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## Control of hyperglycemia in male mice by leflunomide: Mechanisms of action

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### Abstract

p70 S6 kinase (S6K1) is a serine/threonine kinase that phosphorylates the insulin receptor substrate-1 (IRS-1) at serine 1101 and desensitizes insulin receptor signaling. S6K1 hyperactivation due to overnutrition leads to hyperglycemia and type 2 diabetes. Our recent study showed that A77 1726, the active metabolite of the anti-rheumatoid arthritis (RA) drug leflunomide, is an inhibitor of S6K1. Whether leflunomide can control hyperglycemia and sensitize the insulin receptor has not been tested. Here we report that A77 1726 increased AKT<sup>S473/T308</sup> and S6K1<sup>T389</sup> phosphorylation but decreased S6<sup>S235/236</sup> and IRS-1<sup>S1101</sup> phosphorylation in A77 1726-treated 3T3-L1 adipocytes, C2C12 and L6 myotubes. A77 1726 increased insulin receptor tyrosine phosphorylation and binding of the p85 subunit of the PI-3 kinase to IRS-1. A77 1276 enhanced insulin-stimulated glucose uptake in L6 myotubes and 3T3-L1 adipocytes, and enhanced insulin-stimulated glucose transporter type 4 (GLUT4) translocation to the plasma membrane of L6 cells. Finally, we investigated the anti-hyperglycemic effect of leflunomide on *ob/ob* and high-fat diet (HFD)-induced diabetes mouse models. Leflunomide

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treatment normalized blood glucose levels and overcame insulin resistance in glucose and insulin tolerance tests in *ob/ob* and HFD-fed mice but had no effect on mice fed a normal chow diet (NCD). Leflunomide treatment increased AKT<sup>S473/T308</sup> phosphorylation in the fat and muscle of *ob/ob* mice but not in normal mice. Our results suggest that leflunomide sensitizes the IR by inhibiting S6K1 activity *in vitro*, and that leflunomide could be potentially useful for treating patients with both RA and diabetes.

## Keywords

Leflunomide; p70 S6 kinase; Insulin resistance; Insulin receptor substrate; Hyperglycemia

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## Introduction

Type 2 diabetes is a major public health problem (Zimmet *et al.*, 2001; Zimmet *et al.*, 2014). Although many anti-diabetic medications are available, some have intolerable side-effects or lose their therapeutic efficacy after the long-term use (Nathan, 2015). Failure to control hyperglycemia leads to diabetic complications, which account for most diabetes-related morbidity and mortality (Nathan, 2015). Because of physical inactivity and circulating inflammatory cytokines, diabetic patients with rheumatoid arthritis (RA) may have worse hyperglycemia than those without RA (Herlitz-Cifuentes *et al.*, 2015; Pinto *et al.*, 2017). Individuals with RA have a significantly higher risk of developing type 2 diabetes (Jiang *et al.*, 2015) and obesity (Versini *et al.*, 2014) than those without RA. Currently, patients with both RA and diabetes are treated with antidiabetic and anti-RA drugs separately. A drug that controls both RA and hyperglycemia could greatly benefit patients with both problems.

The binding of insulin to its receptor activates the insulin receptor tyrosine kinase, leading to insulin receptor autophosphorylation and the phosphorylation of intracellular protein substrates such as the insulin receptor substrates (IRS) (Fig. 1) (Guo, 2013; Guo, 2014). Tyrosine-phosphorylated IRS interact with the p85 subunit of the PI-3 kinase and activate its catalytic p110 subunit (Fig. 1). PI-3 kinase activation leads to serine phosphorylation and activation of the protein kinase B (AKT) (Guo, 2013; Guo, 2014). AKT activation plays a critical role in glucose metabolism (Copps and White, 2012; Dann *et al.*, 2007). AKT activation stimulates glucose uptake by inducing translocation of the glucose transporter type 4 (GLUT4) to the plasma membrane of both adipose and muscle cells (Fig. 1) (Guo, 2013; Guo, 2014). In addition, AKT also regulates glucose metabolism by stimulating glycogen synthesis and inhibiting gluconeogenesis (Fig. 1) (Guo, 2013; Guo, 2014).

The mechanistic target of rapamycin (mTOR) kinase is a serine/threonine kinase activated by AKT. Overnutrition with high concentrations of amino acids and fatty acids also activates mTOR (Fig. 1). p70 S6 kinase (S6K1), a serine/threonine protein kinase downstream of mTOR, phosphorylates the IRS and subsequently attenuates the activation of the PI3K pathway (Fig. 1) (Fenton and Gout, 2010). Constitutive S6K1 activation by hyperinsulinemia or overnutrition leads to insulin receptor desensitization (Boura-Halfon and Zick, 2009; Copps and White, 2012). S6K1 is also involved in regulating the expression of several energy expenditure-related genes such as the melanocortin-4 receptor (MC4R)

(Um *et al.*, 2006; Um *et al.*, 2004; Xia *et al.*). S6K1<sup>-/-</sup> mice do not develop obesity and hyperglycemia when fed a high-fat diet (HFD) (Um *et al.*, 2004). These mice have a significantly longer life span than the wild-type mice (Selman *et al.*, 2009). Insulin receptor signaling is highly active in the metabolic tissues of the HFD-fed S6K1<sup>-/-</sup> mice, as evidenced by increased AKT phosphorylation in their liver, muscle, and fat (Um *et al.*, 2004). S6K1 is a key kinase driving insulin resistance and inducing obesity under conditions of nutrient overload (Dann *et al.*, 2007).

Leflunomide is an orally administered prodrug proscribed for treating rheumatoid arthritis (RA) (Breedveld and Dayer, 2000). Upon indigestion, it is rapidly and completely (>99%) converted in the gastrointestinal tract, plasma, and liver to its active metabolite, A77 1726 (Fig. 1A) (Breedveld and Dayer, 2000). Once in plasma, A77 1726 is avidly bound to plasma proteins, mainly albumin (Cannon and Kremer, 2004). A77 1726 has a very long half-life of 15.5 days (range 14–18 days) and is cleared after it is metabolized into trifluoromethylaniline-oxanilic acid (60–70%) and excreted into the urine (Breedveld and Dayer, 2000; Cannon and Kremer, 2004). A77 1726 is the only active metabolite of leflunomide and is solely responsible for its therapeutic activity. A77 1726 inhibits the activity of protein tyrosine kinases and dihydroorotate dehydrogenase (DHO-DHase) (Elder *et al.*, 1997; Ruckemann *et al.*, 1998; Siemasko *et al.*, 1998; Siemasko *et al.*, 1996; Xu *et al.*, 1997; Xu *et al.*, 1999; Xu *et al.*, 1995; Xu *et al.*, 1996). The ability of A77 1726 to inhibit DHO-DHase activity (the IC<sub>50</sub> values of approximately 100 nM) is about 10–100 times stronger than its ability to inhibit the activity of protein tyrosine kinases such as p56<sup>lck</sup>, p59<sup>fyn</sup>, and the PDGF receptor (the IC<sub>50</sub> values of approximately 25–50 μM) (Ruckemann *et al.*, 1998; Xu *et al.*, 1997; Xu *et al.*, 1999; Xu *et al.*, 1995; Xu *et al.*, 1996). Inhibition of pyrimidine nucleotide synthesis by leflunomide was thought to be its primary mechanism of action (Bruneau *et al.*, 1998; Williamson *et al.*, 1996). However, addition of exogenous uridine, which normalizes pyrimidine nucleotide levels *in vitro* in cell cultures, only partially antagonizes this anti-proliferative effect. Co-administration of uridine with leflunomide in a lymphadenopathy and autoimmune disease model of MRL/MpJ-*lpr/lpr* mice and in a tumor xenograft model does not abrogate the immunosuppressive and antitumor activities of leflunomide (Xu *et al.*, 1997; Xu *et al.*, 1999). This suggests that leflunomide exerts its anti-proliferative and immunosuppressive activity by other mechanisms (Xu *et al.*, 1997; Xu *et al.*, 1999). Our recent study revealed that leflunomide and A77 1726 directly inhibit the activity of purified S6K1 in an *in vitro* kinase assay and inhibit the activity of S6K1 in cell culture, with an IC<sub>50</sub> value of 50–75 μM (4-fold lower than its plasma levels in patients) (Doscas *et al.*, 2014). Inhibition of S6K1 activity by A77 1726 leads to feedback activation of the PI-3 kinase pathway in tumor cell lines, as evidenced by increased AKT and S6K1 phosphorylation and decreased S6 phosphorylation (Doscas *et al.*, 2014). Here we report that A77 1726 increases S6K1 and AKT phosphorylation, and stimulates GLUT4 translocation to the cell membrane and glucose uptake in myotubes and adipocytes (Fig. 1B). We further show that leflunomide controls hyperglycemia in *ob/ob* mice and in the mice with HFD-induced diabetes but not in normal mice.

## Materials and Methods

### Chemicals, antibodies, and plasmid construct

Leflunomide and A77 1726 were kindly provided by CinKate Corporation (Oak Park, IL). Cytochalasin and rosiglitazone were purchased from Calbiochem (EMD Millipore, Billerica, MA). 3-Isobutyl-1-methylxanthine (IBMX), carboxymethyl-cellulose sodium (CMC), uridine, dexamethasone, and 2-deoxy-glucose (2-DG) were purchased from Sigma Aldrich (St. Louis, MO). 2-DG (5–10 Ci(185–370GBq)/mmol, 1mCi (37MBq) was purchased from PerkinElmer (Waltham, MA). Insulin used in the *in vitro* study was purchased from Invitrogen (Life Technologies, Grand Island, NY). Rapamycin (an mTOR inhibitor), antibodies against AKT, S6K1, S6, IRS-1, p85 of the PI-3 kinase and phospho-antibodies (AKT<sup>S473</sup>, AKT<sup>T308</sup>, S6K1<sup>T389</sup>, S6<sup>S235/236</sup>, IRS-1<sup>S1101</sup>, IR<sup>Y1146</sup>, and IRS-1<sup>S636</sup>) were purchased from Cell Signaling Technology (Danvers, MA). Anti- $\beta$ -actin antibody monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The sources of antibodies and their applications were listed in Table 1. (mCherry-GLUT4-myc expression vector was kindly provided by Dr. Amira Klip (The Hospital for Sick Children, Toronto, Ontario). Use of the radioactive isotope was approved by Rush University Medical Center. All methods were performed in accordance with the relevant guidelines and regulations of Rush University Medical Center and Yangzhou University.

### Cell lines and differentiation

C2C12 (a murine myoblast cell line) and L6 cells (a rat myoblast cell line) were cultured in DMEM supplemented with 10% fetal bovine serum. For myotube differentiation, the confluent monolayers of C2C12 cells were cultured in DMEM containing 10% horse serum for 2 weeks during which the same fresh medium was replenished every two days. For L6 myotube differentiation, the cells were cultured in DMEM containing 2% calf serum for two weeks. The early passages of 3T3-L1 adipocytes (within 15 passages) were differentiated according to a detailed protocol of Zebisch *et al.* (Zebisch *et al.*, 2012). After incubation in insulin-free medium for 7–14 days, more than >95% of the cells exhibited an adipocyte-like phenotype. All three cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

### Glucose uptake

C2C12 myotubes express very low levels of GLUT4 and therefore were not used for glucose uptake experiments. Differentiated 3T3-L1 adipocytes and L6 myotubes were incubated overnight in insulin-free DMEM medium. Cells were starved of serum for 4 hr and then incubated in the absence or presence of A77 1726 (200  $\mu$ M) or rapamycin (20 nM) for 1 hr without or with 2X amino acids in essential balanced salt solution (EBSS) for another 1 hr. Cells were left unstimulated or stimulated with 20 nM insulin for 45 min. Unlabeled 2-DG (0.1 mM) and [<sup>3</sup>H]-DG were added to the cells in the KRP-HEPES buffer (10 mM HEPES, pH7.4, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 2.65 mM CaCl<sub>2</sub>, 2.5 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1% bovine serum albumin) at 37°C for 5 min. The reaction was terminated by adding 10  $\mu$ M cytochalasin B followed by wash with ice-cold PBS three times. The cells were lysed in 0.2 N NaOH. The radioactivity was measured in a liquid scintillation counter. Nonspecific 2-DG uptake was measured by adding cytochalasin B (10  $\mu$ M) into the cells prior to the

addition of 2-DG. The values of 2-DG uptake, after correction by subtracting the value of the non-specific 2-DG uptake, were normalized by protein concentrations, which were quantified by using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

### Western blot

C2C12 myotubes grown in 6-well plates were starved of serum for 4 hr. A77 1726 or rapamycin was added and incubated for 2 hr. Cells were left unstimulated or stimulated with 20 nM insulin for 20 min. Differentiated 3T3-L1 adipocytes and L6 myotubes were treated as described in the glucose uptake experiments. Cell lysates were prepared in NP-40 lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate) and analyzed for protein phosphorylation with the indicated antibodies, followed by reprobing with antibodies against total proteins. The density of the bands was analyzed by using NIH Image-J software and normalized by the arbitrary units of their corresponding total proteins. Quantified results were presented as the mean  $\pm$  standard deviation (SD) from three experiments (Fig. 2–5) in bar graphs.

### Immunoprecipitation

L6 myotubes and 3T3-L1 adipocytes were first starved of serum for 2 hr or overnight, respectively, then incubated either in an amino-acid free medium (EBSS) or EBSS medium containing 2x amino acids (2xAA) in the absence or presence of A77 1726 for 5 hr. Cells were left unstimulated or stimulated with insulin (100 nM) for 10 min. Cell lysates were immunoprecipitated with an anti-p85 rabbit monoclonal antibody followed by probing with anti-p85 and anti-IRS1 antibodies in Western blot.

### Confocal microscopy

Undifferentiated L6 cells seeded on coverslips were transiently transfected with mCherry-GLUT4-myc expression vector DNA using FuGENE6 following the manufacturer's protocol. After incubation for 24 hr, the cells were starved of serum for 2 hr and then incubated in the absence or presence of A77 1726 (200 µM) for 4 hr without or with 2X the amino acid concentrations in essential balanced salt solution (EBSS). Cells were left unstimulated or stimulated with 100 nM insulin for 30 min. The coverslips were collected, fixed, and mounted with 50% glycerin in PBS containing 4,6-diamidino-2-phenylindole (DAPI) (0.5 µg/ml; Sigma Chemical Co.). mCherry-tagged GLUT4 fluorescence was visualized under a Leica LP8 confocal microscope. The percent of cells positive for GLUT4 translocation into the plasma membrane among total mCherry-GLUT4-expressing cells was calculated by counting 10 randomly selected fields from each treatment. The results represent the mean  $\pm$  standard deviation (SD) from one of three experiments with similar results.

### Animals and drug administration

Use of animals and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center and College of Veterinary Medicine, Yangzhou University. All mice were maintained on a 12-hr light/dark cycle and

housed in ventilated cages at an ambient temperature of 23°C. Male *ob/ob* mice (B6.V-Lep<sup>ob</sup>/OlaHsd, male) were purchased from Harlan Laboratories, Inc. These mice were fed *ad libitum* on a normal chow diet (NCD). CMC (1.5% dissolved in distilled water) was used as a vehicle to prepare leflunomide. Mice (8–10-wk-old) were given 1.5% CMC and leflunomide by gavage. Uridine was co-administered with leflunomide to *ob/ob* mice to determine when pyrimidine nucleotide levels in various tissues were normalized, if leflunomide could still control hyperglycemia. The uridine dose was based on our previous studies (Xu *et al.*, 1997; Xu *et al.*, 1999) that 2 g/kg, twice daily is sufficient to normalize or overshoot pyrimidine nucleotide levels in the fast proliferating tumor cells or lymphocytes of *lpr/lpr* mice. Uridine dissolved in saline was given by intraperitoneal (i.p.) injection. *ob/ob* mice were treated with the vehicle (1.5% CMC daily), leflunomide (35 mg/kg/day, daily, gavage), uridine (2 g/kg, twice daily, i.p.) or leflunomide (35 mg/kg/day, daily gavage) + uridine (2 g/kg, twice daily, i.p.) for three days. After fasting 6 hr, blood was sampled from the tail vein and analyzed by a Bayer handheld glucometer. Body weight of mice was measured before and 3 days after treatment. Food intake was measured in the individual cages on day 3 after treatment.

For the glucose tolerance test (GTT) and insulin tolerance test (ITT), mice were treated for 3 days as above. On day 4, mice were fasted and treated with a last dose 6 hr before GTT. Mice were challenged with glucose (1 g/kg) by intraperitoneal injection. For ITT, mice were similarly treated and fasted for 6 hr, followed by intravenous injection of insulin (2.5 unit/kg) into the tail vein. Blood glucose levels were measured at various time points between 0 and 120 min (GTT) or between 0 to 60 min (ITT) by using a Bayer glucometer. AUC (area under curve) of the glucose levels in the GTT and ITT was calculated by using Graphpad Prism 5 software. AUC was plotted as bar graphs.

C57BL/6 male mice were purchased from the Center for Comparative Medicine, Yangzhou University. Mice (5-wks-old) were fed with NCD or HFD (24% fat, 24% protein, 41% carbohydrates, 1% others by weight, which translate to calories percentage as 20% protein, 35% carbohydrate, 45% fat) (Jiangsu Matheson Biological Pharmaceutical Co., LTD) for 10 weeks. Mice were treated with CMC or leflunomide followed by GTT as described as above. ITT in C57BL/6 mice fed either NCD or HFD was conducted similarly as above except insulin (2.5 unit/kg) was intraperitoneally injected.

### In vivo AKT activation

*ob/ob* mice were treated daily for three days. They were given a last dose on day 4 and then fasted for 6 hr. Five minutes after insulin injection (2.5 unit/kg, intravenously), mice were sacrificed. Gastrocnemius muscle, mesenteric visceral white adipose and hepatic tissues (50–100 mg/sample) were collected and immediately homogenized in NP-40 lysis buffer. Protein concentrations were measured using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). AKT<sup>S473/T308</sup>, S6<sup>S235/236</sup>, and IRS-1<sup>S1101</sup> phosphorylation was analyzed by Western blot. The density of the bands was analyzed by using NIH Image-J software and normalized by the arbitrary units of their corresponding total proteins.

## Statistical analysis

Data were presented as mean  $\pm$  standard deviation (SD) (glucose uptake and GLUT4 translation assays) or standard error of the mean (SEM) (blood glucose levels). An unpaired Student *t* test was used to analyze the differences in glucose uptake in 3T3-L1 adipocytes and L6 myotubes in different groups, and the differences in the arbitrary number of Western blot data from the Image J analysis. Differences in blood glucose levels between different treatment groups were analyzed by repeated measures ANOVA (analysis of variance). Differences in blood glucose levels in individual groups before and after treatment were statistically analyzed by a paired Student *t* test. A *p* value  $<0.05$  was considered statistically significant. All statistics was performed with SigmaPlot 11 software (Systat Software, Inc, San Jose, CA).

## Results

### A77 1726 induces feedback activation of the PI-3 kinase pathway

S6K1 inhibition by A77 1726 in tumor cell lines leads to feedback activation of the PI-3 kinase pathway through the insulin-like growth factor 1 (IGF-1) receptor (Doscas *et al.*, 2014). Here we tested if A77 1726 also induced feedback activation of the PI-3 kinase pathway in C2C12 myotubes. As shown in Fig 1, insulin induced the phosphorylation of IRS-1<sup>S636</sup>, AKT<sup>S308</sup>, AKT<sup>S473</sup>, and S6K1<sup>T389</sup> in C2C12 myotubes. A77 1726 enhanced insulin-induced phosphorylation of AKT<sup>S473</sup>, AKT<sup>T308</sup> and S6K1<sup>T389</sup>, but inhibited the phosphorylation of S6<sup>S235/236</sup> and IRS<sup>S1101</sup>, both of which are phosphorylated by S6K1. In contrast, A77 1726 increased the phosphorylation of IRS<sup>S636</sup> (Fig. 2). This site is phosphorylated largely by mTOR. Of note, S6<sup>S235/236</sup> and IRS-1<sup>S1101</sup> were highly phosphorylated in unstimulated C2C12 myotubes. Rapamycin, an inhibitor of mTOR, increased AKT<sup>T308</sup> and AKT<sup>S473</sup> phosphorylation but inhibited IRS<sup>S1101</sup>, IRS<sup>S636</sup>, S6<sup>S235/236</sup>, and S6K1<sup>T389</sup> phosphorylation. Increased S6K1 phosphorylation by A77 1726 is consistent with previous observations with PF-4708671, a specific S6K1 inhibitor (Pearce *et al.*, 2010; Shum *et al.*, 2016).

### A77 1726 enhances insulin receptor signaling

We next examined the effect of A77 1726 on protein phosphorylation in the PI-3 kinase pathway in L6 myotubes and 3T3-L1 adipocytes in the presence of high amino acid concentrations, a condition of insulin resistance. As shown in Fig. 3, insulin induced AKT<sup>S473/T308</sup> phosphorylation in the absence of amino acids in L6 myotubes. Consistent with previous observations (Patti *et al.*, 1998), high amino acids activated S6K1, as revealed by increased S6<sup>S235/236</sup> and IRS-1<sup>S1101</sup> phosphorylation, which is considered the indirect evidence of insulin resistance. A77 1726 induced phosphorylation of S6K1, which is a downstream molecule of mTOR, whereas rapamycin inhibited it. Both A77 1726 and rapamycin inhibited IRS-1<sup>S1101</sup> and S6<sup>S235/236</sup> phosphorylation but increased AKT<sup>S473</sup> and AKT<sup>T308</sup> phosphorylation in L6 myotubes. Similar results were obtained in 3T3-L1 adipocytes (Fig. 4).

Um *et al.* (Um *et al.*, 2004) reported that S6K1 deficiency leads to insulin receptor sensitization, as evidenced by increased insulin receptor tyrosine phosphorylation in the liver

of insulin-treated mice, compared to that in wild-type mice. Here we tested if A77 1726 could indeed enhance insulin receptor signaling. 3T3-L1 adipocytes and L6 myotubes preincubated with A77 1726 in the absence or presence of 2X amino acids, and were left unstimulated or stimulated with insulin for 30 min. As shown in 5A, insulin induced insulin receptor tyrosine phosphorylation in 3T3-L1 adipocytes and L6 myotubes that were cultured in the absence or presence of high concentrations of amino acids. This phosphorylation was further increased by A77 1726. Immunoprecipitation revealed that insulin stimulation increased the binding of the p85 subunit of the PI-3 kinase to IRS-1 in 3T3-L1 adipocytes and L6 myotubes cultured in the absence or presence of 2X amino acids. This binding was further increased by A77 1726 (Fig. 5B). Cell lysates were analyzed by Western blot. Equal levels of IRS-1 and the p85 subunit of the PI-3 kinase were found in the cell lysates used for immunoprecipitation (Fig. 5B).

### **A77 1726 increases GLUT4 translocation to the cell membrane**

AKT activation induces the translocation of GLUT4 from the vesicles into the plasma membrane (Coppes and White, 2012; Dann *et al.*, 2007). GLUT4 was detected in the cytoplasm of unstimulated L6 but was translocated to the plasma membrane in insulin-stimulated cells in the absence of amino acids (Fig. 6). A77 1726 alone slightly increased GLUT4 membrane translocation in L6 cells in the presence of 2x amino acids. Insulin also weakly induced GLUT4 translocation into the plasma membrane in the presence of 2x amino acids, almost as well as in the absence of 2x amino acids, probably due to the use of the undifferentiated L6 myoblast cells in this experiment. Alternatively, the relatively low transfection efficiency and a few GLUT4-positive cells under a high power field may shield the 2x amino acids-mediated inhibition of GLUT4 translocation. Nevertheless, A77 1726 significantly increased insulin-stimulated GLUT4 membrane translocation in L6 cells in the presence of 2x the amino acid concentrations (Fig. 6).

### **A77 1726 increases glucose uptake**

We next determined if insulin receptor sensitization by A77 1726 led to increased glucose uptake. Cells treated with high concentrations of amino acids were considered under the insulin resistance condition. Indeed, 2x the amino acid concentrations significantly decreased the basal level of glucose uptake in L6 myotubes by 29% and reduced insulin-stimulated glucose uptake by 22%, compared to their corresponding controls (Fig. 7A). A77 1726 increased insulin-stimulated glucose uptake in the presence of 2x the amino acid concentrations by 31%. Rapamycin included as a positive control increased glucose uptake by 19% (Fig. 7A) in insulin-stimulated L6 myotubes in the presence of 2x the amino acid concentrations. Slightly better stimulation of insulin-induced glucose uptake by A77 1726 was observed with 3T3-L1 adipocytes (Fig. 7B). Of note, A77 1726 is a cytostatic drug and does not affect the viability and proliferation of differentiated non-dividing cells. Cells were incubated in the presence of A77 1726 only for a few hours. Using an Enhanced Cell Counting Kit-8 (CCK-8), we found that A77 1726 (200  $\mu$ M) did not have any cytotoxicity on 3T3-L1 adipocytes and L6 myotubes (data not shown). Using a CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay, we found that A77 1726 (200  $\mu$ M) reduced the proliferation of 3T3-L1 adipocytes by approximately 20% after incubation for 48 hr (data not shown).



## Control of hyperglycemia by leflunomide

We first assessed the ability of leflunomide to control hyperglycemia in male mice since estrogen in female mice may protect against high-fat diet-induced metabolic syndrome. Female animals are prone to adipose tissue storage and glucose homeostasis, whereas males are predisposed to diabetes. As shown in Table 2, blood glucose levels were very high (>200 mg/dL) in *ob/ob* mice before treatment and remained very high in mice treated with CMC, a vehicle used to dissolve leflunomide. Fasting blood glucose levels decreased to normal levels in mice treated with leflunomide alone or with leflunomide plus uridine (<110 mg/dL). Statistical analysis revealed that leflunomide treatment significantly lowered blood glucose levels ( $p<0.01$ ). Treatment with uridine, a nucleoside used to normalize pyrimidine nucleotide levels *in vitro* and *in vivo* (Xu *et al.*, 1997; Xu *et al.*, 1999), had no effect on fasting blood glucose levels and did not block leflunomide-mediated control of hyperglycemia. These observations suggest that leflunomide controls hyperglycemia independent of its inhibitory effect on pyrimidine nucleotide synthesis. There were no significant differences in food intake and body weight among mice treated with CMC, leflunomide, uridine or leflunomide plus uridine for 3 days (Table 2).

We next conducted GTT to examine the ability of leflunomide to lower blood glucose levels in *ob/ob* mice. Blood glucose levels in mice receiving leflunomide or leflunomide plus uridine were significantly lower than those treated with CMC ( $p<0.001$ ) (Fig. 8A). Uridine treatment alone did not significantly alter blood glucose levels in mice. ITT assay revealed that blood glucose levels were elevated in control mice or in mice treated with uridine 15 min after insulin injection (Fig. 8C). In contrast, insulin did not increase blood glucose levels in leflunomide-treated mice but was able to slightly increase blood glucose levels in mice treated with uridine plus leflunomide (Fig. 8C). Transient increase of blood glucose levels after insulin injection into the tails of control *ob/ob* mice is probably caused by stress or due to the insulin receptor internalization. Transient increase of blood glucose levels after insulin injection is consistent with observations made by others (He *et al.*, 2013). AUC was significantly decreased in leflunomide- or leflunomide plus uridine-treated mice, compared to those treated with CMC or uridine alone (Fig. 8B & D) ( $p<0.01$ ). Consistently, leflunomide treatment significantly decreased blood glucose levels in GTT (Fig. 8E & F) and ITT assays (Fig. 8G & H) in HFD-fed mice, compared to those treated with CMC. Leflunomide appears more effective in lowering blood glucose levels in *ob/ob* mice while only modestly lowering blood glucose levels in the mice with HFD-induced diabetes. Leflunomide treatment did not significantly alter blood glucose levels in mice fed a NCD (Fig. 8E–H).

Finally, we tested if leflunomide treatment enhanced insulin receptor signaling *in vivo* in *ob/ob* mice. As shown in Fig. 9A, insulin significantly increased AKT<sup>S473/T308</sup> phosphorylation in the muscle, fat, and liver of normal mice, compared to the unstimulated controls ( $p<0.05$ ) (Fig. 9C); Leflunomide treatment did not or only slightly enhanced insulin-induced AKT phosphorylation in these insulin-sensitive tissues of control mice; The levels of AKT<sup>S473/T308</sup> phosphorylation in the tissue of the control and leflunomide-treated mice were comparable (Fig. 9A & C) ( $p>0.05$ ). In contrast, insulin poorly induced AKT<sup>S473/T308</sup> phosphorylation in the muscle, fat, and liver of *ob/ob* mice (Fig. 9B).

Densitometry analysis revealed that AKT<sup>S473/T308</sup> phosphorylation in the insulin-sensitive tissue was not significantly different between the untreated and insulin-treated mice ( $p < 0.05$ ) (Fig. 9D). Leflunomide treatment significantly increased insulin-stimulated AKT<sup>S473/T308</sup> phosphorylation in the muscle and adipose tissues, compared to those mice treated with the control vehicle only ( $p < 0.05$ ). AKT<sup>S473/T308</sup> phosphorylation levels appeared to be higher in the liver of leflunomide-treated *ob/ob* mice than that of untreated *ob/ob* mice. However, statistical analysis did not reach significance. Similarly, AKT<sup>S473/T308</sup> phosphorylation levels were also higher in the muscle, fat, and liver of *ob/ob* mice treated with leflunomide plus uridine than those treated with uridine alone (Supplementary figure 1). However, there was no significant difference in S6 and IRS-1 phosphorylation in the muscle, fat, and liver between untreated and leflunomide-treated mice (Supplementary figure 2).

## Discussion

S6K1 hyperactivation has been implicated in insulin resistance and the development of hyperglycemia and obesity (Um *et al.*, 2006; Um *et al.*, 2004). S6K1-deficient mice are resistant to HFD-induced hyperglycemia and obesity (Um *et al.*, 2006; Um *et al.*, 2004). Shum *et al.* (Shum *et al.*, 2016) recently reported that PF-4708671, an S6K1 inhibitor, controls hyperglycemia in HFD-fed mice by sensitizing the insulin receptor. We recently identified S6K1 as a molecular target of leflunomide and A77 1726 (Doscas *et al.*, 2014). Inhibition of S6K1 activity by A77 1726 in A375 melanoma cells leads to the feedback activation of the PI-3 kinase pathway, as evidenced by increased AKT<sup>S473</sup> and decreased S6<sup>S235/236</sup> phosphorylation. Our present study showed that inhibition of S6K1 activity by A77 1726 led to the inhibition of S6<sup>S235/236</sup> and IRS<sup>S1101</sup> phosphorylation in mouse C2C12 and rat L6 myotubes, and in mouse 3T3-L1 adipocytes under normal and/or insulin resistance conditions. A77 1726 enhanced insulin-induced insulin receptor tyrosine phosphorylation, binding of the p85 subunit of the PI-3 kinase to IRS-1 (Fig. 5), and insulin-induced GLUT4 translocation to the plasma membrane in L6 cells in the presence of high amino acid concentrations (Fig. 6). Leflunomide treatment increased AKT<sup>T308/S473</sup> phosphorylation in the muscular and adipose tissues *in vivo*. These observations collectively suggest that inhibition of S6K1 by A77 1726 is primarily responsible for its anti-hyperglycemic effect. Guo *et al.* (Guo *et al.*, 1997) reported earlier that chronic use of leflunomide has no effect on blood glucose levels in healthy rats. Because there is no insulin resistance in the insulin-sensitive tissues of normal animals, leflunomide cannot further sensitize the insulin receptor. Consistently, we found that there was no significant difference in blood glucose levels in NCD-fed lean mice treated with CMC or leflunomide (Fig. 8E–H).

We investigated the inhibitory effect of A77 1726 on the phosphorylation of two relevant substrates of S6K1, S6<sup>S235/236</sup> and IRS-1<sup>S1101</sup>. Due to feedback activation of the PI-3 kinase pathway, S6K1 phosphorylation was increased in A77 1726-treated cells. A77 1726 inhibited S6<sup>S235/236</sup> and IRS-1<sup>S1101</sup> phosphorylation in myotubes and adipocytes *in vitro*. In contrast, leflunomide treatment did not significantly decrease S6<sup>S235/236</sup> and IRS-1<sup>S1101</sup> in the metabolic tissues of insulin-stimulated mice *in vivo* (Supplementary figure 2). Similar to our observations, Shum *et al.* (Shum *et al.*, 2016) reported that PF-4708671, a more potent inhibitor of S6K1 than A77 1726, did not inhibit S6 phosphorylation *in vivo*. These

investigators suggest that compensatory S6K2 activation may shield the inhibitory effect of PF-4708671 on S6 phosphorylation (Shum *et al.*, 2016). Pearce *et al.* (Pearce *et al.*, 2010) reported that PF-4708671 did not inhibit early S6 phosphorylation in 293 cells in the presence of serum. We speculate that inhibition of S6K1 activity by A77 1726 or PF-4708671 may initially inhibit S6 and IRS-1 phosphorylation. However, when S6K1 is further feedback activated, incomplete inhibition of S6 activity may account for lack of inhibition of S6 and IRS-1 phosphorylation. Within a few minutes after insulin injection, leaked S6K1 activity is sufficient to fully phosphorylate S6 and IRS-1. However, once AKT is feedback activated, AKT phosphorylation levels will remain high for a while. Activated AKT continues to regulate glucose metabolism until it is dephosphorylated and inactivated. IRS-1 is phosphorylated at S636 by mTOR (Coppo and White, 2012). Our present study showed that due to feedback activation of mTOR, A77 1726 induced IRS-1<sup>S636</sup> phosphorylation in C2C12 myotubes. Interestingly, IRS-1<sup>S636</sup> phosphorylation by ROCK1 leads to the activation of PI-3 kinase (Coppo and White, 2012). Thus, A77 1726 may improve insulin receptor signaling by inhibiting IRS-1<sup>S1101</sup> and by increasing IRS-1<sup>S636</sup> phosphorylation.

Leflunomide and A77 1726 inhibit S6K1 activity with the IC<sub>50</sub> values of approximately 50–75  $\mu$ M (Doscas *et al.*, 2014). Plasma concentrations of A77 1726 in RA patients treated with leflunomide (20 mg/day) are higher than 200  $\mu$ M (Chan *et al.*, 2005). A77 1726 in the blood of mice treated with leflunomide at a dose of 35 mg/kg had a remarkably long half-life of 15 hr. The blood concentrations of A77 1726 reached a peak of 500  $\mu$ M within 4 hr and remained at 250  $\mu$ M at 24 hr after a single dose of 35 mg/kg of leflunomide in mice (Chong *et al.*, 1999). These data suggest that A77 1726 concentrations in plasma are high enough to inhibit S6K1 activity. Indeed, AKT phosphorylation was increased in the metabolic tissues of leflunomide-treated mice. Blood glucose levels were decreased to normal levels in *ob/ob* mice treated with leflunomide at a dose of 35 mg/kg/day. Co-administration with uridine was unable to block the anti-glycemic effect of leflunomide. An earlier clinical study revealed that the mean glucose levels in the blood of RA patients treated with leflunomide (83 mg/dL) were significantly lower than those treated with other regimens (93 mg/dL) (Rho *et al.*, 2009). About 10% of patients treated with leflunomide undergo weight loss that cannot be attributed to diarrhea or other gastrointestinal side effects and is likely due to an increased metabolic rate (Coblyn *et al.*, 2001). These observations collectively suggest that leflunomide concentrations are high enough to inhibit S6K1 activity, subsequently leading to better control of hyperglycemia. Inflammatory cytokines, in particular TNF- $\alpha$ , can also desensitize the insulin receptor and contribute to the development of hyperglycemia (Coppo and White, 2012). Anti-TNF- $\alpha$  therapy does not lower blood glucose levels in RA patients (Rosenvinge *et al.*, 2007). Many anti-inflammatory drugs, unlike leflunomide, do not lower blood glucose levels (Rho *et al.*, 2009), suggesting that inhibition of inflammation is not a sufficient explanation for better control of hyperglycemia.

We are aware of several weaknesses in our study. First, the evidence supporting the conclusion that leflunomide sensitized the insulin receptor and controlled hyperglycemia by inhibiting the activity of S6K1 *in vivo* is weak. Increased AKT phosphorylation in the tissue of leflunomide-treated mice with insulin resistance indirectly suggests feedback activation of the PI-3 kinase pathway due to the inhibition of S6K1 activity. However, inhibition of S6

and IRS-1 phosphorylation was not seen in the tissue of leflunomide-treated animals. This is consistent with the observations in a study using PF-4708671, a potent S6K1 inhibitor (Shum *et al.*, 2016). Second, leflunomide worked extremely well in lowering blood glucose levels in the *ob/ob* mice, even with a relatively small sample size, the *p* values reached statistically significant levels. However, leflunomide only modestly decreased blood glucose levels in the GTT and ITT in the mouse model of the HFD-induced diabetes. Third, leflunomide treatment significantly enhanced insulin-stimulated AKT phosphorylation in the muscle and adipose tissues of *ob/ob* mice. Leflunomide treatment increased AKT phosphorylation in the liver of *ob/ob* mice, but this was not statistically significant due to a relatively small number of animals and the high variability of AKT phosphorylation. Fourth, since there are already many anti-diabetic drugs available for treating hyperglycemia, leflunomide might be only useful for a certain type of patient population such as those with both diabetes and RA. The diabetes mouse models used in our study do not have RA, thus not completely recapitulating a clinical setting in which leflunomide seems to be used.

Chronic use of rapamycin does not control but rather exacerbates hyperglycemia in mouse models and in patients (Di Paolo *et al.*, 2006; Schindler *et al.*, 2014). Failure of rapamycin to control hyperglycemia is, after chronic use, due to its ability to inhibit the activity of mTORC2 (Fig. 1B), which is responsible for phosphorylating AKT<sup>S473</sup> (Lamming *et al.*, 2012). In the present study, we did not follow up the anti-hyperglycemic effect after chronic use of leflunomide. However, several lines of evidence suggest that chronic use of leflunomide will likely maintain its therapeutic efficacy: 1) Unlike rapamycin, A77 1726 inhibits S6K1 (Doscas *et al.*, 2014), a kinase downstream of mTOR. Therefore, A77 1726 does not act like rapamycin to inhibit mTORC2 (Fig. 1B); 2) The anti-hyperglycemic effect of PF-4708671 after chronic use is sustainable *in vivo* (Shum *et al.*, 2016). Both A77 1726 and PF-4708671 control hyperglycemia by inhibiting S6K1; 3) Blood glucose levels are significantly lower in RA patients chronically treated with leflunomide than those treated with other drugs (Rho *et al.*, 2009). Nevertheless, whether leflunomide can maintain a long-lasting therapeutic effect after chronic use should be verified first in animal models before it can be investigated in patients.

In summary, our present study showed that A77 1726 induced feedback activation of the PI-3 kinase pathway by inhibiting S6K1 activity, and that A77 1726 increased glucose uptake and GLUT4 translocation to the plasma membrane *in vitro* (Fig. 1). A77 1726 may also improve glucose metabolisms by stimulating glycogen synthesis and by inhibiting gluconeogenesis through activated AKT (Fig. 1B). Leflunomide was capable of controlling hyperglycemia and improving insulin sensitivity *in vivo*. Leflunomide could be particularly useful for treating RA patients who also have type 2 diabetes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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Fig. 1A

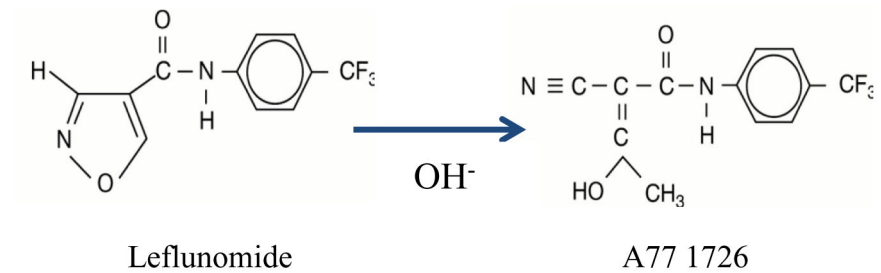
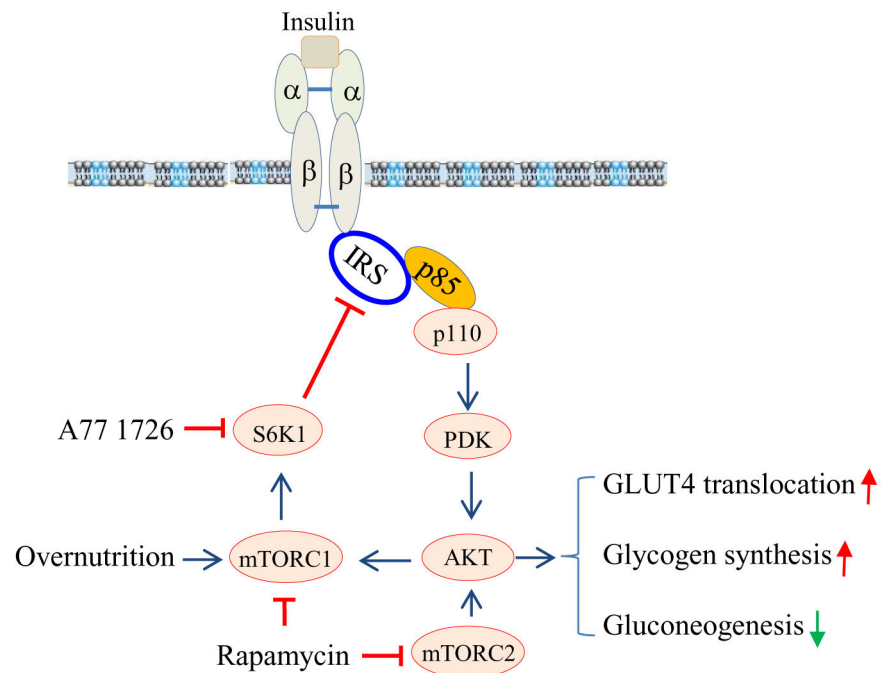


Fig. 1B

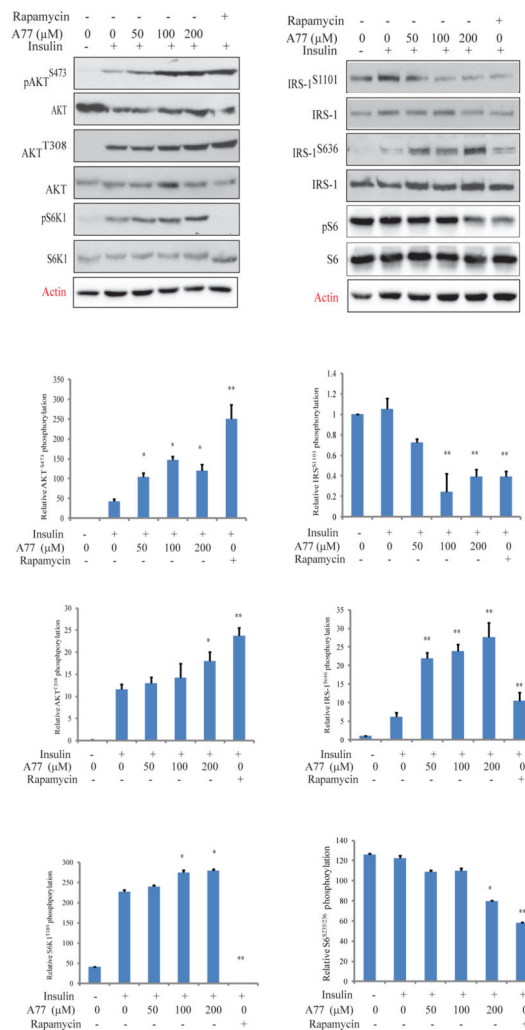
**Fig. 1. The mechanisms of leflunomide-mediated anti-hyperglycemic effect**

(A) Chemical structure of leflunomide and A77 1726. (B) Mode of action of A77 1726.

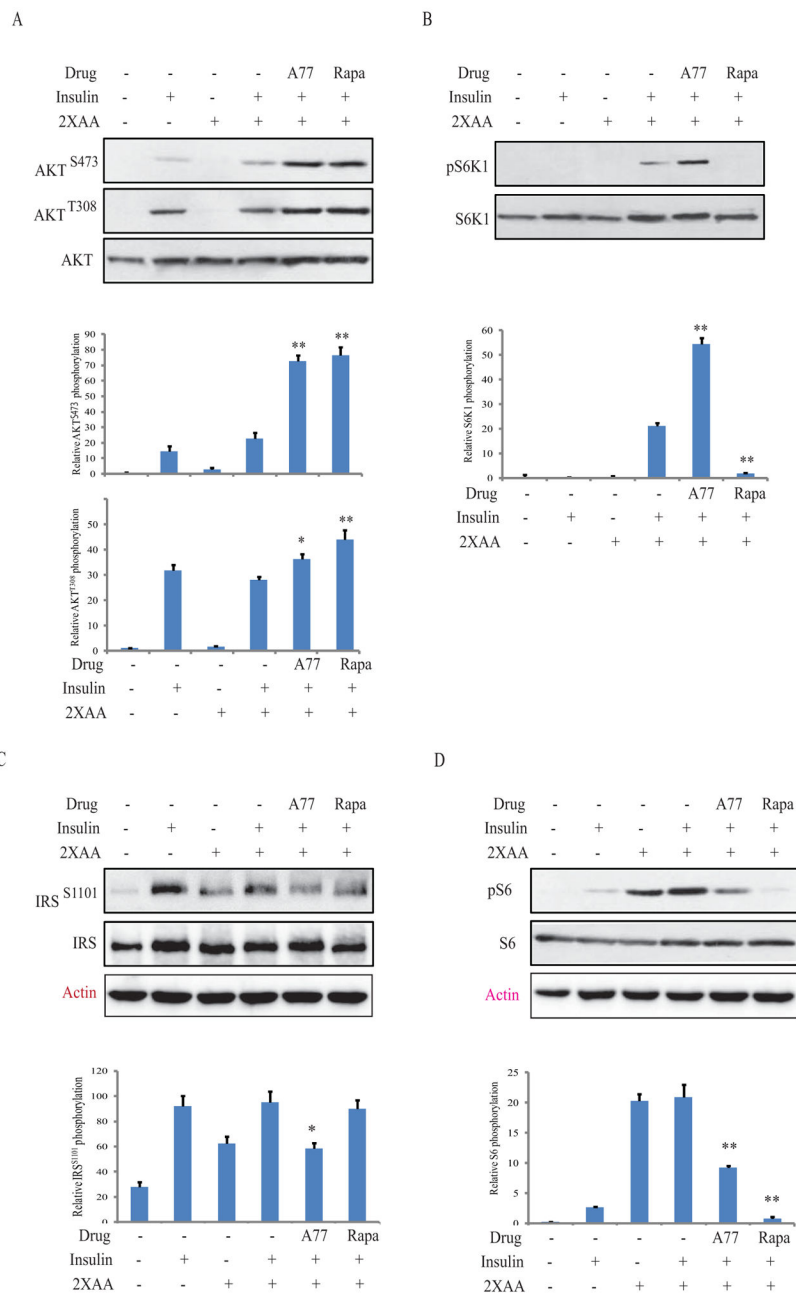
Overnutrition with high concentrations of fatty acids and amino acids leads to constitutive S6K1 activation, which phosphorylates IRS-1<sup>S1101</sup>, leading to poor AKT activation.

Leflunomide and its active metabolite A77 1726 inhibit S6K1 activity, subsequently leading to the inhibition of IRS-1<sup>S1101</sup>. Inhibition of IRS-1<sup>S1101</sup> phosphorylation leads to insulin receptor sensitization, as revealed by increased insulin receptor tyrosine phosphorylation and increased binding of IRS-1 to the p85 subunit of PI-3 kinase. AKT<sup>S473/T308</sup> phosphorylation and activation leads to increased glucose uptake by stimulating GLUT4 membrane translocation, increased glycogen synthesis, and decreased gluconeogenesis. Chronic use of rapamycin leads to inhibition of both mTORC1 and mTORC2, thus exacerbating hyperglycemia.





**Fig. 2. Effect of A77 1726 on protein phosphorylation in the PI-3 kinase pathway**  
 C2C12 myotubes were starved in serum-free medium for 4 hr and then treated with the indicated concentration of A77 1726 or rapamycin (50 nM) for 2 hr. The cells were left unstimulated or stimulated with insulin (20 nM) for 20 min. Cells were harvested and analyzed for the phosphorylation of AKT<sup>S473</sup>, AKT<sup>T308</sup>, and S6K1<sup>T389</sup>, IRS-1<sup>S1101</sup>, IRS-1<sup>S636</sup>, S6<sup>S235/236</sup>, followed by reprobing with their specific antibodies for total protein levels. Relative protein phosphorylation was determined by analyzing the density of bands and presented as bar graphs. The results are the mean  $\pm$  standard deviation (SD) from three experiments. A77, A77 1726; \* $p < 0.05$ ; \*\* $p < 0.01$ , compared to the insulin-stimulated control (no drug treatment).



**Fig. 3. A77 1726 sensitizes insulin receptor in L6 myotubes**

This experiment and the remaining ones (Fig. 4–7) were carried out under the condition of insulin resistance in which the cells were incubated in the presence of high concentrations of amino acids. Briefly, L6 myotubes were first starved of serum for 4 hr and then incubated either in an amino-acid free medium (EBSS) or EBSS medium containing 2x the amino acid concentrations (2xAA) found in MEM in the absence or presence of A77 1726 (200  $\mu$ M) for 2 hr. After stimulation with insulin (20 nM) for 20 min, cells are harvested and analyzed for the phosphorylation of AKT<sup>S473/T308</sup> (A), S6K1<sup>T389</sup> (B), IRS-1<sup>S1101</sup> (C), S6<sup>S235/236</sup> (D), and probed with their specific antibodies for total proteins. Relative protein

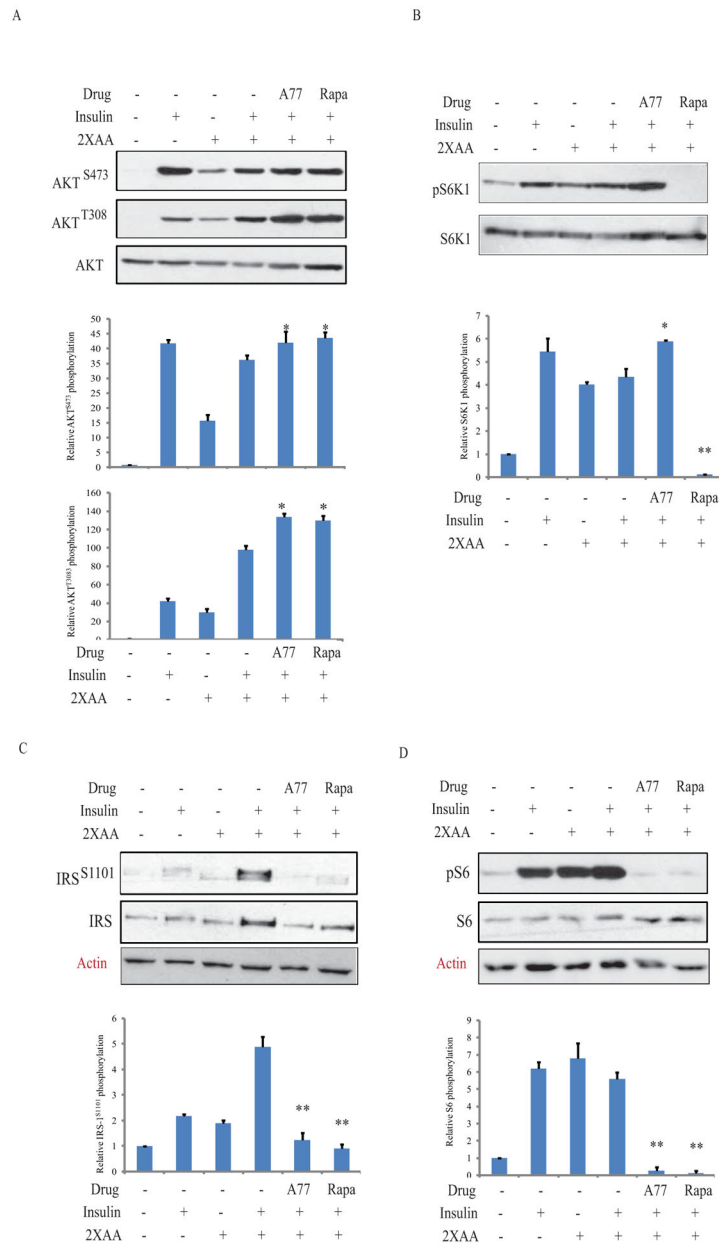
phosphorylation was analyzed by using an Image J software. The results are the mean  $\pm$  SD from three experiments. A77, A77 1726; Rapa, rapamycin. \* $p < 0.05$ ; \*\* $p < 0.01$ , compared to the control (with insulin and 2X amino acids but without drug).

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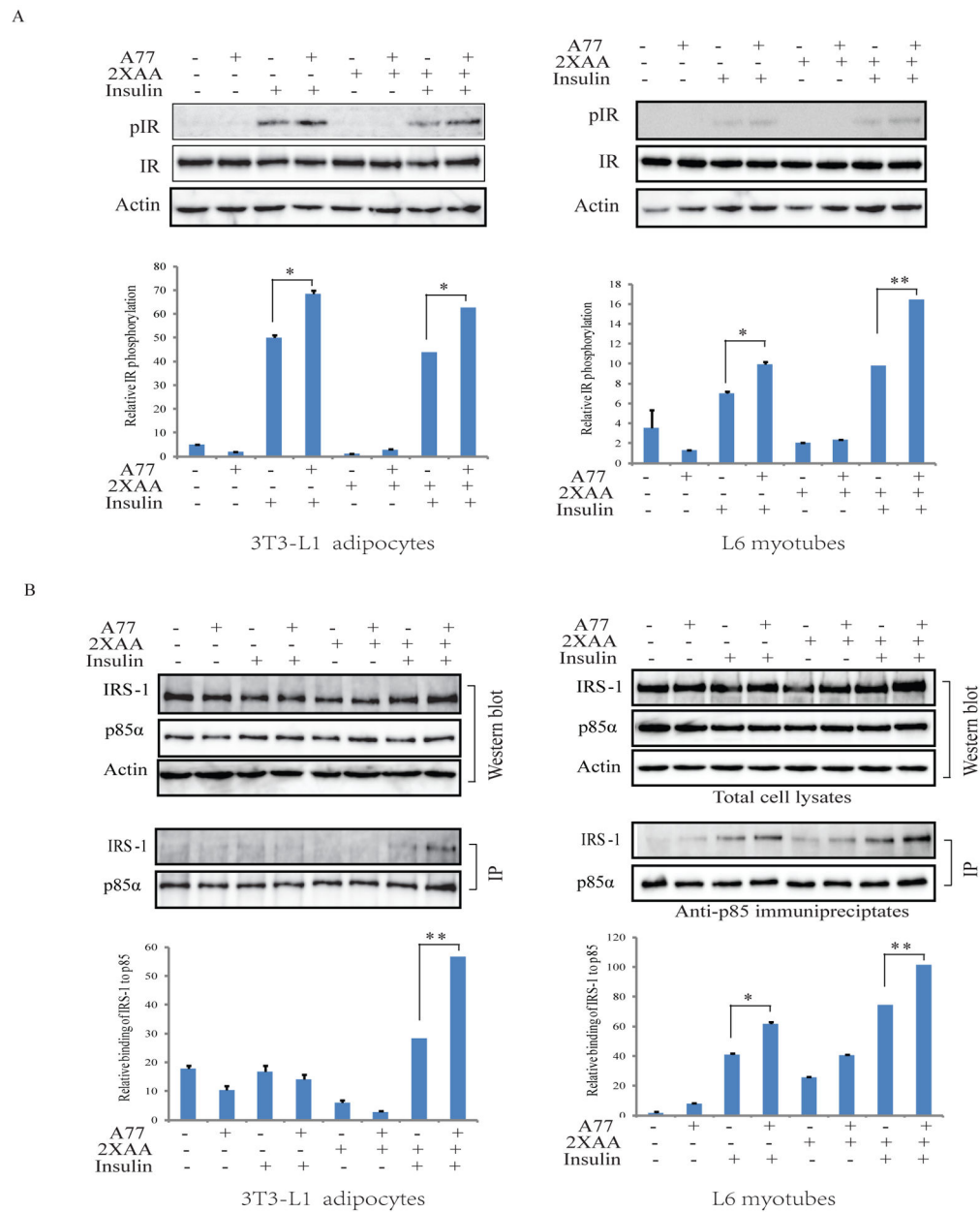
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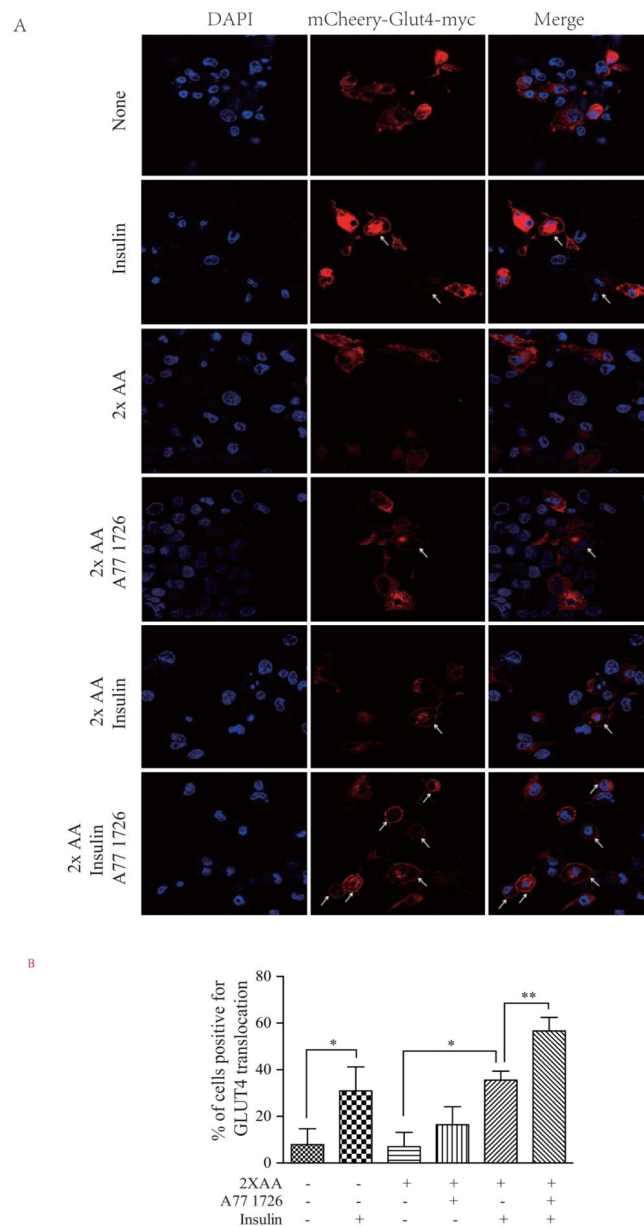


#### Fig. 4. A77 1726 sensitizes insulin receptor in 3T3-L1 adipocytes

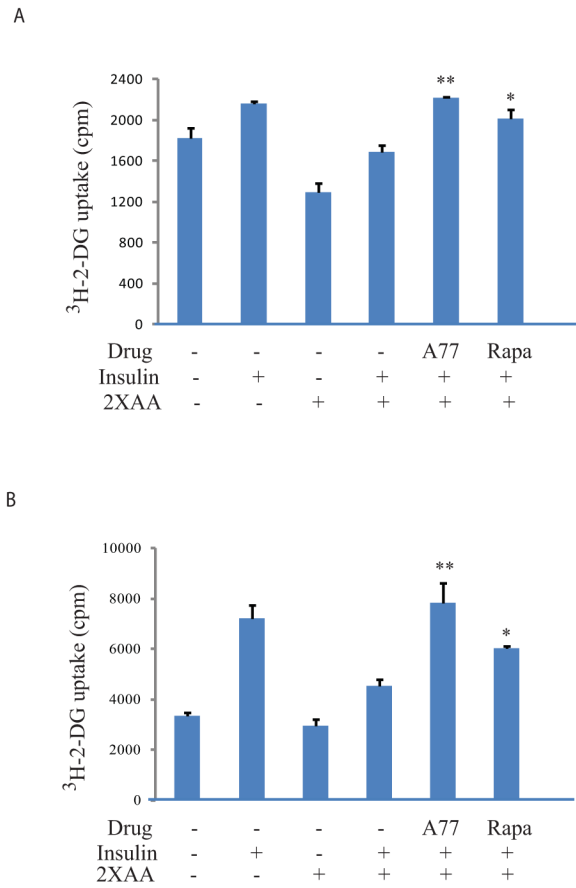
3T3-L1 adipocytes were treated in Fig. 3 and then incubated either in an amino-acid free medium (EBSS) or EBSS medium containing 2x the amino acid concentrations (2xAA) found in MEM in the absence or presence of A77 1726 for 2 hr. Cells were stimulated with insulin (20 nM) for 20 min. The cells were harvested and analyzed for the phosphorylation of AKT<sup>S473/T308</sup> (A), S6K1<sup>T389</sup> (B), IRS-1<sup>S1101</sup> (C), S6<sup>S235/236</sup> (D), and reprobated with their specific antibodies for total proteins. The density of bands was analyzed by using an NIH Image-J software and normalized by the arbitrary units of their corresponding total proteins. The results are the mean  $\pm$  SD from three experiments. A77, A77 1726; Rapa, rapamycin. \* $p < 0.05$ ; \*\* $p < 0.01$ , compared to the control (with insulin and 2X amino acids but without drug).



**Fig. 5. A77 1726 stimulates insulin receptor tyrosine phosphorylation and increases the insulin receptor substrate (IRS-1) binding to the p85 $\alpha$  subunit of the PI-3 kinase**  
**(A)** 3T3-L1 adipocytes and L6 myotubes were similarly treated as in Fig. 3 and analyzed for the phosphorylation of insulin receptor tyrosine phosphorylation at Y1146. **(B)** A77 1726 increases the binding of the p85 subunit of the PI-3 kinase to IRS-1. 3T3-L1 adipocytes and L6 myotubes were treated in Fig. 3. Cell lysates were immunoprecipitated with an anti-p85 antibody followed by probing with anti-p85 and anti-IRS-1 antibodies in Western blot. Relative protein phosphorylation was analyzed by using Image J software. The results are the mean  $\pm$  SD from three experiments. A77, A77 1726; \* $p$ <0.05; \*\* $p$ <0.01

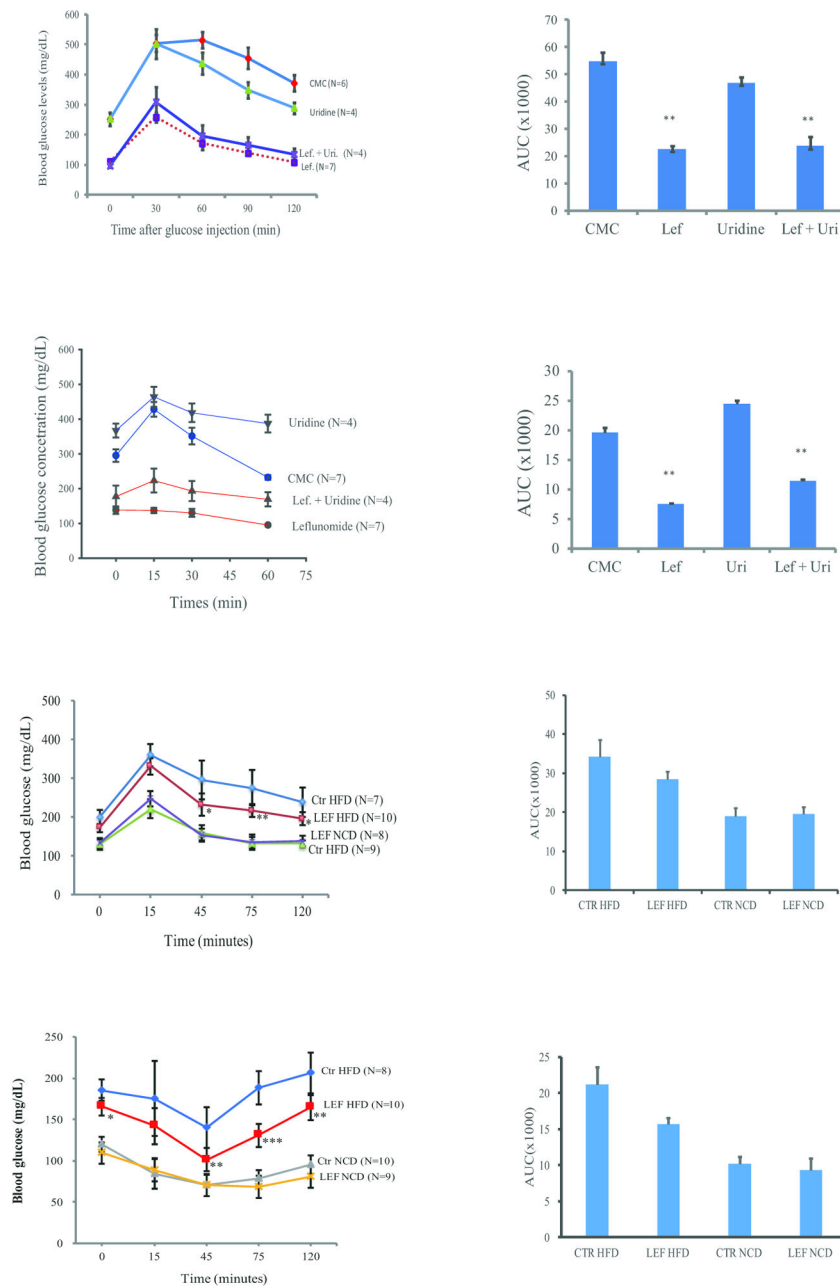


**Fig. 6. A77 1726 enhances insulin-induced GLUT4 translocation to the plasma membrane**  
 (A) GLUT4 translocation to the plasma membrane. mCherry-GLUT4-myc-transfected L6 cells were starved of serum for 4 hr and then incubated in the absence or presence of A77 1726 (200  $\mu$ M) without or with 2X the amino acid concentrations. Cells were left unstimulated or stimulated with 20 nM insulin for 45 min. After fixation in methanol for 10 min, mCherry-tagged GLUT4 fluorescence was visualized under a Leica LP8 confocal microscope. Arrows denote the mCherry-tagged GLUT4 translocation to the cell membrane.  
 (B) Quantification of the GLUT4 translocation to the plasma membrane. The data represent the mean  $\pm$  SD from one of three experiments with similar results. \* $p$ <0.05; \*\* $p$ <0.01



**Fig. 7. A77 1726 increases glucose uptake**

L6 myotubes (A) and 3T3-L1 adipocytes (B) seeded in 24-well plates were starved in serum-free, low glucose DMEM medium for 4 hr. The cells were then incubated in EBSS in the absence or presence of 2xAA and/or A77 1726 (200  $\mu$ M) or rapamycin (50 nM) for 2 hr. The cells were left unstimulated or stimulated with 20 nM insulin for 45 min followed by incubation of [ $^3$ H]-2-DG for 5 min. The data are the mean  $\pm$  SD of the triplicate in one of three experiments with similar results. \* $p$ <0.05; \*\* $p$ <0.01, compared to the control (with insulin and 2X amino acids but without drug).

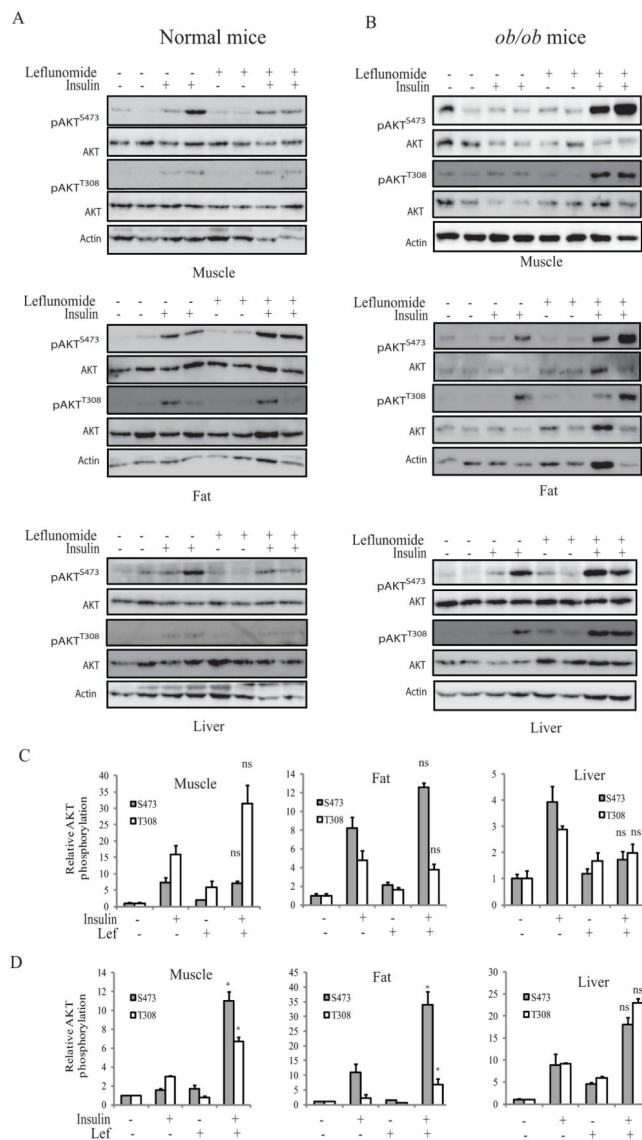


**Fig. 8. Control of hyperglycemia and insulin receptor sensitization by leflunomide**

(A) *ob/ob* mice were treated as described in Materials and Methods. The mice were challenged with glucose (1 g/kg) by intraperitoneal injection for GTT. Blood glucose levels were measured at the indicated time. CMC vs. leflunomide, uridine or leflunomide + uridine,  $p < 0.001$ ; leflunomide vs. leflunomide + uridine,  $p = 0.002$ . Lef, leflunomide; Uri, uridine; The arbitrary values of AUC were calculated and shown in a bar graph (B) \*\*CMC vs. leflunomide or leflunomide + uridine,  $p < 0.001$ ; CMC vs. uridine,  $p = 0.057$ . (C) Control of hyperglycemia by leflunomide in ITT. *ob/ob* mice were treated as described in Materials and Methods. After fasting for 6 hr, insulin (2.5 U/kg) was injected intravenously. Blood



glucose levels were measured at the indicated times. CMC vs. leflunomide, uridine or leflunomide + uridine,  $p < 0.001$ ; leflunomide vs. leflunomide + uridine,  $p = 0.006$ . (D) AUC in the different treatment groups was shown as the bar graph. \*\*CMC vs. leflunomide or leflunomide + uridine,  $p < 0.001$ ; CMC vs. uridine,  $p = 0.115$ . (E & G) C57BL/6 male mice (5w-weeks-old) fed on normal chow diet (NCD) or high-fat diet (HFD) for 10 weeks were treated daily with CMC or leflunomide (35 mg/kg/day) for 3 days and then evaluated for blood glucose levels in GTT (E) and ITT (G). (F & H) AUC in different treatment group was shown as bar graphs. \*\*CMC vs. leflunomide in mice fed with HFD,  $p < 0.001$ ; CMC vs. leflunomide in mice fed with NCD,  $p > 0.05$ .



**Fig. 9. AKT phosphorylation in the metabolic tissues is enhanced by leflunomide** C57BL/6 (A) and *ob/ob* (B) mice were treated daily with CMC (1.5%) or leflunomide (35 mg/kg/day) for 3 days. On day 4, the mice were treated with a last dose and then fasted for 6 hr. Mice were then injected intravenously with saline or insulin (2.5 unit/kg). Five minutes later, the mice were sacrificed. Gastrocnemius muscle, visceral white adipose and liver tissues were harvested and analyzed for AKT<sup>S473/T308</sup> phosphorylation by Western blot with their specific antibodies and reprobated with an antibody against total AKT protein. Immunoblots represent the data from two animals in each group. The experiment was repeated twice. (C & D) The density of the bands in the tissues of normal C57BL/6 mice (C) and *ob/ob* mice (D) was analyzed by using NIH Image-J software and normalized by the arbitrary units of their corresponding total proteins. The results are the mean  $\pm$  SD from four animals in each group. \* $p < 0.05$ ; ns, not significant between CMC/insulin-treated and leflunomide/insulin-treated group.

Table 1

## Antibodies information

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used	RRID (required in revised MSs)
AKT <sup>S473</sup>	Unknown	Phospho-Akt (Ser473)(D9E) Ab	Cell Signaling (#4060)	Rabbit; monoclonal	1:2000	AB_2315049
AKT <sup>T308</sup>	Unknown	Phospho-Akt (Thr308) Ab	Cell Signaling (#9275)	Rabbit; monoclonal	1:1000	AB_329828
AKT	Unknown	Akt Antibody	Cell Signaling (#9272)	Rabbit; monoclonal	1:1000	AB_329827
pS6K1	Unknown	Phospho-p70 S6 Kinase (Thr589)(108D2) Ab	Cell Signaling (#9234)	Rabbit; monoclonal	1:1000	AB_2269803
S6K1	Unknown	p70 S6 Kinase(49D7) Ab	Cell Signaling (#2708)	Rabbit; monoclonal	1:1000	AB_390722
pS6	Unknown	Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) Ab	Cell Signaling (#4858)	Rabbit; monoclonal	1:2000	AB_916156
S6	Unknown	S6 Ribosomal Protein (5G10) Ab	Cell Signaling (#2217)	Rabbit; monoclonal	1:1000	AB_331355
IRS-1 <sup>S1101</sup>	Unknown	Phospho-IRS-1 (Ser1101) Antibody	Cell Signaling (#2385)	Rabbit; monoclonal	1:1000	AB_330363
IRS-1 <sup>S636</sup>	Unknown	Phospho-IRS-1 (Ser636/639) Antibody	Cell Signaling (#2388)	Rabbit; monoclonal	1:1000	AB_330339
IRS-1	Unknown	IRS-1 (59G8) Ab	Cell Signaling (#2390)	Rabbit; monoclonal	1:1000	AB_561122
pIR	Unknown	Phospho-IGF-1 Receptor $\beta$ (Tyr1131)/Insulin Receptor $\beta$ (Tyr1146) Antibody	Cell Signaling (#3021)	Rabbit; monoclonal	1:1000	AB_331578
IR	Unknown	Insulin Receptor $\beta$ (4B8) Ab	Cell Signaling (#3025)	Rabbit; monoclonal	1:1000	AB_2280448
P85 $\alpha$	Unknown	PI3 Kinase p85 (19H8) Ab	Cell Signaling (#4257)	Rabbit; monoclonal	WB:1:1000 IP:1:50	AB_10831521
Actin	Unknown	$\beta$ -Actin Antibody (C4)	Santa Cruz Biotechnology(sc-47778)	Mouse; monoclonal	1:200	AB_626632

Table 2

Control of hyperglycemia by leflunomide in *ob/ob* mice<sup>†</sup>

Drug	Number of mice (N)	Blood glucose concentration (mg/dL)			Body weight (g)			Food intake (g/d)		<i>p</i> value*
		Before	After	<i>p</i> value	Before	After	Change	Before	After	
Vehicle	6	234±16	252±22	0.697	47.8±2.9	48.0±3.1	0.12±0.21	4.9±0.23		
Leflunomide	7	275±12	111±6	0.001	48.4±0.48	48.5±0.51	-0.02±0.13	5.0±0.09		0.687
Uridine	4	210±12	253±15	0.153	48.7±0.68	48.5±0.69	-0.02±0.08	4.9±0.07		1.000
Lef + Uridine	4	222±15	97±15	0.021	48.5±1.21	48.4±1.06	-0.3±0.04	4.8±0.04		0.272

<sup>†</sup> *ob/ob* mice (male, 8–10 wks) were treated with the vehicle (1.5% CMC, daily, gavage), leflunomide (35 mg/kg, daily, gavage), uridine (2 g/kg, twice daily, i.p.) or leflunomide (35 mg/kg, daily, gavage) + uridine (2 g/kg, twice daily, i.p.) for three days. After fasting for 6 hr, blood glucose levels were measured and statistically analyzed by a paired Student *t* test. Body weight of mice were measured before and 3 days after treatment. Food consumption was measured on individual mouse in a separate cage on day 3 after treatment.

\* Compared to the vehicle control.