Orally efficacious novel small molecule 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-α-D-glucopyranose selectively and potently stimulates insulin receptor and alleviates diabetes

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Abstract

Type 2 diabetes (T2D) has become an epidemic worldwide while T1D remains a great medical challenge. Insulin receptor (IR) signaling activators could alleviate hyperglycemia, reduce the burden on the pancreas, and contribute to prevention and treatment of both types of diabetes. Previously, we reported the synthesis and identification of a natural antidiabetic compound α-penta-galloyl-glucose (α-PGG). Subsequent studies led to the identification of an α-P6GG derivative, 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-α-D-glucopyranose (6Cl-TGQ). Here, we report that 6Cl-TGQ not only induced rapid and long-lasting glucose uptake comparable to insulin in adipocytes but also reduced high blood glucose levels to near normal and significantly decreased plasma insulin levels and improved glucose tolerance performance in high-fat diet-induced T2D mice when administered orally at 5 mg/kg once every other day. Moreover, a single gavage of 6Cl-TGQ at 10 mg/kg induced rapid and sharp decline of blood glucose in streptozotocin-induced T1D mice. Our studies further indicated that 6Cl-TGQ activated IR signaling in cell models and insulin-responsive tissues of mice. 6Cl-TGQ-induced Akt phosphorylation was completely blocked by IR and PI3K inhibitors, while the induced glucose uptake was blocked by the same compounds and a Glut4 inhibitor. Receptor binding studies indicated that 6Cl-TGQ bound to IR with a higher affinity than α-PGG. Importantly, 6Cl-TGQ, unlike insulin, selectively induced phosphorylation of IR without activating IGF1R or its signaling and did not increase cancer cell proliferation. These results indicate

Key Words

- polyphenol
- 6Cl-TGQ
- IR
- Akt
- Glut4 translocation
- IGF1R
- diabetes
that 6Cl-TGQ is a potent orally efficacious compound with low carcinogenic potential and may contribute to the prevention and treatment of T1D and T2D.

Introduction

Type 1 diabetes (T1D) and T2D are two major endocrine diseases characterized by insufficient insulin receptor (IR) signaling resulting in hyperglycemia (Zimmet et al. 2001, van Belle et al. 2011, Calhoun et al. 2011, Gunasekaran & Gannon 2011, Polychronakos & Li 2011). However, the causes leading to hyperglycemia in the two diseases are different. The key problem in T2D is insulin resistance or ineffective IR signaling (Saltiel & Kahn 2001, Zimmet et al. 2001, Gunasekaran & Gannon 2011). The pancreas has to produce and secrete more and more insulin (hyperinsulinemia) to compensate for insulin resistance. Eventually, insulin production drops due to excessive workload on the pancreas (Gunasekaran & Gannon 2011). In T1D, β-cells in the pancreas are erroneously destroyed by the host immune system leading to an absolute shortage of insulin (van Belle et al. 2011, Gunasekaran & Gannon 2011). In principle, any drug able to activate IR signaling and increase glucose uptake in insulin-responsive cells is beneficial to diabetic patients because it is able to at least partially replace the function of insulin, lowering demand for pancreatic insulin production and secretion.

The IR is a transmembrane heterotetrameric receptor tyrosine kinase functioning as a signal initiator and transmitter for glucose transport and metabolism. Activation of the IR signaling pathway involves auto-phosphorylation of IR, initiated by a conformational change after ligand binding, followed by activation of the downstream signaling cascade involving IRS, PI3K, and Akt (Saltiel & Kahn 2001). The final step in the signaling pathway of IR-mediated glucose transport is Glut4 translocation from the cytoplasm to the plasma membrane of insulin-responsive cells including muscle, fat, and liver cells (Pessin & Saltiel 2000, Chiang et al. 2001, Saltiel & Kahn 2001). Increasing IR signaling-mediated Glut4 translocation is key to alleviating hyperglycemia in both types of diabetes. In both T1D and T2D patients, an IR signaling activator can function as an insulin substitute to partially correct hyperglycemia (Saltiel & Kahn 2001, Taylor 2012).

Studies in recent years documented that the use of some antidiabetic drugs, such as insulin and its analog glargine, is associated with increased cancer rates (Gunter et al. 2009, Hemkens et al. 2009, Wilson 2011). This can be explained by the fact that insulin-like growth factor 1 receptor (IGF1R) is highly homologous to IR and the two signaling pathways share large similarities. Both IR and IGF1R signaling pathways consist of two major branches, the PI3K–Akt pathway and the MAPK pathway. Any IR signaling activator also tends to activate oncogenic IGF1R signaling (Higashi et al. 2010, Lewis et al. 2010, Wu et al. 2011, Huang et al. 2012, Ma et al. 2012). Few agents are known to selectively activate IR signaling without triggering IGF1R signaling. So far, only four nonpeptidyl fungal metabolites and derivatives have been characterized as selective IR activators (Zhang et al. 1999, Liu et al. 2000, Qureshi et al. 2000, Salituro et al. 2001).

We previously identified α-penta-galloyl-glucose (α-PGG) as a natural potent IR signaling activator and glucose transport stimulator (Li et al. 2005). Animal studies showed that α-PGG reduces blood glucose levels in diabetic and obese mice (Li et al. 2005). Subsequent structure–activity relationship studies led to the synthesis and identification of an α-PGG derivative, 6-chloro-6-deoxy-1,2,3,4-tetra-O-galloyl-α-D-glucopyranose (6Cl-TGQ), which showed much enhanced glucose transport-stimulating activity in a 10-min glucose uptake assay (Ren et al. 2006). But the antidiabetic activities of 6Cl-TGQ were not systematically studied. Here, we examined the long-term glucose uptake stimulatory activity of 6Cl-TGQ and evaluated the effectiveness of 6Cl-TGQ in vivo using both T1D and T2D animal models. We further studied the pharmacological working mechanism of 6Cl-TGQ and assessed its oncogenic potential.

Materials and methods

Compounds and cell lines

Compounds 6Cl-TGQ and α-PGG were synthesized as described previously (Ren et al. 2006). Compound solutions were prepared by dissolving the compounds in sterile ddH2O. Bovine insulin was purchased from Sigma–Aldrich and human IGF1 was purchased from
Abcam (Cambridge, MA, USA). A 200 μM IR inhibitor HNMPA-(AM)₃ (Biomol, Plymouth Meeting, PA, USA), 200 nM PI3K inhibitor wortmannin (Sigma–Aldrich), and 5 μM Glut4 inhibitor cytochalasin B (Santa Cruz Biotechnology) were used as described in Li et al. (2005). 3T3-L1 preadipocytes were purchased from ATCC (Rockville, MD, USA) and were maintained in ATCC-recommended cell culture media and conditions. 3T3-L1 preadipocytes were differentiated to adipocytes as previously reported (Liu et al. 2001). IR overexpressing CHO cells (Frattali et al. 1991) and IGF1R overexpressing NIH-3T3 cells (Dupont et al. 2001a) were maintained as described in the references.

**Glucose uptake assay**

The glucose transport stimulatory activity of compounds was analyzed by measuring the 3T3-L1 adipocyte uptake of 2-deoxy-D-(3H)-glucose (PerkinElmer Life Sciences, Waltham, MA, USA) as described previously (Li et al. 2005, Ren et al. 2006).

**High-fat diet-induced T2D animal model**

Healthy C57BL/6j male mice 3 weeks of age from Jackson Laboratory (Bar Harbor, ME, USA) were fed on either high-fat diet (HFD, 60% fat; Research Diets, New Brunswick, NJ, USA) or regular low-fat diet (LFD, 15% fat; Research Diets), until body weights and blood glucose levels of the mice on the two diets were statistically different (more than 4 weeks). HFD-induced diabetic and obese (Steppan et al. 2001, Araujo et al. 2007) mice were then assigned into different treatment groups by randomization, ten mice per group (n=10). The mice remained on assigned diets throughout the study. Compound treatment started after the T2D model was stable on ‘week 0’ and continued for 10 weeks. Blood glucose levels of these mice were measured immediately before the start of compound treatment for each week. T2D mice were treated with 6Cl-TGQ at a dose of 5 mg/kg every other day by oral delivery (gavage) of 200 μl aqueous compound solution. Healthy controls were gavaged with the same volume of ddH₂O. Blood glucose, body weight, and food intake of the treated mice were measured once a week. Plasma samples of the treated mice were collected on selected weeks for the measurement of plasma insulin. At the end of the study with HFD-induced T2D mice, 6Cl-TGQ-treated mice were subjected to a glucose tolerance test. Glucose was injected into mice at time zero, and blood glucose levels were monitored for about 3.5 h until blood glucose levels returned to basal levels. The next day, mice were killed after being fasted for 4 h. The liver and heart were removed and weighed immediately.

**Streptozotocin-induced T1D animal model**

Healthy CD-1 mice (~25 g) on regular diet were acclimated for at least 5 days and were then injected with 250 mg/kg streptozotocin (STZ; Sigma–Aldrich) via tail vein to induce death of the pancreatic β-cells and hyperglycemia (Xiang et al. 2010, Denroche et al. 2011). At a minimum of 4 days after STZ injection, blood glucose measurements were taken and animals with blood glucose levels in the range of 300–550 mg/dl were chosen for study. The number of mice ranged from one to four per treatment group. For determining the effect of 6Cl-TGQ in T1D mice, 6Cl-TGQ was dissolved in ddH₂O before the gavage treatment at a dose of 10 mg/kg body weight. After gavage, blood glucose levels of the treated mice were monitored for 3 h. Mice with ddH₂O gavage served as a negative control. T1D mice used for Akt activation analysis were killed 30–45 min after i.v. injection of 6Cl-TGQ at a dose of 10 mg/kg, ddH₂O or 450 ng/kg insulin-injected mice were used as controls. The muscle tissues were removed and the protein samples from the tissues were carefully prepared.

**Healthy mice**

Seven C57BL/6j male mice 10 weeks of age from Jackson Laboratory were fed on a regular diet. They were fasted overnight before experiments. In the Akt activation study, mice were killed 30–45 min after i.p. injection of 6Cl-TGQ at a dose of 10 mg/kg, ddH₂O or 450 ng/kg insulin-injected mice were used as controls. The muscle, fat, and liver tissues were removed and total proteins were isolated for Akt phosphorylation analysis.

**Animal studies**

All animal studies above were conducted in accordance with the NIH and Ohio University IACUC guidelines for the use and care of laboratory animals.

**Western blot**

Cells were starved for 2 h with serum-free DMEM and 0.5 h with KRP buffer followed by treatment with different conditions for 15 min unless otherwise indicated. Western blot analyses were performed using the standard protocol. Antibodies against phosphor-IR
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(Tyr1146), phosphor-IGF1R (Tyr1131), phosphor-Akt (Ser473), phosphor-MAPK (Thr202/Tyr204), and the loading controls β-tubulin and β-actin were from Cell Signaling Technology (Danvers, MA, USA).

GLUT4 translocation

The Glut4 membrane translocation study was performed as described previously (Li et al. 2005). Briefly, adipocytes, incubated in the presence or absence of 6Cl-TGQ at 37 °C for 15 min, were immunostained with anti-GLUT4 antibody (IF8, Biogenesis Nutraceuticals, Mill Creek, WA, USA) followed by a treatment with secondary fluorescein (FITC)-conjugated affiniPure F(ab’)2 fragment donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Crove, PA, USA). The stained cells were visualized and photographed with a Zeiss confocal microscope at a magnification of 630×.

IR binding assay: BIAcore

Surface plasmon resonance (Wong et al. 1999) was used to determine the binding affinity (Kd) of 6Cl-TGQ and α-PGG with the pure recombinant glycosylated IR (R&D Systems, Inc., Minneapolis, MN, USA) using a BIAcore X system (BIACore, Inc., GE Healthcare, New York, NY, USA) as described previously (Li et al. 2005). Briefly, IR was bound to an NTA chip via IR’s His tag. Increasing concentrations of 6Cl-TGQ or α-PGG were injected into the system to allow interaction with the immobilized IR on the chip surface. After sensograms of 6Cl-TGQ-IR or α-PGG-IR binding at different concentrations were recorded, the binding affinity was analyzed using BIAevaluation Software. Flow cells or channels without IR were used as negative controls and α-PGG was used as a positive control.

Insulin displacement assay

The assay was performed as described previously (Li et al. 2005). Briefly, total protein, with IR as a predominant receptor protein, was prepared from CHO-IR cells and used to bind the wells of FlashPlates pre-coated with wheat germ agglutinin (WGA; PerkinElmer Life Sciences). After removal of unbound proteins and washes, fixed amounts of insulin labeled with 125I (Amersham, GE Healthcare) were added to wells with mock, or various concentrations of α-PGG or 6Cl-TGQ. Following overnight incubation and removal of unbound radioactive ligands and washes, the bound ligands were eluted from the receptors with 0.2 M glycine (pH 2.8) and measured for its radioactivity using a Beckman Coulter LS 6400 Multi-Purpose Scintillation Counter. WGA wells coated with protein isolated from regular CHO cells served as controls for nonspecific ligand binding. The receptor binding results were analyzed with the software GraphPad Prism. (GraphPad Software Inc., La Jolla, CA, USA)

Cancer cell study

Human colon cancer RKO cells or human breast cancer MCF7 cells were treated with either mock (H2O) or different concentrations of 6Cl-TGQ for 48 h. MTT assay was carried out to determine the number of viable cells after treatment as described in Cao et al. (2011).

Statistical analysis

All relevant assays and animal study data were analyzed with one-way ANOVA accompanied by Tukey’s test to identify significant intergroup differences. In all figures, values are mean ± s.d. unless otherwise indicated. Except for animal studies, samples were replicated either in duplicate or in triplicate in each experiment, which was repeated at least three times. P<0.05 was set as the level of statistical significance. *P<0.05; **P<0.01; and ***P<0.001.

Results

6Cl-TGQ induced long-lasting glucose uptake in 3T3-L1 adipocytes, comparable to insulin and with a higher efficacy and potency than α-PGG

The compound 6Cl-TGQ shares the basic structure of α-PGG but with a Cl group replacing the galloyl group on carbon 6 of the glucose core of α-PGG (Fig. 1A). As a result, 6Cl-TGQ (807 Da) has a smaller molecular weight than α-PGG (940 Da). In glucose uptake assays, 6Cl-TGQ was found to be more potent than α-PGG. 6Cl-TGQ was capable of inducing the same amount of glucose transport at a lower concentration and inducing more glucose transport than α-PGG of the same concentration at nearly all concentrations tested (Fig. 1B, C and D). The profile of the dose curve of 6Cl-TGQ is similar to that of insulin (Fig. 1B), suggesting that 6Cl-TGQ induces glucose transport by the same mechanism as insulin. Although the maximal glucose uptake induced by 30 μM 6Cl-TGQ within 10 min after the start of the assay (Fig. 1B) was lower than the maximal induction by insulin, the glucose uptake induced by 30 μM 6Cl-TGQ within 60 min after the start of the assay was almost as high as the maximal.
induction by insulin (Fig. 1C). These results were confirmed by the time course analysis (Fig. 1D). Both 6Cl-TGQ and α-PGG induced glucose transport within the first minutes of the assay, almost as fast as insulin (Fig. 1D).

**Oral administration of 6Cl-TGQ significantly alleviated hyperglycemia and glucose intolerance in HFD-induced T2D mice**

As 6Cl-TGQ showed high efficacy in stimulating glucose transport in 3T3-L1 adipocytes, we hypothesized that it is able to reduce blood glucose levels in diabetic mouse models. To test this hypothesis, an animal study was conducted using HFD-induced T2D mice, which mimics T2D induction in humans. Repeated 6Cl-TGQ treatments by oral delivery (gavage) at a dose of 5 mg/kg every other day resulted in a significant reduction of blood glucose levels (Fig. 2A). Specifically, the blood glucose levels of 6Cl-TGQ-treated T2D mice declined dramatically within the first 3 weeks after 6Cl-TGQ treatment and remained at levels close to those of the healthy mice but statistically lower than those of the untreated T2D mice throughout the following weeks (Fig. 2A). Repeated 6Cl-TGQ treatments also lowered the plasma insulin levels throughout the treatment period (Fig. 2B). Compared with the untreated T2D mice, 6Cl-TGQ-treated T2D mice showed a significantly reduced peak level of blood glucose surge in a glucose tolerance test (Fig. 2C).

A single 6Cl-TGQ administration led to rapid reduction of blood glucose levels in STZ-induced T1D mice

Because 6Cl-TGQ was found to be quite effective in both 3T3-L1 adipocytes and a T2D mouse model, we extended our study to see whether 6Cl-TGQ is also effective in a T1D mouse model. The T1D mice model was established using STZ injection. In 75% of all cases, a single oral delivery of 6Cl-TGQ at 10 mg/kg was found to induce a rapid and sharp decline of blood glucose levels when compared with mock controls (Fig. 2D).

**Oral administration of 6Cl-TGQ for 10 weeks did not reduce food intake or body weight in HFD-induced T2D mice**

In order to determine whether 6Cl-TGQ persistently reduced the blood glucose levels of HFD-induced T2D mice
by decreasing the appetite or nutrient absorption of mice, the monitored food intake and body weight of mice were analyzed. No significant changes in food intake (Fig. 3A) or body weight (Fig. 3B) were found between 6Cl-TGQ-treated and -untreated T2D mice. Also, no significant changes in the liver (Fig. 3C) and heart (Fig. 3D) weights were observed between 6Cl-TGQ-treated and -untreated T2D mice at the end of the 10-week treatment.

6Cl-TGQ activated IR signaling in both cell models

As 6Cl-TGQ was found to stimulate potent glucose transport in 3T3-L1 adipocytes and significantly reduced glucose levels in both T1D and T2D mice models, it was hypothesized that 6Cl-TGQ was influencing the IR signaling pathway. To determine whether 6Cl-TGQ could activate IR signaling, the signaling that regulates glucose transport in insulin-responsive cells, CHO-IR stable cells and 3T3-L1 adipocytes were treated with 6Cl-TGQ for various lengths of time. Insulin- and α-PGG-treated cells were used for comparison. Phosphorylation of IR was detected after the first minute of 6Cl-TGQ treatment and the effect lasted for at least 60 min in CHO-IR cells (Fig. 4A). Phosphorylation of the key IR downstream signaling factor Akt by 6Cl-TGQ was detected 5 min after the initiation of compound treatment and Akt phosphorylation remained robust 60 min after 6Cl-TGQ treatment in both CHO-IR and 3T3-L1 cells (Fig. 4A). The IR and Akt activation patterns of 6Cl-TGQ resemble those of insulin and α-PGG (Fig. 4A). But 6Cl-TGQ induced stronger activation than α-PGG at almost all time points (Fig. 4A). Remarkably, 6Cl-TGQ-induced Akt phosphorylation was almost as robust as that induced by insulin 5–60 min after the initiation of the treatments in 3T3-L1 adipocytes and 60 min after the initiation of the treatments in CHO-IR cells (Fig. 4A). These results are consistent with the time-dependent glucose uptake assay in which the glucose transport induced by 6Cl-TGQ was slower than that induced by insulin in the first few minutes but caught up later (Fig. 1D). Importantly, 6Cl-TGQ-induced Akt...
phosphorylation in 3T3-L1 cells was blocked by both the IR inhibitor HNMPA-(AM)₃ and the PI3K inhibitor wortmannin (Fig. 4B), suggesting that 6Cl-TGQ activates Akt through the IR–PI3K pathway. Like insulin and α-PGG (Li et al. 2005), 6Cl-TGQ induced Glut4 translocation from the cytosol to the plasma membrane in 3T3-L1 adipocytes (Fig. 4C). Moreover, glucose uptake induced by 6Cl-TGQ was effectively blocked by the inhibitors for IR, PI3K, or Glut4 movement (Fig. 4D).

6Cl-TGQ activated the IR signaling in mice

6Cl-TGQ can not only activate IR signaling in cell models but can also induce potent Akt phosphorylation in mice with an intact IR signaling system (Fig. 5). A single injection of 6Cl-TGQ at 10 mg/kg resulted in Akt phosphorylation in the muscle of STZ-induced T1D mice (Fig. 5A), and in the muscle, fat, and liver tissues of healthy mice (Fig. 5B, C and D), strongly supporting that 6Cl-TGQ activated IR signaling to reduce blood glucose in vivo.

6Cl-TGQ bound to IR with a higher affinity than α-PGG

A receptor binding study with the BIACore system showed that 6Cl-TGQ bound IR with a $K_d$ of about 19 μM, which is about 2.3-fold smaller than that of α-PGG (Fig. 6A; Wanant & Quon 2000). The insulin displacement assay revealed that the $K_i$ of 6Cl-TGQ for IR was ~1.8 μM, which is about 3.3-fold lower than that of α-PGG for IR (Fig. 6B). The higher receptor binding affinity of 6Cl-TGQ is consistent with its higher glucose uptake stimulatory activity (Fig. 1B, C and D) and stronger IR signaling activation activity (Fig. 4A).

6Cl-TGQ did not activate IGF1R signaling or stimulate cancer growth

IGF1R is highly homologous with IR and intimately involved in cancer progression (Higashi et al. 2010, Lewis et al. 2010, Wu et al. 2011, Huang et al. 2012, Ma et al. 2012). A serious problem for IR signaling activators is that they might not distinguish IGF1R from IR, activating IGF1R and increasing cancer rates (Gunter et al. 2009, Hemkens et al. 2009, Wilson 2011). To determine whether 6Cl-TGQ activates IGF1R signaling, IGF1R overexpressing NIH-3T3 cells were treated with 6Cl-TGQ. In contrast to IGF1 treatment, 6Cl-TGQ did not induce the phosphorylation of IGF1R or the key downstream factors Akt and MAPK (Fig. 7A). Furthermore, 6Cl-TGQ did not promote RKO or MCF7 cancer cell proliferation at all tested
concentrations (Fig. 7B and C). Instead, it slightly decreased cancer cell viability 48 h after the start of the treatment (Fig. 7B and C).

Discussion

6CI-TGQ is a derivative of α-PGG (Ren et al. 2006). Together, with α-PGG, it belongs to a large group of polyphenolic compounds called tannins (Li et al. 2005, Ren & Chen 2007). Tannins are broadly distributed in plants as part of the plant’s natural defense system (Bennick 2002). It is believed that tannins benefit human health as antioxidants and anticancer reagents (Chung et al. 1998). α-PGG and its anomer β-PGG are key hydrolysable tannins (Liu et al. 2005). Hydrolysable tannins have shown effectiveness in correcting hyperglycemia in diabetes patients (Gin et al. 1999). And both α-PGG (Li et al. 2005) and β-PGG (Liu et al. 2005) have been found to activate IR signaling to induce glucose uptake in insulin-responsive cells. α-PGG could even reduce blood glucose levels in diabetic mice (Li et al. 2005). Recently, α-PGG was also found to induce an insulin-like, IR-mediated antiplatelet aggregation reaction both in vitro and in vivo (Perveen et al. 2011). 6CI-TGQ is a synthetic novel small compound, with a molecular weight significantly lower than α-PGG and a glucose uptake stimulatory activity much higher than α-PGG as shown in a 10-min glucose uptake assay (Ren et al. 2006). The smaller molecular weight (807 Da) makes 6CI-TGQ a more easily deliverable compound with high bioavailability.
Indeed, 6Cl-TGQ is highly stable, soluble in H2O, and very effective in reducing blood glucose levels in T1D and T2D mice when orally delivered (Fig. 2A, C and D). Importantly, 6Cl-TGQ showed much higher IR binding affinity than α-PGG (Fig. 6) and higher potency and efficacy than α-PGG in stimulating glucose uptake at all time points of the 1-h glucose uptake assays (Fig. 1B, C and D). Strikingly, the maximal cumulative glucose uptake induced by 6Cl-TGQ was similar to that induced by insulin at 1 h after the start of the glucose uptake assays (Fig. 1C and D).

Like insulin, 6Cl-TGQ is rapid acting. It activated IR signaling (Fig. 4A) and stimulated glucose uptake (Fig. 1D) within the first few minutes of the assays. Just like regular insulin, which begins to reduce blood glucose within 30 min of injection, a single dose of 6Cl-TGQ activated IR signaling (Fig. 5) and started to reduce the blood glucose levels (Fig. 2D) within 30 min of administration. Also like insulin, 6Cl-TGQ has a long-lasting effect. It stimulated IR signaling (Fig. 4A) and induced glucose uptake (Fig. 1C and D) for at least 1 h in cell studies. Our animal studies showed that the peak activity of a single dose of 6Cl-TGQ appeared at 1–2 h after oral administration and lasted for at least an hour (Fig. 2D). This is similar to but slightly slower than regular insulin, which reaches a peak effect within 1 h after injection. Repeated dosing made the antidiabetic function of 6Cl-TGQ even more long lasting.

6Cl-TGQ could effectively reduce the fasting basal blood glucose levels of T2D mice even when being orally delivered once every other day at a concentration of 5 mg/kg (Fig. 2A). Polyphenols may reduce nutrient absorption (Bennick 2002). However, the long-lasting antidiabetic function of 6Cl-TGQ was not caused by reduced appetite or nutrient absorption after compound treatment (Fig. 3). Rather, it was possibly due to 6Cl-TGQ’s long-lasting blood glucose-lowering effects or its other potentially beneficial biological activities such as antioxidant effects. Importantly, 6Cl-TGQ consistently reduced the plasma insulin levels in T2D mice (Fig. 2B). A single dose of 6Cl-TGQ lowered plasma insulin levels 40–90 min after injection (X Chen 2012, unpublished observation). These results could be important as they showed the potential of 6Cl-TGQ to replace some functions of insulin and decrease the insulin production burden on β-cells. But detailed studies are needed to further assess the potential of 6Cl-TGQ in reducing insulin levels and preserving β-cell functions. It is also important that 6Cl-TGQ appeared to be a safe agent. During our animal studies, 6Cl-TGQ was not observed to have acute toxic effects except for some hypoglycemic effects much milder than those observed for insulin (Y Cao, J Kim & X Chen 2012, unpublished observation).
results). 6CI-TGQ also did not reduce the appetite, body weight, liver weight, or heart weight of mice when compared with the untreated animals (Fig. 3).

One of the key concerns for the use of IR signaling activators as antidiabetic agents is their potential to also activate the IGF1R signaling. IGF1R and IGF1R signaling resembles IR and IR signaling respectively. Despite the significant structural and signaling cascade similarities between IGF1R and IR, they are not redundant. The physiological functions of their signaling could be distinct. For instance, cDNA microarray analysis of NIH-3T3 cells indicated that more than half of the genes upregulated by IGF1R activation are associated with mitogenesis and differentiation, whereas none of the genes specifically upregulated by IR activation are associated with these processes, suggesting that IGF1R activation is more mitogenic than IR activation (Dupont et al. 2001b). Indeed, IGF1R activation has been found to be intimately associated with cancer development (Higashi et al. 2010, Lewis et al. 2010, Wu et al. 2011, Huang et al. 2012, Ma et al. 2012). There is evidence showing that using insulin and insulin analogs to treat diabetes increases cancer rates as they may also activate IGF1R signaling (Gunter et al. 2009, Hemkens et al. 2009, Wilson 2011). The problem is compounded by a nationwide cohort study showing that most glucose-lowering antidiabetic medications, including thiazolidinediones and sulfonylureas, increase cancer risk (Higashi et al. 2010, Andersson et al. 2012). Therefore, efforts are needed to avoid these side effects.

Figure 6
6CI-TGQ bound IR with a higher affinity than α-PGG. IR binding studies were performed as described in the Materials and methods section.
(A) IR binding by 6CI-TGQ as determined by a BIAcore system. (B) Insulin displacement from IR by 6CI-TGQ as determined by an in-solution receptor binding competition assay.

Figure 7
6CI-TGQ did not activate IGF1R signaling or promote cancer cell growth.
(A) 6CI-TGQ did not induce phosphorylation of IGF1R, Akt, or MAPK in IGF1R overexpressing cells. NIH-3T3 cells overexpressing IGF1R were treated with 30 µM 6CI-TGQ or 0.1 µM IGF1 for 15 min. Proteins were isolated from the treated cells and analyzed for the phosphorylation of IGF1R, Akt, and MAPK. β-Actin and β-tubulin were used as internal protein controls.
6CI-TGQ did not promote the growth of (B) the human colon cancer RKO cells and (C) the human breast cancer MCF7 cells. RKO and MCF7 cells were treated with various concentrations of 6CI-TGQ for 48 h. ddH2O-treated cells were used as controls. Cell viability was measured by an MTT cell proliferation assay after the compound treatment. Results were presented as mean viability of cells treated with a certain concentration of 6CI-TGQ relative to mean viability of cells treated with ddH2O.
to find new glucose-lowering agents with low carcinogenicity and other side effects. At present, only four nonpeptidyl fungal metabolite and derivatives were characterized as selective IR activators (Zhang et al. 1999, Liu et al. 2000, Qureshi et al. 2000, Salituro et al. 2001). But their potential as new antiidiabetic medication has not been further evaluated. 6Cl-TGQ was found to bind IGFR1 (X Chen 2005, unpublished results), which initially raised concerns about its potential to activate mitogenic signaling. Our studies with the IGFR1 over-expressing NIH-3T3 cells indicated that, although 6Cl-TGQ binds to IGFR1, the binding does not activate IGFR1 or IGFR1-mediated signaling that induces cell growth and proliferation (Fig. 7A). These results suggested that 6Cl-TGQ is capable of distinguishing IR from IGFR1, binding to both but only activating the former. The carcinogenic potential of 6Cl-TGQ was further evaluated using the human colon cancer cell line RKO and the human breast cancer cell line MCF7 as insulin and insulin analogs were found to increase colon and breast cancer rates (Gunter et al. 2009, Hemkens et al. 2009, Wilson 2011). A 48-h treatment with 6Cl-TGQ did not increase cancer cell growth at all tested concentrations (Fig. 7B and C). The non-carcinogenicity of 6Cl-TGQ is in agreement with our recent finding that α-PGG, a structural and functional analog of 6Cl-TGQ, could also distinguish IR signaling from IGFR1 signaling and induce apoptosis in human cancer cells (Cao et al. 2011).

In summary, 6Cl-TGQ demonstrated glucose transport stimulatory activity and IR binding affinity significantly stronger than those of the natural lead compound α-PGG. 6Cl-TGQ (30 μM) could induce a transport of almost as much glucose as the maximum induced by insulin in 1 h as shown in the glucose uptake assays. Oral delivery of 6Cl-TGQ resulted in a long-term reduction of blood glucose in HFD-induced T2D mice and induced rapid and sharp blood glucose decline in STZ-induced T1D mice. 6Cl-TGQ activated IR signaling without activating IGFR1 signaling, reducing the concern that 6Cl-TGQ may possess insulin-like carcinogenic potential. All these results indicate that 6Cl-TGQ is a true low-molecular weight, orally deliverable, potent, and selective IR signaling activator that may be valuable for the prevention and treatment of T1D and T2D and may contribute to the discovery of more novel glucose-lowering agents.

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