7-But-2-ynyl-9-(6-methoxy-pyridin-3-yl)-6-piperazin-1-yl-7,9-dihydro-purin-8-one Is a Novel Competitive and Selective Inhibitor of Dipeptidyl Peptidase IV with an Antihyperglycemic Activity

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ABSTRACT

7-But-2-ynyl-9-(6-methoxy-pyridin-3-yl)-6-piperazin-1-yl-7,9-dihydro-purin-8-one (ER-319711) is a novel dipeptidyl peptidase (DPP-IV) inhibitor discovered in our laboratories. In this study, we have characterized this DPP-IV inhibitor in vitro and in vivo as an antidiabetic agent. The trifluoroacetate salt form of ER-319711, ER-319711-15, inhibited human DPP-IV with an IC$_{50}$ value of 0.089 µM, whereas its IC$_{50}$ values toward human DPP8 and DPP9 were >100 µM. Inhibition kinetic pattern analysis indicated that ER-319711-15 inhibited DPP-IV in a competitive manner. ER-319711-15 (1 mg/kg) reduced glucose excursion in an oral glucose tolerance test (OGTT) using Zucker fafa rats, with significant increases in plasma insulin and active glucagon-like peptide-1 levels. In an OGTT using mice fed a high-fat diet in which ER-319711-15 (0.1–10 mg/kg) was orally administered at 0 h, and glucose was loaded at 0 and 5 h, this compound improved glucose tolerance dose dependently at both 0- and 5-h glucose loading. Next, we compared efficacy of ER-319711-15, E3024, a competitive DPP-IV inhibitor having an imidazopyridazinone structure, or vildagliptin, a slow-binding and long-acting DPP-IV inhibitor, at the same dose, 10 mg/kg, in the same procedures. At the first glucose challenge, all compounds lowered area under the curve (AUC) values of delta blood glucose between 0 and 2 h significantly to the same degree. At the second glucose load, the AUC values between 5 and 7 h were significantly decreased by ER-319711-15 and vildagliptin, but not by E3024. Therefore, ER-319711 might be a potent, competitive, and selective DPP-IV inhibitor with an antihyperglycemic activity.

Dipeptidyl peptidase (DPP)-IV degrades active glucagon-like peptide-1 (GLP-1) [GLP-1(7-36)amide and GLP-1(7-37)], which is an incretin released from L cells in the intestine after meal intake that enhances insulin secretion in a glucose-dependent manner. GLP-1 has an antidiabetic action in patients with type 2 diabetes (Nauck et al., 1993; Gutniak et al., 1994). DPP-IV cleaves GLP-1 rapidly, so the half-life of GLP-1 is only 1 to 2 min. Accordingly, the prevention of GLP-1 inactivation by DPP-IV inhibition is currently being actively explored as a novel approach to the treatment of type 2 diabetes (Deacon et al., 2004). DPP-IV inhibition leads to blood glucose-lowering effects in animal models of diabetes (Pederson et al., 1998; Reimer et al., 2002; Burkey et al., 2005) and in patients with type 2 diabetes (Ahrén et al., 2002, 2005).

ER-319711 is a novel DPP-IV inhibitor discovered in our laboratories. Demuth et al. (2005) categorized DPP-IV inhibitors based on their mode of inhibition and structures as follows: reversible product analog inhibitors (e.g., P32/98; Sorbera et al., 2001), covalently modifying product analog inhibitors [e.g., vildagliptin (LAF237); Villhauer et al., 2003], and reversible nonpeptidic heterocyclic inhibitors [e.g., sitagliptin (MK-0431); Kim et al., 2005]. ER-319711 belongs to the third group, and is a novel, 8-oxo-purine derivative.

In this study, we have characterized the inhibition mode...
for DPP-IV and enzyme selectivity toward DPP-IV, DPP8, and DPP9 of the newly discovered DPP-IV inhibitor, and we have investigated its antihyperglycemic activity using Zucker fa/fa rats and mice fed a high-fat diet. The high-fat diet-fed mouse model is considered to be a robust model for impaired glucose tolerance and early type 2 diabetes (Winzell and Ahrén, 2004), both of which are targets of DPP-IV inhibitors.

**Materials and Methods**

**Chemicals.** The trifluoroacetate salt form of ER-319711 (ER-319711-15), E3024, and 1-[[3-hydroxy-1-adamantyl]amino][acetetyl]-2-cyano-(S)-pyrrolidine (vildagliptin) were synthesized in our laboratories. Lys[[NO$_2$]] pyrrolidide was purchased from Bachem (Bubendorf, Switzerland), which is a nonselective DPP-IV/DPP8/DPP9 inhibitor (Lankas et al., 2005), used as a reference. The chemical structures of these compounds are shown in Fig. 1.

**Inhibitory Effects toward DPP-IV, DPP8, and DPP9.** Purified human recombinant DPP-IV was purchased from R&D Systems (Minneapolis, MN). Recombinant human DPP8 and DPP9 were expressed by baculovirus and purified. An enzyme was mixed with ER-319711-15 or Lys[[NO$_2$]] pyrrolidide in an assay buffer (10 mM phosphate-buffered saline and 0.1% bovine serum albumin, pH 7.4). The enzyme reaction was started by an addition of 2 mM glycyl-L-proline, and the changing of absorbance at 405 nm was monitored for 20 min with a microplate spectrophotometer (SpectraMax; Molecular Devices, Sunnyvale, CA). Three separate experiments were performed, and means of IC$_{50}$ values and 95% confidence interval (CI) were calculated.

**Inhibitory Kinetic Analysis.** Recombinant human DPP-IV activity was measured at varied concentrations of ER-319711-15 (0.1, 1, 10, and 100 μM). For each concentration, measurements were conducted in the presence of varied concentrations of glycyl-L-proline p-nitroanilide tosylate (0.006, 0.024, 0.10, 0.39, 1.56, 6.25, 25, and 100 mM). Three separate experiments were performed in triplicate. The inhibitory pattern was evaluated by the Eadie-Hofstee plot using a curve-fitting program (GraphPad Software Inc., San Diego, CA).

**Animals.** Male Crlj:ZUC-Lepr$_{fa}$ (Zucker fa/fas) rats and male C57Bl/6NcrCrlJ mice were purchased from Charles River Japan (Tokyo, Japan). The rats and mice were provided with a commercial diet (MF, Oriental Yeast, Tokyo, Japan) and water ad libitum, and they were kept under conventional conditions of controlled temperature, humidity, and lighting (22 ± 2°C, 55 ± 5%, and a 12-h light/dark cycle with lights on at 7:00 AM). All procedures were conducted according to the Eisei Animal Care Committee’s guidelines.

**Plasma Insulin and Active GLP-1 Levels in an Oral Glucose Tolerance Test OGTT Using Zucker fa/fa Rats.** ER-319711-15 (1 mg/kg) or vehicle (0.5% methyl cellulose; MC) was orally administered to overnight-fasted Zucker fa/fa rats 0.5 h before oral glucose load (2 g/kg). Blood samples were drawn from the tail vein 0, 0.5, 1, 2, and 3 h after the glucose load. In addition, approximately 250 μl of blood samples was collected with heparinized capillary tubes at 0, 0.5, 1, and 2 h. After centrifugation, supernatants were assayed for plasma insulin and active GLP-1 levels. Plasma insulin levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Kanagawa, Japan) and rat insulin as a standard with the microplate spectrophotometer. Plasma active GLP-1 levels were determined using an ELISA kit (GLP-1(7-36) Active ELISA kit; Linco Research, Inc., St. Charles, MO).

**OGTT Using Mice Fed a High-Fat Diet: Dose Dependence of Efficacy of ER-319711-15.** Mice were fed a high-fat diet (D12492 Rodent Diet with 60 kcal% fat) for 4 weeks from 11 weeks of age, and 42 mice were selected based on absolute body weight and randomly divided into seven groups. ER-319711-15 (0.1, 0.3, 1.3, and 10 mg/kg), vildagliptin (10 mg/kg) as a positive control, or vehicle (0.5% MC) alone was orally administered to overnight-fasted mice at the same time of oral glucose administration (2 g/kg) (0 h). After 5 h, glucose was orally administered again. Blood samples were collected from the tail vein 0, 0.5, 1, 2, 5, 5.5, 6, and 7 h after the compound treatment.

**OGTT Using Mice Fed a High-Fat Diet: Comparison of Efficacy among E3024, ER-319711-15, and Vildagliptin.** Mice were fed a high-fat diet (D12492 Rodent Diet with 60 kcal% fat); Research Diets, Inc., New Brunswick, NJ) for 4 weeks from 11 weeks of age, and 24 mice were selected based on body weight and randomly divided into four groups. E3024 (10 mg/kg), ER-319711-15 (10 mg/kg), vildagliptin (10 mg/kg), or vehicle (0.5% MC) alone was orally administered to overnight-fasted mice at the same time of oral glucose administration (2 g/kg) (0 h). After 5 h, glucose was orally administered again. Blood samples were collected from the tail vein 0, 0.5, 1, 2, 5, 5.5, 6, and 7 h after the compound treatment.

**Blood Glucose Determination.** Blood samples (10 μl) were collected from the tail vein and mixed with 140 μl of 0.6 M perchloric acid. After centrifugation, the supernatants were assayed for glucose using an enzymatic assay kit (Glucose CII-test WAKO; Wako Pure Chemicals, Osaka, Japan) with the microplate spectrophotometer.

**Statistical Analysis.** Data are expressed as the mean ± S.E.M. To determine the integrated glucose response to the glucose challenge, the area under the curve (AUC) of delta blood glucose after the glucose load was calculated using a trapezoidal rule (between 0 and 2 h and between 5 and 7 h). Differences in the AUC values of delta blood glucose in an OGTT using mice between the vehicle- and E3024- treated groups were determined by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test.

Differences in the AUC values of delta blood glucose in an OGTT using mice between the groups treated with vehicle or ER-319711 at several doses were determined by one-way ANOVA, followed by
DPP-IV inhibition kinetic pattern of ER-319711.

Inhibition kinetics of dipeptidyl peptidase IV by ER-319711-15 (the trifluoroacetate salt form of ER-319711). Different concentrations of ER-319711-15 (0, 0.03, 0.1, 0.3, 1, and 3 μM) were incubated in the presence of various concentrations of glycyl-L-proline p-nitroanilide tosylate (0.006, 0.024, 0.10, 0.39, 1.56, 6.25, 25, and 100 mM). Initial rates of the reaction were measured, and the results were expressed as the Eadie–Hofstee plot. Data represent the mean ± S.E.M. of three separate experiments performed in triplicate.

ER-319711-15 caused significant decreases in AUC values of delta blood glucose from 1 mg/kg. The AUC values of 3 to 10 mg/kg ER-319711-15 were similar to that of 10 mg/kg vildagliptin. At the second glucose load, a significant decrease in the AUC value from 3 mg/kg ER-319711-15 was observed, and efficacy of 10 mg/kg ER-319711-15 and 10 mg/kg vildagliptin was almost identical. Results of regression analysis indicated that ER-319711-15 lowered the AUC in a dose-dependent manner at both 0- and 5-h glucose administration.

In an OGTT using mice fed a high-fat diet, we examined the efficacy of antihyperglycemic effects between ER-319711-15 (10 mg/kg), E3024 (10 mg/kg), and vildagliptin (10 mg/kg) (Fig. 5, A and B). At simultaneous administration of a compound and glucose, ER319711-15, E3024, and vildagliptin lowered glucose excursions to almost the same degree. At 5-h postglucose load, ER-319711-15 and vildagliptin reduced the AUC significantly, but a decrease in the AUC by E3024 was not significant.

**Discussion**

GLP-1, which is secreted in a nutrient-dependent manner, stimulates glucose-dependent insulin secretion and regulates glycemia. However, half-life of active GLP-1 is very short due to degradation by DPP-IV, and the actions of GLP-1 do not last long. Then, development of DPP-IV inhibitors has been active worldwide, which is expected to control blood glucose levels by enhancement of the action of GLP-1 for the treatment of diabetes. In a course of research of novel DPP-IV inhibitors in our laboratories, we discovered a new series of DPP-IV inhibitors with potent and efficacious antihyperglycemic effects.
8-oxo-purine-derived DPP-IV inhibitors. In this study, we report in vitro and in vivo characteristics of a representative of 8-oxo-derivative compounds, ER-319711-15.

Kinetics study indicated that ER-319711-15 inhibited DPP-IV competitively. In an OGTT using Zucker fa/fa rats, we confirmed that ER-319711-15 improved glucose tolerance, accompanied with increases in both plasma insulin and intact GLP-1 levels. These are characteristic actions of DPP-IV inhibitors. In addition, ER-319711-15 ameliorated glucose tolerance in mice fed a high-fat diet, and this effect was dose-dependent.

A recent focus of research of DPP-IV inhibitors is on long-acting inhibitors, aiming at less dosing frequency. Vildagliptin, which is a slow-binding DPP-IV inhibitor, was dosed to patients with type 2 diabetes once daily, thereby improving glycemic control (Ristic et al., 2005). Following vildagliptin, K579 (Takasaki et al., 2004) and saxagliptin (BMS-477118) (Augeri et al., 2005) have been reported to be long-acting DPP-IV inhibitors, both of which also show slow-binding inhibition. In this study, we investigated efficacy of ER-319711-15, in comparison with E3024 and vildagliptin, in 0- and 5-h postdose OGTT using mice fed a high-fat diet, at the same dose of 10 mg/kg. E3024 is a selective and competitive DPP-IV inhibitor with an imidazopyridazinone structure discovered in our laboratories (Yasuda et al., 2006). In an OGTT with a simultaneous administration of the compound and glucose (0 h), all compounds showed drastic decreases in blood glucose to the same degree. In a 5-h postdose OGTT, we observed an equal, significant glucose-lowering effect between 10 mg/kg ER-319711-15 and 10 mg/kg vildagliptin. E3024, however, did not manifest significant decrease in AUC values. Thus, ER-319711-15 might be a longer acting competitive DPP-IV inhibitor than E3024.

DPP-IV is a member of a family of serine peptidases including quiescent cell proline dipeptidase, DPP8, and DPP9. Acute and chronic administration of a DPP-IV inhibitor with DPP8/9 inhibition activity caused thrombocytopenia and splenomegaly in rats, and bloody diarrhea in dogs (Lankas et al., 2005). A selective DPP8/9 inhibitor caused the same signs, whereas no toxicity was observed in rats or dogs treated with a selective DPP-IV inhibitor, suggesting that inhibition of DPP8/9 leads to profound toxicity in preclinical

**Fig. 3.** Effects of ER-319711 on blood glucose, plasma insulin and active glucagon-like peptide-1 (GLP-1) levels in an oral glucose tolerance test in Zucker fa/fa rats. ER-319711-15 (the trifluoroacetate salt form of ER-319711; 1 mg/kg) or vehicle was orally administered 0.5 h before glucose load (2 g/kg). Changes of blood glucose (A) and AUC values of delta blood glucose between 0 and 3 h (B). Changes of plasma insulin levels (C) and delta plasma insulin levels between 0 and 0.5 h (D). Changes of plasma active GLP-1 levels (E) and delta plasma active GLP-1 levels between 0 and 0.5 h (F). Values are expressed as the mean ± S.E.M. *, p < 0.05 by Student’s t test (B and D). #, p < 0.05 by Mann-Whitney U-test (F).

**Fig. 4.** Changes of blood glucose levels (A) and AUC values of delta blood glucose between 0 and 2 h and between 5 and 7 h (B) in an oral glucose tolerance test in mice fed a high-fat diet. ER-319711-15 (the trifluoroacetate salt form of ER-319711; 0.1, 0.3, 1, 3, and 10 mg/kg), vildagliptin (10 mg/kg), or vehicle was orally administered at 0 h with glucose (2 g/kg). At 5 h, glucose (2 g/kg) was orally loaded again. Values are expressed as the mean ± S.E.M. *, p < 0.05 versus vehicle group by Dunnett’s multiple comparison test. #, p < 0.05 versus vehicle group by Student’s t test.
Lys[Z(NO2)] pyrrolidide inhibited not only DPP-IV but also antihyperglycemic activity. ER-319771-15 might be a novel potent DPP-IV inhibitor with an effect lasts longer than the effect of E3024. Thus, DPP-IV inhibitor with an 8-oxo-purine structure. Its glucose-lowering effect lasts longer than the effect of E3024. Thus, ER-319771 might be a novel potent DPP-IV inhibitor with an antihyperglycemic activity.

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References

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