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One-step modification to identify dual-inhibitors targeting both pancreatic triglyceride lipase and Niemann-Pick C1-like 1

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ABSTRACT

Pancreatic triglyceride lipase (PTL) and Niemann-Pick C1-like 1 (NPC1L1) have been identified as attractive therapeutic targets for obesity and hypercholesteremia, respectively. Obesity and hypercholesteremia usually co-exist, however no dual-inhibitors against PTL and NPC1L1 were reported for the treatment of obesity patients with hypercholesteremia so far. In this work, molecular hybridization-based one-step modification screening identified a potent dual-inhibitor against PTL and NPC1L1. Compound P1-11 has IC_{50} values of 2.1 μM against PTL through covalent binding, as well as significantly reduces cholesterol absorption in a non-competitive inhibitory manner. Molecule docking and molecular dynamics studies revealed the reason of its activity to both PTL and NPC1L1. Moreover, the gene and protein expression levels of PTL and NPC1L1 were also determined respectively after the treatment of P1-11. Development of dual-inhibitors against PTL and NPC1L1 could provide novel treatment options for obesity patients with hypercholesteremia. The results of current research would great support the development of dual-inhibitors against PTL and NPC1L1.

1. Introduction

Obesity and hypercholesteremia have been now regarded as major health issues worldwide. Additionally, they are recognized to be important risk factors for many diseases including type II diabetes, hypertension and even cancer [1–3]. Further, obesity and hypercholesteremia are usually combined in some diseases of cardiovascular system and endocrine system [4–6]. Therefore, prevention and treatment of obesity and hypercholesteremia are also the key to reducing the prevalence and mortality of chronic metabolic diseases. Excessive intake of fat and cholesterol is the major cause of these diseases. Decreasing absorption of dietary fat and cholesterol is an effective way to reduce obesity and hypercholesteremia. Among them, lifestyle modification is the preferred. However, medication also offers an ideal option for patients who are reluctant to make lifestyle changes.

There are two main types of anti-obesity drugs on the market, appetite inhibitors that act on central nervous system and PTL inhibitors [7]. The application of appetite inhibitors is restricted due to their side effects for nervous system. PTL is considered as the safest target for diet-induced anti-obesity drug development [8]. PTL could hydrolyze triglycerides into diglycerides that are further hydrolyzed into monoglycerides and fatty acids, which are then absorbed by small intestinal epithelial cells. It is responsible for the hydrolysis and absorption of approximately 50% of total dietary fats in the intestinal lumen [9]. Thus, PTL inhibition could be an effective strategy for anti-obesity. A number of PTL inhibitors, including synthetic and natural small molecules, have been discovered and structure-based design (Fig. 1). For example, Carnosol, Broussonone A, Vibralactone and Echitamine were derived from natural products and have also been proved to be active against PTL in micromolar rang [10–13]. Based on the analysis of published literatures, natural products with lactone ring structure or phenolic hydroxyl group might exhibit moderate PTL inhibitory activity. Some PTL inhibitors were artificially designed to mimic lactone ring structure that could covalently interact with Ser152 of PTL. Compound 1, containing a diamide bond designed to mimic the lactone ring structure, exhibited potent inhibit activity against PTL with $IC_{50} = 4.8$ μM [14].

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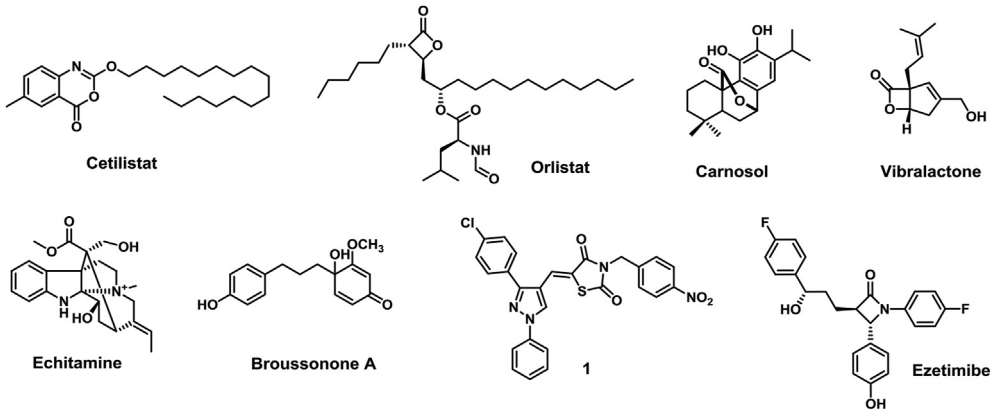

Fig. 1. Structures of inhibitors against PTL or NPC1L1.

Additionally, some PTL inhibitors showed significantly inhibit activity for triglyceride elevation in mice model. Moreover, PTL inhibitors, orlistat and cetilistat, have been approved for the treatment of obesity in 1998 and 2003 respectively [15,16].

Medications used for hypercholesterolemia include: cholesterol biosynthetic inhibitors (statins), cholesterol absorption inhibitors, proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, and bile acid chelating agents. The combination of cholesterol biosynthetic inhibitors and absorption inhibitors is the first choice for the treatment of hypercholesterolemia. Targets for cholesterol absorption inhibitors (*e.g.*, ezetimibe) have been identified as Niemann-Pick C1-like 1 (NPC1L1) that is specific transporters for cholesterol [17]. NPC1L1 has attracted more and more attention due to its own characteristics. It is abundantly expressed in the small intestine of humans, and responsible for transporting cholesterol to across the apical membrane of enterocytes [18]. In genetically NPC1L1-deficient mice, a 70% reduction in intestinal cholesterol absorption was seen, which indicated that NPC1L1 plays an essential role in promoting intestinal cholesterol uptake [19]. Reducing absorption of cholesterol by inhibiting NPC1L1 also has the added advantage that there was no effect on the absorption of fat-soluble nutrients such as fat-soluble vitamins, triglycerides, or bile acids [20]. Thus, it has been identified as an attractive therapeutic target to lower cholesterol blood levels. There are few reports of NPC1L1 inhibitors at present. The most classic NPC1L1 inhibitor is ezetimibe, which has gained approval from Food and Drug Administration in 2002 for the treatment of dyslipidemia (*e.g.* hypercholesteremia) [21,22]. As a potent cholesterol absorption inhibitor, it could effectively prevent the absorption of cholesterol, thus lower circulating plasma cholesterol in humans by 15−20% [23].

It is important to note that obese patients often exhibit high levels of plasma cholesterol. In other word, hypercholesteremia and obesity are often co-exist. However, as far as we know, there are no drugs designed to treat both hypercholesteremia and obesity at the same time. Although some studies have demonstrated that the anti-obesity drug orlistat also showed effect of lowering cholesterol level when compared to patients receiving placebo, orlistat is still used as an anti-obesity drug until now, not used for the treatment of cholesterol-lowering like statins (inhibitors of cholesterol synthesis) [24−26]. As important therapeutic targets for obesity and hypercholesteremia, PTL and NPC1L1 are of great significance for the design and development of new therapeutic drugs. Active compounds that target both PTL and NPC1L1 have the potential to treat obesity and hypercholesteremia simultaneously. Thus, dual-target inhibitors for both PTL and NPC1L1 would provide new options for obese patients with hypercholesterolemia. However, as far as we know, there were no dual-target inhibitors for PTL and NPC1L1 up to today. In the present study, dual-target inhibitors for PTL and NPC1L1 were designed, and dual-target effect were demonstrated in enzyme and cell level.

2. Design and synthesis of dual-inhibitors

The design strategy of dual-inhibitors is based on the structure alignment of classic drugs and computer molecular docking simulation (Fig. 2). Orlistat and ezetimibe were selected as the starting molecules for the dual-target inhibitor design based on the following three reasons: (1) their structural commonality (tetra-atomic ring); (2) the reduction of orlistat on cholesterol levels in obese patients has been demonstrated previously [27], as well as ezetimibe was shown to reduce the level of triglycerides in patients with hyperlipidemia [28]; (3) proven clinical effectiveness [16,22]. In addition, it was worth noting that Saeed Alqahtani et al.'s study has proved that orlistat could decrease dietary cholesterol absorption, which was achieved in part by inhibition of NPC1L1 [29]. Their study demonstrated that it was feasible to design NPC1L1 inhibitors based on orlistat. Considering the catalytic serine could reach the solvent and react with substrate only when the surface loop is open (Fig. S1), the crystal structure of PTL (PDB: 1LPB) with open conformation was used to simulate the interaction with inhibitors in computer molecular docking and molecular dynamics (MD) studies [30]. Additionally, crystal structure of NPC1L1 in complex with an ezetimibe from Ching-Shin Huang et al.'s study (PDB: 6V3H), revealed mechanisms of cholesterol transport and ezetimibe inhibition, and therefore was utilized in our present work for docking and MD studies [31].

The design process is following: (1) Orlistat is the typical representative of PTL inhibitors, which contains β-lactone that could form a covalent bond with the active serine residue site (Ser152) of PTL, and thus leading to PTL inactivate. Interestingly, ezetimibe also contains a quaternary cycloamide structure similar to orlistat. The β-lactone has higher reactivity with the hydroxyl of serine compared to the lactam structure, so it was used as a starting component of the dual-target inhibitors. (2) Two alkyl chains of orlistat fit into a hydrophobic groove of PTL and are thought to thus mimic the interaction between the leaving fatty acid of a triglyceride substrate and the PTL [30]. Alkyl chains (C-6, C-13) are also considered as the key groups to achieve PTL inhibition. Thus, two alkyl chains were also retained in designed dual-inhibitors. (3) For ezetimibe, the most significant feature is the presence of three substituted benzene rings, except for the lactam ring. In contrast, orlistat has three distinct alkyl chains, beside β-lactone ring. Therefore, we hypothesized that the modification with substituted

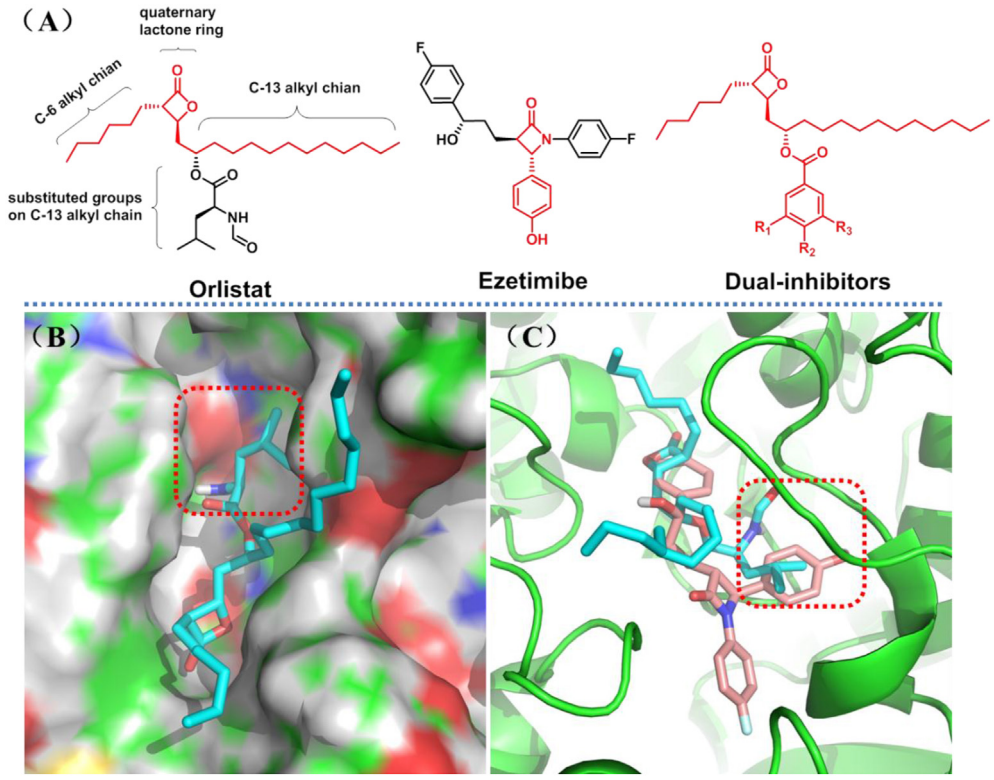

Fig. 2. Design strategy of dual-inhibitors. (A) Structures of Orlistat, Ezetimibe and dual-inhibitors. (B) Binding model of orlistat with PTL (PDB: 1LPB). PTL is shown by surface based on the color of atoms. The substituent on the C-13 alkyl chain is marked in the red box. (C) Orlistat and ezetimibe are docked into the pocket of NPC1L1 (PDB: 6V3H). The substituent on the C-13 alkyl chain of orlistat and the 3-hydroxyphenol of ezetimibe are marked in the red box.

benzene ring on existing lactone ring and alkyl chain may increase inhibitory activity against NPC1L1. Firstly, we examined the interaction of the substituted groups on orlistat C-13 chain with PTL through molecular docking (Fig. 2B red box, Fig. S2A). The result showed that the substituents on the C-13 chain are located on one side of the PTL binding pocket, and more importantly, larger substituents might be accommodated at this position (Fig. 2B red box). Second, the molecular docking between inhibitors (orlistat and ezetimibe) and NPC1L1 (PDB: 6V3H) indicated that the substituted groups on the C-13 chain were located at the same binding site with the *p*-hydroxyphenyl group on ezetimibe (Fig. 2C red box). These results provided theoretical support for benzene ring substitution on the C-13 chain. Finally, based on the above analysis, novel dual-inhibitors were designed, which consists three parts: β-lactone, two alkyl chains and a substituted benzene ring (Fig. 2A, right).

Dual-inhibitors designed in present work were list in Fig. 3. Compounds P1-10, P1-11, P1-15 and P1-16 contained phenolic hydroxyl group, which was used to mimic phenolic hydroxyl group of ezetimibe. The effect of quantity and location of hydroxyl groups was also taken into account. In addition, compounds P1-8, P1-12, P1-13 and P1-14 contained different numbers of methoxy substitutions on the benzene ring were also designed. Compound P1-17 and P1-18 were designed to verify whether the presence of substituted benzene ring affects the covalent binding of lactone ring to the active site of PTL. The synthetic method of intermediates (substituted benzoic acid) referred to the study of Somin Park et al., and the synthetic route of targeted compounds followed the work of Giorgio Ortar et al. [32,33] They were showed in Schemes S1–S2.

The labeling principle of covalent binding was shown in Fig. 4A, with compound P1-17 as an example. Compound P1-17 (and P1-18) contained an acetylene group, which could click with a fluorescent molecule containing azide groups (*e.g.*, TAMRA-PEG3-N_3). Therefore, PTL could be fluorescently labeled through click chemistry only when inhibitors covalently bind to PTL [34]. The labeled PTL could be imaged through SDS-PAGE/in-gel fluorescence scanning in indicated wavelength (FITC channel). The control group did not add inhibitors before the click chemistry (Fig. 4B, line 6), or was directly imaged using unprocessed PTL through SDS-PAGE/in-gel fluorescence scanning (Fig. 4B, line 1). The result from Fig. 4B indicated that P1-17 and P1-18 could covalent bind to PTL. However, P1-17 showed higher binding efficiency than that of P1-18, which may be due to steric hindrance, since P1-18 contained larger substituent group that may affect the binding of P1-18 to PTL. More importantly, the results suggested that the substitution of benzene ring on C-13 alkyl chain did not change the binding mode of target compounds and PTL, but the covalent binding efficiency was obviously affected by the size of substituent.

3. Activity and mechanism evaluation

After the biocompatibility of the target compounds were proved, their inhibition activity against PTL was firstly evaluated using colorimetric assay (Fig. S4). The inhibitory rate was measured at concentration of 50 μM, and the compounds with good inhibition rate (>50% at 50 μM) were selected for IC_{50} assay. The results of the enzymatic assay were shown in Table 1. Compounds (P1-13, P1-14, P1-15, P1-16 and P1-18), which contain 2 or 3 substituent groups on benzene ring, did not exhibit PTL inhibitory activity with the inhibition rate < 50% at 50 μM. The targeted compounds (P1-8, P1-11, P1-12 and P1-17) with one substituent group on benzene ring were potent inhibitors of PTL with IC_{50} values 2.1–5.9 μM. This result was consistent with that from Fig. 4, that large substituent groups affect the interaction between inhibitors and PTL, thus affecting activity of inhibitors. Interestingly, compound P1-10 with

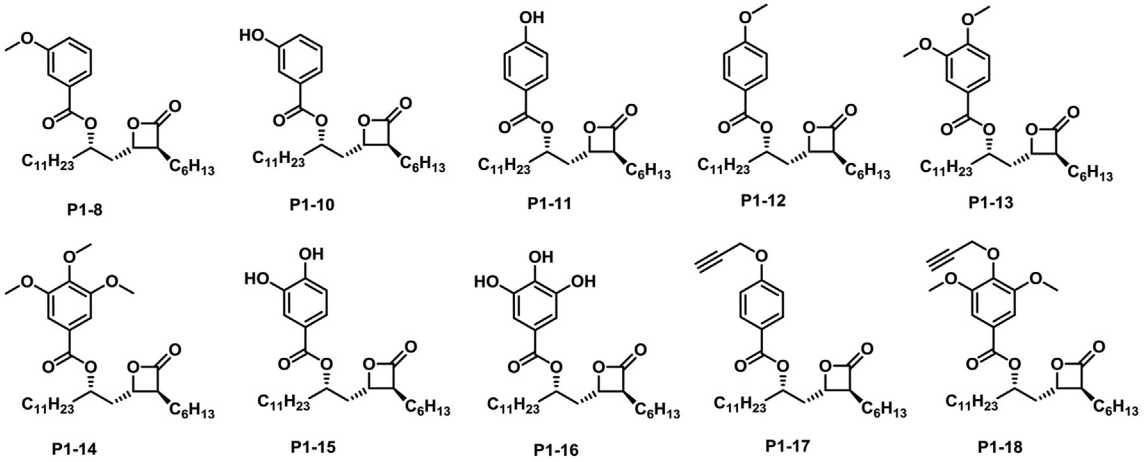

Fig. 3. Structures of novel dual-inhibitors against PTL and NPC1L1.

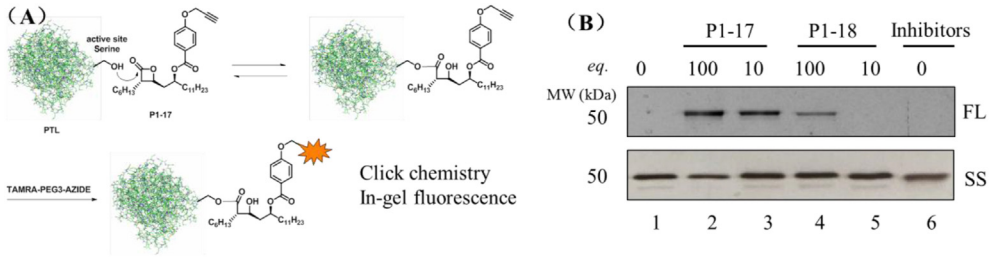

Fig. 4. The labeling of PTL in the presence of inhibitors through covalent binding. (A) Principle of covalent binding, with P1-17 as an example. (B) PTL was labeled by P1-17 prior to being clicked with TAMRA-PEG3-N_3, following SDS-PAGE/in-gel fluorescent scanning (FL, indicating only labeled PTL) and silver staining (SS, indicating total PTL).

Table 1
Inhibition activity assay of targeted compounds against PTL (purified protein), together with the positive control orlistat and ezetimibe.

compd	Inhibition (%) (at 50 μM)	IC_{50} (μM)	compd	Inhibition (%) (at 50 μM)	IC_{50} (μM)
P1-8	73.6 ± 3.8	4.5 ± 1.7	P1-15	48.1 ± 4.5	–
P1-10	24.6 ± 5.7	–	P1-16	35.6 ± 2.6	–
P1-11	69.3 ± 2.6	2.1 ± 0.5	P1-17	71.4 ± 5.4	4.1 ± 2.7
P1-12	78.2 ± 4.1	5.9 ± 1.2	P1-18	28.3 ± 2.5	–
P1-13	41.2 ± 1.2	–	Orlistat	89.5 ± 4.2	0.78 ± 0.8
P1-14	32.4 ± 3.3	–	Ezetimibe	23.5 ± 4.9	–

only one substituent on benzene ring did not show inhibitory activity against PTL at 50 μM with the inhibition rate <50%. On the contrary, the counterpart P1-8 with methoxy substitution showed ideal activity against PTL with IC_{50} = 4.5 μM. After further verification of the structure and activity testing process of compound P1-8 and P1-10, it was difficult for us to give a completely convincing explanation. However, molecular docking given some tips that the 3-hydroxybenzene ring of compound P1-10 was located in the active site of the PTL, while the lactone ring, which was supposed to bind to Ser152, was pushed outside of the binding pocket (Fig. S6). In other words, the presence of 3-hydroxyl group on the benzene ring may interfere with the covalent binding of the compound P1-10 to the PTL. In contrast, the 3-methoxy group of compound P1-8 was located outside of the binding pocket due to hydrogen bond interaction being not involved, as well as lactone ring was closer to the active site of PTL (Fig. S7).

As a transporter protein, NPC1L1 has no catalytic activity, so it is impossible to evaluate inhibitory activity of targeted compounds against NPC1L1 by colorimetric assay. To investigate the active of targeted compounds for NPC1L1, the surface plasmon resonance (SPR) was used to analyse their binding to NPC1L1. SPR could characterize the interaction between target proteins and drugs, and has been exploited as a powerful tool for drug discovery. The response values and SPR traces were presented in Fig. 5. The response of ezetimibe to NPC1L1 was normalized as 1.0. Although no binding was observed in most compounds, compound P1-10 and P1-11 containing one hydroxyl group on benzene ring showed potent binding to NPC1L1. Additionally, orlistat also exhibited moderate binding to NPC1L1, which was consistent with previous study [29]. The molecular docking results showed that the substituted benzene ring of compound P1-10 and P1-11 and the *p*-hydroxybenzene ring of ezeimibe were located in the same position of the NPC1L1 binding cavity (Fig. S8, Fig. S10B). It was worth noting that the response value of P1-11 to NPC1L1 was about 4-folds of that of orlistat, suggested that the activity of P1-11 to NPC1L1 was significantly superior to orlistat. This was consistent with the binding pattern that we assumed in molecular design, which further confirmed the feasibility of design strategy in present study. In addition, the response values and SPR traces of inhibitors for PTL were displayed in Fig. S5. The result was consistent with the activity

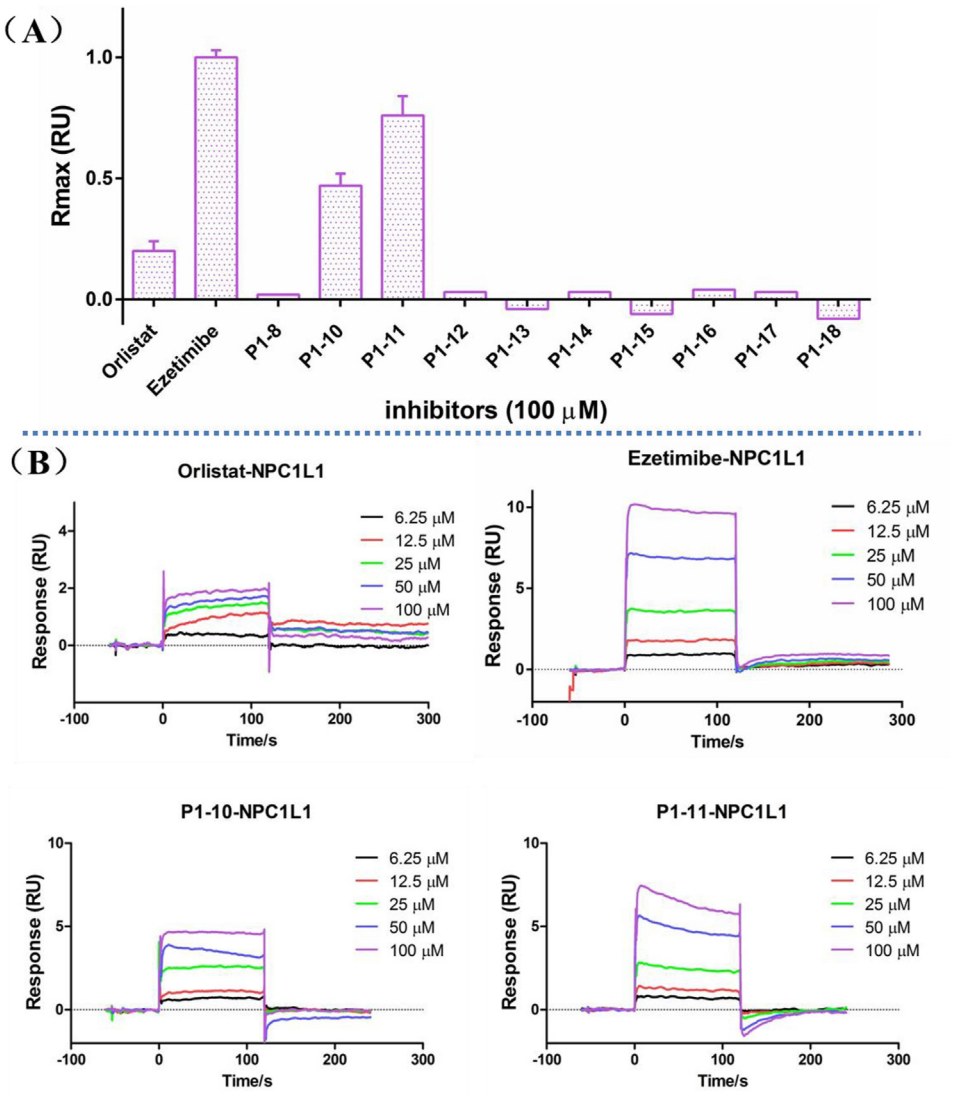

Fig. 5. (A) Interaction assay of inhibitors with NPC1L1 by SPR. Data is presented as relative maximum binding in 100 μM. The binding of ezetimibe to NPC1L1 were normalized as 1.0. (B) Binding sensorgram for inhibitors (orlistat, ezetimibe, P1-10, P1-11) interaction with immobilized NPC1L1. The concentrations of inhibitors were 6.25, 12.5, 25, 50, and 100 μM.

data determined by colorimetry except for compound P1-10. Combining all above results showed that compound P1-11 had the highest inhibitory activity against PTL and NPC1L1 among all targeted compounds, thus it was used for further evaluation.

After testing the inhibitory activity of the target compounds against PTL and NPC1L1 at protein level, their activity against SW1990 and Caco2 cells was also determined respectively. The result of Western blotting showed that PTL was highly expressed in Caco2 and SW1990 cells, while NPC1L1 was only highly expressed in Caco2 cells (Fig. 6A). Thus, SW1990 cells were used to evaluate the inhibitory activity of compounds against PTL, as well as Caco2 was used to evaluate activity against NPC1L1 at the cellular level. Cellular PTL activity assay revealed that SW1990 lysate (total protein 100 μg/mL) showed higher activity than 600 U μg^{-1} (Fig. 6B). Compound P1-11 could significantly inhibit PTL activity of SW1990 lysate at 100 μM. In addition, compounds (P1-8, P1-12 and P1-17), which showed inhibitory activity against pure PTL, also showed moderate activity against SW1990 cell lysate. The inhibition rate of these compounds including positive control orlistat on SW1990 lysate was less than 50% at 50 μM, thus further IC_{50} evaluation was not performed. HPLC-MS was used to determine the uptake rate of cholesterol-d6 in Caco2 cells in presence of inhibitors. Regression curve of the measured abundance versus the concentration of cholesterol-d6 was given in Fig. S12. Chromatograms were shown in Fig. S13. The result suggested that P1-11 exhibited obvious inhibitory effect for uptake of cholesterol, which was equivalent to that of ezetimibe but was better than that of orlistat (Fig. 6C). Considering that cholesterol-d6 could also into cell through free diffusion except for transporting through NPC1L1, over 30% reduction of cell uptake was considered a good effect. Furthermore, kinetic study on the inhibition of cellular uptake of cholesterol-d6 by P1-11 was examined through Lineweaver–Burk double reciprocal plot. The result confirmed that P1-11 was a non-competitive inhibitor showed similar inhibition mode with ezetimibe reported in previous study (Fig. 6D) [29]. Molecular docking also demonstrated that P1-11 and ezetimibe block cholesterol transport by occluding the tunnel instead of competing with cholesterol binding site (Fig. S10, Fig. S11) [31].

Molecular docking simulation was performed to explain inhibition activity of P1-11 against PTL and NPC1L1. The results of

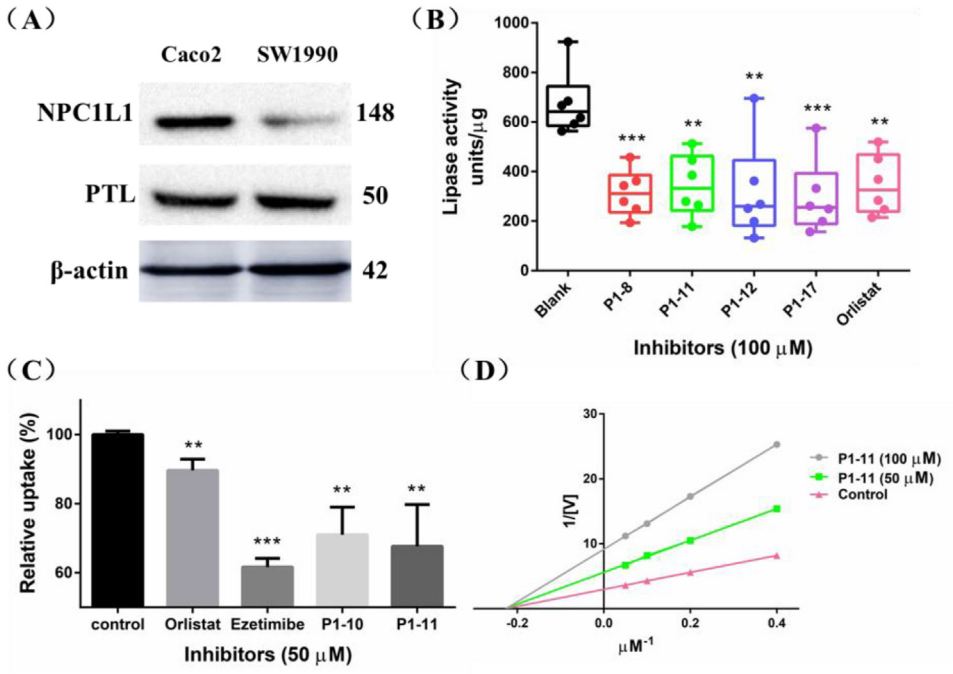

Fig. 6. (A) Expression of PTL and NPC1L1 in Caco2 and SW1990 cells. (B) Lipase activity assay of SW1990 cell lysates (n = 6) in presence of target compounds, together with the positive control orlistat (100 μM). (C) Assay the affection of inhibitors (50 μM) on cellular uptake of cholesterol-d6 through HPLC-MS. Each value represents the mean ± S.D. (n = 3 independent experiments). (D) Lineweaver–Burk plot in the presence of inhibitors at different concentrations. (**P < 0.01, ***P < 0.001 compared to control).

docking were displayed in Fig. 7. Since the covalent binding mode of P1-11 to PTL has been confirmed in Fig. 4, here we utilized the non-covalent docking mode to observe the possible binding driving forces. Fig. 7A showed that Phe77 and Leu153 may be advantageous for lactone ring of P1-11 to be close to Ser152 in active site by hydrogen interaction, which could promote the covalent binding reaction between the hydroxyl group of Ser152 and lactone ring of P1-11. The substituted benzene ring group on C-13 alkyl chain was located in one side of the binding pocket, and that validated what we had anticipated in molecular design (Fig. S9A). The super-imposed images of P1-11 and orlistat in binding cavity of PTL also clearly indicated that their lactone rings were close to Ser152 (Fig. S9B). Furthermore, the docking simulation of P1-11 and NPC1L1 showed that hydroxyl on benzene ring formed hydrogen bond with amino acid residues Gly1028, which was consist with that of ezetimibe (Fig. 7B, Fig. S3 and Fig. S10). All molecular docking results, combining Fig. 4 and reports on orlistat action model [35], suggested that the binding model of P1-11 and PTL (or and NPC1L1) was similar to that of orlistat and ezetimibe, respectively.

MD studies could be better simulated the dynamic process of ligand-protein binding, which take into account the influence of water molecules and ions et al. The result suggested that hydrogen bonds between receptors (PTL, NPC1L1) and P1-11 were always present throughout the MD simulation. The stability of these hydrogen bonds was evaluated by calculating the evolution of distances between H-bond interacting atoms in 50 ns MD simulations (Fig. 8A–C). The distances of Phe77 and P1-11 have a narrow

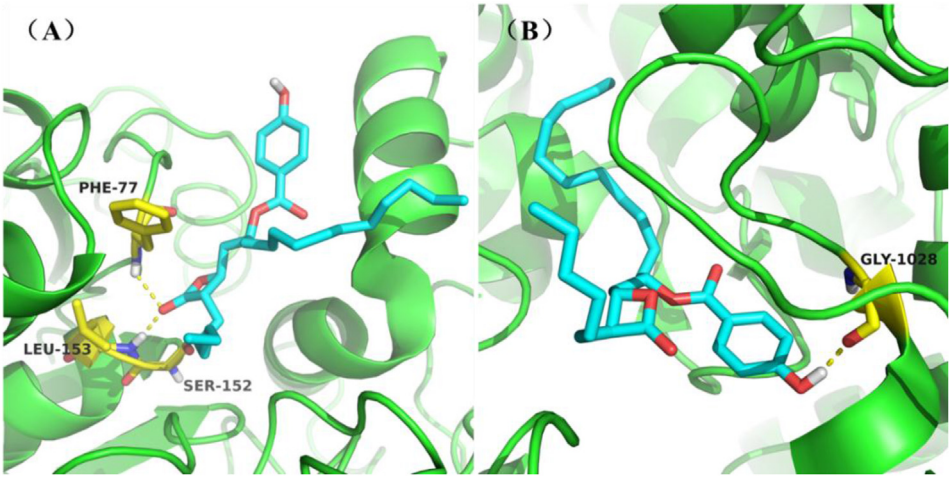

Fig. 7. Binding modes of the P1-11 to PTL (PDB: 1LPB) (A) and NPC1L1 (PDB: 6V3H) (B). The inhibitor P1-11 was shown with color by element (carbon in cyan). The H-bonds were displayed using yellow dashed lines. The key amino acid residues were shown with color by element (carbon in yellow).

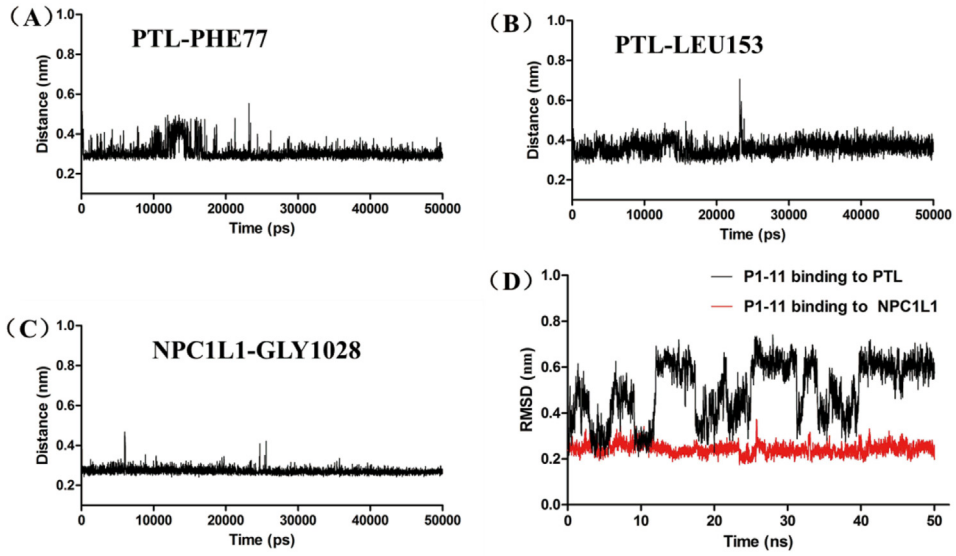

Fig. 8. Evolution of the distance between the pairwising interacting atoms that can form H-bonds from P1-11 and residues of PTL (PDB: 1LPB) (A, B) or NPC1L1 (PDB: 6V3H) (C). (D) RMSD of P1-11 binding to PTL (1LPB, in black) and NPC1L1 (6V3H, in red) in MD simulation.

range fluctuated at the beginning of the MD, afterwards, the distances reached equalization and maintained constant in the last 30 ns. Additional, the distance between Leu153 and P1-11 in complex PTL/P1-11, as well as between Gly1028 and P1-11 in complex NPC1L1/P1-11 were almost no fluctuation. It meant that these hydrogen bonds were stable. Furthermore, the root-mean-squared deviation (RMSD) is widely used to assess the distance between two aligned objects. The RMSD value of P1-11 in process of MD simulation was shown in Fig. 8D. After 40 ns simulation, the conformation of P1-11 bound to PTL began to remain stable, while that of P1-11 bound to NPC1L1 always remained stable in total process. In addition, the change of protein conformation reflects stability of complex in MD simulation. The crucial loop of PTL (PDB: 1LPB) has always been in open position before and after dynamic simulation, which was good for ligand binding (Fig. 9A). There was also no significant difference in the superposed images consisting of NPC1L1 in before and after dynamics simulation, indicated that the conformation of NPC1L1 was relatively stable within 50 ns (Fig. 9B). The backbone RMSD value of PTL and NPC1L1 was always in 0.2–0.4, which also demonstrated the stability of protein conformation during dynamic simulation (Fig. 9C). All the above observations (and Fig. 4) suggested that the interaction between P1-11 and PTL (or and NPC1L1) was potent and stable. That may be the reason, at least in part, why the compound P1-11 displayed significant activity against PTL and NPC1L1.

The inhibitory activity of P1-11 against PTL and NPC1L1 has been demonstrated using above several methods. Then whether P1-11 could affect the protein expression of PTL and NPC1L1? Before measuring the expression levels of PTL and NPC1L1, the gene expression of PTL and NPC1L1 was determined through qRT-PCR (Fig. 10). Similar to orlistat, P1-11 also reduced the expression of PTL gene in Caco2 cells. Meanwhile, ezetimibe also showed the similar effect. Furthermore, neither P1-11 nor ezetimibe affected the expression of NPC1L1 gene. Afterwards, Western blot was performed to test the amount of PTL and NPC1L1 in Caco2 cells (Fig. 11A). Meanwhile protein band intensities were quantified by densitometric analysis (Fig. 11B–D). Western blot analysis results showed that the protein expression of PTL was decreased after treatment with P1-11 at 100 μM in Caco2 cells. Orlistat and ezetimibe also could reduce the protein expression of PTL. The results were consistent with the level of gene expression of PTL. Moreover, the protein expression of NPC1L1 was not altered by orlistat at tested concentrations (1, 10 and 100 μM). This result was also consistent with the study from Saeed Alqahtani et al. [29] It was noted that both P1-11 and ezetimibe increased the protein expression of NPC1L1 after 24 h of treatment. Feedback regulation

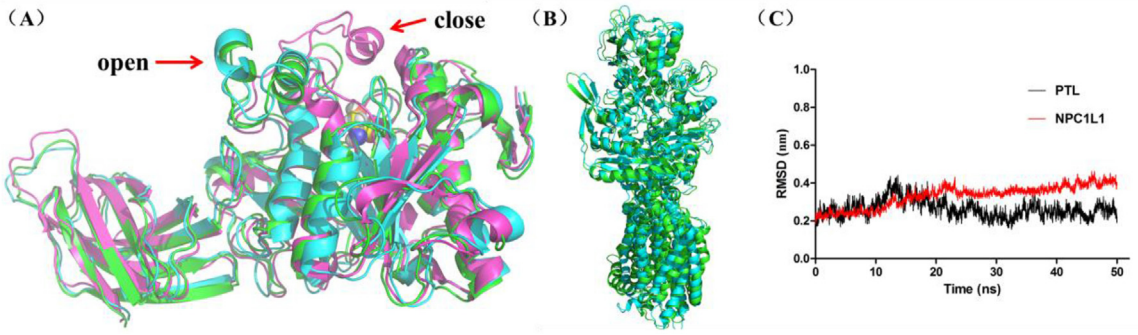

Fig. 9. (A) PTL (PDB: 1LPB, in green) with open conformation and PTL (PDB: 1N8S, in magenta) with closed conformation were aligned to PTL itself (PDB: 1LPB) after dynamic simulation (in cyan). (B) NPC1L1 (PDB: 6V3H, in green) was aligned with itself after dynamic simulation (in cyan). (C) RMSD of PTL (PDB: 1LPB, in black) and NPC1L1 (PDB: 6V3H, in red) in MD simulation.

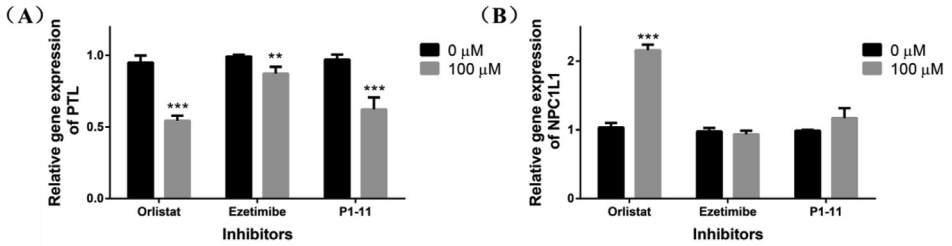

Fig. 10. The relative expression levels of PTL gene (A) and NPC1L1 gene (B) after treatment with inhibitors (100 μM) for 24 h in Caco2 cells. **P < 0.01, ***P < 0.001 compared to control.

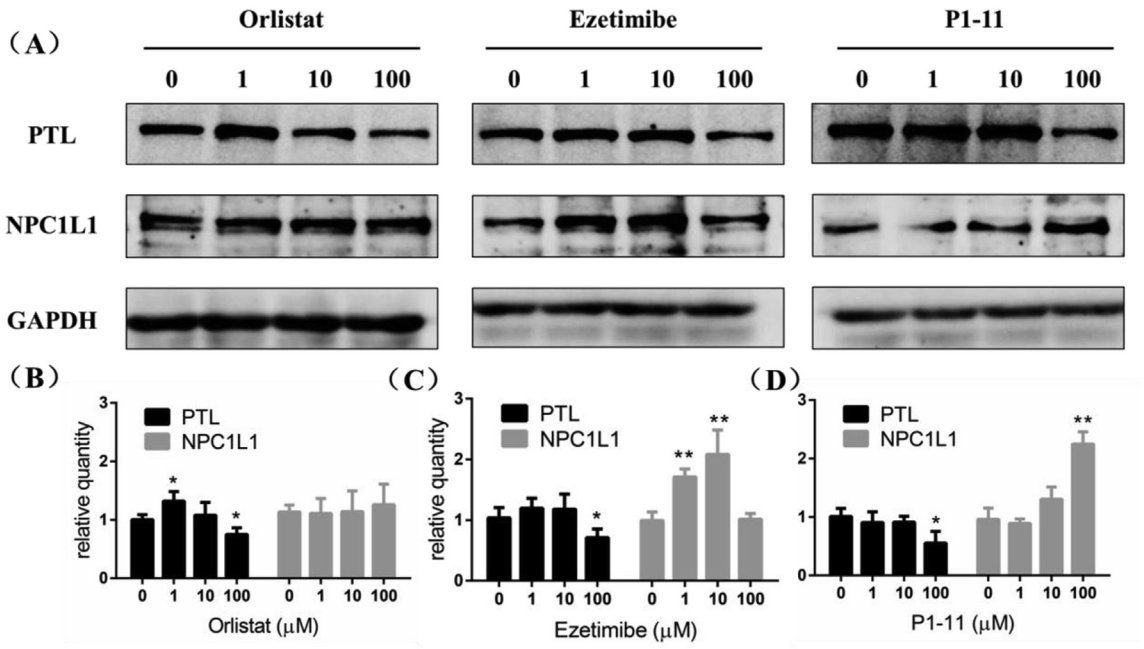

Fig. 11. The effect of inhibitors on the protein expression of PTL and NPC1L1 in Caco2 cell. (A) Western blot analysis of cellular PTL and NPC1L1 after treatment with inhibitors at 0, 1, 10 and 100 μM for 24 h. Densitometry analyses of the blots showed changes in the expression of PTL and NPC1L1 following orlistat (B), ezetimibe (C) and P1-11 (D) treatment when compared to control. (*P < 0.05, **P < 0.01).

may be a plausible explanation. The addition of NPC1L1 inhibitors leads to a reduction in cellular cholesterol uptake. As a protective mechanism, cells may increase the expression of NPC1L1 in order to absorb more cholesterol. From another perspective, the effect of P1-11 on NPC1L1 protein expression was similar to that of ezetimibe, which could indirectly reflect the potential of P1-11 for the treatment of hypercholesteremia.

4. Conclusion

In summary, the key functional group grafting and computer simulation analysis led to the discovery of dual-inhibitors targeting PTL and NPC1L1. Inhibitor P1-11, which incorporated *p*-hydroxyphenyl group to the C-13 alkyl chain, emerged as the most potent dual-inhibitor in all targeted compounds with IC_{50} values of 2.1 μM against PTL, as well as with the excellent inhibition against NPC1L1 which was comparable to that of ezetimibe. SDS-PAGE/in-gel fluorescence scanning indicated that P1-11 could inhibit PTL through covalently binding. Meanwhile, Lineweaver-Burk double reciprocal plot suggested that P1-11 was a non-competitive NPC1L1 inhibitor. Molecular docking and MD studies were used as an auxiliary means to demonstrate the interaction between P1-11 and PTL (or and NPC1L1). QRT-PCR and Western blot indicated that the inhibition of P1-11 on PTL was similar to that of orlistat, while the inhibition of P1-11 on NPC1L1 was similar to that of ezetimibe, in gene and protein expression level. Further studies on these compounds in cellular activity, cell permeability and selectivity would be necessary to provide PTL/NPC1L1 dual-inhibitors suitable for in vivo proof of animal studies in obesity accompanied hypercholesteremia.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113358.

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