Ceftezole, a cephem antibiotic, is an α-glucosidase inhibitor with *in vivo* anti-diabetic activity

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Abstract. Using a high throughput-compatible assay to screen for potential α-glucosidase inhibitors, we found that the ß-lactam antibiotic ceftezole exhibited potent α-glucosidase inhibitory activity. In *in vitro* α-glucosidase assays, ceftezole was shown to be a reversible, non-competitive inhibitor of yeast α-glucosidase with a K_i value of 5.78×10^{-7} M when the enzyme mixture was pretreated with ceftezole. Using an *in vivo* streptozotocin-induced mouse model, we confirmed that blood glucose levels decreased by 30% 20 min after ceftezole treatment (10 mg/kg/day). Expression levels of glycogen synthase kinase-3, peroxisome proliferator-activated receptor-γ, and uncoupling protein-3 mRNA were also slightly decreased compared to controls following ceftezole treatment. Taken together, these *in vivo* and *in vitro* results suggest that ceftezole may be a clinically useful anti-diabetic compound.

Introduction

Diabetes, which is one of the most severe metabolic disorders in humans, is characterized by hyperglycemia as a result of a relative or an absolute lack of insulin or insulin activity on target tissue, or both (1). Diabetes is actually a heterogeneous group of metabolic disorders caused by both genetic and environmental factors that result in glucose intolerance (2). It originates from a biochemical imbalance in the pancreatic ß-cell environment and is caused by mutations in a variety of genes, suggesting that the majority of diabetic disorders are multifactorial. Diabetic disorders are classified into two major groups: type 1 or insulin-dependent diabetes mellitus (IDDM), and type 2 or non-insulin-dependent diabetes (NIDDM) (3). Type 1 diabetes represents ~2-5% of all cases, and the major susceptibility gene for IDDM maps to the HLA region of chromosome 6, while type 2 diabetes represents ~95% of all cases and affects ~215 million people worldwide (4,5). Both forms of diabetes are associated with major long-term complications, including cardiomyopathy, angiopathy, neuropathy, nephropathy, and digestive insufficiencies. IDDM is caused by auto-immune destruction of pancreatic ß-cells, which renders the pancreas unable to synthesize and secrete insulin (6). For the majority of IDDM and NIDDM cases, the precise biochemical defects, genetic causes, and other contributing factors are not fully understood (7).

Here, we present data indicating that ceftezole exhibits both *in vitro* α-glucosidase inhibition activity and *in vivo* streptozotocin (STZ)-induced diabetic activity. We identified ceftezole using a high throughput-compatible α-glucosidase assay and verified its effectiveness by performing an oral glucose tolerance test in STZ-induced diabetic mice and RT-PCR of their blood cells. These data suggest that ceftezole has potential as an anti-diabetic drug.

Materials and methods

Reagents. α-Glucosidase (from bakers yeast), ß-glucosidase (from almond), α-mannosidase (Jack beans), and ß-mannosidase (from snail acetone powder), *p*-nitrophenyl (PNP) glycosides, and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, MO). Ceftezole and GlucoDoctor were obtained from KyungBo Chemical (Seoul, Korea) and All Medicus (Seoul, Korea), respectively.

High throughput-compatible α-glucosidase assays. To screen agents that potently inhibit various commercially available glycosidases, a high-throughput enzyme assay was performed as described previously with a slight modification (8) (data

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not shown). The high throughput-compatible assay was performed using a CyBi-Well 2000 liquid handler (CyBio, Jena, Germany) and a Victor2 multi-label counter (Wallac, Turku, Finland). In brief, α-glucosidase and the other glycosidases were assayed using 50 mM phosphate buffer at pH 6.7, and 1 mM *p*-nitrophenyl glycoside in a 96-well plate was used as a substrate. The concentration of enzymes used in each experiment is described in the figure legends. Ceftezole at the designated concentrations was added to the enzyme/buffer solution and incubated at 30˚C for 1 h, and the substrate was then added to start the enzyme reaction. When pre-treatment was not specified, mixtures of substrate and ceftezole at various concentrations were prepared beforehand and added to the enzyme solution. Enzyme reactions were performed at 30˚C for 30 min, and 3 vol of 1 M sodium carbonate were then added to stop the reaction. The total reaction volume was 100 μl. Enzymatic activity was quantified by measuring the absorbency at 405 nm. One unit of α-glucosidase and other glycosidases is defined as the amount of enzyme liberating 1.0 μmole PNP per min under the assay conditions. The high throughput-compatible protocol, which consisted of 80 steps using the liquid handler was performed with a slight modification as described in the manufacturer's manual (CyBio). After liquid handling in each well, plates were transferred to the multilabel counter to measure the optical density using a robotic arm (Thermo CRS, Burlington, Canada).

Kinetics of enzyme inhibition. The enzyme reaction was performed according to the above reaction conditions with inhibitors at various concentrations. Inhibition strengths for the inhibitors were determined by Dixon plot and its replot of slope versus the reciprocal of the substrate concentration (9).

RNA preparation and RT-PCR. Total cellular RNA was extracted from blood cells using the TRIzol reagent (Gibco BRL, Grand Island, NY). cDNAs were synthesized from 1 μg total RNA using Oligo(dT) reverse transcriptase (iNtRON Biotechnology, Sungnam, Korea) and were PCR-amplified with specific primers: mouse aldose reductase (496-bp long) forward, 5'-ATC TGG AAC TCA ACA ACG GC-3' and reverse, 5'-CCT CTC AAT CTG AAG AGG GT-3'; GSK-3 (996-bp long) forward, 5'-GAG GAG CTA TCT TCG GAT AG-3' and reverse, 5'-GCG TTC CTA GTA CCT TGA TG-3'; PPAR-γ (532-bp long) forward, 5'-CCA CTC GCA TTC CTT TGA CA-3' and reverse, 5'-TCA GCT GGT CGA TAT CAC TG-3'; PTP-1B (633-bp long) forward, 5'-CGG AAC AGG TAC CGA TAT GT-3' and reverse, 5'-GCA TCT CCA GCA GTA CTT TC-3'; and UCP-3 (659-bp long) forward, 5'-GAA GCA AAA GATT GCC AGG C-3' and reverse, 5'-CAC CAT CTC AGC ACA GTT GA-3'. GAPDH (371-bp long) forward 5'-ATG TTC CAG TAT GAC TCC AC-3' and reverse, 5'-GCC AAA GTT GTC ATG GAT GA-3' was used as an internal control. The resulting PCR products were analyzed by 1.2% TAE agarose gel electrophoresis and visualized by ethidium bromide staining.

Streptozotocin exposure and diabetic mice. Diabetes was induced by intravenous STZ injections [30 mg/kg in freshly prepared 10 mM sodium citrate buffer (pH 4.5)] into veins of
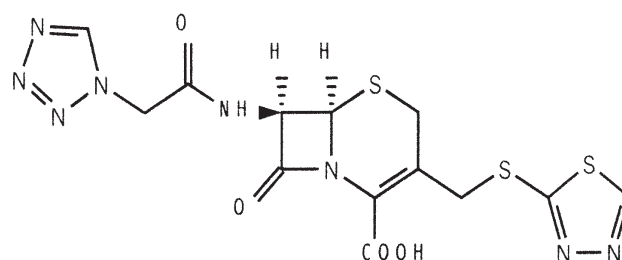

Figure 1. Structure of ceftezole.

mice in one diabetic control group and one treatment group (10). Diabetic female mice (C57BL/6Nkist) were identified as those having blood glucose levels >250 mg/dl using a GlucoDoctor kit. After 20 days of STZ injections, we confirmed that diabetic mice (n=10) exhibited significantly increased blood glucose levels (266.7±15.8 mg/dl) compared with the control mice (104.1±12.9 mg/dl). Ceftezole or vehicle (distilled water) was given intraperitoneally (30 mg/kg/day) every day for 14 days. Twenty-four hours after the final ceftezole treatment, mice were anesthetized with pure diethyl ether inhalation, and blood analysis was carried out. Tissues were collected for RT-PCR. Remaining mice were subjected to an oral glucose tolerance test. Animal husbandry and experimentation were performed in compliance with the Guiding Principles in the Care and Use of Animals and the Animal Welfare Committee of the Korean Research Institute of Bioscience and Biotechnology (KRIBB). C57BL/6NKist mice were housed in specific pathogen-free (SPF) barrier facilities of KRIBB, a sub-center of the International Council for Lab Animal Science (ICLAS; code no. Kist). All mice were fed Purina rat chow (#5012, Harlan, Indianapolis, IN) and water *ad libitum*. Animals were maintained in a room under standard laboratory conditions (23±1˚C and 50±5% humidity) with a 12 h/12 h dark/light cycle. Animal care guidelines and rules for animal experiments, including ethical care, were strictly followed under the guidance of the committee.

Oral glucose tolerance test. Oral glucose tolerance was tested with a slight modification of the protocol described previously (11). Briefly, 20 days after STZ exposure, animals underwent an oral glucose tolerance test (OGTT). The oral blood glucose concentration was determined using the GlucoDoctor glucose testing system based on a glucose oxidase assay.

Results

High throughput-compatible screening. While screening potential α-glucosidase inhibitors from various sources, we developed a high throughput-compatible assay protocol for α-glucosidase inhibition (data not shown). Using this protocol, we tested ~11,800 chemicals and 1,450 unfractionated plant extracts. As a result, we isolated and identified the ß-lactam antibiotic ceftezole as a potential α-glucosidase inhibitor (Fig. 1 and data not shown).

Ceftezole inhibits α-glucosidase in vitro. Of the enzymes tested, α-glucosidase was the most sensitive to ceftezole; the
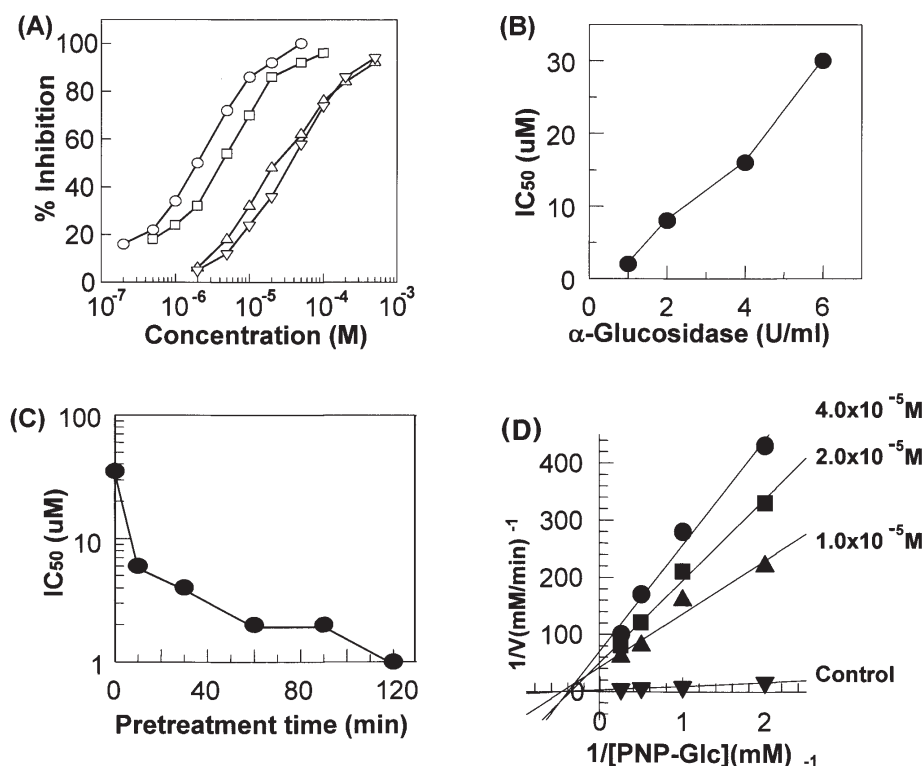
Figure 2. Ceftezole is an α-glucosidase inhibitor *in vitro*. (A) Inhibition of various glucosidases by ceftezole. Enzyme solutions were treated with various concentrations of ceftezole: 1 U/ml α-glucosidase (○), 0.5 U/ml ß-glucosidase (▽), 0.5 U/ml α-mannosidase (□), and 0.1 U/ml ß-mannosidase (△). (B) The IC_{50} value for α-glucosidase inhibition varied depending on the concentration of α-glucosidase and the time of ceftezole pretreatment. Different concentrations (1, 2, 4, and 6 U/ml) of α-glucosidase were treated with ceftezole (10 μM) for 1 h. (C) Pretreatment effect of ceftezole. α-Glucosidase (1 U/ml) was pretreated with ceftezole (10 μM) for 0-120 min in phosphate buffer [50 mM (pH 6.7)] at 30˚C. After pretreatment, PNP-α-glucopyranoside was added to the mixture to initiate the enzyme reaction. IC_{50} values were determined by quantifying the amount of PNP liberated. (D) Double reciprocal plots of the inhibition kinetics of yeast α-glucosidase by ceftezole. Following ceftezole treatment for 1 h at 30˚C, 50 μl α-glucosidase (10 U/ml) was treated with a mixture of each 50 μl of the designated concentration of PNP-α-glucopyranoside to initiate the enzyme reaction.

concentration required for 50% inhibition (IC_{50}) was 2.1×10^{-6} M (2.1 μM) (Fig. 2A). α-Glucosidase activity was reduced by ceftezole in a dose-responsive manner. The IC_{50} values for other glycolytic enzymes including α-mannosidase, ß-mannosidase, and ß-glucosidase, were 4.1×10^{-6} M (4.1 μM), 2.2×10^{-5} M (22.0 μM) and 3.3×10^{-5} M (33.0 μM), respectively. We next examined the effects of ceftezole on α-glucosidase inhibition (Fig. 2B and C). Following the addition of 2 mM glucose to the reaction mixture, the IC_{50} value increased >5-fold, indicating that ceftezole either acts as an analog of glucose or binds to the glucose-binding site of α-gluco-sidase. Fig. 2B shows the effect on IC_{50} values when the amount of α-glucosidase in the reaction mixture was raised from 1.0 to 6.0 units/ml. During the reaction, the IC_{50} value was increased from 2.2×10^{-6} M (2.2 μM) to 3.0×10^{-5} M (30.0 μM). The α-glucosidase inhibitory activity of ceftezole was increased by preincubating ceftezole with α-gluco-sidase (Fig. 2C). When the substrate (1 mM) and ceftezole (10 μM) were added simultaneously, the IC_{50} was $\sim3.5 \times 10^{-5}$ M (35.0 μM). This value was decreased 17.5-fold ($\sim2.0 \times 10^{-6}$ M) when α-glucosidase was treated with ceftezole at 30˚C for 1 h before the enzyme reaction. Double-reciprocal plots of α-glucosidase kinetics with ceftezole revealed that α-gluco-sidase activity was also inhibited noncompetitively when the enzyme was pretreated with ceftezole (10 μM) for 1 h, with a K_i value of 5.78×10^{-7} M (0.578 μM) (Fig. 2D).

Ceftezole exhibits an in vivo anti-diabetic profile. To confirm the effectiveness of ceftezole in an *in vivo* model, we analyzed whether or not ceftezole treatment decreased oral blood glucose levels in STZ-induced, diabetic C57BL/6NKist mice. Using GlucoDoctor, we determined the blood glucose levels of STZ-induced animals and considered mice with blood glucose levels >250 mg/dl (266.7±15.8 mg/dl) to be diabetic. Thereafter, these animals received ceftezole (30 mg/kg) one time per day for 14 days, and oral glucose levels were immediately assessed. Following ceftezole treatment, blood glucose levels decreased by as much as 30% (195.3±22.4 mg/dl) compared to control animals (Fig. 3A). These results indicate that ceftezole inhibits the blood glucose increase that occurs following glucose uptake.

To determine how ceftezole affects the expression of various proteins, we performed RT-PCR of some biomarkers that are involved in diabetes. From the blood cells of STZ-induced control or ceftezole-treated mice before OGTT, total RNA was isolated and extracted. After isolation of mRNA, RT-PCR was carried out as described in Materials and methods. As shown in Fig. 3B, mRNA expression levels of GSK-3, PPAR-γ and UCP-3 were slightly decreased compared to that of the internal control GAPDH. The expression levels of other biomarkers for diabetes, including aldose reductase and PTP-1B, were not significantly changed by ceftezole treatment. Collectively, these data show that ceftezole exhibits anti-diabetic activity *in vivo* as well as *in vitro*.
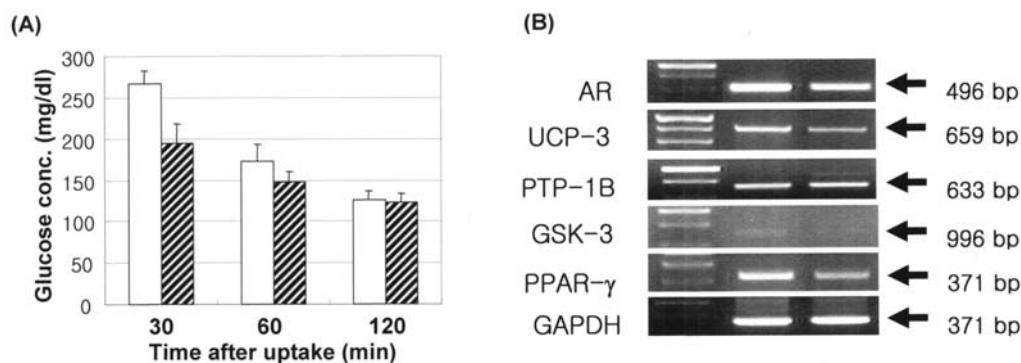
Figure 3. Ceftezole decreases blood glucose levels *in vivo*. (A) An STZ-induced animal model showed that ceftezole exhibits anti-hyperglycemic effects *in vivo*. The detailed procedure is described in Materials and methods. Briefly, after 20 days of STZ injections, ceftezole or vehicle was given intraperitoneally (30 mg/kg/day) every day for 14 days. Twenty-four hours after the final ceftezole treatment, the remaining mice were subjected to an oral glucose tolerance test as described previously (11). The means and standard errors were generated and significant differences were determined by the Student's t-test. Significance was established at p<0.05. White bars, vehicle; hatched bars, ceftezole-treated. (B) mRNA expression patterns following ceftezole treatment in STZ-induced diabetic mice before OGTT. Primers are listed in Materials and methods. Tissues were lysed with TRIzol and collected supernatants were used for RT-PCR.

Discussion

Glycosidases, which are located in the brush-border surface membrane of intestinal cells (12), are essential for proper carbohydrate digestion (13). These enzymes are known to be adaptive and are specifically stimulated by certain dietary sugars. Carbohydrates have been shown to increase glucose metabolism in diabetic patients. Therefore, it is recommended that diabetic patients avoid simple carbohydrates (e.g., sucrose, maltose) in favor of polysaccharide alternatives (14,15).

Glycosidase inhibitors are important tools for studying the mechanisms of action of glycosidases and are also prospective therapeutic agents for some degenerative diseases, including diabetes, viral attachment, and cancer (16,17). Some researchers have reported that oral administration of specific α-glucosidase inhibitors can effectively improve hyperglycemia as well as diabetic complications (18).

Acarbose is a pseudo-tetrasaccharide consisting of an unsaturated cyclitol unit that is a potent competitive inhibitor of α-glucosidase. Acarviosine-glucose has been shown to be a potent inhibitor of baker's yeast α-glucosidase that inhibits α-glucosidase activity 430-fold stronger than acarbose (19). Other anti-diabetic agents derived from natural plant sources, including *Myrcia multiflora*, *Aralia elata*, *Beta bulgaris*, and *Kochia scoparia*, are currently being investigated in clinical trials (20).

In performing high-throughput screening for α-glucosidase inhibitors, we recently identified the semi-synthetic antibiotic ceftezole (Fig. 1), as a potentially potent compound. The current study confirmed that ceftezole is a reversible and noncompetitive inhibitor of yeast α-glucosidase with a K_i value of 5.78×10^{-7} M when the enzyme mixture is pretreated with ceftezole. More importantly, an STZ-induced diabetic mouse model revealed that ceftezole can be clinically used to treat diabetic symptoms, suggesting that the present agent can be used in diabetic complications (10,21,22).

Cephem antibiotics are very important for the treatment of a variety of bacterial infections and represent a new generation of ß-lactam antibiotics (23). Ceftezole has an excellent anti-biological profile against a broad spectrum of organisms, including gram-negative bacteria (24). The antibiotic is produced by *Streptomyces clavurigrus*, *Streptomyces limpanii*, *Streptomyces lactamdurans*, *Emericellopsis*, and *Paecilomyces sp.*, but these strains do not produce sufficient amounts for large-scale application. Therefore, the microbial production of ceftezole has been substituted with chemical synthesis, beginning with cephalosporin C; however, use of this agent has resulted in minor immunotoxicity in mice (25).

We found that α-glucosidase activity was inhibited by ceftezole in a dose-responsive manner (Fig. 2B). We concluded that ceftezole may bind to α-glucosidase, and that α-glucosidase inhibitory activity by ceftezole was increased by preincubating it with the enzyme (Fig. 2C). Comparison of the Dixon plots indicated that an ~20-fold-higher concentration of dialyzed ceftezole-treated α-glucosidase was required to attain the same level of enzyme activity as dialyzed untreated α-glucosidase (data not shown). These data suggested that the mechanism of action of α-glucosidase inhibition may be reversible. Double reciprocal plots of α-glucosidase with ceftezole revealed that this process was non-competitive when the enzyme was pretreated with ceftezole for 1 h.

An *in vivo* STZ-induced diabetic model revealed that ceftezole may be clinically used for managing diabetic complications. We found that administration of ceftezole (10 mg/kg) decreased oral blood glucose levels by up to 30% compared to those of control-treated animals. Although we did not show the time course data, ceftezole treatment resulted in decreased glucose levels between 10 and 60 min of oral glucose administration. Together, these *in vitro* and *in vivo* assays revealed that ceftezole exerts its anti-diabetic activity by inhibiting α-glucosidase and by decreasing blood glucose levels.

In conclusion, the above results suggest that ceftezole may potentially be clinically useful as a potent inhibitor of α-glucosidase in patients with diabetes and other metabolic diseases. The inhibition studies described here provide useful information for designing new, potent glycosidase inhibitors.

Yeast α-glucosidase is known to be very different from mammalian digestive enzymes, indicating that ongoing research should focus on the inhibitory activity of ceftezole against mammalian intestinal α-glucosidases. Studies to elucidate the molecular mechanisms of action of more potent ceftezole derivatives as well as potential synergistic effects of ceftezole and the previously reported α-glucosidase inhibitor genistein (8) are currently underway. Further studies with ceftezole should be performed in primates to determine any possible *in vivo* side effects of this agent and, moreover, to overcome drug resistance in the event of long-term use.

Acknowledgements

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