Novel Liver-targeted conjugates of Glycogen Phosphorylase Inhibitor PSN-357 for the Treatment of Diabetes: Design, Synthesis, Pharmacokinetic and Pharmacological Evaluations

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PSN-357, an effective glycogen phosphorylase (GP) inhibitor for the treatment for type 2 diabetics, is hampered in its clinical use by the poor selectivity between the GP isoforms in liver and in skeletal muscle. In this study, by the introduction of cholic acid, 9 novel potent and liver-targeted conjugates of PSN-357 were obtained. Among these conjugates, conjugate 6 exhibited slight GP inhibitory activity (IC50 = 31.17 μM), good cellular efficacy (IC50 = 13.39 μM) and suitable stability under various conditions. The distribution and pharmacokinetic studies revealed that conjugate 6 could redistribute from plasma to liver resulting in a considerable higher exposure of PSN-357 metabolizing from 6 in liver (AUC_liver/AUC_plasma ratio was 18.74) vs that of PSN-357 (AUC_liver/AUC_plasma ratio was 10.06). In the in vivo animal study of hypoglycemia under the same dose of 50 mg/kg, conjugate 6 exhibited a small but significant hypoglycemic effects in longer-acting manners, that the hypoglycemic effects of 6 is somewhat weaker than PSN-357 from administration up to 6 h, and then became higher than PSN-357 for the rest time of the test. Those results indicate that the liver-targeted glycogen phosphorylase inhibitor may hold utility in the treatment of type 2 diabetes.

Type 2 diabetes mellitus (T2DM) continue to expand at epidemic rates and new medicines targeting novel mechanisms are urgently needed. Glycogen phosphorylase (GP) is the key enzyme that catalyzes glycogenolysis, leading to the release of glucose from glycogen. Three isoforms of GP have been identified that located within different metabolically active tissues for different physiological functions. The muscle isoform provides energy for muscle contraction, the brain isoform provides an emergency supply of glucose during periods of anoxia or severe hypoglycemia, and the liver isoform regulates glucose release from hepatic glycogen stores. In addition, it has been demonstrated that the inhibition of GP is involved in the promoting of glycogen synthesis in liver. Thus, GP, especially the isoform in liver has received great recent interest as potential target for T2DM. Although liver GP inhibition is regarded as an excellent therapeutic target for the treatment of diabetes, one very important factor relating to the relevance and importance of isoform specificity with this new therapeutic remains to be proven. As previously stated, brain, liver and skeletal muscle isoforms demonstrate 80% homology in their structures, thus finding 100% specific inhibitors of the liver isoforms has proved difficult. Therefore, drug development programmes must consider the potential side effects of such compounds in relevant models. For example, inhibition of skeletal muscle GP when the liver isoform is the primary target could have devastating effects on maintaining...

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uptake systems in the liver and the ileum. All the facts definitely indicate that cholic acid-drug conjugates revealed that hybrid molecules formed by covalent linkage of a drug to cholic acid are recognized by the bile acid

mediate

41

40

with TFA furnished intermediate, which was directly reacted with cholic acid to give the inter-

group from

40

. Removal of the protected

PSN-357

of the resulting amine with FmocCl produced dipeptide intermediate

39

over Pd/C in MeOH furnished

37

, followed by protection

in 79% yield. Hydrogenolysis of

37

and DIPEA furnished

PSN-357

with the carboxylic acid derivative

13

16

the amine hydrochloride salt

16

. Reaction of

13

with NaOH afforded the carboxylic acid derivative

12

in 98% yield. Deprotection of the N-Boc group from

15

S

hand, for segment coupling, (Fig. 2 18. Commercially available 2-chloro-4-methyl-5-nitropyridine (Fig. 6). Treatment of N6-Cbz-L-lysine methyl ester was prepared from cholic acid and sarcosine methyl ester.

26–29

afforded hydrochloride salts with cholic acid in the presence of HATU and DIPEA. Cleavage of the protecting groups in intermediates

22–25

Boc-Asp(OtBu)-OH) produced intermediates

22–25

PSN-357

in 52% yield. Subsequent deprotection

32

31

with LiOH provided Boc-val-ala-OH

31

in 63% yield. Hydrolyzed of

12

with NaOH afforded the carboxylic acid derivative

13

in excellent yield. On the other hand, for segment coupling, (S)-N-Boc-4-fluorophenylalanine (14) coupled to the 4-hydroxypiperidine in the presence of HATU and DIPEA to give

15

in 98% yield. Deprotection of the N-Boc groups from

15

with HCl gave the amine hydrochloride salt

16

. Reaction of

16

with the carboxylic acid derivative

13

in the presence of HATU and DIPEA furnished

PSN-357

in 69% yield (Fig. 2).

The synthesis of cholate conjugate

1

and

2

are shown in Fig. 3. Reaction of cholic acid (17) with glycine methyl ester provided amide derivative

18

in 63% yield. Hydrolyzed of

18

with LiOH afforded the glycine conjugate

19

in 76% yield. Compound

19

was treated with

PSN-357

in the presence of DCC and DMAP to obtain conjugate

1

. In the same fashion, conjugate

2

was prepared from cholic acid and sarcosine methyl ester.

The synthesis of conjugates

3–6

was carried out starting from

PSN-357

(Fig. 4). Reaction of

PSN-357

with various protected amino acids (N-Boc-L-Ala-OH, N-Boc-L-Val-OH, N-Boc-O-TBS-L-Ser-OH, Boc-Asp(OtBu)-OH) produced intermediates

22–25

. Cleavage of the protecting groups in intermediates

22–25

afforded hydrochloride salts

26–29

. Direct coupling of

26–29

with cholic acid in the presence of HATU and DIPEA gave the expected conjugates

3–6

.

As shown in Fig. 3, the preparation of conjugate

7

via a multistep process by modifying the well-known synthesis method reported in the literature. Coupling of N-Boc-L-Val-OH with L-alanine methyl ester hydrochloride yielded N-protected dipeptide ester

31

. Hydrolyzed of

31

with LiOH provided Boc-val-ala-OH

32

. The dipeptide derivative

32

was treated with

PSN-357

to obtain compound

33

in 52% yield. Subsequent deprotection of compound

33

using TFA gave compound

34

, which was reacted with cholic acid through the action of HATU and Et,N to give the conjugate

7

.

Conjugate

8

was obtained in a similar manner using dipeptide linkage prepared from the protected lysine ester (Fig. 6). Treatment of N6-Chz-L-lysine methyl ester

35

with Boc-L-phenylalanine in the presence of EDCI in DMF at room temperature afforded dipeptide ester

36

. Hydrolyzed of

36

with NaOH in MeOH/H2O gave dipeptide derivative

37

in 79% yield. Hydrogenolysis of

37

over Pd/C in MeOH furnished

38

, followed by protection of the resulting amine with FmocCl produced dipeptide intermediate

39

. Conjugation of

39

with

PSN-357

in the presence of 1-propanephenosphoric acid cyclic anhydride (T3P) gave the compound

40

. Removal of the protected group from

40

with TFA furnished intermediate

41

, which was directly reacted with cholic acid to give the intermediate

42

in a similar reaction pattern as conjugate

7

. Deprotection of the Fmoc group of

42

with piperidine afforded the target conjugate

8

.

The complete synthesis of conjugate

9

containing an aromatic azo-linkage is depicted in Fig. 7. Reduction of

o-nitrocinnamic acid

43

under H2 atmosphere with Pd/C in aqueous NaOH gave the amine intermediate

44

.
It was necessary to perform the reduction under basic condition in order to inhibit cyclization of itself.
Intermediate 44 was oxidized using Oxone to give nitroso acid intermediate 45. The condensation between intermediate 45 and tert-butyl 4-aminobenzoate was performed in glacial acetic acid at room temperature to give the compound 46. Treatment of 46 with PSN-357 using the Steglich reaction afforded compound 47. Deprotection of the tert-butyl group from 47 using TFA gave the intermediate carboxylic acid 48 in high yield. On the other hand, coupling of cholic acid with N-Boc-ethylenediamine in the presence of DEPC and Et3N in DMF gave compound

Figure 1. Chemical Structures of 9 novel cholic acid conjugates of PSN-357.

X =

1: \( \text{N-BOC} \)
2: \( \text{N-BOC} \)
3: \( \text{N-BOC} \)
4: \( \text{N-BOC} \)
5: \( \text{N-BOC} \)
6: \( \text{N-BOC} \)
7: \( \text{N-BOC} \)
8: \( \text{N-BOC} \)
9: \( \text{N-BOC} \)
Deprotection of the Boc group was performed with 6 M methanolic HCl to yield the cholic acid derivative 50. Compound 50 was attached to the corresponding carboxylic acid 48 under amide-bond-forming condition in the presence of HATU to give the conjugate 9.

Enzyme Assay and SAR Analysis. All the compounds were evaluated for their inhibitory activity against rabbit muscle GPα (RMGPa). The activity of RMGPa was measured through detecting the release of phosphate from glucose-1-phosphate in the direction of glycogen synthesis20. CP-91149, a well-known allosteric GP inhibitor, which shares the same binding site with PSN-357, was used as the positive control and the results are summarized in Table 121. The assay results showed that most of the synthesized compounds exhibited moderate to good inhibitory activities against RMGPa. Compared to PSN-357 (IC₅₀ = 0.42 μM), introduction of cholic acid group to PSN-357 resulted in a great loss of activity (1–3, 5–9) or no activity (4). This remarkable loss of activity was probably due to the direct steric interference by such bulky substituent of cholic acid group. Among the compounds, conjugate 1 (IC₅₀ = 5.9 μM) displayed the most efficient inhibition. While the GP inhibition of
the intermediates (e.g. 25–28, 33, 40, 42, 48) seemed much acceptable, indicating that introduction of different substitutions in small size on the hydroxyl moiety of PSN-357 resulted in a slight loss of potency. The results is consistent with the SARs of PSN-357 that the hydroxyl of piperidine is not an essential group for the GP inhibitory activity that could be modified slightly. In general, the L-serine derivative 28 (IC50 = 0.52 μM) was the most active of the series, being approximately 1.25-fold less potent than that of PSN-357.

**Cell Assay and SAR Analysis.** To evaluate the effects of all compounds in cells, the glycogenolysis assays were established in both rat and human liver cells based on the published method. These results are summarized in Table 2. Two of the conjugates revealed excellent inhibitory activity in the cellular assays. Of these, conjugate 9 showed the best activity in isolated rat hepatocytes and HepG2 cells, with IC50 value of 12.3 μM and 6.4 μM, respectively. Likewise, conjugate 6 showed IC50 value of 13.4 μM in isolated rat hepatocytes and 6.1 μM in HepG2 cells, respectively. The inhibitory activities of the derivatives of PSN-357 (e.g. 25–28) were also explored. It is not surprising that the derivatives still retained micromolar inhibitory activities. Introduction of steric bulks to the hydroxyl group (e.g. 33, 40, 42, 48) led to a complete loss of activity. Data analysis indicated no clear SAR for the substituents in the cell-based assays.

**In Vitro Stability studies.** All the conjugates were tested for their chemical and metabolic stability in multiple assays, including simulated gastric fluid (SGF), simulated intestinal fluid (SIF), mouse plasma, and mouse liver microsome (MLM).

For the stability in SGF (Fig. 8) and SIF (Fig. 9), the conjugates could be divided into three groups: (1) stable conjugates 2, 6 and 9. All of them were stable in SGF for 24 h with almost no detectable PSN-357, but in SIF, conjugate 6, being stable up to 24 h, were much more stable than 2 and 9. Still, 2 and 9 were relatively stable than other conjugates that no more than 20% of the two compounds were degraded during the 24 h incubation in SIF; (2) unstable conjugates 1 and 7. The two conjugates were degraded within 6 h and 1 h in SGF and SIF, respectively, with almost no conjugates at the end of the 24 h incubation in both SGF and SIF; (3) complicated conjugates 3, 4, 5 and 8. They were relatively stable in SGF over 24 h of incubation, but degraded rapidly in SIF within 1 h.
As shown in Fig. 10, PSN-357 was relatively stable in mouse plasma within the 120-min test, while the conjugates degraded and declined in a mono-exponential model, except conjugates 4 and 6. Conjugates 4 and 6 exhibited considerable stability in mouse plasma as PSN-357 with approximately 4% and 12% degradation, respectively.
Additionally, the compounds’ stability in microsome were evaluated by measuring the rate of compounds consumption in MLM and the results are shown in Table 3. PSN-357 demonstrated good metabolic stability in MLM with longer half life ($t_{1/2}$ > 145 min) and slower elimination rate ($CL_{int}$ < 9.6 $\mu$L/min/mg protein, $CL$ < 38.0 $\mu$L/min/mg protein). Conjugates 6, 8 and 9 showed considerable metabolic stability with $t_{1/2}$ over a range of 84.5 to 91.2 min and $CL_{int}$ over a range of 15.2 to 16.4 $\mu$L/min/mg protein. Conjugates 1–5, exhibited poor metabolic stability resulting in a short elimination half-life and a high systemic clearance relative to conjugates 6, 8 and 9. In addition, conjugate 7, it metabolized fastest in MLM, with a $t_{1/2}$ of 4.7 min and displayed very high intrinsic hepatic clearance ($CL_{int}$ of 297.4 $\mu$L/min/mg), suggesting the potential for unacceptably high hepatic clearance. Those results indicate that the release of PSN-357 from the conjugates were greatly affecting by the linkers.

Biodistribution and Pharmacokinetic Studies for Compound PSN-357 and Conjugate 6 in Mice. Based on the results of the potency and in vitro stability studies, conjugate 6 was selected for in vivo pharmacokinetic analysis following a single intravenous injection of 5 mg/kg in male C57 BL/6. Doses of PSN-357 intravenous were used as standard regimens for comparison. The results are shown in Table 4 and Fig. 11. In plasma, the concentration of PSN-357 reached the C$_{max}$ of 628 ng/mL at 5 min and then sharply decreased during 30 min after dosing, but it gradually increased to reach a secondary peak at 60 min perhaps due to enterohepatic circulation, and the value of AUC$_{0-t}$ is 965 ng/mL.h. For conjugate 6, the concentration reached the C$_{max}$ of 726 ng/mL at 5 min and then rapidly decreased to 97 ng/mL at 240 min after dosing. In addition, the concentration of major metabolite PSN-357 released from 6 reached the C$_{max}$ of 98 ng/mL at 120 min with the AUC$_{0-t}$ of 691.67 ng/mL.h, about 6 and 1.4-fold lower than that of PSN-357, respectively. On the other hand, in livers, conjugate 6 did not show the

![Figure 5. Synthesis of conjugate 7.](image-url)
highest concentration at 5 min after administration as compared with PSN-357. The concentration of 6 is dramatically increased to reaching a mean $C_{\text{max}}$ of 3094.55 ng/mL at about 15 min after dosing, suggesting the redistribution from plasma or other tissues to livers may have happened, then 6 was eliminated during 30–240 min. For PSN-357 released from conjugate 6, the value of $C_{\text{max}}$ is 1023 ng/mL, about 6-fold lower than that of PSN-357, but the value of $AUC_{0-t}$ is 12960 ng/mL$\cdot$h, about 1.4-fold higher than that of PSN-357 (9711 ng/mL$\cdot$h), further, $(AUC_{\text{liver}}/AUC_{\text{plasma}})$ of PSN-357 metabolizing from 6 is 18.74, about 2-fold higher than that of PSN-357 (10.06). Those results suggested that conjugate 6 exhibited some targeting effect to liver that might enrich and display a longer duration of action in liver in comparison with PSN-357.

**In Vivo Efficacy of Compound PSN-357 and Conjugate 6.** In vivo efficacy of compound PSN-357 and conjugate 6 were studied on leptin-deficient $ob/ob$ mice. Metformin was chosen as a positive control. Figure 12 showed that treatment with PSN-357 (50 mg/kg) could significantly reduce the blood glucose (BG) to a nadir of 6.36 ± 1.46 mg/dl at 1 h min vs 12.80 ± 1.58 in control $ob/ob$ mice ($p < 0.005$), with significant effects also being evident at 2 h ($p < 0.005$) and 3 h ($p < 0.005$) vs controls. A similar but somewhat weaker hypoglycemic effect was observed for the conjugate 6 under the same dosage. Glucose lowering was statistically significant at 1 ($p < 0.01$), 2 ($p < 0.005$), 3 ($p < 0.005$), 6 ($p < 0.005$) and 24 h ($p < 0.05$), with the largest drop (BG level is around 7.79 mmol/L) occurring at 3 h. Especially, there was still significant decrease in BG levels by conjugate 6 up to 6 h.
after administration. The effect might be due to the fact that 6 acts in a sustained release and longer acting manners. It is noteworthy that the findings are highly consistent with the results from in vivo pharmacokinetic studies.

Conclusions
In summary, though a strategy of bile acid conjugation, it has been found possible to prepare liver-selective conjugates of PSN-357. The in vitro biological and stability studies of these conjugates were evaluated to supporting the selection of a conjugate candidate for in vivo pharmacokinetics and pharmacological evaluation. Among the conjugates, conjugate 6 exhibited moderate enzyme inhibitory activity, suitable cellular activity and acceptable stability in various biological fluids. This compound is preferentially distributed into liver and possesses a longer

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**Table 1. RMGPa inhibition assay for conjugates 1–9 and some intermediates.** Each value represents the mean ± S.D. of three determinations. NI means no inhibition. CP-91149 was used as positive control.

| Compound | IC_{50} (μM) | Compound | IC_{50} (μM) |
|----------|-------------|----------|-------------|
| 1        | 5.94 ± 0.74 | 27       | 0.88 ± 0.17 |
| 2        | 26.01 ± 13.9| 28       | 0.52 ± 0.01 |
| 3        | 40.53 ± 15.7| 33       | 27.03 ± 6.39|
| 4        | NI          | 40       | 3.44 ± 0.22 |
| 5        | 81.94 ± 14.4| 42       | 15.42 ± 0.89|
| 6        | 31.17 ± 11.1| 47       | NI          |
| 7        | 6.07 ± 0.27 | 48       | 3.11 ± 0.05 |
| 8        | 10.74 ± 5.6 | 49       | NI          |
| 9        | 51.69 ± 4.1 | 50       | NI          |
| 17       | NI          | PSN-357  | 0.42 ± 0.01 |
| 25       | 0.81 ± 0.077| CP-91149 | 0.09 ± 0.04 |
| 26       | 0.56 ± 0.043|          |             |
duration of action than PSN-357 at the same dose. Moreover, conjugate 6 was able to maintain acceptable antidiabetic effects relative to PSN-357. These results implied that the development of liver-selective conjugates might offer a potential opportunity to overcome the muscles side-effects caused by sequence homology of three GP isoforms.

Materials and General Methods
Chemistry section. (The detailed information is in Supplementary information).

Enzyme Kinetics. The inhibitory activity of the test compounds against rabbit muscle glycogen phosphorylase a (GPa) was monitored using microplate reader (BIO-RAD) based on the published method.

Table 2. Glycogenolysis inhibition assay for conjugates 1–9 and some intermediates in liver cells. Each value represents the mean ± S.D. of three determinations. bNI means no inhibition. cCP-91149 was used as positive control.
In brief, GPa activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate. Each test compound was dissolved in DMSO and diluted at different concentrations for IC50 determination. The enzyme was added into 100 μL of buffer containing 50 mM Hepes (pH = 7.2), 100 mM KCl, 2.5 mM MgCl2, 0.5 mM glucose-1-phosphate, 1 mg/mL glycogen and the test compound in 96-well microplates (Costar). After the addition of 150 μL of 1 M HCl containing 10 mg/mL ammonium molybdate and 0.38 mg/mL malachite green, reactions were run at 22 °C for 25 min, and then the phosphate absorbance was measured at 655 nm. The IC50 values were estimated by fitting the inhibition data to a dose-dependent curve using a logistic derivative equation.

Glycogenolysis Inhibition in Rat Hepatocytes and HepG2 cells. The inhibition of hepatic glycogenolysis was monitored by the measurement of liver glycogen, which was done quantitatively by the anthrone reagent (Sigma) colorimetric method based on the published method20. Isolated rat hepatocytes or HepG2 cells (Sigma) were treated with the test compound or DMSO solvent (final concentration, 0.10%), followed by 60-min incubation with 0.3 nM glucagon (GGN). Assays were terminated by centrifugation, and cells were digested with 30% KOH followed by glycogen determination. The IC50 values were estimated by fitting the inhibition data to a dose-dependent curve using a logistic derivative equation.

Table 3. In vitro T1/2 data and intrinsic clearance values for conjugates 1–9 in mouse liver microsomes (n = 3).

| Compound | Mouse liver microsomes | | | |
|----------|------------------------|--------|--------|--------|
|          | t1/2 (min)              | CLint (μL/min/mg) | CL (mL/min/kg) |
| 1        | 31.9                   | 43.4               | 171.9            |
| 2        | 24.9                   | 55.6               | 220.2            |
| 3        | 27.4                   | 50.6               | 200.0            |
| 4        | 36.5                   | 38.0               | 150.5            |
| 5        | 40.8                   | 34.0               | 134.6            |
| 6        | 84.5                   | 16.4               | 64.9             |
| 7        | 4.7                    | 297.4              | 1177.7           |
| 8        | 85.6                   | 16.2               | 64.2             |
| 9        | 91.2                   | 15.2               | 60.2             |
| PSN-357  | ≥145                   | <9.6               | <38.0            |
| Diclofenac (PC) | 26.3               | 52.6               | 208.3            |

Table 4. Pharmacokinetic parameters in plasma and liver tissue of PSN-357 and conjugate 6 by 5 mg/kg intravenous administration in mice.

| Tissue | PSN-357 | Conjugate 6 | Metabolite PSN-357 of conjugate 6 |
|--------|---------|-------------|---------------------------------|
|        | AUC0-t (ng/mL·h) | Cmax (ng/mL) | MRT0-t (h) | AUC0-t (ng/mL·h) | Cmax (ng/mL) | MRT0-t (h) | AUC0-t (ng/mL·h) | Cmax (ng/mL) | MRT0-t (h) |
| Plasma | 965.16  | 628.35      | 3.18     | 1518.40 | 726.2         | 7.52     | 691.67 | 98.33 | 10.55 |
| Liver  | 9711.94 | 6677.85     | 4.52     | 3981.99 | 3094.55      | 4.42     | 12960.21 | 1023.14 | 9.97 |

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Stability Tests of the Conjugates. Stability in Simulated Gastrointestinal Fluids. The simulated gastrointestinal fluids were prepared according to USP specifications. For SGF, NaCl (0.12 g) and pepsin (0.18 g, from porcine stomach mucosa) was dissolved in HCl (0.42 mL) and sufficient H2O was added to make 60 mL. The pH of the test solution was determined as 1.21 by pH meter. For the SIF, KH2PO4 (0.408 g) and pancreatin (0.6 g, from porcine pancreas) was dissolved in H2O (60 mL). The pH of the test solution was determined as 6.79 by pH meter. Each test compound was dissolved in DMSO and diluted to a final concentration of 2 μM in SIF and SGF. The final concentrations of DMSO and CH3CN in the incubation mixture were 0.2% and 0.45%. Aliquots of these solutions were pipetted into glass tubes, and placed in a 37 °C shaking water bath. Test samples at corresponding time point (1, 2, 6, 24 h) were removed at the end of incubation time and immediately mixed with 800 μL of cold acetonitrile containing 500 ng/mL tolbutamide (internal standard). Samples were subjected to centrifuge at 4000 rpm, 4 °C for 20 min. Aliquots 60 μL of supernatant was combined with 120 μL water for LC-MS/MS analysis.

Stability in Plasma. Frozen plasma from male C57 BL/6 mice was incubated for 5 min at 37 °C before the addition of the test compound. Then prepared 2μM incubation sample, and aliquots of the incubation mixtures (100 μL) were taken at predetermined time points (10, 30, 60, 120 min) at 37 °C. After incubation, the mixtures was added 400 μL of 50% ACN/50% MeOH containing internal standard (IS, 200 ng/mL Tolbutamide and 20 ng/mL Buspirone) to each sample tube and then centrifuged at 13000 rpm for 8 min. Blank incubations in the absence of the test compound were also performed. The analyses of test compounds were performed by LC-MS/MS analysis.

Stability in Liver Microsomes. The microsomal pellet was suspended in potassium phosphate buffer (100 mM, pH 7.4), 10 μM test compound or positive control (diclofenac) was added. Liver microsomes from C57BL/6 mice (BD Gentest) were pooled. The mixture of microsome solution and compound was incubated at 37 °C for about 10 min in the presence of a NADPH-regenerating system, consisting of 0.02 M DL-isocitric acid (trisodium salt), 0.1 mg/mL isocitrate dehydrogenase and 1 mM NADPH. The addition of ice-cold CH3CN (including 100 ng/mL tolbutamide as internal standard) terminated the reaction. The mixture was vortexed for approx (30 s), centrifuged (20 min, 4000 rpm) and the supernatant was collected and analysed by LC-MS/MS method.
Tissue Distribution and Pharmacokinetic Parameters for Compound PSN-357 and Conjugate 6.
The in vivo experiments of compound PSN-357 and conjugate 6 were determined as described below. The animals were housed and cared for in accordance with the guidelines established by the National Science Council of the Republic of China. All experimental protocols were approved by Animal Care and Use Committee of Chengde Medical University, and all experiments were performed in accordance with the approved guidelines.

Male C57 BL/6 mice (18–20 g, 5–6 weeks old) were provided by the Model Animal Research Center of Nanjing University, and housed under standard animal care practices with ad libitum access to food and water throughout the procedures. After 1 week acclimation, blood was collected from the retroorbital sinus for plasma glucose determination, and mice were randomized to groups with similar mean body weight. Blood and liver samples were taken at 0.0833, 0.25, 0.5, 1, 2, 3, 4, 6, and 24 hours following intravenous administration and stored at −80 °C until analysis. Aliquots of all biological matrices were deproteinized with a mixture of methanol/acetonitrile (1:1). The suspension was vortexed, mixed, and centrifuged at 13000 rpm for 10 min. The organic phase was injected into the LC-MS/MS system. PK calculations and statistical comparisons on PK data were performed according to a non-compartmental kinetic model with validated software (Kinetica™ version 5.1, Thermo Electron Corporation, USA).

Glucose Lowering in Vivo. Five- to six-week old obese, diabetic ob/ob mice (male C57BL/6J) and their lean, nondiabetic male C57BL/6J littermates were provided by the Model Animal Research Center of Nanjing University, and housed under standard animal care practices with ad libitum access to food and water throughout the procedures. After 1 week acclimation, blood was collected from the retroorbital sinus for plasma glucose determination, and mice were randomized to groups with similar mean ± SD. Mice were then dosed p.o. daily for 4 days with vehicle consisting of DMSO/PEG400/H2O (1:3:6, v/v/v). On day 5, mice were treated p.o. with PSN-357 (50 mg/kg) or conjugate 6 (50 mg/kg) or vehicle, then bled at 0, 1, 2, 3, 6, and 24 hours post-dose for plasma glucose determination. Statistical analysis of the hypoglycemic effect was determined by unpaired t test with the ob/ob mice vehicle treated group.

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Author Contributions
Conceived and designed the study: L.Z. and C.S. Performed the experiments: L.Z., C.S., G.M., L.Z., Z.Y., J.L. and Y.W. Analyzed the data: G.M., Z.Y. and Y.W. Contributed reagents/materials/analysis tools: L.Z., C.S., G.M., L.Z., Z.Y., J.L. and Y.W. Wrote the manuscript: L.Z.

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