Herbal Constituent Sequoyitol Improves Hyperglycemia and Glucose Intolerance by Targeting Hepatocytes, Adipocytes, and β cells

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Abstract

The prevalence of insulin resistance and type 2 diabetes increases rapidly; however, treatments are limited. Various herbal extracts have been reported to reduce blood glucose in animals with either genetic or dietary type 2 diabetes; however, plant extracts are extremely complex and leading compounds remain largely unknown. Here we show that 5-O-methyl-myoinositol (also called sequoyitol), a herbal constituent, exerts anti-diabetic effects in mice. Sequoyitol was chronically administrated into ob/ob mice either orally or subcutaneously. Both oral and subcutaneous administrations of sequoyitol decreased blood glucose, improved glucose intolerance, and enhanced insulin signaling in ob/ob mice. Sequoyitol directly enhanced insulin signaling, including phosphorylation of IRS1 and Akt, in both HepG2 cells (derived from human hepatocytes) and 3T3-L1 adipocytes. In agreement, sequoyitol increased the ability of insulin to suppress glucose production in primary hepatocytes and to stimulate glucose uptake into primary adipocytes. Furthermore, sequoyitol improved insulin signaling in INS-1 cells (a rat β cell line) and protected INS-1 cells from streptozotocin (STZ)- or H2O2-induced injury. In mice with STZ-induced β cell deficiency, sequoyitol treatments increased plasma insulin levels and decreased hyperglycemia and glucose intolerance. These results indicate that sequoyitol, a natural, water-soluble small molecule, ameliorates hyperglycemia and glucose intolerance by increasing both insulin sensitivity and insulin secretion. Sequoyitol appears to directly target hepatocytes, adipocytes, and β cells. Therefore, sequoyitol may serve as a new oral diabetes medication.
Introduction

Glucose is a primary metabolic fuel. Blood glucose levels are maintained within a narrow range through the action of insulin and other metabolic hormones (18, 34). Insulin decreases blood glucose by stimulating glucose uptake into adipose tissue and skeletal muscle and by suppressing hepatic glucose production (34). Insulin resistance is the primary risk factor for type 2 diabetes (42). Additionally, pancreatic β cell function is also impaired in type 2 diabetes and unable to secrete sufficient insulin to compensate for insulin resistance (26). Relative insulin deficiency, due to a combination of insulin resistance and impaired pancreatic β cell function, contributes to hyperglycemia and glucose intolerance in type 2 diabetes. Small molecules with the capability to improve insulin sensitivity and/or β cell function have a great therapeutic potential for the treatment of type 2 diabetes.

Growing evidence indicate that herbal constituents hold a great promise for the treatment of type 2 diabetes, and extracts of numerous plants have been reported to reduce blood glucose (6, 8, 17, 22, 41). In search for small compounds with an anti-diabetic property, we examined natural compounds in plant extracts, focusing on inositol derivatives. Inositols consist of nine isomeric forms (myo-, scyllo-, epi-, allo-, cis-, neo-, muco-, D-chiro-, L-chiro-inositol) and are synthesized from glucose-6-phosphate, a glycolytic metabolite (28). Many forms of inositols and inositol derivatives are essential components of membrane phospholipids in animals and humans (28, 39). These phospholipids not only play an important structural role but also mediate cell signaling (11, 39, 40). Interestingly, insulin stimulates production of inositol phosphoglycans (IPG) (21, 29, 36). IPG is believed to act as an intracellular second messenger to mediate insulin metabolic action (21, 23). Moreover, chiro-inositol levels are decreased in type 2 diabetes (3), and increased myo-inositol/chiro-inositol ratios are associated with type 2 diabetes and insulin resistance in both animals and humans (20, 23). D-chiro-inositol and 3-O-methyl-D-chiro-inositol (D-pinitol) have been reported to act as an insulin mimetic to improve hyperglycemia in both mice and humans with type 2 diabetes (4, 7, 10, 19, 35). It is important to identify additional inositol
derivatives, to evaluate their therapeutic potentials, and to elucidate the cellular mechanisms of their actions.

In this study, we have identified 5-O-methyl-myoinositol (sequoyitol) as a new natural compound with anti-diabetic properties. Sequoyitol is a herbal constituent. Both oral and subcutaneous administrations of sequoyitol ameliorate hyperglycemia and glucose intolerance in ob/ob mice with insulin resistance. Chronic sequoyitol treatments also improve hyperglycemia and glucose intolerance in streptozocin (STZ)-treated mice with insulin deficiency. Additionally, sequoyitol directly improves insulin sensitivity in cultured hepatocytes, adipocytes and β cells, and protects β cells against oxidative injury. These results suggest that sequoyitol has a therapeutic potential for the treatment of diabetes.

Research Design and Methods

Animals. ob/ob and C57BL/6 mice were purchased from the Jackson laboratory (Bar Harbor, ME). Mice were housed on a 12-h light/12-h dark cycle in the Unit for Laboratory Animal Medicine at the University of Michigan or in the animal facility at the Institute for Nutritional Sciences, SIBS, CAS, Shanghai, China. Mice were fed a normal chow ad libitum with a free access to water. Animal experiments were conducted following the protocols approved by the University Committee on the Use and Care of Animals at the University of Michigan Medical School and by the Institutional Animal Care and Use Committees at the Institute for Nutritional Sciences, SIBS, CAS.

Sequoyitol administration, glucose tolerance tests (GTT), and insulin tolerance tests (ITT). For oral administration, ob/ob mice (8-9 weeks) received sequoyitol (98% purity, Xiangbei Welman Pharmaceutical Co., Ltd, Changsha, China) by gastric gavage (40 mg/kg body weight, twice daily). For subcutaneous administration, ob/ob mice (10 weeks) were anesthetized with 2-4% isoflurane, and osmotic minipumps (model 2002; Alzet, Cupertino, CA) were implanted subcutaneously. Minipumps were prefilled with either a sterile 0.9% NaCl vehicle or sequoyitol. A sequoyitol-filled pump released sequoyitol at 0.5 nmol/h. For streptozotocin (STZ) treatments, C57BL/6 males (9 weeks) were intraperitoneally injected with STZ (80 mg/kg body weight once a day for 2 days). The mice were fed either tap water (control) or water supplemented with sequoyitol (7 mg/ml). Blood samples were
collected from tail veins, and blood glucose and plasma insulin were measured as described previously (31). For GTT, mice were fasted overnight (or 6 h) and intraperitoneally injected with D-glucose (0.6-0.8 g/kg body weight), and blood glucose levels were monitored after glucose injection. For ITT, mice were fasted for 5-6 h and intraperitoneally injected with human insulin and blood glucose levels were monitored after glucose injection.

**Immunoprecipitation and immunoblotting.** Mice were fasted overnight, anesthetized, and administrated with insulin via inferior vena. Livers were homogenized in a lysis buffer (50 mM Tris HCl, pH 7.5, 1.0% NP-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na$_2$VO$_4$, 100 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) as described previously (32, 44). Liver extracts were immunoprecipitated and immunoblotted with the indicated antibodies. Liver extracts were also immunoblotted with antibodies against phospho-Akt pSer$^{473}$ (Cell Signaling) and pThr$^{308}$ (Santa Cruz) or total Akt (Santa Cruz). HepG2 cells, 3T3-L1 adipocytes, and INS-1 cells were treated with the indicated ligands. Cell extracts were prepared and subjected to immunoprecipitation and immunoblotting assays.

**Adipocyte glucose uptake.** 3T3-L1 preadipocytes were differentiated into adipocytes as described previously (9). The adipocytes were incubated with sequoyitol for 6 or 12 h and subjected to glucose uptake assays using 2-[³H]deoxy-D-glucose (9). C57BL/6 males were fed a high fat diet (45% fat) for 8 weeks, and epididymal fat depots were isolated, minced, and digested in 1 mg/ml Type II collagenase (Worthington Biochem, Lakewood, NJ) at 37°C for 40 min. The adipocytes were dispersed, filtered through 2 layers of cloth, washed 3 times with Krebs-Ringer buffer (KRB) (130 mM NaCl, 5 mM KCl, 1.3 mM CaCl$_2$, 1.3 mM MgSO$_4$, 25 mM HEPES, pH 7.4) containing 4% bovine serum albumin (BSA), and cultured in KRB with 4% BSA. Primary adipocytes were treated with sequoyitol (100 μM) for 3 h and stimulated with 5 nM insulin for 30 min; 2-[³H]deoxy-D-glucose was added during the last 10 min incubation. To stop glucose uptake, adipocytes were mixed with dinonyl phthalate oil (Sigma-Aldrich, St Louis, MO) and centrifuged at 10,000 rpm for 1 min. Adipocytes were on the top of the organic phase and
transferred into a scintillation vial and solubilized in 0.5% SDS buffer. 2-[3H]deoxy-D-glucose uptake was determined by scintillation counting and normalized to total adipocyte numbers.

**Primary hepatocyte glucose production:** Primary hepatocytes were prepared by liver-perfusion with type II collagenase, grown on collagen-coated plates, and subjected to glucose production assays as described previously (9, 44). Briefly, primary hepatocytes were pretreated with sequoyitol (100 μM) or vehicles overnight, and subsequently incubated for 4 h in an assay buffer (118 mM NaCl, 2.5 mM CaCl₂, 4.8 mM KCl, 25 mM NaHCO₃, 1.1 mM KH₂PO₄, 1.2 mM MgSO₄, 10 μg ZnSO₄, 0.6% BSA, 10 mM HEPES, 5 mM lactate, 5 mM pyruvate, and pH 7.4). To increase glucose production, cells were treated with 10 μM N⁶,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (DB-cAMP) and 100 nM dexamethasone; to decrease glucose production, insulin was added into the assay buffer. Glucose in culture medium was measured and normalized to total protein levels, and the normalized values were used as an index to estimate glucose production.

**INS-1 cell viability:** Rat INS-1 cells were grown in RPMI-1640 supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium-pyruvate and 0.05 mM β-mercaptoethanol as described previously (14). Cells were pretreated with or without sequoyitol (0, 5, or 10 mg/ml) for 6 h, and then treated with STZ (0.5 mM) or H₂O₂ (10 μM) in the presence or absence of sequoyitol for additional 12 h. Cell viability was measured using 3 (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assays.

**Statistical Analysis.** Data were presented as means ± SEM. Differences between groups were analyzed by two-tailed Student’s t test. P< 0.05 was considered statistically significant.

**Results**

**Subcutaneous administration of sequoyitol improves insulin resistance and glucose intolerance in ob/ob mice.** We examined the anti-diabetic effect of herbal extracts and identified sequoyitol as an insulin sensitizer. Sequoyitol is a 5-O-methyl derivative of myo-inositol (Fig. 1A). To determine whether sequoyitol reduces blood glucose under insulin resistant conditions, it was
subcutaneously administrated into ob/ob male mice (10 weeks) via osmotic minipumps (0.5 nmol/h) for 23 days. ob/ob mice are deficient of leptin and commonly used as a genetic model of obesity and type 2 diabetes. Sequoyitol treatments did not alter body weights (Fig.1B). Fourteen days after treatments, blood glucose levels were significantly lower in sequoyitol-treated than in vehicle-treated mice (Fig. 1C).

Sequoyitol treatments also significantly reduced plasma insulin levels (by 29%) (Fig. 1C). HOMA index, a commonly used parameter to estimate insulin sensitivity, was reduced by 44% in sequoyitol-treated mice than in vehicle-treated mice (saline: 76.1 ± 6.5, n=6; sequoyitol: 42.3 ± 16.0, n=7; p=0.0006). To further analyze insulin sensitivity and glucose metabolism, we performed glucose tolerance tests (GTT) (17 days after treatments) and insulin tolerance tests (ITT) (10 days after treatments). Sequoyitol treatments significantly reduced blood glucose levels 30 min after glucose injection during GTT, and AUC decreased by 34% in sequoyitol-treated mice (Fig. 1D). Sequoyitol treatments also increased the ability of insulin to reduce blood glucose levels, and AUC decreased by 15% in sequoyitol-treated mice (Fig. 1E). These results demonstrate that chronic sequoyitol treatments ameliorate hyperglycemia, hyperinsulinemia, insulin resistance, and glucose intolerance in mice with obesity and insulin resistance.

**Subcutaneous administration of sequoyitol enhances insulin signaling in ob/ob mice.** To determine whether sequoyitol treatments enhance insulin signaling, we examined insulin-stimulated phosphorylation of insulin receptors (IR), IRS1, Akt (pSer<sup>473</sup> and pThr<sup>308</sup>), and ERK1/2 in the liver. ob/ob mice were chronically treated with saline or sequoyitol (0.5 nmol/h) via osmotic minipumps for 23 days. Mice were fasted overnight and treated with insulin (3 units/kg body weights) for 5 min, and liver extracts were immunoprecipitated with antibodies against IR and IRS1 and immunoblotted with anti-phosphotyrosine (αPY), IR or IRS1 antibodies. In parallel experiments, liver extracts were immunoblotted with the indicated antibodies. In vehicle-treated control mice, insulin stimulated tyrosine phosphorylation of IR, modestly stimulated tyrosine phosphorylation of IRS1 and Ser<sup>473</sup>/Thr<sup>308</sup> phosphorylation of Akt, but did not stimulate ERK phosphorylation, in agreement with insulin resistance in these mice (Fig. 2A).

Sequoyitol treatments significantly increased the ability of insulin to stimulate Akt phosphorylation (Fig. 2A). Ser<sup>473</sup> and Thr<sup>308</sup> phosphorylation was 68% and 118% higher in sequoyitol-treated mice than in
vehicle-treated mice, respectively (Fig. 2B). Sequoyitol also modestly increased insulin-stimulated
tyrosine phosphorylation of IRS1 and ERK phosphorylation (Figs. 2A-B). Ser^{307} phosphorylation of IRS1
is believed to negatively regulate insulin sensitivity (2, 33). To measure Ser^{307} phosphorylation, mice
were treated with saline or sequoyitol for 23 days as described above, and liver extracts were
immunoprecipitated with anti-IRS1 antibody and immunoblotted with anti-phospho-IRS1(pSer^{307})
antibody. Sequoyitol treatments significantly decreased Ser^{307} phosphorylation of IRS1 (Fig. 2C). These
data suggest that sequoyitol acts as an insulin sensitizer to improve glucose metabolism in ob/ob mice.

**Oral administration of sequoyitol improves insulin sensitivity and glucose metabolism in ob/ob mice.** To verify the anti-diabetic effect of sequoyitol, ob/ob male and female mice (8-9 weeks) were
administrated with sequoyitol (40 mg/kg body weight, twice daily) by oral gavage. Sequoyitol treatments
(17 days) did not alter body weight in either males (Con: 45.7 ± 0.7 g, n=5; Seq: 47.4 ± 0.6, n=5; p=0.1026) or females (Con: 44.7 ± 1.1 g, n=5; Seq: 43.9 ± 1.3, n=6; p=0.6286). As expected, sequoyitol
treatments (18 days) reduced blood glucose in both males and females (Fig. 3A). Oral administration of
sequoyitol also markedly improved glucose intolerance during GTT (Fig. 3B). Surprisingly, plasma
insulin levels were similar between control and treated groups in both males (Con: 4.4 ± 0.4 ng/ml, n=5; Seq: 5.1 ± 0.1, n=5; p=0.173) and females (Con: 4.9 ± 0.1 ng/ml, n=5; Seq: 4.7 ± 0.2, n=6; p=0.483).
These data suggest that blood insulin levels do not contribute to the hypoglycemic effect of sequoyitol
under these conditions. In ITT, blood glucose levels were significantly lower 15, 30, and 60 min after
insulin injection in both males and females (Fig. 3C). These results suggest that sequoyitol has a potential
to be used as an oral diabetes medication to improve hyperglycemia and glucose intolerance.

**Sequoyitol directly enhances insulin signaling and suppresses glucose production in hepatocytes.** To determine whether sequoyitol directly targets hepatocytes, we examined the effect of
sequoyitol on insulin signaling in HepG2 cells, human hepatoblastoma cells. HepG2 cells were pretreated
with or without sequoyitol and subsequently stimulated with insulin. Cell extracts were
immunoprecipitated with anti-IRS1 antibody and immunoblotted with anti-phospho-tyrosine antibody.
Cell extracts were also immunoblotted with anti-phospho-Akt (pSer^{473}) antibody. Insulin stimulated
phosphorylation of IRS1 and Akt in control cells, and sequoyitol further enhanced insulin-stimulated phosphorylation of both IRS1 and Akt (Fig. 4A). We quantified IRS1 and Akt phosphorylation and observed that sequoyitol increased insulin-stimulated phosphorylation of IRS1 by 114% and Akt by 54% (Fig. 4B). Because tumor necrosis factor (TNF)α contributes to insulin resistance in obesity (1, 15, 16, 30, 37, 38), we examined the possibility that sequoyitol may counteract TNFα-induced insulin resistance. HepG2 cells were pretreated with TNFα in the presence or absence of sequoyitol, and the pretreated cells were stimulated with insulin. TNFα suppressed insulin-stimulated tyrosine phosphorylation of insulin receptors (IR) and IRS1 and Akt phosphorylation as expected (Fig. 4C, lanes 5 vs 2). Sequoyitol significantly increased insulin-stimulated phosphorylation of IR (by 46%), IRS1 (by 48%), and Akt (pSer473) (by 61%) (Fig. 4C). We also examined the effect of myo-inositol, a potential derivative of sequoyitol in vivo, on insulin signaling in hepatocytes. Like sequoyitol, myo-inositol pretreatments also improved insulin-stimulated tyrosine phosphorylation of IR and IRS1 and Ser473 phosphorylation of Akt and counteracted TNFα inhibition of insulin signaling (Fig. 4D). These data suggest that sequoyitol and its derivatives sensitize insulin responses in hepatocytes.

To determine whether sequoyitol suppresses hepatic glucose production, primary hepatocytes were pretreated without or with sequoyitol (100 μM) for 16 h and subsequently subjected to glucose production assays. Basal glucose production was similar between control and sequoyitol-pretreated cells (Fig. 4E). DB-cAMP, a cAMP analog, stimulated glucose production by 49% in control cells but only by 19% in sequoyitol-pretreated hepatocytes (Fig. 4E). These data indicate that sequoyitol is able to directly suppress hepatic glucose production in an insulin-independent manner. Either sequoyitol or insulin alone partially suppressed glucose production in DB-cAMP-stimulated hepatocytes; however, sequoyitol and insulin in combination completely inhibited DB-cAMP-stimulated glucose production (Fig. 4E). These data suggest that sequoyitol and insulin act additively or synergistically to suppress hepatic glucose production.
Sequoyitol directly enhances insulin signaling and glucose uptake in adipocytes. To determine whether sequoyitol directly targets adipocytes, 3T3-L1 adipocytes were pretreated with or without sequoyitol prior to insulin stimulation. Insulin stimulated tyrosine phosphorylation of IR and IRS1 in control cells, and sequoyitol further increased insulin-stimulated phosphorylation of IR by 31% and IRS1 by 73% (Figs. 5A-B). To determine whether sequoyitol counteracts TNFα inhibition of insulin signaling, 3T3-L1 adipocytes were pretreated with TNFα in the presence or absence of sequoyitol. TNFα inhibited insulin signaling in control cells as expected (Fig. 5C, lanes 5 vs 2). Sequoyitol increased insulin-stimulated phosphorylation of IR by 53%, IRS1 by 69%, and Akt by 74% in TNFα-treated 3T3-L1 adipocytes (Fig. 5C). Similarly, myo-inositol, a potential sequoyitol derivative, also enhanced insulin-stimulated phosphorylation of IR, IRS1 and Akt and counteracted TNFα inhibition of insulin signaling in adipocytes (Fig. 5D). These data suggest that sequoyitol and its derivatives directly enhance insulin sensitivity in adipocytes.

To determine whether sequoyitol regulates glucose uptake, 3T3-L1 adipocytes were pretreated with sequoyitol for 6 or 12 h prior to insulin stimulation. Insulin stimulated glucose uptake in control adipocytes as expected, and sequoyitol further increased insulin-stimulated glucose uptake by 34% and 81% 6 h and 12 h after pretreatments, respectively (Fig. 5E). These data provide additional evidence supporting sequoyitol as an insulin sensitizer. Prolonged sequoyitol treatments (12 h) alone also increased basal glucose uptake (Fig. 5E). In agreement, sequoyitol increased both basal and insulin-stimulated glucose uptake in mouse primary adipocytes (Fig. 5F). These data suggest that sequoyitol promotes glucose uptake into adipocytes by both insulin-dependent and insulin-independent mechanisms.

Sequoyitol protects islet β cells against oxidative injury. To determine whether sequoyitol directly targets β cells, INS-1 cells, derived from rat β cells, were pretreated without (control) or with sequoyitol prior to STZ (0.5 mM) or H₂O₂ (10 μM) treatments, and cell viability was measured using MTT assays. Both STZ and H₂O₂ markedly reduced the viability of control INS-1 cells, and sequoyitol dose-dependently increased the viability of STZ- and H₂O₂-treated INS-1 cells (Fig. 6A). These data
indicate that sequoyitol protects against β cell injury and death under oxidative stress conditions. To determine whether sequoyitol improves insulin signaling in INS-1 cells, INS-1 cells were pretreated with or without 10 mg/ml sequoyitol for 6 h prior to insulin stimulation (50 nM for 15 min). Sequoyitol pretreatments increased the ability of insulin to stimulate phosphorylation of both IR and Akt (pSer473) in three repeated experiments (Fig. 6B). To determine whether sequoyitol improves β cell function in animals, C57BL/6J males were injected with STZ, a β cell toxin, and divided into control and sequoyitol-treated groups. Sequoyitol was administrated in drinking water (70-100 mg kg⁻¹ day⁻¹). Body weights were similar between these two groups during the treatments (Fig. 7A). STZ treatments progressively increased blood glucose in the control group, and sequoyitol markedly attenuated STZ-induced hyperglycemia in the sequoyitol-treated group (Fig. 7B). Blood glucose levels decreased by 26% 31 days after sequoyitol treatments. Sequoyitol also increased plasma insulin levels by 155% (Fig. 7C) and significantly improved glucose intolerance (Fig. 7D). These data raise the possibility that sequoyitol may protects against β cell injury.

**Discussion**

Herbal extracts have been reported to reduce blood glucose in animals (6, 8, 17, 22, 41); however, these extracts are extremely complex and the leading compounds that exert anti-diabetic effects remain poorly understood. The cellular targets of these herbal extracts and the molecular basis of their actions are unknown. Lack of these important information limits our ability to use herbal therapies to treat diabetes. In this work, we have identified sequoyitol, a natural compound present in many plants (e.g. *Amentotaxus yunnanensis*, *Aristolochia arcuata*, and *Crossostephium chinese* (12, 24, 43), as a novel anti-diabetic small molecule (molecular weight: 194).

We demonstrated that chronic administration of sequoyitol significantly improved hyperglycemia and glucose intolerance in *ob/ob* mice. Sequoyitol treatments did not alter body weights, indicating that the improvement in metabolism is unlikely to be secondary to an alteration in energy balance and adiposity. In agreement, sequoyitol not only suppressed glucose production in primary hepatocytes but
also promoted glucose uptake in both 3T3-L1 adipocytes and primary adipocytes. These observations
suggest sequoyitol attenuates hyperglycemia and glucose intolerance in type 2 diabetes at least in part by
directly targeting hepatocytes and adipocytes and improving the metabolic function of these two cell
types. Sequoyitol is water-soluble; additionally, both oral and subcutaneous administration of sequoyitol
improves hyperglycemia and glucose intolerance. These properties make sequoyitol an appealing oral
diabetes medication candidate.

To gain insight into the molecular mechanisms of sequoyitol action, we examined insulin signaling. We
observed that sequoyitol directly enhanced insulin-stimulated phosphorylation of IR, IRS1, and Akt in
hepatocytes, adipocytes, and/or INS-1 β cells. It also counteracted TNFα inhibition of insulin signaling
in hepatocytes and adipocytes. Subcutaneous administration of sequoyitol improved insulin signaling in
ob/ob mice. Chronic sequoyitol treatments reduced hyperinsulinemia and increased the hypoglycemic
effect of insulin in these mice. These observations suggest that sequoyitol ameliorates hyperglycemia in
part by functioning as an insulin sensitizer. The direct molecular targets of sequoyitol are currently
unknown. Sequoyitol may serve as a precursor of inositolts, phosphatidylinositols, and other inositol
derivatives, and these inositol derivatives may mediate sequoyitol’s anti-diabetic effect. Indeed, like
sequoyitol, myo-inositol enhanced insulin signaling in both hepatocytes and adipocytes.

Sequoyitol alone suppressed glucose production in primary hepatocytes and stimulated basal
glucose uptake in adipocytes, suggesting that sequoyitol is able to improve hyperglycemia and glucose
intolerance in ob/ob mice by an insulin-independent mechanism. Interestingly, sequoyitol alone does not
stimulate Akt phosphorylation, suggesting that the Akt pathway may not mediate the sequoyitol action
under these conditions. Inositol phosphoglycans (IPGs), endogenous inositol derivatives, are believed to
act as intracellular mediator of insulin metabolic action (7, 21, 23, 29, 36). IPGs appear to regulate
glucose metabolism by activating pyruvate dehydrogenase phosphatases and protein phosphotase 2C (5,
13, 23, 25, 27). Sequoyitol (5-O-methyl-myo-inositol) may promote IPG production by serving as a
precursor or a regulator. Additionally, hyperglycemia is associated with both deficiency of chiro-inositol
and increased myo-inositol/chiro-inositol ratios (3, 7, 20, 23). Sequoyitol may promote chiro-inositol
production and decrease myo-inositol/chiro-inositol ratios, thus improving hyperglycemia and glucose
tolerance in mice with obesity.

Sequoyitol also improved hyperglycemia and glucose intolerance in STZ-treated mice. STZ
promotes hyperglycemia and glucose intolerance by destroying islet β cells. Sequoyitol treatments
markedly increased plasma insulin levels (by 155%), suggesting that sequoyitol preserves β cell viability
and/or function in vivo. In agreement, sequoyitol enhanced insulin signaling in INS-1 cells and directly
protected against STZ- and H2O2-induced INS1 β cell death. In supporting these findings, a herbal
extract containing sequoyitol has been reported to improve insulin secretion from rat islets (45). The
molecular basis of sequoyitol’s cytoprotection is unclear. D-pinitol, another methyl derivative of inositol,
acts as an antioxidant to protect against hepatocyte injury (35). Sequoyitol may similarly protect β cells
from oxidative injury by acting as an antioxidant.

In summary, we have identified sequoyitol as a new herbal constituent with an anti-diabetic
property. Both oral and subcutaneous administrations of sequoyitol improve hyperglycemia and glucose
intolerance in both ob/ob and STZ-treated mice. Sequoyitol directly improves glucose metabolism in
hepatocytes and adipocytes by both insulin-dependent and insulin-independent mechanisms. It also
protects β cells from oxidative injury. Thus, sequoyitol has a therapeutic potential and may serve as an
oral diabetes medication.

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Disclosures

Ting Wang is an employee of Xiangbei Welman Pharmaceutical Co., Ltd. which provides sequoyitol. The other authors have nothing to declare.

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Fig. 1. Subcutaneous administration of sequoyitol decreases blood glucose and plasma insulin and improves glucose intolerance in \textit{ob/ob} mice. \textbf{A}: sequoyitol structure. \textbf{B}: \textit{ob/ob} males (10 weeks) were subcutaneously administrated with sequoyitol (0.5 nmol/h) (n=7) or a saline vehicle (n=6) via osmotic minipumps. Body weights were monitored daily. \textbf{C}: Fasting blood glucose and plasma insulin levels 14 days after sequoyitol treatments. \textbf{D}: GTT. Mice (17 days after treatments) were fasted for 16 h and intraperitoneally injected with D-glucose (0.8 g/kg body weight). Blood glucose levels were monitored. a.u.: arbitrary units. \textbf{E}: ITT. Mice (10 days after treatments) were fasted for 5 h and intraperitoneally injected with insulin (4 units/kg body weight). Blood glucose levels were measured and normalized to initial values. Error bars represent SEM. *\(P < 0.05\).

Fig. 2. Sequoyitol improves insulin signaling in \textit{ob/ob} mice. \textit{ob/ob} mice (10 weeks) were subcutaneously administrated with sequoyitol (0.5 nmol/h) or saline via osmotic minipumps. Twenty-three days after treatments, mice were fasted overnight and treated with insulin (3 units/kg body weight for 5 min) via inferior vena. \textbf{A}: Liver extracts were immunoprecipitated with anti-IR and anti-IRS1 antibodies and immunoblotted with anti-phospho-tyrosine (\(\alpha\)PY), IR, or IRS1 antibodies. In parallel, liver extracts were immunoblotted with anti-phospho-Akt (\(\alpha\)pSer473 or \(\alpha\)pThr308), Akt, phospho-ERK, and ERK antibodies. \textbf{B}: Akt or ERK phosphorylation was quantified using densitometry and normalized to total Akt or ERK levels (Con: n=3; Seq: n=3). \textbf{C}: Liver extracts (from the insulin treated groups) were immunoprecipitated with anti-IRS1 antibody and immunoblotted with anti-phospho-IRS1 (pSer\textsuperscript{307}) or anti-IRS1 antibodies. Phosphorylation of Ser\textsuperscript{307} was quantified and normalized to total IRS1 levels. Error bars represent SEM. *\(P < 0.05\).

Fig. 3. Oral administration of sequoyitol improves hyperglycemia and glucose intolerance in \textit{ob/ob} mice. \textit{ob/ob} mice (8-9 weeks) were administrated by oral gavage without (males: n=5; females: n=5) or with sequoyitol (40 mg/kg body weight, twice daily; males: n=5; females: n=6). \textbf{A}: Fasting (6 h) blood glucose 18 days after treatments. \textbf{B}: GTT. Mice (31 days after treatments) were fasted for 6 h and intraperitoneally injected with D-glucose (0.75 g/kg body weight). Blood glucose levels were monitored.
C. ITT. Mice (27 days after treatments) were fasted for 6 h and intraperitoneally injected with insulin (1.5 units/kg body weight). Blood glucose levels were measured. Error bars represent SEM. * \( P < 0.05 \), ** \( P < 0.01 \).

**Fig. 4. Sequoyitol enhances insulin signaling and suppresses glucose production in hepatocytes.**

A: HepG2 cells were pretreated without or with sequoyitol (100 \( \mu \)M) for 12 h prior to insulin (10 nM) stimulation (5 min). Cell extracts were immunoprecipitated with \( \alpha \)IRS1 and immunoblotted with \( \alpha \)PY.

The blots were reprobed with \( \alpha \)IRS1. Cell extracts were also immunoblotted with \( \alpha \)pSer473 or \( \alpha \)Akt.

B: Phosphorylated IRS1 and Akt were quantified by densitometry and normalized to total IRS1 and Akt levels, respectively (Con: n=3; Seq: n=3). a.u.: arbitrary units.

C: HepG2 cells were treated with or without sequoyitol (100 \( \mu \)M) in the presence or absence of TNF\( \alpha \) (10 ng/ml) for 12 h, and then stimulated with insulin (10 nM for 5 min). Cell extracts were immunoprecipitated with \( \alpha \)IR and \( \alpha \)IRS1 and immunoblotted with \( \alpha \)PY. The same blots were reprobed with \( \alpha \)IR or \( \alpha \)IRS1, respectively. Cell extracts were also immunoblotted with \( \alpha \)pSer473 or \( \alpha \)Akt. IR, IRS1, and Akt phosphorylation was quantified and normalized to total IR, IRS1, and Akt protein levels, respectively (Con: n=3; Seq: n=3).

D: HepG2 cells were pretreated with myo-inositol (100 \( \mu \)M) in the presence or absence of TNF\( \alpha \) (10 ng/ml) for 12 h, and then stimulated with insulin (10 nM for 5 min). Cell extracts were immunoprecipitated with \( \alpha \)IR and \( \alpha \)IRS1, and immunoblotted with \( \alpha \)PY, \( \alpha \)IR, or \( \alpha \)IRS1, respectively. Cell extracts were also immunoblotted with \( \alpha \)pSer473 or \( \alpha \)Akt.

E: Primary hepatocytes were prepared from C57BL/6 males, treated without or with sequoyitol (100 \( \mu \)M) overnight, stimulated with a vehicle, DB-cAMP (10 \( \mu \)M), or DB-cAMP plus insulin (100 nM), and subjected to glucose production assays. Glucose production was normalized to total hepatocyte protein levels (Con: n=4; Seq: n=4). Each experiment was performed three times. Data are presented as mean ± SEM. * \( p<0.05 \).

**Fig. 5. Sequoyitol enhances insulin signaling and increases glucose uptake in adipocytes.**

A: 3T3-L1 preadipocytes were fully differentiated into adipocytes (day 8). The adipocytes were pretreated without or with sequoyitol (100 \( \mu \)M) for 12 h and then stimulated with insulin (10 nM) for 5 min. Cell extracts were
immunoprecipitated with αIR or αIRS1 and immunoblotted with αPY. The same blots were reprobed with αIR or αIRS1. B: Phosphorylated IRS1 and Akt were quantified by densitometry and normalized to total IRS1 and Akt levels, respectively (Con: n=3; Seq: n=3). C: 3T3-L1 adipocytes were treated with or without sequoyitol (100 μM) in the presence or absence of TNFα (10 ng/ml) for 12 h, and then stimulated with insulin (10 nM) for 5 min. Cell extracts were immunoprecipitated with αIR and αIRS1 and immunoblotted with αPY, αIR, or αIRS1, respectively. Cell extracts were also immunoblotted with αpSer473 or αAkt. IR, IRS1, and Akt phosphorylation was quantified and normalized to total IR, IRS1, and Akt protein levels, respectively (Con: n=3; Seq: n=3). D: 3T3-L1 adipocytes were treated with or without myo-inositol (100 μM) in the presence or absence of TNFα (10 ng/ml) for 12 h, and then stimulated with insulin (10 nM) for 5 min. Insulin signaling was examined as described in C. E: 3T3-L1 adipocytes were pretreated without or with sequoyitol (100 μM) for 6 or 12 h. The cells were subsequently stimulated with or without insulin (10 nM) and subjected to glucose uptake assays. F: Primary adipocytes were isolated from C57BL/6 males fed a high fat diet for 8 weeks, treated without or with sequoyitol (100 μM) for 3 h, stimulated with or without insulin (5 nM), and subjected to glucose uptake assays. Each experiment was performed three times. Data are presented as mean ± SEM. *p<0.05.

Fig. 6. Sequoyitol protects against STZ- and H2O2-induced INS1 β cell death. A: INS-1 β cells were incubated with sequoyitol for 3 h, and then with STZ or H2O2 in the presence of sequoyitol for additional 12 h. Cell viability was measured using MTT assays (n=4 for each group). B: INS-1 cells were deprived of serum overnight, pretreated with or without 10 mg/ml sequoyitol for 6 h, and then stimulated with 50 nM insulin for 15 min. Cell extracts were immunoprecipitated with αIR and immunoblotted with αPY or αIR. Cell extracts were also immunopblotted with αpSer473 or αAkt. Data are presented as mean ± SEM. *p<0.05.

Fig. 7. Sequoyitol decreases hyperglycemia and glucose intolerance and increases plasma insulin in STZ-treated mice. C57BL/6 males (9 weeks) were intraperitoneally injected with STZ (160 mg/kg body weight) and randomly divided into control and sequoyitol-treated groups. Cont (n=8): mice had a free
access to water; Seq (n=8): mice had a free access to water supplemented with sequoyitol (7 mg/ml). A: Growth curves. B: Randomly-fed blood glucose (10:00-11:00 am). C: Plasma insulin levels 14 days after sequoyitol treatments (Con: n=7; Seq: n=7). D. GTT (glucose: 0.6 g/kg body weight) 29 days after sequoyitol treatments. Data are presented as mean ± SEM. *p<0.05.
Shen H., Fig. 2

A

IP: αIR

αPY

αIR

αPython

αIRS1

IP: αIRS1

αPY

αIR

αPython

αIRS1

Blot

Phosphorylation (a.u.)

Con

Seq

Insulin:

Con

Seq

B

pAkt (pThr308)

pAkt (pSer473)

pERK1/2

Con

Seq

Phosphorylation (a.u.)

C

IP: αIRS1

αSer307

αIRS1

Phospho-Ser307 (a.u.)

Con

Seq

*
Shen H., Fig. 3

A

Males

Females

B

GTT

C

ITT

Blood Glucose (mg/dl)

Time (min): 0 15 30 60 90
Glucose Production (µg/mg/h)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Basal</th>
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<tr>
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<td>50</td>
<td>60</td>
<td>70</td>
</tr>
</tbody>
</table>

Shen H., Fig. 4
Shen H., Fig. 5

**A**
- IP: aIRS1
- Blot: aPY, aR
- Insulin: - + - +
- Seq: - +

**B**
- Con Seq
- piR piRS1
- Phosphorylation (a.u.)

**C**
- IP: aR
- Blot: aPY, aR
- IP: aIRS1
- Blot: aPY, aIRS1
- Lysates
- aPY aR aIRS1 aAkt
- Phosphorylation (a.u.)

**D**
- IP: aR
- Blot: aPY, aR
- IP: aIRS1
- Blot: aPY, aIRS1
- Lysates
- Myo-inositol: - - + + + +
- Insulin: - + + + + +
- TNFα: - - - - + +

**E**
- Con Seq (6 h) Seq (12 h)
- Glucose uptake (pmol/mg/min)

**F**
- Con Seq
- Glucose uptake (10^-7 pmol/cell/min)
Figure 6

A. INS-1 Cell Viability (%)

B. Western Blot Analysis

Seq (mg/ml): 0 0 5 10
STZ: 0.5 mM
H₂O₂: 10 μM

* indicates statistically significant differences.