X-3, a mangiferin derivative, stimulates AMP-activated protein kinase and reduces hyperglycemia and obesity in db/db mice

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ARTICLE INFO

Article history:
Received 12 May 2014 
Received in revised form 12 January 2015 
Accepted 6 February 2015 
Available online 11 February 2015

Keywords: 
AMP-activated protein kinase 
Animal experiment 
Anti-diabetic 
Compounds

Diabetes mellitus is a major health concern, affecting nearly 10% of the population. Here we describe a potential novel therapeutic agent for this disease, X-3, a derivative of mangiferin. Therapeutic administration of X-3 significantly and dose-dependently reduced plasma glucose and triglycerides in db/db mice following 8 week-treatments. Treatment with X-3 dose-dependently increased the number of insulin-positive β-cell mass. Importantly, X-3 did not cause any death or signs of toxicity in acute toxicity studies. Study of mechanism of action revealed that X-3 increased glucose uptake in parallel with increased phosphorylation of AMP-activated protein kinase (AMPK) in 3T3-L1 cells. It activates AMPK in both LKB1-dependent and -independent manner. Furthermore, administration of X-3 resulted in activation of AMPK and its downstream target, acetyl-CoA carboxylase (ACC) in the hypothalamus, liver, muscle and adipose tissues of C57BL/6 mice. An 80 mg/kg X-3 was more potent than metformin at 500 mg/kg in the hypothalamus, and interscapular fat tissues, potent than MF at the same dose in the liver. Thus, we conclude that X-3 is a promising new class of AMPK activating drug, and can potentially be used in the treatment of type 2 diabetes.

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1. Introduction

Mangiferin (MF) is a xanthonoid found in mangoes and Anemarrhena asphodeloides rhizomes (Miura et al., 2001). Mangiferin has been used in India for the treatments of arteriosclerosis, coronary heart disease and diabetes. It has been shown that MF exhibits antidiabetic (Ichiki et al., 1998; Muruganandan et al., 2005; Yoshikawa et al., 2001), hypolipidemic and antiatherogenic properties (Guo et al., 2011; Muruganandan et al., 2002, 2005) by reducing plasma total cholesterol, triglycerides, low density lipoprotein-cholesterol (LDL-C) and increasing high density lipoprotein (HDL-C) (Muruganandan et al., 2005). Niu et al. has recently reported that MF decreased plasma FFA in hyperlipidemic rats and activated AMPK in liver, whereas there is no sufficient evidence to show that MF could alone activate AMPK at the cellular level (Niu et al., 2012). Moreover the major shortcomings of MF are its poor solubility and oral bioavailability (Cai et al., 2010).

AMPK is a major cellular energy sensor and a master regulator of metabolic homeostasis (Viollet et al., 2009; Zhang et al., 2009). AMPK is a heterotrimeric protein kinase consisting of a catalytic (α) and two regulatory subunits (β and γ) (Hardie et al., 2006; Viollet et al., 2006). AMPK are activated by two distinct signals: a Ca 2+-dependent pathway mediated by calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) and an AMP-dependent pathway mediated by LKB1 (Sanders et al., 2007). Under conditions of energy depletion, AMPK inhibits ATP-consuming pathways (e.g., fatty acid synthesis, cholesterol synthesis, protein synthesis and gluconeogenesis) and stimulates ATP-generating processes (e.g., fatty acid oxidation and glycolysis), thus restoring overall cellular energy homeostasis (Carling, 2004; Zhang et al., 2009). In addition, AMPK activation acutely increases glucose uptake (via GLUT1 and GLUT4) and glycolysis (Hue et al., 2002; Jing and Ismail-Beigi, 2006; Jones and Dohm, 1997). In lipid metabolism, AMPK activation results in the phosphorylation and inactivation of ACC (Carling et al., 1987), a direct AMPK substrate, leading to decreased conversion of acetyl-CoA to malonyl CoA. Malonyl CoA allosterically inhibits carnitine palmitoyl-CoA transferase (CPT1), the rate-limiting step in transport of long chain acyl-CoAs into mitochondria for oxidation (Lochhead et al., 2000; McGarry and Brown, 1997). Therefore, a reduction in malonyl CoA levels increases fatty acid oxidation.

AMPK, independently of insulin, is able to phosphorylate Akt substrate AS160, while inhibition of AS160 is believed to allow increased GLUT4 membrane localization and glucose uptake (Sano et al., 2007).
In addition, AMPK inhibits the mTOR/p70S6K pathway and reduces IRS-1 phosphorylation on serine, resulting in the overphosphorylation of Akt and AS160, and then, increasing insulin sensitivity (Lansey et al., 2012; Sano et al., 2007; Wang et al., 2007).

Given the aforementioned critical actions of AMPK on glucose and lipid metabolism, targeting AMPK activation appears to be an attractive therapeutic strategy for the treatment of type 2 diabetes mellitus (T2DM) and related metabolic disorders. Two classes of commonly used insulin-sensitizing drugs, thiazolidinediones and biguanides, exert their therapeutic effects, at least in part, by activating AMPK (Nawrocki et al., 2010; Zhou et al., 2001). A number of natural products, including alkaloids, bitter melon extracts, berberine, and resveratrol, have been also found to activate AMPK (Hay and Sonenberg, 2004). Resveratrol has been shown to lower the blood sugar levels in both Phase Ib and Phase Ila clinical trials (Sirtris Pharmaceuticals, 2008, 2009).

In the current study we explored whether derivatization of MF, X-3, could improve in vivo effects in the treatment of insulin-resistant diabetic mice and delineated modes of action.

2. Materials and methods

2.1. Reagents

Mangiferin, 5-aminoimidazole-4-carboxamide ribonucleoside, Compound C, STO-609, and pioglitazone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Wortmannin was from Selleck Chemicals (Houston, TX, USA). AMPK, pAMPK (Thr 172), ACC, pACC (Ser 79), AKT, pAKT (Ser 473) antibodies were from Cell Signaling Technology (Danvers, MA, USA). GLUT1, GLUT4 antibodies were from Abcam PLC (Cambridge, UK).

2.2. Animal studies

Mouse experiments were conducted in 6- to 8-week-old female db/db (C57BL/KsJ) and db/+(C57BL/KsJ) mice (Qingzilan Technology Co. Ltd, Nanjing, China) and normal lean littermates (C57BL/6J). Experimental animals unless noted were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China). All animal procedures were performed in accordance with the guidelines of the institutional animal care and use committee of the Second Military Medical University. X-3 was administered once daily by oral gavage for 8 weeks at 40, 80, and 120 mg/kg, respectively. One percent carboxymethyl cellulose sodium in distilled water was used as the vehicle. Pioglitazone (Pio) 75 mg/kg/d was used as a positive control. Blood samples were obtained by tail snipping. Six-hour fasting plasma glucose, body weight, and food consumption were monitored weekly. Plasma insulin levels were measured by enzyme-linked immunosorbent assay using kits from Mercodia (St. Charles, MO). Serum non-esterified fatty acid (NEFA) was measured by tail bleeds.

2.3. Intraperitoneal glucose tolerance tests

Mice were fasted for 6 h before glucose tolerance tests. Intraperitoneal glucose load was administered at 2 g/kg of body weight. Glucose levels were measured by tail bleed.

2.4. Determination of serum and liver triglyceride content

Liver triglyceride content was assayed as described (Atkinson et al., 2003). Liver and serum triglyceride contents were quantified colorimetrically with the enzymatic assay kit L-Type Triglyceride M (Wako Pure Chemical Industries, Richmond, VA).

2.5. Determination of adipocyte size

Total adipocyte area was manually traced and analyzed with image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). White adipocyte area was measured in more than 200 cells per mouse in each group according to methods described previously (Kubota et al., 1999).

2.6. Immunohistochemistry

A commercial staining kit was utilized following the manufacturer's instructions (SuperPicture™ 3rd Gen IHC Detection Kit, Invitrogen). Primary antibodies: Guinea Pig anti-insulin (DAKO Co., Carpenteria, CA, USA) was used at 1:50 for immunohistochemistry. Morphometric evaluation of the β-cell area was performed on insulin-stained sections using Image-Pro Plus 6.0 as described (Conarello et al., 2003).

2.7. Acute Toxicity Study

Experiments were conducted in ICR mice (18–22 g, n = 20) and Wistar rats (180–220 g, n = 20), half male and half female. Mice were treated with X-3 (3.57 g/kg/d p.o.), rats were given X-3 (1.785 g/kg/d p.o.). Vehicle control animals were administered the same volume of 0.5% carboxymethyl cellulose sodium in distilled water. The treatment period is 7 days. All animals were observed daily for mortality and signs of toxicity such as changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, autonomic activity, changes in gait, posture and response to handling, as well as the bizarre behavior during the entire period of the study.

2.8. Cell lines and cell culture

Murine 3T3-L1 preadipocytes, LKB1-deficient Hela cells, and HEK293 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

2.9. The 3T3-L1 differentiation and Oil Red O staining

Culture and differentiation of 3T3-L1 cells were described previously (Choi et al., 2009). In brief, the cells were grown and maintained in high-glucose DMEM containing 10% FBS in a 5% CO2 environment. The cells were allowed to grow for 2 days postconfluence and then differentiated by the addition of IBMX (500 μM), dexamethasone (1 μM), and insulin (10 μg/ml) for 3 days. The medium was changed every 2 days (Supplementary Fig. S1).

Fat staining with Oil Red O was described previously (Choi et al., 2009). 3T3-L1 cells were fixed with 10% formaldehyde for 30 min and then stained with Oil Red O for 2 h, followed by washing with 60% methanol.

2.10. Glucose uptake assay

2-Deoxyglucose uptake was estimated in a 96-well plate by an enzymatic NADPH amplifying system assay (ab136955, Abcam, UK). Briefly, 3T3-L1 preadipocytes or adipocytes were serum starved for overnight, and then the cells were incubated with 100 μl KRPH buffer containing 2% BSA for 40 min, then treated with indicated compounds for 2 h at 37 °C. Compound C (CC, 40 μM) was added 30 min before the initiation of treatment. Wortmannin (W, 300 nM) was added 1 h before the end of the treatment. After the addition of 100 μl low-glucose DMEM (21885, Life Technologies) containing 1 mM 2-deoxyglucose for 20 min, cells were washed 3 times with PBS, and lysed to prepare an 50 μl reaction system. For 3T3-L1 preadipocytes, cell lysates were diluted 1:1, for adipocytes, they were diluted 1:10. After a series of reactions, samples were measured at
OD$_{412nm}$ A standard curve was generated by placing 2-DG6P standard solutions in wells of the culture plate that had been prepared without cells.

2.11. Cell viability assessment

Cell viability assessment was performed by using Cell Counting Kit (CCK-8), which was purchased from Dojindo Laboratory, Japan. 3T3-L1 preadipocytes or adipocytes were serum-starved overnight, and then treated with indicated compounds for 24 h. Cell viability assessment was guided by the CCK-8 technical manual (downloadable at http://www.dojindo.com/newimages/CCK-8TechnicalInformation.pdf).

2.12. Protein analysis

Western blot analysis were performed by standard methods with antibodies directed against total AMPK, phosphorylated AMPK, total ACC, phosphorylated ACC, total AKT, or phosphorylated AKT. The phospho-AMPK antibody detects endogenous AMPKα1 and α2 when phosphorylated at Thr172. For GLUT1 and GLUT4, cells in 6-well plate were serum starved overnight, then incubated with 1.5 ml KRPH buffer containing 2% BSA for 40 min, and then exposed to indicated compounds for 6 h or 24 h. After the addition of 1.5 ml low-glucose DMEM for 20 min, cells were washed 3 times with PBS and lysed, plasma membrane fractions (10 μg protein) were prepared using Protein Extraction Reagent Kit (89826, Pierce, USA), and then were probed with anti-GLUT1 or anti-GLUT4.

2.13. Measurement of intracellular ATP levels

3T3-L1 preadipocytes were serum-starved overnight, and then the medium was changed to DMEM supplemented with 10% FBS. Two hours later, the cells were treated with indicated compounds for 30 min. The ATP levels in cell extracts were measured as previously described (Fryer et al., 2002).

2.14. Statistics

Unless otherwise indicated, data were expressed as means ± SE. For multiple comparisons, statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. X-3 reduces hyperglycemia in db/db mice

Aiming to improve the in vivo efficacy of MF, we prepared X-3, a derivative of MF. As shown in Fig. 1, the calculated lipophilicities (logP(o/w), SlogP) (Tetko et al., 2009) of X-3 are quite higher than that of MF, indicating a better absorption and permeation of X-3.

To fully characterize the effects of X-3 on metabolic control, we performed an 8-week study in db/db mice. We chose Pio as a positive control, because Pio as an AMPK activator (Nawrocki et al., 2010; Zhou et al., 2001) was shown to be superior to metformin (Met) in improving postload glycemia and composite insulin sensitivity index (C ISI) in patients with type 2 diabetes (Ceriello et al., 2005; Schernthaner et al., 2004). As Fig. 2 shows, db/db mice had significantly higher glucose levels than db/+ mice. After 8 weeks of therapy with X-3 (40, 80, 120 mg/kg/day) or Pio (75 mg/kg/day), postprandial blood glucose levels were measured. Both agents effectively reduced the postprandial blood glucose levels. Moreover, X-3 dose-dependently decreased postprandial blood glucose levels. Significant changes occurred in the 80 and 120 mg/kg groups (Fig. 2A).

Next the effects of X-3 on 6 h fasting blood glucose levels were determined. X-3 markedly reduced 6 h fasting blood glucose levels at all 3 doses in mice under 2-week or 8-week treatment. Mice treated with X-3 120 mg/kg/day or Pio 75 mg/kg/day for 2 weeks showed 6 h fasting blood glucose levels were reduced by 48% or 34%, respectively ($P < 0.05$, Fig. 2B).

Furthermore, we investigated the effects of X-3 on glucose changes using intraperitoneal glucose tolerance test at the end of treatments. During glucose tolerance test, blood glucose levels were dramatically increased in db/db mice compared to db/+ mice, while X-3 and Pio significantly prevented the blood glucose level increases, especially at 30 min time point ($P < 0.05$, Fig. 2C). Comparing the area under the curve (AUC) among groups, X-3 groups showed dose-dependent reductions of AUC, and the significant reductions were found in X-3 80 mg/kg/day, 120 mg/kg/day and Pio 75 mg/kg/day treated groups (Fig. 2D). The results shown earlier suggested that X-3 improved glucose tolerance.

The effects of X-3 on plasma insulin levels were determined. X-3 significantly reduced plasma insulin levels (Fig. 2E). Thus, X-3 effectively reduced hyperglycemia and hyperinsulinemia in this db/db mouse model of type 2 diabetes.

3.2. X-3 suppresses obesity in db/db mice

The anti-obesity effect of X-3 was examined in db/db mice. Type 2 diabetes is associated with high levels of TGs and NEFAs, both of which play a role in the development of peripheral insulin resistance. We evaluated the effects of X-3 on TG and NEFA levels in db/db mice. Compared with db/+ mice, diabetic db/db mice had 1.3-, 1.4-, and 1.5-fold elevation in peripheral plasma (PP) NEFA, TG or liver TG levels, respectively. Similar to Pio, X-3 effectively decreased PP NEFA, TG and liver TG levels in a dose-dependent manner (Fig. 2F, G, and H).

Consistent with previous reports (Jung et al., 2006), db/db mice exhibited a significant increase in the rate of body weight gain when compared with age- and gender-matched db/+ mice. As depicted in Fig. 3A, B, X-3 treatments result in small and statistically insignificant decreases in body weight and food intake. In contrast, Pio-treated mice gained significantly more body weight. These results suggest that X-3 and Pio have different mechanisms of action. Pio-induced weight gain is related to proliferator-activated receptor-γ (PPARγ). Specific knockout of PPARγ in the collecting duct prevented TZD-induced fluid retention and weight gain (Li et al., 2010; Shaw et al., 2005). So X-3 may act through PPARγ-independent pathway.
Fig. 2. Effects of X-3 on glucose, insulin, NEFA and triglyceride levels in db/db mice. db/db mice were treated with X-3 at indicated dosages orally for 8 weeks. Postprandial blood glucose levels (A), and 6-h fasting blood glucose (B) were measured at the indicated week 24 h after last dose. Plasma glucose (C), the area under the curve (AUC, D) responses to an intraperitoneal glucose challenge (2 g/kg) after 6 h of food deprivation, serum insulin (E), NEFA (F), plasma triglycerides (G), and liver triglycerides levels (H) were measured at the end of treatment. Age- and sex-matched lean littermates (db/+ ) were used as nondiabetic controls. Data are means ± SE of 4–7 mice in each group. *P < 0.05 vs. the vehicle-treated group. **P < 0.01 vs. the vehicle-treated group.
We next determined the effect of different treatment regimens on the morphology of abdominal white adipose tissue (ABAT). Histological analysis revealed a marked hyperplasia of adipocytes in the db/db mice (Fig. 3C). The size of ABAT adipocytes of diabetic db/db mice was significantly larger than that of the db/+ mice. As shown in Fig. 3D, X-3 dose-dependently decreased the adipose tissue content. Treatment with 120 mg/kg X-3 or 75 mg/kg Pio for 8 weeks significantly reduced the adipocyte size of db/db mice to a similar level (Fig. 3D). These findings were consistent with previous results that X-3 effectively decreased plasma and liver TG levels in this db/db mouse model of type 2 diabetes. However, decreased weight gain was insignificant for X-3 treatments, the possible cause is redistribution of adipose depots (Hirosumi et al., 2002). Further study is needed to examine total body composition.

3.3. X-3 increases β-cell mass in db/db mice

In patients destined to develop type 2 diabetes, however, β-cell function deteriorates progressively and >50% of β-cell function is typically lost by the time hyperglycemia is diagnosed. This loss of β-cell function and/or mass leads to rising blood glucose levels and to frank diabetes. Fig. 4A shows the insulin immunostaining of pancreatic tissues in db/+ and db/db mice. Morphologically, islets in the db/db pancreas generally showed hypertrophy, having various sizes and shapes, and most of the β-cells were extensively degranulated as compared to islets in normal littermates. Similar to Pio, both the size and the number of islets, as well as insulin immunopositivity, were dose-dependently increased in X-3 treated db/db mice, while the most significant changes occurring in X-3 C120 mg/kg group. (Fig. 4B), demonstrating beneficial effects on
increasing islet neogenesis and differentiation. These findings have engendered significant interest in the potential of X-3 to enhance β-cell function and thereby modify the course of disease in subjects with type 2 diabetes.

3.4. Acute toxicity studies of X-3 in mice and rats

In the acute study, X-3 was administered orally. The LD50 values of X-3 were estimated more than 3570 mg/kg in mice, more than 1785 mg/kg in rats. No obvious clinical signs of toxicity or mortality were observed in mice and rats with any of the X-3 doses until the end of the observation period of 7 days.

3.5. X-3 enhances basal glucose uptake in 3T3-L1 cells

To investigate the mechanism of action for the antidiabetic activity of X-3, we evaluated its glucose uptake activity in 3T3-L1 preadipocytes and adipocytes. As Fig. 5A shows, X-3 and MF dose-dependently enhanced glucose transport activities both in 3T3-L1 preadipocytes and adipocytes. The significant enhancements were found in X-3 at 3 assayed dosages in 3T3-L1 preadipocytes and at 70 μM in 3T3-L1 adipocytes. X-3 was more potent than MF at 25 and 50 μM (P<0.05). Dose-dependent experiment showed the stimulation of 2-deoxyglucose uptake was maximal with 70 μM X-3 or MF, which condition was employed in subsequent experiments to test the effect of X-3. To identify a potential mechanism at the molecular level by which X-3 or MF increases glucose uptake, we examined whether it modulates the expression levels of the glucose transporters GLUT1 and GLUT4 in 3T3-L1 preadipocytes and adipocytes. As Fig. 5B, C shows, 70 μM X-3 or MF treatment led to a significant increase in GLUT1 and GLUT4 membrane protein, indicating that X-3 and MF increased GLUT1- and GLUT4-mediated glucose uptake. Cell viability assessment (Fig. 5D) revealed the very low acute toxicity following 24 h exposure of 3T3-L1 cells to X-3 or MF (70 μM, <4%), suggesting that the effect of X-3 and MF on glucose uptake could not be explained by cell toxicity. In addition, since 3T3-L1 preadipocytes is not inferior to adipocytes in evaluating X-3 and MF's glucose transport activities, 3T3-L1 preadipocytes were applied to subsequent experiments.

3.6. X-3 stimulates AMPK in vitro

To further examine the effect of X-3 or MF on GLUT1- and GLUT4-mediated glucose transport, we analyzed the phosphorylation of AMPK by western blotting. Using an AMPK activator AICAR as a positive control (Sakoda et al., 2002), we found that X-3 or MF treatment resulted in a significant elevation of the phosphorylation at Thr-172 on the AMPKα2 subunit in 3T3-L1 preadipocytes (Fig. 5E). X-3 or MF also increased the phosphorylation of the AMPK target ACC at similar concentrations at which glucose uptake was stimulated (Fig. 5A). Using an AMPK inhibitor compound C, the ability of MF or X-3 to stimulate the phosphorylation of AMPK and ACC was diminished (Fig. 5B). Moreover, compound C partially blocked the stimulation effect of X-3 on glucose uptake in 3T3-L1 cells (Fig. 5E), suggesting a direct involvement of AMPK in enhancing glucose transport by X-3. Together, these data suggest that X-3 and MF primarily activate AMPK by increasing its phosphorylation at Thr-172.

3.7. X-3 promotes LKB1-dependent and -independent phosphorylation of AMPK

AMPK is primarily activated by two kinases, LKB1 and CaMKKβ. Previous report showed that AICAR induced phosphorylation of AMPK in an ataxia telangiectasia mutated (ATM)-dependent, LKB1-independent manner (Sun et al., 2007). Consistent with the previous report, we found AICAR stimulated AMPK and ACC phosphorylation in the LKB1-deficient HeLa cells, and MF also activated AMPK and ACC in a LKB1-independent fashion. In contrast, the ability of X-3 to induce AMPK and ACC phosphorylation in HeLa cells was substantially reduced (Fig. 6C). In addition, X-3 was shown to decrease the intracellular ATP level at 70 μM in 3T3-L1 preadipocytes (Fig. 6D). So it is possible that X-3 initiates an elevation of the AMP:ATP ratio, which results in LKB1-dependent phosphorylation and activation of AMPK. In 3T3-L1 preadipocytes and Hela cells, a relevant cell system for calcium-dependent activation of AMPK, we found that the CaMKKβ inhibitor STO-609, at a nontoxic concentration, reduced the effect of X-3 or MF on AMPK and ACC phosphorylation (Fig. 6B), but the inhibition was not as complete as in the control cells, especially in 3T3-L1 preadipocytes. Collectively, this suggests that MF activated AMPK and ACC in a LKB1-independent fashion, while X-3 did in a LKB1-dependent and -independent manner.

3.8. X-3 activates AMPK in vivo

To extend our in vitro study, we tested whether X-3 could activate AMPK in the hypothalamus, liver, muscle and adipose tissues in mice. Western blot analysis revealed that a single administration of X-3, MF or Met resulted in an increased AMPK and ACC phosphorylation in the hypothalamus, liver, muscle and adipose tissues within 2 h (Fig. 7). It was shown that 80 mg/kg X-3 was more effective than Met at 500 mg/kg in the hypothalamus, and interscapular fat tissues, and MF at the same dose in the liver. On average, X-3 (80 mg/kg) increased activity of AMPK with 1.4 times the potency of Met (500 mg/kg), and 1.3 times the potency of MF (80 mg/kg). Taken together, these results indicate that X-3 also activates AMPK in vivo and this might result in the phosphorylation and presumed inhibition of ACC. Previous studies revealed that AMPK activation in vivo, especially in liver and muscle, leads to improved metabolic control, which include modulation of lipid metabolism, reduction in blood glucose, and improvement in insulin sensitivity (Zhang et al., 2009), so the results discussed earlier may account for X-3's antidiabetic effects in db/db mice.

3.9. X-3 activates Akt in 3T3-L1 preadipocytes

It has been shown that Met can activate Akt through alternate, AMPK-independent pathway(s) (Janjetovic et al., 2011), so we examined Akt phosphorylation by X-3 in 3T3-L1 preadipocytes. Similar to Met, X-3 or MF alone could stimulate Akt phosphorylation. Surprisingly, X-3 was shown to be more potent than MF or Met for stimulating Akt phosphorylation in the absence of insulin (Fig. 8). The Akt phosphorylation was significantly increased when insulin was used in combination with X-3 or MF. PI3K inhibitor wortmannin partially blocked the effects of X-3, MF, Met alone or in combination with insulin on Akt phosphorylation. Furthermore, wortmannin partially blocked the effects of X-3 or MF on glucose uptake (Fig. 5E, F), these results suggest that activation of Akt by X-3 or MF represents an alternative mechanism to improve glucose uptake in 3T3-L1 cells.

4. Discussion

In the current study, we have described an analog of MF, X-3, as a potential anti-diabetic agent. We have shown that X-3 improved glucose and lipid metabolism (Figs. 2 and 3), and increased the number of insulin-positive β-cell mass (Fig. 4). Further study revealed that X-3 exerted its effects by promoting GLUT1 and GLUT4-mediated glucose uptake (Fig. 5) by activating AMPK (Figs. 6 and 7), and Akt (Fig. 8). However, MF activated AMPK in a LKB1-independent manner, while X-3 did in a LKB1-dependent and independent fashion.
In line with the characteristics of AMPK activators (Nawrocki et al., 2010; Zhou et al., 2001), MF and X-3 activated AMPK both in vitro and in vivo (Figs. 6 and 7). This is consistent with the report that MF activated AMPK in liver of hyperlipidemic rats (Niu et al., 2012). AMPK specific inhibitor Compound C could block MF or X-3-induced AMPK and ACC phosphorylation. Most importantly, X-3 exhibited improved in vivo effects in terms of counteracting hyperglycemia, increased adiposity, tissue triglyceride accumulation, and insulin resistance in db/db mice (Figs. 2–4). Taken together, we believe that the antidiabetic effects of X-3 are mediated by its activation of AMPK.

LKB1-dependent pathway, but not AMPK, plays a critical role in the liver in the control of gluconeogenesis. AMPKα1α2LS−/− mice displayed normal fasting glucose level and glucose tolerance, while mice with LKB1-deficiency exhibited hyperglycemia and glucose intolerance (Shaw et al., 2005). Our results showed that X-3 activated AMPK mainly by the LKB1 pathway, while MF utilized an LKB1-independent pathway. Though MF was shown to have antidiabetic effects in vivo...
Guo et al., 2011; Ichiki et al., 1998; Miura et al., 2001; Muruganandan et al., 2002, 2005; Niu et al., 2012; Yoshikawa et al., 2001), there are also negative reports. In a study, treatment with 15 or 45 mg/kg of MF for 12 weeks was unable to lower blood glucose in streptozotocin-induced diabetic rats (Li et al., 2010). Interestingly, our results showed X-3 dose-dependently reduced 6 h fasting blood glucose levels. The hypoglycemic effects of treatment with 120 mg/kg of X-3 for 2 weeks were even superior to that of 75 mg/kg Pio (P < 0.05, Fig. 2B). More evidence is needed to clarify the differences between MF and X-3 in the control of gluconeogenesis.

Fig. 6. X-3 and MF promote phosphorylation of AMPK and ACC in vitro. (A) X-3 and MF induces phosphorylation of AMPK and ACC in 3T3-L1 preadipocytes. Cells were stimulated with X-3, MF or AICAR (positive control) for 2 h. Then cells were lysed, and phospho-specific antibodies were used to determine phosphorylation of AMPK and ACC in immunoblots. Equal proteins were applied to immunoblotting analyses. Densitometric analyses and quantification of the ratios pAMPK/AMPK and pACC/ACC from experiments. a.u., arbitrary units. (B, C) Compound C or STO-609 decreased phosphorylation of AMPK and ACC stimulated by X-3 or MF. 3T3-L1 preadipocytes (B) or Hela cells (C) were treated with indicated compound in the presence or absence of compound C or STO-609. Immunoblotting analyses were used to measure protein levels. ##P < 0.01 vs. basal levels. *P < 0.05, **P < 0.01 vs. cells treated with the indicated compound in the absence of Compound C or STO-609. (D) Intracellular ATP levels in 3T3-L1 preadipocytes. Cells were starved, then treated for 0.5 h with indicated compounds. **P < 0.01 vs. basal levels.
We also investigated whether the effects of X-3 or MF could be due to an alternative, AMPK-independent pathway. Indeed, X-3 or MF alone or in combination with insulin induced PKB/Akt phosphorylation. The PKB/Akt phosphorylation could be partially blocked by pretreatment with PI3K inhibitor wortmannin (Fig. 8). Further, wortmannin partially blocked the effects of X-3 or MF on glucose uptake (Fig. 5E, F), supporting activation of Akt by X-3 or MF represents an alternative mechanism to improve glucose uptake in 3T3-L1 cells, and indicating that PI3K has an important permissive effect on AKT phosphorylation. But PI3K/PDK1 is unlikely to be the direct upstream enzyme for LKB1 (Beauloye et al., 2002; Zou et al., 2003).

How PI3K leads to the AMPK-LKB1 complex remains unknown. The regulation of glucose transport is complex and requires multiple signaling inputs that converge on GLUT1 and GLUT4 vesicle transport (Badr, 2009; Mezei et al., 2003). As a metabolic sensor, AMPK regulates several intracellular systems including the cellular uptake of glucose. We found that X-3 or MF-induced glucose uptake was partly inhibited by Compound C in 3T3-L1 preadipocytes (Fig. 5E). It is therefore possible that, in 3T3-L1 cells, a small residual activation of endogenous AMPK is sufficient to cause a partial stimulation of glucose uptake. Similarly, in the individual AMPKα2 knockout mice, there is a compensatory increase in the expression of AMPKα1 (Viollet et al., 2003), and this might be sufficient to stimulate glucose uptake in the muscle. In a previous study, overexpression of a dominant-negative AMPK under the control of a muscle creatine kinase promoter (Mu et al., 2001) only partially inhibited glucose uptake, suggesting that AMPK-independent pathway(s) also regulate contraction-induced glucose uptake.

Examination of the structure of X-3 reveals that it has higher logP and SlogP values compared with MF (Fig. 1), which was predicted to open up the structure, making it more amenable for uptake. Furthermore, activation by X-3 in hypothalamus indicates it may cross the blood–brain barrier and could be active in the central nervous system (CNS). Therefore, X-3 has physicochemical parameters predicting acceptable aqueous solubility, intestinal permeability, and brain penetration, which is good for experimental evaluation in vivo for oral bioavailability and CNS bioactivity. In addition, we investigated the toxicological effects of X-3 in animals. X-3 did not cause any death or signs of toxicity in acute toxicity studies.

In summary, we have identified a novel derivative of mangiferin, X-3. Our results indicate that X-3 mediates activation of AMPK and AKT and improves overall glucose and lipid metabolism. Therefore, X-3 is a new AMPK activating agent and can be potentially developed into a new anti-diabetic drug.

**Acknowledgments**

The authors thank Professor Xinmin Liu and Jun Wang for their technical assistance. The authors thank Professor Junping Zhang, Zhenlin Hu, and the Ivy Club for revising the manuscript. This study...
levels in the presence of insulin.


Fig. 8. X-3 and MF enhance P3-kinase/Akt signaling. 3T3-L1 preadipocytes were starved, and then treated with indicated compounds for 4 h, insulin (10^{-5} M) was added 30 min before the end of the treatment, whereas wortmannin (300 nM) was added 1 h before the end of the treatment. Phospho-Akt and Akt levels in protein lysates were detected by Western analysis (A). Densitometric analyses and quantification of the ratios pAKT/AKT were shown (B). I, insulin; W, wortmannin. ^P<0.05, ^^P<0.01. ##P<0.01 vs. basal levels in the absence of insulin.

was supported by National Science Foundation of China 81325024, Shanghai Science and Technology Committee 12431900805.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.02.008.

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