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Original article

Poliothrysoside and its derivatives as novel insulin sensitizers potentially driving AMPK activation and inhibiting adipogenesis☆

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ABSTRACT

In our efforts to develop safe and active chemical entities from nature, we first identified poliothrysoside (**1**), a phytoconstituent isolated from *Flacourtia indica*, possessing antidiabetic potential. Subsequently, fifteen derivatives (**2**–**16**) were synthesized to assess the activity profile of this class. All the compounds were analyzed for their glucose uptake potency in chronic insulin-induced insulin resistant 3T3-L1 adipocytes. Interestingly, compound **2** exhibited strong ability to increase the insulin sensitivity, primarily activating the AMPK signaling pathway and also inhibited the adipogenesis in 3T3-L1 adipocytes, in concentration dependent manner. Overall, these studies suggest the potential of poliothrysoside and its derivatives as promising leads for non-insulin dependent type 2 diabetes (T2D).

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1. Introduction

Diabetes mellitus is a progressive metabolic disease and represents a huge social and healthcare problem owing to the burden of its long term complications [1]. It is characterized by dysregulation of glucose homeostasis manifesting as hyper-glycemia, abnormalities in lipid and protein metabolism, which primarily affects three major organs – liver, skeletal muscle and adipocytes [2]. People suffering from diabetes are at high risk for macrovascular complications such as myocardial ischemia, stroke, adverse cerebrovascular events, and hypertension [3–5]. Non-insulin dependent type 2 diabetes (T2D) is the most prevalent form of the disease, affecting more than 90% of the diabetic patients. Clinical treatment of T2D heavily relies on oral hypoglycemic agents which mimic insulin in its action or enhance the sensitivity of insulin, and thus there has been considerable interest in insulin-sensitizers to counteract insulin resistance for the treatment of this metabolic syndrome [6]. Adipose tissue that comprises of one sixth of body weight and once considered as lipid storing inert cells are now considered as major endocrine organ by virtue of its capacity to secret various adipokines and cytokines, and contributes greatly to overall energy homeostasis [7]. Adipocyte insulin resistance is considered as early events before development of Type 2 diabetes and associated complications.

Metformin (a biguanide) has been used as the first line treatment for T2D which activates AMP-activated protein kinase (AMPK) [8,9]. AMPK is a key sensor enzyme that regulates intracellular and whole body energy metabolism, that has been implicated as a potential target in T2D [10]. It is activated by increase in the cellular AMP: ATP ratio caused by metabolic stresses that deplete cellular ATP supplies such as low glucose, hypoxia, ischemia, and heat shock. As a result it regulates signaling pathways that replenish cellular ATP supplies. Activation of this heterotrimeric kinase enhances both the transcription and translocation of GLUT4 (a glucose transporter protein), resulting in an increase in insulin-stimulated glucose uptake. Activated AMPK stimulates fatty acid oxidation, glycolysis and enhances insulin sensitivity, and inhibits fatty acid and cholesterol synthesis, resulting in restoration of energy homeostasis [11,12]. Thus, AMPK is considered to be a key therapeutic target that could provide a multifaceted approach to the treatment of obesity and type II diabetes mellitus. Rosiglitazone (a thiazolidinedione), another well known marketed hypoglycemic drug has also been reported to mediate its activity through the

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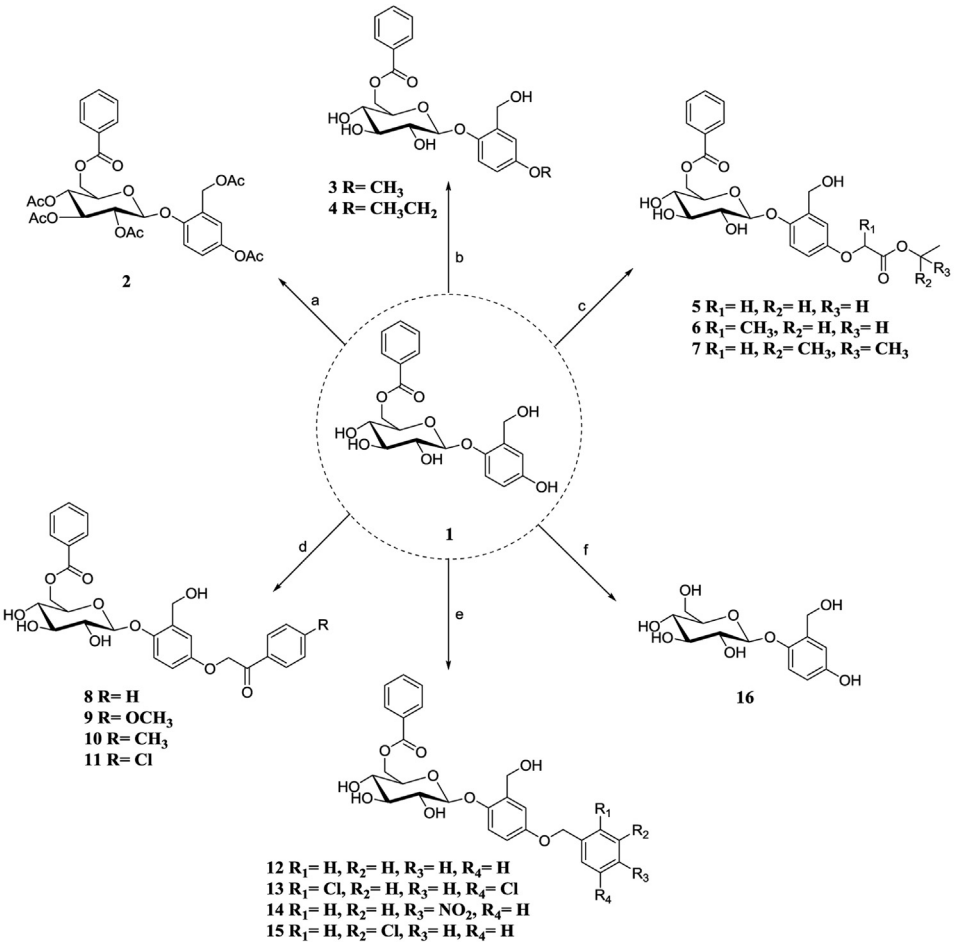

Reagent and conditions: (a) Ac_2O, pyridine, rt, 95% yield; (b) Me_2SO_4 or $(C_2H_5)_2SO_4$, anhyd. K_2CO_3, dry CH_3CN, reflux, 47% and 25 % yield, respectively; (c) α-bromoester, anhyd. K_2CO_3, dry CH_3CN, reflux, 5 h, 32-52% yield; (d) 4-substituted phenacylbromide, anhyd. K_2CO_3, dry CH_3CN, reflux, 4 h, 54-74% yield; (e) substituted benzylbromide, anhyd. K_2CO_3, dry CH_3CN, reflux, 5 h, 20-41% yield; (f) i. $Ba(OH)_2.8H_2O$, water, reflux, 2 h. ii. H_2SO_4, followed by $BaCO_3$, 99% yield.

Scheme 1. Chemical transformations of phenolic glycoside, poliothrysoside (**1**).

activation of AMPK [13]. However, adverse side effects like gastrointestinal problems, lactic acidosis, increased risk of heart failure and stoke associated with these two class of anti-diabetic drugs is a matter of serious concern [14,15]. Thus, medicinal chemists globally are in fray to develop more potent hypoglycemic agents with a good safety and tolerability profile, that can improve insulin sensitivity or act as insulin mimetics [16–18].

Natural products have been the most consistent source of drug leads. Biguanides that are derived from guanidine compounds, were originally isolated from *Galega officinalis*, a traditional plant used in the treatment of diabetes [19]. Berberine, an alkaloid has been shown to improve insulin sensitivity in animal models of insulin resistance by activating AMPK in multiple cell types [20]. Recent studies have suggested resveratrol as a new therapeutic agent to treat T2D and lipid induced insulin resistance, which may act through activation of AMPK [21,22]. AMPK activators on other hand are also known to decrease lipid droplet formation and thereby decrease adipogenesis [23,24]. There are several traditional medicines that have been reported to have antidiabetic effects [25], but the molecular targets of such compounds have not been revealed, and their mode of action in animal models has not been undertaken.

The plant *Flacourtia indica* Merr. has been reported to possess a wide range of pharmacological properties. In a recent study, the ethanolic extract of *F. indica* leaves exhibited interesting antidiabetic potential in STZ induced hyperglycemic rats. In particular, it significantly reduced elevated blood glucose, serum TG, TC and LDL-C level in the diabetic rats [26]. The plant is rich in phenolic glycosides but none of the constituent ever accounted for its antidiabetic potential, though several of them are known for antimalarial, anti-inflammatory, and inhibitory activity on snake venom phosphodiesterase I and HIV-1 RNase H [27]. There have been reports regarding the potential of phenolic glycosides as antidiabetic agents [28,29] which encouraged us to identify the active principle of *F. indica* responsible for its antidiabetic effects.

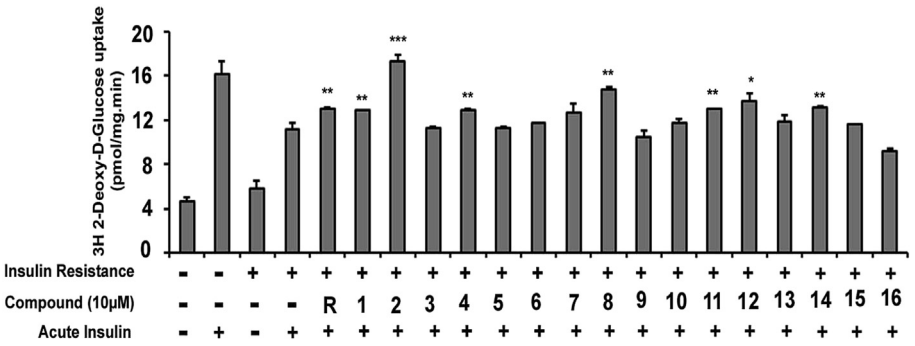

Fig. 1. Effect of compounds (**1**–**16**) on glucose uptake in chronic insulin induced insulin resistance in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated in DMEM containing 10% fetal bovine serum in the absence or presence of chronic insulin (10 nM) as mentioned in Experimental section. Glucose uptake was determined by the addition of [3H] 2-deoxyglucose (1 μCi/mL). Cells were lysed in 0.1 N NaOH solution and cell lysates were added to scintillation fluid for CPM counts. Data are expressed as means (±SD) of $n = 3$ independent experiment. $^{***}P < 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

As part of our drug discovery programme from Indian medicinal plants, we carried out phytochemical investigation of *F. indica* leaves and twigs and isolated one of the major phenolic glycoside, poliothrysoside (**1**) [30]. To study the unexplored antidiabetic strength of **1**, it was chemically modified to give another fifteen derivatives which together were evaluated for their potential to increase glucose uptake and inhibit insulin resistance.

2. Results and discussion

2.1. Chemistry

Poliothrysoside (**1**) was acylated with acetic anhydride in the presence of pyridine to afford poliothrysoside pentaacetate (**2**) in quantitative yields. The phenolic hydroxy group at C-5 underwent *O*-alkylation reactions with different alkylating agents using K_2CO_3 in dry acetonitrile (Scheme 1). Reaction conditions such as choice of alkylating agent, solvent, catalyst, and reaction time were optimized for the best results. Methyl ether (**3**) and ethyl ether derivative (**4**) were obtained by refluxing **1** with dimethyl sulphate and diethyl sulphate, respectively. **1** was further refluxed with three different α-bromoesters under the optimized conditions to afford compounds **5**–**7**. Similarly, alkylation with different 4-substituted phenacyl bromides yielded respective analogs **8**–**11**, while substituted benzyl bromides afforded compounds **12**–**15** under the same reaction conditions. Substitution with phenacyl bromides was straightforward, with good yields (54–74%) while benzyl bromides gave low yields (20–41%). The targeted compounds were purified by column chromatography using methanol–chloroform solvent gradient. Furthermore, compound **1** was subjected to mild basic hydrolysis in barium hydroxide to afford the debenzoyl poliothrysoside (**16**). All compounds were characterized using ^{1}H and ^{13}C NMR, mass spectrometry, IR, and elemental analysis methods.

2.2. Glucose uptake in chronic insulin resistant 3T3-L1 adipocytes

Insulin resistance is a manifestation of T2D that was developed in 3T3-L1 adipocytes with physiologically relevant concentration of insulin. With the emergence of insulin resistance, the adipocytes become desensitized to the biological effects of insulin, which is reflected in a reduction of the efficacy of insulin to stimulate glucose transport. Thus, in order to check the therapeutic potential of phenolic gylcoside derivatives (**1**–**16**) in T2D complications, they were evaluated for their ability to enhance glucose uptake in fully differentiated chronic insulin induced insulin resistance in 3T3-L1 adipocytes in the presence of insulin [31,32]. The study revealed that the tested compounds at 10 μM concentration were able to increase insulin sensitivity which was manifested in the form of enhanced glucose uptake against the resistance developed (Fig. 1). Poliothrysoside **1** and derivatives **2**, **4**, **8**, **11**, **12**, and **14** gave significant protection against insulin resistance. Derivative **2** showed remarkable sensitizing effects to insulin resistance with the maximum glucose uptake of 17.35 ± 0.49 pmol/mg min which was far better than well known insulin sensitizer drug rosiglitazone. Being the most active compound among the derivatives, **2** was chosen for further studies.

To optimize the results for compound **2**, it was evaluated for concentration dependent response in insulin resistant adipocytes with or without insulin stimulation. This experiment was necessary to assess inherent glucose uptake increase potential of compound **2**. The compound **2** addition inhibited insulin resistance development in concentration dependent manner, showing insulin sensitivity comparable to non-insulin resistant adipocytes at 10 μM concentration. This experiment also demonstrated that compound alone also increases glucose uptake in insulin resistant adipocytes without insulin pulse (Fig. 2).

Structure activity relationship studies revealed that benzoyl group at C-6 of sugar moiety was essential for insulin sensitizing effects as compound **16** did not show effective activity. Substitution with alkyl, phenacyl or benzyl group at C-5 hydroxy enhance the insulin sensitizing ability while acetylation of hydroxy groups boasts of significant increase in the activity of phenolic glycoside **1**.

2.3. Signaling pathway studies

Glucose uptake is activated by several mechanisms into the cell. One of the major signaling pathways contributing to intracellular glucose uptake is phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway. Insulin accelerates glucose transport *via* phosphorylation and activation of AKT which in turn leads to GLUT4 translocation to plasma membrane [33]. The other major pathway is AMPK signaling pathway which is proposed to directly stimulate GLUT4 translocation independent of PI3K-AKT pathway. As glucose uptake in adipocyte was observed in concentration dependent manner without any acute insulin stimulation, it was interesting to study the glucose uptake pathways. Hence to investigate the factor responsible for increasing the glucose uptake, the adipocytes were treated with selected compound **2** at 10 μM and a western blot was performed to check phosphorylation of AMPK (Thr172) and AKT (Ser473). From the western blot obtained, it was observed that compound **2** caused AMPK activation by phosphorylation like standard compound metformin, while AKT phosphorylation remained unchanged with the treatment. A comparison

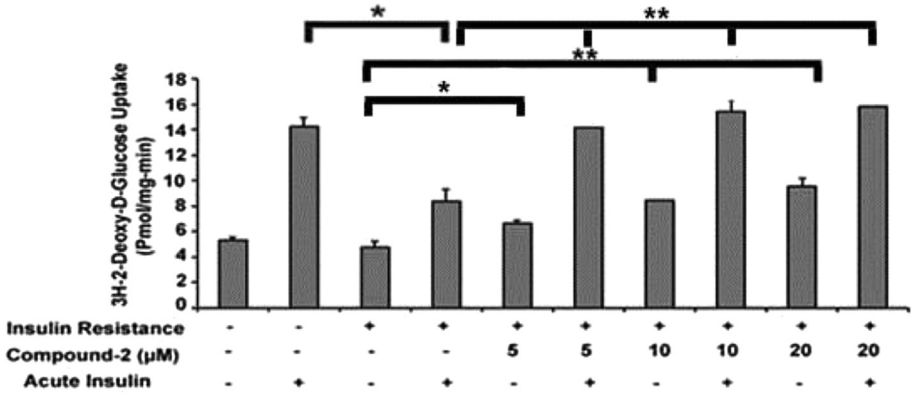

Fig. 2. Concentration dependent effect of compound **2** on glucose uptake in chronic insulin induced insulin resistance in 3T3-L1 adipocytes. Experiments were carried out as mentioned in Experimental section. Data are expressed as means (±SD) of $n = 3$ independent experiment. $^{***}P < 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

with standard drug metformin at 1 mM concentration showed that this derivative was a potent activator of AMPK. The results clearly indicated that the glucose uptake by compound **2** is at least in part caused by the activation of AMPK and not by PI3K-AKT (Fig. 3).

In order to validate, whether the uptake was indeed *via* AMPK activation, an AMPK inhibitor compound C (10 μM) was used half an hour before addition of 10 μM compound **2**. The co-incubation of compound C was given for 6 h along-with the compound incubation. Then glucose uptake studies were performed. Studies demonstrate that the increased glucose uptake caused due to addition of compound was reduced in the presence of AMPK inhibitor compound C thereby confirming the signaling pathway (Fig. 4).

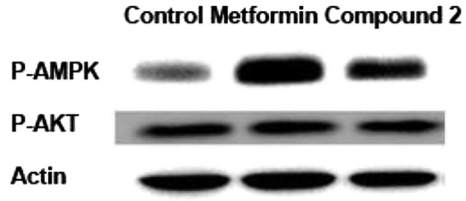

Fig. 3. Effect of compound **2** on phosphorylation of AMPK and AKT. 3T3-L1 adipocytes were treated with compound **2** at 10 μM or Metformin at 1 mM (standard) in serum free high glucose DMEM medium with 0.5% BSA for 24 h. Whole cells were lysed in lysis buffer and protein quantified with BCA. Actin was used as a loading control. Data is representative of three independent experiments that showed same tendency.

2.4. Inhibition of adipogenesis

It has been reported previously that AMPK activation is accompanied by the inhibition of adipogenesis [23,24], and compound **2** had been shown to act *via* AMPK activation, thus its effect on adipogenesis was also evaluated. Our results demonstrated that compound **2** was well capable of inhibiting adipogenesis as evidenced by microscopic observation. The extracted Oil-Red O accumulated in the lipid droplets showed ~20% and 64% inhibition in the increased lipid accumulation induced by differentiation inducers, in the presence of 20 and 50 μM compound concentration, respectively. Although at lower concentration (10 μM), it exhibited insulin resistance reversal, at higher concentrations compound **2** inhibited lipid droplet formation significantly in concentration dependent manner without any toxicity (Fig. 5(A) and (B)).

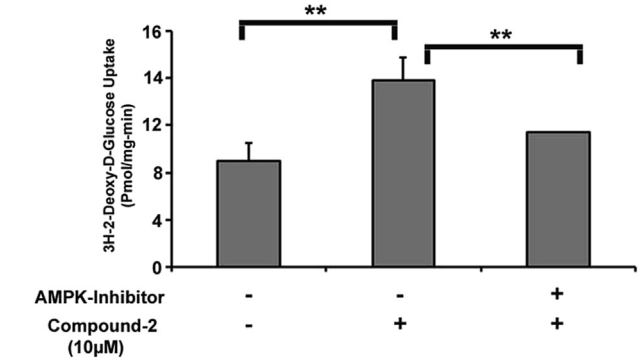

Fig. 4. Effect of compound **2** on AMPK mediated glucose uptake. 3T3-L1 adipocytes were treated with compound **2** for 24 h alone or pre-treated with compound C for 30 min before compound **2** treatment. Data are expressed as means (±SD) of $n = 3$ independent experiment. $^{***}P < 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

3. Conclusion

The major phenolic glycoside poliothrysoside (**1**) was chemically modified to give fifteen derivatives (**2**–**16**) and these compounds were analyzed for their glucose uptake potency in chronic insulin-induced insulin resistant 3T3-L1 adipocytes. The results indicated that the derivatives were able to prevent development of insulin resistance in the presence of chronic insulin. Further, they were shown to activate the glucose uptake through activation of AMPK. Derivative **2** was a lead molecule showing strong insulin sensitizing effects with significant inhibition of adipogenesis in concentration dependent manner as compared to the standard drug rosiglitazone. Thus, compound **1** and its derivatives have emerged as potent insulin sensitizers which might act through the activation of AMPK pathway. Taken together, these studies suggest the potential of poliothrysoside and its derivatives as promising leads for non-insulin dependent type 2 diabetes (T2D).

4. Experimental section

4.1. General experimental procedures

Synthesis of derivatives was carried out using lab reagents. Melting points were recorded on Stuart SMP 30 capillary melting point apparatus and are uncorrected. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR experiments were recorded on Bruker Avance DRX spectrometer, using $CDCl_3$, CD_3OD or DMSO-d_6 as solvent and TMS (trimethyl silane) as internal standard. All chemical shift values are expressed in ppm (δ), coupling constants (J) are presented in Hz,

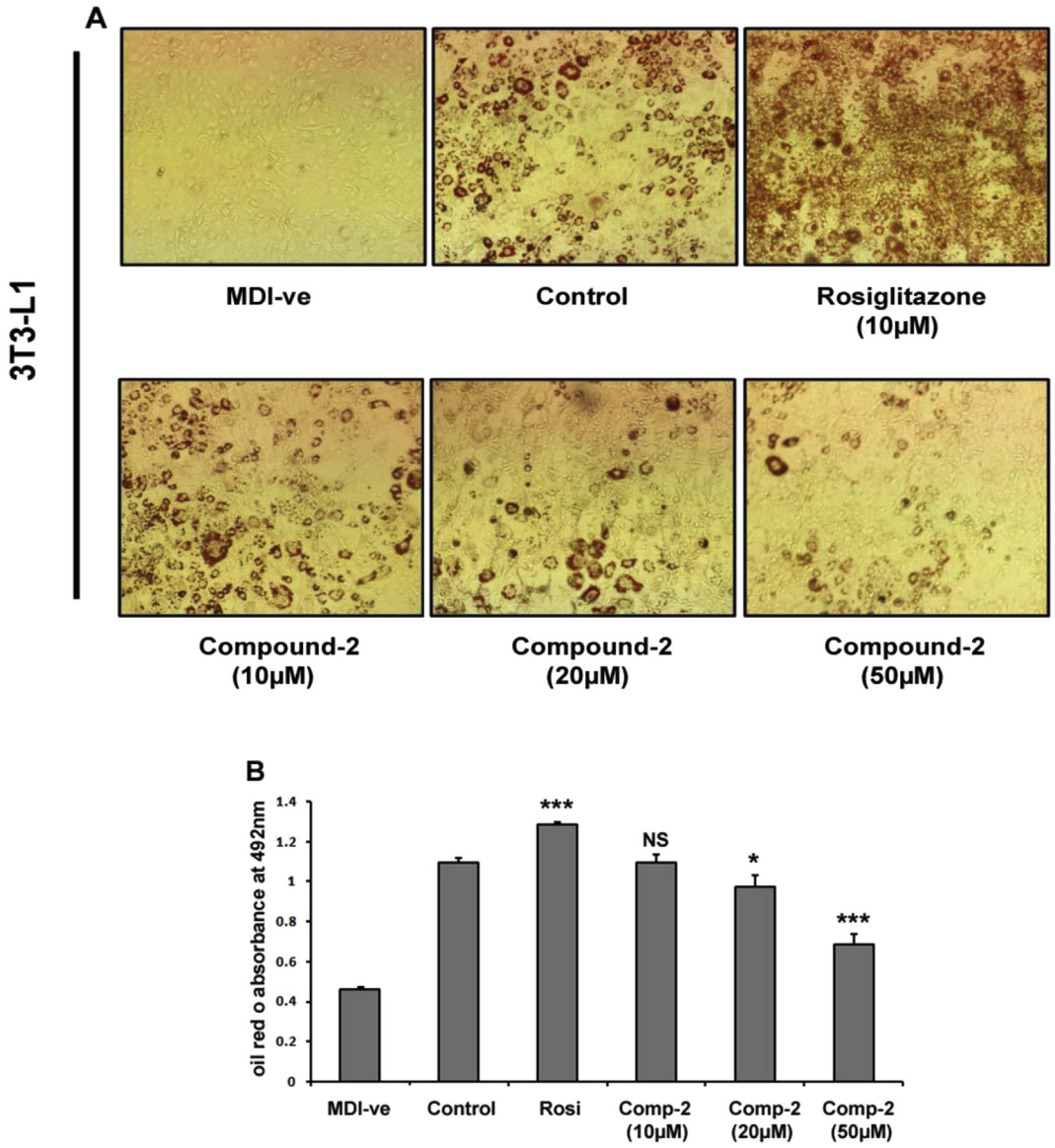

Fig. 5. Concentration dependent effect of compound **2** on lipid accumulation in 3T3-L1 cells. (A) Lipid accumulation was observed microscopically after Oil Red-O staining. (B) Oil Red-O was quantified spectrophotometrically at 492 nm. Data are expressed as means (±SD) of $n = 3$ independent experiment. $^{***}P < 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

and peak patterns are reported as broad (br), singlet (s), doublet (d), triplet (t), quartet (q), double doublet (dd), and multiplet (m). ESIMS spectra were recorded on ACQUITY UPLC equipped with TQD mass spectrometer. Elemental analyses were performed on Vario EL-III C, H, N, S Elemental Analyzer. Column chromatography (CC) was carried out on silica gel (100–200 mesh sizes, Merck). Analytical and preparative TLC were performed on precoated silica gel $60F_{254}$ aluminum and glass plates (Merck, 0.25 and 1 mm), respectively. Spots were visualized on TLC by UV light or by I_2 vapors or by charring on hot plate after spraying with 1% vanillin (w/v) in 10% H_2SO_4 (v/v) solution. Synthesized compounds were of requisite purity (>95%) as evidenced from the elemental analysis data. All organic solvents were distilled prior use.

4.2. Chemical modification

4.2.1. Poliothrysoside pentaacetate (**2**)

To a solution of **1** (50 mg, 0.12 mmol) in pyridine (1 mL), acetic anhydride (1 mL) was added and the reaction mass was stirred overnight at room temperature. It was diluted with water and extracted three times with EtOAc. Organic layer was washed with acidified water, dried over anhyd. Na_2SO_4 and then concentrated to give pure compound **2** (gum, 70 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 2.04–2.28 (15H, s, 5 × C$\underline{H}_3$CO), 3.99 (1H, m, H-5′), 4.38 (1H, dd, J = 6.1, 12.1 Hz, H-6′a), 4.55 (1H, dd, J = 2.1, 12.1 Hz, H-6′b), 5.04–5.34 (6H, m, H-1′, 2′, 3′, 4′, 7), 6.81 (1H, dd, J = 2.6, 8.7 Hz, H-4), 7.07 (1H, d, J = 2.6 Hz, H-6), 7.12 (1H, d, J = 8.8 Hz, H-3), 7.46 (2H, t, J = 7.4 Hz, H-3″), 7.59 (1H, t, J = 7.4 Hz, H-4″), 8.05 (2H, d, J = 7.2 Hz, H-2″); ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 20.7–21.2 (5C$\underline{H}_3$CO), 60.7 (C-6′), 62.8 (C-7), 68.9 (C-4′), 71.3 (C-2′), 72.4 (C-5′), 72.9 (C-3′), 100.0 (C-1′), 117.4 (C-3), 122.2 (C-6), 122.4 (C-4), 127.9 (C-1), 128.7 (C-3″), 129.7 (C-1″), 129.9 (C-2″), 133.5 (C-4″), 146.5 (C-5), 152.1 (C-2), 166.2 (C-7″), 169.5–170.7 (5 × C$\underline{H}_3$C$\underline{O}$); ESIMS m/z 634 $[M+NH_4]^+$; Anal. Calcd for $C_{30}H_{32}O_{14}$: C, 58.44; H, 5.23; Found: C, 58.24; H, 5.20.

4.2.2. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-methoxybenzyl alcohol (**3**)

A mixture of **1** (50 mg, 0.12 mmol), Me_2SO_4 (0.02 mL, 0.2 mmol) and anhyd. K_2CO_3 (30 mg, 0.22 mmol) in dry CH_3CN (10 mL) was

refluxed for 5 h. Reaction mass was filtered on Whatman filter paper and the filtrate was concentrated. Crude mass thus obtained was purified over silica gel CC in MeOH: $CHCl_3$ (0–8%) to furnish white solid **3** (m.p. 136–138 °C, 24 mg). ^{1}H NMR (300 MHz, CD_3OD) δ_H 3.48 (3H, m, H-2′, 3′, 4′), 3.70 (3H, s, OCH_3), 3.74 (1H, m, H-5′), 4.44–4.55(2H, m, H-6′), 4.67–4.76 (3H, m, H-1′, 7), 6.47 (1H, dd, J = 2.9, 8.8 Hz, H-4), 6.89 (1H, d, J = 2.8 Hz, H-6), 7.07 (1H, d, J = 8.8 Hz, H-3), 7.49 (2H, t, J = 7.7 Hz, H-3″), 7.63 (1H, t, J = 7.3 Hz, H-4″), 8.04 (2H, d, J = 7.1 Hz, H-2″); ^{13}C NMR (75 MHz, CD_3OD) δ_C 56.0 (OCH_3), 60.8 (C-7), 65.3 (C-6′), 72.0 (C-4′), 75.0 (C-2′), 75.6 (C-5′), 78.0 (C-3′), 104.2 (C-1′), 114.0 (C-4), 115.0 (C-6), 119.2 (C-3), 129.6 (C-3″), 130.6 (C-2″), 131.3 (C-1″), 134.0 (C-1), 134.3 (C-4″), 150.5 (C-2), 156.8 (C-5), 167.7 (C-7″); ESIMS m/z 443 $[M+Na]^+$; Anal. Calcd for $C_{21}H_{24}O_9$: C, 59.99; H, 5.75; Found: C, 59.64; H, 5.68.

4.2.3. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-ethoxybenzyl alcohol (**4**)

A mixture of **1** (30 mg, 0.07 mmol), $(C_2H_5)_2SO_4$ (0.014 mL, 0.11 mmol) and anhyd. K_2CO_3 (20 mg, 0.14 mmol) in dry CH_3CN (10 mL) was refluxed for 16 h. On completion of the reaction, same procedure followed as done for **3**, to furnish white solid **4** (8 mg; m.p. 132–134 °C). ^{1}H NMR (300 MHz, CD_3OD) δ_H 1.34 (3H, t, J = 6.9 Hz, CH_3), 3.49 (3H, m, H-2′, 3′, 4′), 3.72 (1H, m, H-5′), 3.91 (2H, q, J = 6.9 Hz, OCH_2CH_3), 4.44–4.76 (5H, m, H-1′, 6′, 7), 6.47(1H, dd, J = 2.8, 8.8 Hz, H-4), 6.88 (1H, d, J = 2.5 Hz, H-6), 7.06 (1H, d, J = 8.8 Hz, H-3), 7.49 (2H, t, J = 7.7 Hz, H-3″), 7.63 (1H, t, J = 7.3 Hz, H-4″), 8.04 (2H, d, J = 7.1 Hz, H-2″); ESIMS m/z 457 $[M+Na]^+$; Anal. Calcd for $C_{22}H_{26}O_9$: C, 60.82; H, 6.03; Found: C, 60.52; H, 6.12.

4.2.4. General procedure for the synthesis of 5-ether derivatives (**5**–**15**)

A mixture of **1** (1 equiv), respective 2-bromo compound (1.5–2.0 equiv), and anhyd. K_2CO_3 (1.5–2.0 equiv) in dry CH_3CN (10 mL) were refluxed for 4–5 h, till the completion of the reaction as monitored by TLC. The reaction mass was filtered on Whatman filter paper and the filtrate was concentrated under vacuum to dryness. The crude mass thus obtained was subjected to CC over silica gel eluted with gradient of MeOH: $CHCl_3$ (0–10%) to obtain pure compounds in quantitative yields.

4.2.4.1. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(2-ethoxy-2-oxoethoxy) benzyl alcohol (**5**).

White solid; yield 0.52 equiv; m.p. 126–128 °C; ^{1}H NMR (300 MHz, CD_3OD) δ_H 1.26 (3H, t, J = 7.1 Hz, OCH_2CH_3), 3.50 (3H, m, H-2′, 3′, 4′), 3.73 (1H, m, H-5′), 4.21 (2H, q, J = 7.1 Hz, OCH_2CH_3), 4.47 (2H, m, H-6′), 4.57 (2H, s, OCH_2CO), 4.67–4.75 (3H, m, H-1′, 7), 6.50 (1H, dd, J = 2.9, 8.8 Hz, H-4), 6.93 (1H, d, J = 2.8 Hz, H-6), 7.07 (1H, d, J = 8.9 Hz, H-3), 7.49 (2H, t, J = 7.7 Hz, H-3″), 7.63 (1H, t, J = 7.3 Hz, H-4″), 8.04 (2H, d, J = 7.1 Hz, H-2″); ^{13}C NMR (75 MHz, CD_3OD) δ_C 14.4 (OCH_2CH_3), 60.6 (C-7), 62.3 (OCH_2CH_3), 65.2 (C-6′), 66.7 (OCH_2CO), 71.9 (C-4′), 75.0 (C-2′), 75.5 (C-5′), 77.9 (C-3′), 103.9 (C-1′), 114.8 (C-4), 115.8 (C-6), 118.9 (C-3), 129.6 (C-3″), 130.6 (C-2″), 131.3 (C-1″), 134.1 (C-1), 134.3 (C-4″), 151.1 (C-2), 155.0 (C-5), 167.7 (C-7″), 171.0 (OCH_2CO); ESIMS m/z 510 $[M+NH_4]^+$; Anal. Calcd for $C_{24}H_{28}O_{11}$: C, 58.53; H, 5.73; Found: C, 58.48; H, 5.65.

4.2.4.2. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(1-ethoxy-1-oxopropan-2-yloxy)benzyl alcohol (**6**).

White solid; yield 0.32 equiv; m.p. 120–122 °C; ^{1}H NMR (300 MHz, CD_3OD) δ_H 1.21 (3H, t, J = 7.2 Hz, OCH_2CH_3), 1.54 (3H, d, J = 6.8 Hz, $OCH(CH_3)CO$), 3.48 (3H, m, H-2′, 3′, 4′), 3.73 (1H, m, H-5′), 4.15 (2H, q, J = 7.1 Hz, OCH_2CH_3), 4.46 (2H, m, H-6′), 4.68–4.77 (4H, m, H-1′, 7, $OCH(CH_3)CO$), 6.49 (1H, dd, J = 2.8, 8.8 Hz, H-4), 6.90 (1H, d, J = 2.8 Hz, H-6), 7.08 (1H, d, J = 8.8 Hz, H-3), 7.51 (2H, t, J = 7.4 Hz, H-3″), 7.65 (1H, t, J = 7.1 Hz, H-4″), 8.05 (2H, d, J = 7.7 Hz, H-2″); ^{13}C NMR (75 MHz, CD_3OD) δ_C 14.4 (OCH_2CH_3), 18.8 ($OCH(CH_3)CO$), 60.7 (C-7), 62.3 (OCH_2CH_3), 65.3 (C-6′), 72.0 (C-4′), 74.3 ($OCH(CH_3)CO$), 75.0 (C-2′), 75.6 (C-5′), 78.0 (C-3′), 104.1 (C-1′), 114.1 (C-4), 115.4 (C-6), 119.0 (C-3), 129.7 (C-3″), 130.6 (C-2″), 131.3 (C-1″), 134.1 (C-1), 134.4 (C-4″), 151.2 (C-2), 154.6 (C-5), 166.9 (C-7″), 171.1 ($OCH(CH_3)CO$); ESIMS m/z 529 $[M+Na]^+$; Anal. Calcd for $C_{25}H_{30}O_{11}$: C, 59.28; H, 5.97; Found: C, 59.18; H, 5.92.

4.2.4.3. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(2-tert-butoxy-2-oxoethoxy)benzyl alcohol (**7**).

Gum; yield 0.37 equiv; ^{1}H NMR (300 MHz, CD_3OD) δ_H 1.47 (9H, s, $3CH_3$), 3.49 (3H, m, H-2′, 3′, 4′), 3.73 (1H, m, H-5′), 4.46 (2H, s, OCH_2CO), 4.54–4.76 (5H, m, H-1′, 6′, 7), 6.49 (1H, dd, J = 2.8, 8.8 Hz, H-4), 6.91 (1H, d, J = 2.7 Hz, H-6), 7.08 (1H, d, J = 8.8 Hz, H-3), 7.51 (2H, m, H-3″), 7.64 (1H, m, H-4″), 8.05 (2H, d, J = 7.8 Hz, H-2″); ESIMS m/z 543 $[M+Na]^+$; Anal. Calcd for $C_{26}H_{32}O_{11}$: C, 59.99; H, 6.20; Found: C, 60.97; H, 6.88.

4.2.4.4. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(2-oxo-2-phenylethoxy)benzyl alcohol (**8**).

White solid; yield 0.68 equiv; m.p. 138–140 °C; ^{1}H NMR (300 MHz, DMSO-d_6 + D_2O) δ_H 3.28 (3H, m, H-2′, 3′, 4′), 3.73 (1H, m, H-5′), 4.27–4.60 (4H, m, H-6′, 7), 4.69 (1H, d, J = 6.7 Hz, H-1′), 5.40 (2H, s, OCH_2COPh), 6.35 (1H, dd, J = 2.8, 8.8 Hz, H-4), 6.92 (2H, m, H-3, 6), 7.45 (3H, m, Phenyl), 7.58 (2H, t, J = 7.6 Hz, H-3″), 7.71(1H, t, J = 7.2 Hz, H-4″), 7.94 (2H, d, J = 6.2 Hz, Phenyl), 7.99 (2H, d, J = 7.6 Hz, H-2″); ESIMS m/z 542 $[M+NH_4]^+$; Anal. Calcd for $C_{28}H_{28}O_{10}$: C, 64.12; H, 5.38; Found: C, 63.78; H, 5.12.

4.2.4.5. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(2-(4-methoxyphenyl)-2-oxoethoxy)benzyl alcohol (**9**).

White solid; yield 0.57 equiv; m.p. 134–136 °C; ^{1}H NMR (300 MHz, DMSO-d_6 + D_2O) δ_H 3.30 (3H, m, H-2′, 3′, 4′), 3.68 (1H, m, H-5′), 3.84 (3H, s, OCH_3), 4.27–4.59 (4H, m, H-6′, 7), 4.68 (1H, d, J = 6.7 Hz, H-1′), 5.32 (2H, s, OCH_2COPh), 6.33(1H, dd, J = 2.8, 8.8 Hz, H-4), 6.92 (2H, m, H-3, 6), 7.10 (2H, d, J = 8.7 Hz, 4-methoxyphenyl), 7.46 (3H, m, H-3″, 4″), 7.94 (2H, d, J = 7.2 Hz, 4-methoxyphenyl), 7.98 (2H, d, J = 8.8 Hz, H-2″); ESIMS m/z 572 $[M+NH_4]^+$; Anal. Calcd for $C_{29}H_{30}O_{11}$: C, 62.81; H, 5.45; Found: C, 62.68; H, 5.39.

4.2.4.6. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(2-oxo-2-p-tolylethoxy)benzyl alcohol (**10**).

White solid; yield 0.74 equiv; m.p. 136–138 °C; ^{1}H NMR (300 MHz, DMSO-d_6 + D_2O) δ_H 2.38 (3H, s, CH_3), 3.28 (3H, m, H-2′, 3′, 4′), 3.67 (1H, m, H-5′), 4.27–4.58 (4H, m, H-6′, 7), 4.68 (1H, d, J = 6.8 Hz, H-1′), 5.34 (2H, s, OCH_2COPh), 6.31 (1H, dd, J = 2.8, 8.8 Hz, H-4), 6.91 (2H, m, H-3, 6), 7.39 (2H, d, J = 8.2 Hz, p-tolyl), 7.44 (3H, m, H-3″, 4″), 7.88 (2H, d, J = 8.1 Hz, p-tolyl), 7.93 (2H, d, J = 7.7 Hz, H-2″); ESIMS m/z 556 $[M+NH_4]^+$; Anal. Calcd for $C_{29}H_{30}O_{10}$: C, 64.68; H, 5.61; Found: C, 64.55; H, 5.49.

4.2.4.7. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(2-(4-chlorophenyl)-2-oxoethoxy)benzyl alcohol (**11**).

White solid; yield 0.54 equiv; m.p. 144–146 °C; ^{1}H NMR (300 MHz, DMSO-d_6 + D_2O) δ_H 3.29 (3H, m, H-2′, 3′, 4′), 3.73 (1H, m, H-5′), 4.27–4.60 (4H, m, H-6′, 7), 4.69 (1H, d, J = 6.9 Hz, H-1′), 5.39 (2H, s, OCH_2COPh), 6.37 (1H, dd, J = 2.6, 8.7 Hz, H-4), 6.94 (2H, m, H-3, 6), 7.47 (3H, m, H-3″, 4″), 7.65 (2H, d, J = 8.5 Hz, 4-chlorophenyl), 7.94 (2H, d, J = 6.7 Hz, H-2″), 8.00 (2H, d, J = 8.5 Hz, 4-chlorophenyl); ESIMS m/z 576 $[M+NH_4]^+$; Anal. Calcd for $C_{28}H_{27}ClO_{10}$: C, 60.17; H, 4.87; Found: C, 60.02; H, 4.49.

4.2.4.8. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-benzyloxybenzyl alcohol (**12**).

White solid; yield 0.41 equiv; m.p. 128–130 °C; ^{1}H NMR (300 MHz, CD_3OD): δ_H 3.51 (3H, m, H-2′, 3′, 4′), 3.72 (1H, m, H-5′), 4.43–4.76 (5H, m, H-1′, 6′, 7), 4.96 (2H, s, OCH_2Ph), 6.54 (1H, dd, J = 2.8, 8.8 Hz, H-4), 6.98 (1H, d, J = 2.8 Hz, H-6), 7.06 (1H, d, J = 8.8 Hz, H-3), 7.28–7.40 (5H, m, Phenyl), 7.45 (2H, t, J = 7.7 Hz, H-3″), 7.60 (1H, t, J = 7.3 Hz, H-4″), 8.03 (2H, d, J = 7.2 Hz, H-2″); ESIMS

m/z 514 $[M+NH_4]^+$; Anal. Calcd for $C_{27}H_{28}O_9$: C, 65.31; H, 5.68; Found: C, 65.02; H, 5.52.

*4.2.4.9. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(2, 5-dichlorobenzyloxy)benzyl alcohol (**13**).* White solid; yield 0.28 equiv; m.p. 138–140 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 3.33 (3H, m, H-2′, 3′, 4′), 3.71 (1H, m, H-5′), 4.29–4.63 (4H, m, H-6′, 7), 4.74 (1H, d, J = 6.6 Hz, H-1′), 5.04 (2H, s, OC$\underline{H}_2$Ph), 6.52 (1H, dd, J = 2.8, 8.7 Hz, H-4), 7.04 (1H, d, J = 2.8 Hz, H-6), 7.19 (1H, d, J = 8.8 Hz, H-3), 7.44 (1H, dd, J = 2.8, 8.7 Hz, Phenyl), 7.56 (4H, m, Phenyl, H-3″), 7.67 (1H, t, J = 7.3 Hz, H-4″), 8.00 (2H, d, J = 7.8 Hz, H-2″); ESIMS *m/z* 587 $[M+Na]^+$; Anal. Calcd for $C_{27}H_{26}Cl_2O_9$: C, 57.36; H, 4.64; Found: C, 57.97; H, 5.06.

*4.2.4.10. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(4-nitrobenzyloxy) benzyl alcohol (**14**).* White solid; yield 0.40 equiv; m.p. 186–188 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 3.34 (3H, m, H-2′, 3′, 4′), 3.70 (1H, m, H-5′), 4.28–4.62 (4H, m, H-6′, 7), 4.72 (1H, d, J = 6.6 Hz, H-1′), 5.02 (2H, s, OC$\underline{H}_2$Ph), 6.51 (1H, dd, J = 3.3, 9.1 Hz, H-4), 7.02 (1H, d, J = 3.3 Hz, H-6), 7.17 (1H, d, J = 8.9 Hz, H-3), 7.48 (2H, d, J = 8.0 Hz, Phenyl), 7.54 (2H, t, J = 7.3 Hz, H-3″), 7.67 (1H, m, H-4″), 8.00 (2H, d, J = 7.8 Hz, H-2″), 8.19 (2H, d, J = 8.1 Hz, Phenyl); ESIMS *m/z* 559 $[M+NH_4]^+$; Anal. Calcd for $C_{27}H_{27}NO_{11}$: C, 59.89; H, 5.03; N, 2.59; Found: C, 60.17; H, 5.53; N, 2.60.

*4.2.4.11. 2-(6-Benzoyl-β-glucopyranosyloxy)-5-(3-chlorobenzyloxy) benzyl alcohol (**15**).* White solid; yield 0.20 equiv; m.p. 132–134 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 3.33 (3H, m, H-2′, 3′, 4′), 3.70 (1H, m, H-5′), 4.28–4.62 (4H, m, H-6′, 7), 4.72 (1H, d, J = 6.6 Hz, H-1′), 5.18 (2H, s, OC$\underline{H}_2$Ph), 6.53 (1H, dd, J = 3.0, 8.6 Hz, H-4), 7.04 (1H, d, J = 2.9 Hz, H-6), 7.17 (1H, d, J = 8.6 Hz, H-3), 7.51–7.56 (3H, m, Phenyl), 7.67–7.70 (4H, m, Phenyl, H-3″, H-4″), 7.98 (2H, d, J = 7.3 Hz, H-2″); ESIMS *m/z* 548 $[M+NH_4]^+$; Anal. Calcd for $C_{27}H_{27}ClO_9$: C, 61.08; H, 5.13; Found: C, 61.85; H, 5.48.

*4.2.5. 2-β-Glucopyranosyloxy-5-hydroxybenzyl alcohol (**16**)*

A mixture of **1** (50 mg, 0.12 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (80 mg, 0.25 mmol) in water (10 mL) was refluxed for 2 h. The resultant solution was acidified with sulphuric acid to precipitate $BaSO_4$. It was filtered off and then extracted with ether to remove benzoic acid thus formed. Further, the aqueous layer was neutralized with $BaCO_3$, filtered and then concentrated to give pure debenzoyl poliothrysoside **16** (gum, 37 mg). ^{1}H NMR (300 MHz, CD_3OD) δ_H 3.34–3.46 (4H, m, H-2′, 3′, 4′, 5′), 3.68 (1H, m, H-6′a), 3.90 (1H, d, J = 11.5 Hz, H-6′b), 4.54 (1H, d, J = 12.9 Hz, H-7a), 4.69 (2H, m, H-7b, H-1′), 6.65 (1H, dd, J = 2.8, 8.7 Hz, H-4), 6.79 (1H, d, J = 2.8 Hz, H-6), 7.08 (1H, d, J = 8.7 Hz, H-3); ^{13}C NMR (75 MHz, CD_3OD) δ_C 61.0 (C-7), 62.5 (C-6′), 71.4 (C-4′), 75.0 (C-2′), 77.9 (C-5′), 78.0 (C-3′), 104.6 (C-1′), 115.8 (C-4), 116.4 (C-6), 119.4 (C-3), 133.7 (C-1), 150.2 (C-2), 154.1 (C-5); ESIMS *m/z* 325 $[M+Na]^+$; Anal. Calcd for $C_{13}H_{18}O_8$: C, 51.65; H, 6.00; Found: C, 51.58; H, 5.78.

4.3. Cell culture and adipogenic differentiation

3T3-L1 pre-adipocytes cells were obtained from American Type Culture Collection (ATCC). Cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and antibiotic penicillin and streptomycin (complete media). For differentiation induction, two day post confluent cells were given differentiation media (containing insulin 5 μg/mL, IBMX 0.5 mM and Dexamethasone 250 nM). After completing 72 h time period, media was replaced with complete media containing insulin 5 μg/mL. This media was further replaced after 48 h and cells were then maintained in complete media till complete adipogenesis. More than 90% differentiated cells were used for experiments.

4.4. Chronic insulin induced insulin resistance

To validate 3T3-L1 adipocytes as a model for analyzing insulin resistance, the compounds used in the study were solubilized in DMSO, control cells were treated with matching concentrations of DMSO with compound. For insulin resistance development, fully differentiated adipocytes were treated with 10 nM insulin at 12:00, 16:00, and 20:00 h on day 1 and 08:00 h on the subsequent day. Compounds were present throughout the experiments along with insulin. At 12:00 h on the second day, cells were washed 4–5 times with Krebs Ringer HEPES buffer (NaCl-121 mM, KCl-4.9 mM, $CaCl_2$-0.33 mM, $MgSO_4$-1.2 mM, HEPES-12mM and pH 7.4) supplemented with 5 mM D-glucose and 0.05% BSA before acute insulin treatment for 30 min. For glucose uptake determination cells were given 100 μM 2-deoxy D-glucose and 1 μCi 2-deoxy-D-[3H]-glucose/mL for 5 min in KRH buffer. Finally, cells were washed three time with ice-cold Phosphate buffer saline to stop the reaction and cells were lysed in 0.1 N NaOH. A fraction of lysates were taken for protein estimation by Bicinchoninic acid assay method (Sigma). Remaining lysates were added in 3 mL scintillation fluid (cocktail W). CPM counts readings were taken on scintillation counter (Perkin Elmer). CPM count of radioactivity was normalized with protein content and expressed in pmol/mg min.

4.5. Western blotting

Following treatment, cell lysates were prepared in mammalian cell lysis buffer (G-Biosciences Cat. No# 786-180) containing 0.5 M EDTA, protease and phosphatise arrest (Sigma). Protein concentrations were estimated using BCA kit (Sigma). Lysates of equal proteins were heated in Laemmli sample (containing 10% β-mercaptoethanol) buffer at boiling temperature for 5 min. Proteins were resolved on 12% SDS gel and electro-transferred on nitrocellulose membrane. Membrane was blocked with 5% skimmed milk (Sigma). After blocking, membrane was incubated with indicated antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies. Immunoblots were developed with Immobiline western Chemiluminescence substrate (Millipore) on Image Quant LAS4000 (GE Healthcare Life Sciences). β-actin was used as housekeeping gene for loading control.

4.6. Triglyceride assay and Oil-Red O staining

After completing the differentiation of 3T3-L1 cells with or without compound at given concentration, cells were rinsed in phosphate buffered saline (pH 7.4). After those adipocytes lipid globules were stained with Oil-Red O (0.36% in 60% isopropanol) for 20 min. Cells were washed twice with phosphate buffer saline and images were acquired on Nikon Eclipse Ti microscope at 10× magnification. After complete removal of PBS from the wells, 100% isopropanol was used to extract the dye and absorbance was measured at 492 nm.

4.7. Statistical analysis

Data were expressed as mean (±SD) from '*n*' numbers of separate experiments. Student's *t*-test was used for comparisons of measured individual parameters. '*P*' values <0.05 were considered to be statistically significant. Data was analyzed on Graph Pad Prism (Version3.00, Graph pad Software Inc. San Diego, CA, USA).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.09.015.

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