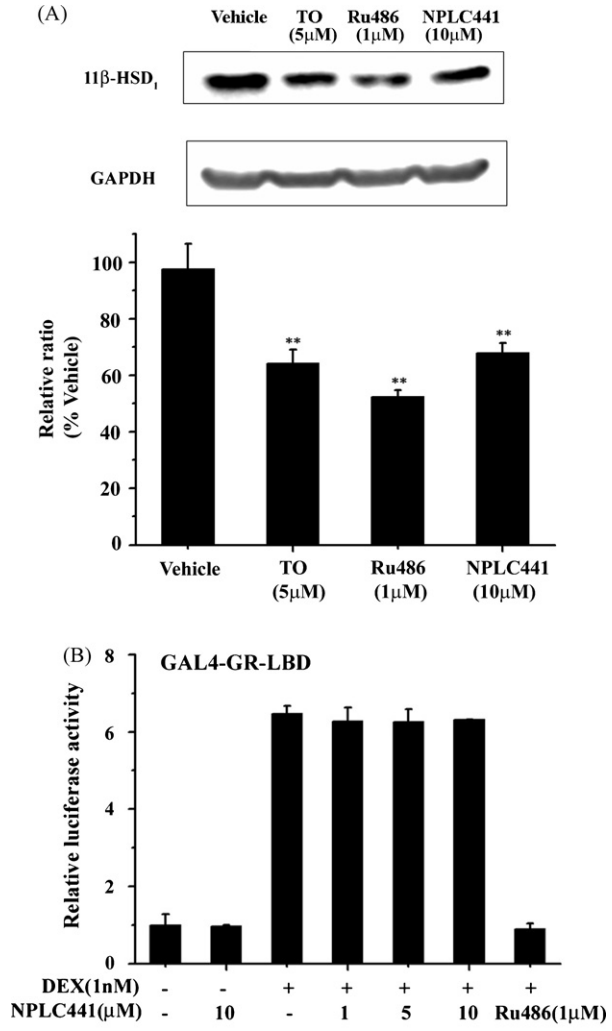

Fig. 9 – NPLC441 suppressed 11β-HSD$_1$ expression in HepG2 cells. (A) NPLC441 suppressed 11β-HSD$_1$ expression in HepG2 cells. HepG2 cells were treated with TO901317 (TO, 5 μM), Ru486 (1 μM) and NPLC441 (10 μM), respectively in α-MEM media containing 10% charcoal-stripped serum. After 48 h, cells were lysed and the lysates were analyzed by Western blot. The immunoblots are representative of three separate experiments, and the values shown in the bar graph below the representative blots are means ± S.E., $n = 3$. $^{**}P < 0.01$ *vs.* vehicle. (B) NPLC441 rendered no effect on GR-LBD in mammalian one-hybrid assay. HEK-293T cells were transiently co-transfected with UAS-TK-Luc reporter and the fusion construct of Gal4DBD-GR-LBD. After 5-h transfection, cells were treated with compound(s) or vehicle (−) in the absence or presence of 1 nM DEX for 18 h. The transactivation activities of GR-LBD were subsequently evaluated by measuring the luciferase activities. Values are means ± S.E., $n = 3$. vehicle = 1.

could reduce serum glucose levels in rats given an oral glucose load [48]. In addition, much target information for their beneficial effects on diabetes have been also identified during the past decade, such as PPARγ [49], α-glucosidase [45] and glycogen phosphorylase [46]. In the current work, we identified a novel OA derivative NPLC441 (Fig. 1), which functions as a multi-target performer and displays more potent biological activities than OA. As a selective PTP1B inhibitor, it could enhance insulin-stimulated activation of IR and AKT in HepG2 cells. NPLC441 is also an RXRα antagonist that can selectively activate LXRα:RXRα heterodimer, suppress the expression of 11β-HSD$_1$ in HepG2 cells and up-regulate GLUT4 expression in 3T3-L1 adipocytes. Further research indicates that NPLC441 could effectively stimulate glucose uptake in 3T3-L1 adipocytes.

By molecular and enzymatic assay, we have shown that NPLC441 displays ~2-fold inhibitory activity against PTP1B than OA. In addition, NPLC441 bound strongly to RXRα-LBD ($K_D = 0.72$ μM) as compared to OA which had a weak binding affinity ($K_D = 321$ μM). Moreover, in transient transfection assays, OA had no effect on the transactivation of either LXRα:RXRα heterodimer or RXRα-LBD, while NPLC441 functioned effectively on both of them. All these obtained results thus revealed that NPLC441 was intracellularly stable and showed more potent relevant pharmacological functions compared with OA.

PTP1B has been considered as an attractive therapeutic target for the treatment of type 2 diabetes and obesity. However, as PTPases regulate multiple important signaling pathways and a single pathway may be controlled by several PTPases, non-selective PTP1B inhibitors might lead to unwanted side effects [20]. Our work indicated that NPLC441 displays ~3-fold selectivity over CD45 and TC-PTP (the closest structural homologue of PTP1B), and shows no inhibition against LAR or PTPα, suggesting its good inhibition preference for PTP1B *versus* other examined PTPases (Table 1).

Selective inhibition of PTP1B was previously shown to stimulate GLUT4 translocation from intracellular vesicle pool to the plasma membrane through PI3K/AKT pathway [16]. In the present work, NPLC441 as a selective PTP1B inhibitor significantly enhanced insulin-stimulated phosphorylation of IR and AKT. Due to the cellular toxicity in CHO-K1 cells, NPLC441 was failed to be observed in the stimulation of GLUT4 translocation. Although GLUT4 translocation is one of the major factors responsible for the whole-body glucose uptake, the amount of stored GLUT4 protein is also an important determinant [28]. As reported, overexpression of GLUT4 protein in muscle could increase basal and insulin-stimulated whole-body glucose disposal in conscious mice [50], and the synthetic LXRα agonist TO901317 could increase basal glucose uptake in 3T3-L1 adipocytes [51]. In our work, we demonstrated that NPLC441 as an agonist against LXRα:RXRα heterodimer dose-dependently increased the basal glucose uptake in 3T3-L1 adipocytes, while it failed to stimulate the insulin-induced glucose uptake. Similarly, the ursolic acid (an isomer of OA) has been identified to be a PTP1B inhibitor ($IC_{50} = 3.08$ μM), but there is no further report related to its ability to stimulate insulin-induced glucose uptake [52]. It is tentatively supposed that this unusual discrepancy might be possibly due to some C2-ceramide-like factors that mediate the blunted GLUT4 translocation response to insulin [53].

Physiologically, LXRα functions as transcriptional activation factor by forming obligate heterodimer with RXRα. The RXR-heterodimers can be divided into two types:

nonpermissive heterodimers that can only be activated by ligands of partners, such as RXR:RAR (retinoic acid receptor), and permissive heterodimers that can be activated by either RXR or partner-specific ligand, such as LXRα:RXRα [23,24]. Here, we have shown that NPLC441 as an RXRα antagonist could activate the LXRα:RXRα heterodimer. The transient transfection assays indicated that NPLC441 (15 μM) could effectively activate the promoter activities of the two known LXRα target genes ABCA1 gene (1.26-fold) and ABCG1 gene (3.16-fold). In addition, this compound is inactive on the other permissive RXRα-heterodimers, including PPARγ:RXRα and FXR:RXRα in transient transfection assays. To our knowledge, NPLC441 might be the first RXRα antagonist that selectively activates LXRα:RXRα heterodimer. Our results may reinforce the concept that the activity of RXR ligand can be restricted to specific permissive RXR-heterodimer, following the discovery that the RXR antagonist LG100754 could selectively activate PPARγ:RXRα heterodimer [24]. The noted affinity of 0.72 μM for the RXRα-LBD is 10–25-fold from concentrations used in the experiments 4–6, whereas it correlates to that used in mammalian one-hybrid assay, suggesting that the K_D value of the RXR ligands may not be always consistent with the concentrations used in the assays related to RXR-heterodimers.

11β-HSD_1 has been recognized as a promising drug target for the treatment of metabolic syndromes [30,54]. Recently, efforts have been made to study the regulation of 11β-HSD_1 gene expression. For direct regulation, CCAAT enhancer-binding protein (C/EBP) and GR were reported to directly regulate 11β-HSD_1 expression by binding to the promoter of 11β-HSD_1 gene sequence [43,55,56]. For indirect interfere, some ligand-dependent transcription factors such as LXRα, PPARγ and PPARα, were found to tissue-specifically down-regulate the expression of 11β-HSD_1, and these effects were further proved to be mediated by GR [29,57–60]. In our work, we have shown that NPLC441 is neither an LXRα nor a GR ligand, however, as an LXRα:RXRα heterodimer agonist, it could suppress 11β-HSD_1 expression in HepG2 cells. In addition, the GR antagonist Ru486 was previously shown to block the corticosterone-induced 11β-HSD_1 expression in cultured hippocampal neurons [42], here we demonstrated that Ru486 treatment alone could also significantly decrease the expression of 11β-HSD_1 in HepG2 cells.

Recently, combination therapy using the mixture of monotherapy or individual multi-target agent has been extensively applied to combat multigenic diseases such as cancers, and the diseases that might affect multiple tissues or cell types such as diabetes [61]. Taking advantage of the multifunctional nature of the triterpenoids, many synthetic OA derivatives have been applied for cancer therapy, for example, the synthetic OA derivatives CDDO and its methyl ester (CDDO-Me) are currently in phase I clinical trials for the treatment of leukaemia and solid tumours [6]. NPLC441 in our work has displayed multi-target information against type 2 diabetes, suggesting its potency for further applications.

In summary, our work has identified a novel OA derivative NPLC441 as both a selective PTP1B inhibitor and an LXRα:RXRα heterodimer agonist. The obtained results might provide a new insight into the anti-diabetic application of OA derivative via multi-target mechanisms.

Acknowledgements

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