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A novel SIRT1 activator E6155 improves insulin sensitivity in type 2 diabetic KKA$_y$ mice

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ABSTRACT

Sirtuin 1 (SIRT1) is an NAD$^+$-dependent protein deacetylase that plays a critical role in controlling energy metabolism, stress response and aging. Hence, enhancing SIRT1 activity could be a potential therapeutic strategy to treat metabolic diseases such as diabetes. However, pharmacological activators for SIRT1 are scarce to date. In this study, using the optimized high throughput screening, we identified E6155, a piperazine 1, 4- diamide compound, as a new small molecular activator of SIRT1. We further found that E6155 significantly upregulated glucose uptake in cultured normal liver cells and skeletal muscle cells through increasing SIRT1 deacetylase activity. In type 2 diabetic KKA$_y$ mice, E6155 treatment markedly decreased the level of fasting glucose. Moreover, E6155 improved oral glucose tolerance and insulin tolerance. Euglycemic clamp and the homeostasis model assessment of insulin resistance index showed that E6155 ameliorated the insulin resistance and increased insulin sensitivity in diabetic mice. Mechanistically, we observed that the antidiabetic effects of E6155 were involved in SIRT1 dependent activation of LKB1/AMPK and IRS1/AKT pathways. In conclusion, our findings identified E6155 as a novel SIRT1 activator and suggested that E6155 could be a promising drug candidate for treating insulin resistance and diabetes.

1. Introduction

Calorie restriction has pleiotropic effects on cell metabolism and extended lifespan in various species [1]. It has been proposed that the beneficial effects of calorie restriction is partially through silent information regulator 2 (Sir2), a NAD$^+$-dependent histone deacetylase [2]. Sirtuin 1 (SIRT1) belongs to mammals' sirtuins family, which shares the highest sequence homology with yeast silent information regulator-2 (ySir2), is the most extensively studied member of the class III histone deacetylase [3].

Recently, many studies have demonstrated that SIRT1 activation could improve insulin sensitivity and modulate glucose metabolism [4–6]. SIRT1 has been shown to have benefits in improving insulin resistance and regulating insulin-stimulated glucose uptake by AMP-activated kinase (AMPK)-dependent and insulin receptor substrate 1 (IRS1)/AKT-dependent pathways [7,8]. Accumulating evidence suggested that AMPK activation could ameliorate insulin resistance and prevent the pathologies of type 2 diabetes [9]. In addition, overexpression of SIRT1 could activate liver kinase B1 (LKB1) and subsequently activate AMPK by decreasing lysine acetylation of LKB1, which caused the translocation of LKB1 from the nucleus to the cytoplasm [10]. The IRS1/AKT pathway is important in mediating Glucose transporter type 4 (Glut4) translocation and glucose transport, however, the signaling is impaired in the conditions of insulin resistance [7,11]. Also, it was demonstrated that resveratrol, a SIRT1 activator, promotes SIRT1 through LKB1-dependent AMPK activation [12] and IRS1/AKT activation by increasing the phosphorylation of IRS1 and AKT [13]. Therefore, SIRT1 was thought to be a promising therapeutic target for the treatment of metabolic disorders and insulin resistance-related diseases.

In this study, through high throughput screening (HTS), we discovered a novel SIRT1 activator E6155, which is a piperazine 1, 4-diamide compound. We further evaluated the beneficial effects of

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E6155 on glucose metabolism and explored the possible mechanism *in vitro* and *in vivo*. Our findings demonstrated that E6155 improved insulin sensitivity and prevented insulin resistance in KKA_y mouse model of type 2 diabetes, and thus E6155 might be potentially used in treating diabetes caused by insulin resistance.

2. Materials and methods

2.1. Materials

The small-molecule compound library was maintained by the National Laboratory for Screening New Microbial Drugs, CAMS&PUMC (Beijing, China). E6155 for the animal experiments was ordered from J&K Scientific (Beijing, China). 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) was ordered from Invitrogen (MA, USA). EX527 was purchased from Selleckchem (TX, USA). Insulin, Doxorubicin and Palmitate were obtained from Sigma Aldrich (MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (MA, USA).

2.2. Cell culture

HepG2 cells, L02 cells and L6 cells were purchased from National Infrastructure of Cell Line Resource (Beijing, China). HepG2 cells, L02 cells and L6 cells were cultured in DMEM with 10% FBS (v/v) in 37 °C, 5% CO_2 incubator. For L6 myoblast differentiation, after the cells met 80% confluence, the medium was changed to DMEM with 2% FBS for 7 days.

2.3. HTS assay for SIRT1 activators

In order to identify SIRT1 activators from the synthetic and natural compound library of our laboratory, we established an HTS assay using the purified recombinant human SIRT1 and HTRF SIRT1 assay purchased from Cisbio bioassays (Parc Marcel Boiteux, France). Reactions were conducted in 384-well white-bottom plates which contains 0.025 U purified SIRT1 enzyme, 1 mM dithiothreitol, 150 μM NAD^+, and 6 nM SIRT1-specific deacetylation substrate-d2, DMSO (final concentration 1%, negative control) or a test compound (final DMSO concentration 1%) each well. Enzyme control wells did not contain SIRT1 enzyme and negative control wells did not contain test compound. After mixing the reaction components, plates were incubated at room temperature for 2 h. Then Anti-acetyl MAb-cryptate was added for additional 5 h. Fluorescence at 620 nm and 665 nm of each well was measured using a fluorometric plate reader (PerkinElmer). Sample activity was defined as the percentage of signal increase relative to the signal window, according to the following formula: ratio = (absorbance at 665 nm/absorbance at 620 nm) × 10^4; deacetylation of substrate (%) = 100- ($ratio_{sample}/ratio_{Enzyme\ control} \times 100$); upregulating activity regulatory = $ratio_{sample}/ratio_{negative\ control} \times 100\%$. A value ≥ 150% was considered to have a potential activation effect. After validated as potential activators, EC_{50} values were calculated by dose-dependent curve using GraphPad Prism 5 software.

2.4. Western blotting

Western blotting was performed as previously described [14]. The primary antibodies include Acetyl-p53 (#2570, Cell Signaling Technology), p53 (#2524, Cell Signaling Technology), Phospho-AMPKα (#2531, Cell Signaling Technology), AMPKα (#5832, Cell Signaling Technology), Phospho-AKT (#9271, Cell Signaling Technology), AKT (#4691, Cell Signaling Technology), and GAPDH (#AB2302, EMD Millipore). All western blot experiments were repeated at least three times and representative images were shown.

2.5. Glucose uptake assay

Glucose uptake activity of the cells after the treatment of E6155 with or without EX527 was assayed by 2-NBDG as described previously [15]. L02 or L6 myotubes were incubated with E6155 (0.1 μM) with or without EX527 (10 μM) in 37 °C for 18 h. After that, cells were stimulated with 100 nM insulin for 30 min. Then, insulin was replaced with 50 μM of 2-NBDG. After 1h, the cells were washed three times and lysed in lysis buffer for 10 min. The fluorescence intensity of supernatants which containing equal amount of protein were measured using a fluorometric plate reader (PerkinElmer) at 485 nm excitation and 535 nm emission, respectively. Each glucose uptake assay was performed three times.

2.6. Glucose homeostasis analysis in type 2 diabetic KKA_y mice

Female KKA_y mice (16-weeks old, 30–35 g) were purchased from the Institute of Laboratory Animal Sciences, CAMS, PUMC. Animal care and experimental procedures were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of the Institute of Medicinal Biotechnology Institute. In all groups, the mice were fed a high-fat diet to render the mice diabetic (fasting blood glucose>11.1 mmol/L). The model group mice were treated with 0.5% carboxymethylcellulose sodium (CMC-Na). E6155 group were treated with E6155 (10 mg/kg) in CMC-Na by oral gavage once daily for 3 weeks. At day 0, 4, 7, 11, 17 and 20 of the treatment, the mice were fasted for 12 h and the fasting blood glucose was measured using the blood samples from the tail vein. At the end of the experiment, the mice were anesthetized and blood samples were collected from the retro-orbital plexus after fasted for 12 h. Serum insulin, glucose, leptin and adiponectin were measured using commercially available kits (Nanjing SenBeiJia, China) according to manufacturers' protocol.

2.7. Oral glucose tolerance test (OGTT)

After 2 weeks of E6155 or vehicle treatment, mice were fasted for 6 h but not limited to the water. 2 g/kg body weight glucose was given by gavage and glucose levels were detected at 0, 30, 60, 90 and 120 min after the glucose treatment [16].

2.8. Insulin tolerance test (ITT)

After 2 weeks of E6155 or vehicle treatment, another cohort of mice was fasted for 12 h but have free access to water. Then the mice were subcutaneous injected with insulin solution at 0.75 IU/kg body weight. After 0, 40, 90 and 120 min injection, the blood samples from the tail vein of each mouse were used to measure blood glucose levels [17].

2.9. Hyperinsulinemic–euglycemic clamp analysis

After E6155 or vehicle treated for 3 weeks, hyperinsulinemic–euglycemic clamp experiments were performed [18]. Mice were fasted for 12 h and anesthetized before the study. The right jugular vein was catheterized the Y shape pipe for the infusion of glucose and insulin. After the adaptation period, human insulin was infused by syringe infusion pump (KD Scientific, USA) at the rate of 20 mIU/kg/min. When the blood glucose levels decreased to below 6.0 mmol/L, infusion of glucose was started and monitored the blood glucose level every 5 min. The glucose infusion rate (GIR) of five time points after the glucose levels