Effects of the Combination of a Dipeptidyl Peptidase IV Inhibitor and an Insulin Secretagogue on Glucose and Insulin Levels in Mice and Rats

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ABSTRACT

Several combination therapies have been tried for treating type 2 diabetes to control more effectively fasting hyperglycemia and postprandial hyperglycemia. In this study, we have examined the effects of combining a novel, selective, and competitive dipeptidyl peptidase IV (DPP-IV) inhibitor, 3-but-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4H-imidazo[4,5-d]pyridazin-4-one tosylate (E3024), with a representative of one of two types of insulin secretagogues, i.e., either glybenclamide (a sulfonylurea) or nateglinide (a rapid-onset/short-duration insulin secretagogue), on glucose and insulin levels in an oral glucose tolerance test (OGTT) using mice fed a high-fat diet. In addition, we have investigated the effects of these combinations on blood glucose levels in fasting rats. Two-way analysis of variance showed that the combination of E3024 and glybenclamide improved glucose tolerance additively and also caused a synergistic increase in insulin levels in the OGTT in mice fed a high-fat diet. In a similar way, the combination of E3024 and nateglinide ameliorated glucose tolerance additively and raised insulin levels additively. In fasting rats, coadministration of E3024 with glybenclamide or nateglinide treatment did not affect the glucose-lowering effects of the insulin secretagogues. Therefore, a DPP-IV inhibitor in combination with glybenclamide or nateglinide may be a promising option for the treatment of type 2 diabetes, and particularly, for controlling postprandial hyperglycemia in the clinic.

Dipeptidyl peptidase IV (DPP-IV) degrades active glucagon-like peptide (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 promotes 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 latter’s half-life 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).

E3024 is a novel, selective and competitive DPP-IV inhibitor, discovered in our laboratories (Yasuda et al., 2006). Demuth et al. (2005) have classified 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). E3024 belongs to the third group and has a novel, imidazopyridazinone structure.

Insulin secretagogues used in the clinic are categorized into the following two groups: sulfonylureas and rapid-onset/short-duration insulinotropic agents (Krentz and Bailey, 2005). The former class includes glybenclamide and glyburide, whereas the latter class includes nateglinide and repaglinide. The sulfonylureas target fasting hyperglycemia, whereas the rapid-acting insulin secretagogues improve postprandial hyperglycemia (Van Gaal and De Leeuw, 2003).

Recently, the importance of reducing not only fasting hyperglycemia but also postprandial hyperglycemia has been demonstrated. Fasting hyperglycemia increases a risk of mi-

ABBREVIATIONS:
DPP-IV, dipeptidyl peptidase IV; GLP, glucagon-like peptide; E3024, 3-but-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4H-imidazo[4,5-d]pyridazin-4-one tosylate; GIP, glucose-dependent insulinotropic polypeptide; OGTT, oral glucose tolerance test; MC, methylcellulose; AUC, area under the curve; ANOVA, analysis of variance; P32/98, di-[(S,S)-2-amino-3-methyl-pentanoic-1,3-thiazolidide] fumarate; K579, (S)-1-[4-methyl-1-(2-pyrimidinyl)-4-piperidyldiamino]acetyl-2-pyrrolidinocarbonitrile; LAF237, vildagliptin; MK-0431, sitagliptin.

Dipeptidyl peptidase IV (DPP-IV) degrades active glucagon-like peptide (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 promotes 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 latter’s half-life 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).

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Microvascular complications (UK Prospective Diabetes Study (UKPDS) Group, 1998), whereas postprandial hyperglycemia has shown to be an independent risk factor for the development of macrovascular complications (Ceriello, 2000; Monnier, 2000; Temelkova-Kurktschiev, 2000). Several combination therapies using agents with complementary mode of actions have been examined to address improvement of fasting hyperglycemia and postprandial hyperglycemia. In this study, we examined the effects of a combination of E3024 and glybenclamide and that of E3024 and nateglinide on glucose and insulin levels in an oral glucose tolerance test (OGTT) in mice fed a high-fat diet, and we further studied the effects of these combinations on blood glucose levels in fasting rats to explore their possibilities as novel combination therapies.

**Materials and Methods**

**Chemicals.** E3024 was synthesized in our laboratories. Glybenclamide [4-[[2-(5-chloro-2-methoxybenzoylamino)ethyl](N-cyclohexyl-...
carbamoyl)benzene sulfonamide] was purchased from Sigma-Aldrich (St. Louis, MO). Nateglinide [(−)-N-(trans-4-isopropylcyclohexane-carbonyl)-D-phenylalanine] was purchased from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). The chemical structure of E3024 is indicated in Fig. 1. GLP-1 (7-36)amide and human glucose-dependent insulinotropic polypeptide (GIP) were purchased from Bachem AG (Bubendorf, Switzerland) and Peptide Institute, Inc. (Osaka, Japan), respectively.

Animals. Ten-week-old male C57BL/6NCrj mice and 5-week-old male Wistar rats were purchased from Charles River Japan (Tokyo, Japan). The mice and rats were provided with a commercial diet (MF, Oriental Yeast, Tokyo, Japan) and water ad libitum and 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 Eisai Animal Care Committee’s guidelines.

Effects of the Combination of E3024 and Glybenclamide or Nateglinide on Blood Glucose Levels in an OGTT. 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 28 mice were selected based on body weight and randomly divided into four groups. The reason why we used the high-fat diet-fed mouse model is that this model is considered to be a robust model for impaired glucose tolerance and early type 2 diabetes (Winzell and Ahren, 2004), both of which are targets of DPP-IV inhibitors. E3024 (3 mg/kg), glybenclamide (1 mg/kg), a mixture of E3024 (3

Fig. 3. Effects of a combination of E3024 (3 mg/kg) and nateglinide (50 mg/kg) on blood glucose (A and B) and plasma insulin levels (C) in an oral glucose tolerance test using mice fed a high-fat diet. The compound(s) and glucose (2 g/kg) were orally administered at 0.5 and 0 h, respectively. Changes of blood glucose levels and AUC values of delta blood glucose values between 0 and 2 h are indicated in A and B, respectively. Values are expressed as the mean ± S.E.M. of seven mice. Results of two-way ANOVA on the AUC values and plasma insulin levels are indicated in insets (B and C, respectively). Results of the Tukey multiple comparison test are as follows: *, p < 0.05 versus vehicle group; #, p < 0.05 versus E3024 group; and $, p < 0.05 versus nateglinide group.
mg/kg) and glybenclamide (1 mg/kg), or vehicle (0.5% methylcellulose; MC) alone was orally administered to overnight-fasted mice 0.5 h before an oral glucose load (2 g/kg). Blood samples were collected from the tail vein 0.5 h before the glucose load and 0, 0.5, 1, and 2 h after the glucose load, and blood glucose and plasma insulin levels were determined. The same OGTT procedure was performed for the combination of E3024 and nateglinide, but the dose of nateglinide was 50 mg/kg.

**Effects of the Combination of Glybenclamide and Nateglinide on Blood Glucose Levels in the OGTT.** Mice were fed a high-fat diet for 5 weeks from 11 weeks of age, and 20 mice were selected based on body weight and randomly divided into four groups. Glybenclamide (1 mg/kg) nateglinide (50 mg/kg), a mixture of glybenclamide (1 mg/kg) and nateglinide (50 mg/kg), or vehicle (0.5% MC) alone was orally administered to overnight-fasted mice 0.5 h before the oral glucose load (2 g/kg). Blood samples were collected from the tail vein 0.5 h before the glucose load, and 0, 0.5, 1, and 2 h after the glucose load, and blood glucose levels were determined.

**Effects of the Combination of Glybenclamide and GLP-1 on Blood Glucose Levels in the OGTT.** Mice were fed a high-fat diet for 6 weeks from 11 weeks of age, and 24 mice were selected based on body weight and randomly divided into four groups. Before the glucose load (2 g/kg), glybenclamide (1 mg/kg) or vehicle (0.5% MC) alone was orally administered to overnight-fasted mice. GLP-1 (7-36)amide (1 μg) or saline was intraperitoneally injected immediately after the glucose administration. Blood samples were collected from the tail vein 0.5 h before the glucose load and 0, 0.5, 1, and 2 h after the glucose load, and blood glucose levels were determined.

**Effects of the Combination of Glybenclamide and GIP on Blood Glucose Levels in the OGTT.** Mice were fed a high-fat diet for 6 weeks from 11 weeks of age, and 20 mice were selected based on body weight and randomly divided into four groups. Before the glucose load (2 g/kg), glybenclamide (1 mg/kg) or vehicle (0.5% MC) alone was orally administered to overnight-fasted mice. GIP (1 μg) or saline was intraperitoneally injected immediately after the glucose administration. Blood samples were collected from the tail vein 0.5 h before the glucose load and 0, 0.5, 1, and 2 h after the glucose load, and blood glucose levels were determined.

**Effects of the Combination of E3024 and Glybenclamide or Nateglinide on Fasting Blood Glucose Levels in Normal Rats.** Six-week-old rats were used. E3024 (3 mg/kg), glybenclamide (1 mg/kg), a mixture of E3024 (3 mg/kg), and glybenclamide (1 mg/kg), or vehicle (0.5% MC) alone was orally administered to overnight-fasted rats. Blood samples were collected from the tail vein before (0 h) and 1, 2, and 3 h after the drug administration to determine the blood glucose levels. The same procedures were performed for the combination of E3024 and nateglinide, but the dose of nateglinide was 50 mg/kg.

**Blood Glucose and Plasma Insulin Determination.** Blood samples (10 μl) were collected from the tail vein before (0 h) and 1, 2, and 3 h after the drug administration to determine the glucose levels. The same procedures were performed for the combination of E3024 and nateglinide, but the dose of nateglinide was 50 mg/kg.

**Blood Glucose and Plasma Insulin Determination.** Blood samples (approximately 50 μl) were collected from the tail vein with heparinized capillary tubes and centrifuged (7000 rpm for 5 min at 4°C).

**Fig. 4.** Effects of a combination of glybenclamide (1 mg/kg) and GLP-1 (1 μg) on blood glucose levels in oral glucose tolerance test using mice fed a high-fat diet. Glybenclamide was orally administered at 0.5 h, and glucose (2 g/kg p.o.) and GLP-1 (1 μg i.p.) treatment were conducted at 0 h. Changes of blood glucose values and AUC values of delta blood glucose values between 0 and 2 h are indicated. Values are expressed as the mean ± S.E.M. of six mice. Results of two-way ANOVA on the AUC values are indicated in an inset. Results of the Tukey multiple comparison test are as follows: *, p < 0.05 versus vehicle + saline group; #, p < 0.05 versus vehicle + GLP-1 group; and $, p < 0.05 versus glybenclamide + saline group.
The supernatants were assayed for plasma insulin levels using an enzyme-linked immunosorbent assay kit (Ultra sensitive rat insulin ELISA kit; Morinaga Institute of Biological Science, Kanagawa, Japan) and mouse insulin (Morinaga Institute of Biological Science) as a standard with the microplate spectrophotometer.

**Statistical Analysis.** Data are expressed as the mean ± S.E.M. To determine the integrated glucose response to the oral glucose challenge, the area under the curve (AUC) of delta blood glucose after the oral glucose load was calculated using a trapezoidal rule. To examine main effects and interaction in the combination studies, two-way analysis of variance (ANOVA) was performed on the AUC values and plasma insulin levels at 0.5 h. The Tukey multiple comparison test was used to examine the difference between the two groups.

In the studies on effects of the combination of E3024 and glybenclamide or nateglinide on fasting blood glucose levels, we performed two-way ANOVA with respect to the values of delta blood glucose at 2 h (that is, the differences in blood glucose levels between 0 and 2 h) in the combination with glybenclamide, and those at 1 h in the combination with nateglinide.

A probability (p) value <0.05 (two-sided) was considered statistically significant. Statistical analyses were performed using an SAS software package version 8.1 (SAS Institute Japan Ltd., Tokyo, Japan).

**Results**

Figure 2 illustrates the changes of blood glucose levels (Fig. 2A) and the AUC of delta blood glucose (B) in the OGTT using mice fed the high-fat diet that were treated with E3024 (3 mg/kg) and/or glybenclamide (1 mg/kg). Two-way ANOVA indicated significant main effects on the AUC values. No significant interaction was seen between the effects of E3024 and glybenclamide (Fig. 2B). E3024 and glybenclamide significantly reduced the AUC values, compared with that of the vehicle treatment. The AUC value of the combination group was significantly lower than those of the other groups. Figure 2C shows the changes of plasma insulin levels in the mice. Two-way ANOVA indicated significant main effects of E3024 and glybenclamide on plasma insulin levels 0.5 h after the oral glucose load. In addition, a significant interaction was detected between the effects of E3024 and glybenclamide. The insulin level of the E3024 plus glybenclamide treatment was significantly higher than those of the other three groups.

Figure 3 shows the changes of blood glucose levels (Fig. 3A) and the AUC of delta blood glucose (Fig. 3B) in the OGTT using mice fed the high-fat diet that were treated with E3024 (3 mg/kg) and/or nateglinide (50 mg/kg). Two-way ANOVA indicated significant main effects of both E3024 and nateglinide on the AUC values. No significant interaction was seen between the effects of E3024 and nateglinide (Fig. 3B). E3024 and nateglinide significantly decreased the AUC values in comparison with that of the vehicle treatment. The AUC value of the combination group was significantly lower than those of the vehicle- and nateglinide-treated groups. Figure 3C shows the changes of plasma insulin levels in the mice.

**Fig. 5.** Effects of a combination of glybenclamide (1 mg/kg) and GIP (1 μg) on blood glucose levels in oral glucose tolerance test using mice fed a high-fat diet. Glybenclamide was orally administered at −0.5 h, and glucose (2 g/kg p.o.) and GIP (1 μg i.p.) treatment were conducted at 0 h. Changes of blood glucose levels (A) and AUC values of delta blood glucose values between 0 and 2 h (B) are indicated. Values are expressed as the mean ± S.E.M. of six mice. Results of two-way ANOVA on the AUC values are indicated in an inset. Results of the Tukey multiple comparison test are as follows: *, p < 0.05 versus vehicle + saline group; and #, p < 0.05 versus vehicle + GIP group.
Two-way ANOVA indicated significant main effects of both E3024 and nateglinide on plasma insulin levels 0.5 h after the oral glucose load. No significant interaction between the effects of E3024 and nateglinide was seen. The combination resulted in levels significantly higher than any other group.

Effects of the combination of glybenclamide and GLP-1 on glucose excursions in the OGTT are shown in Fig. 4A. Two-way ANOVA indicates significant main effects of both glybenclamide and GLP-1 on the AUC values, but there was no significant interaction between the effects of glybenclamide and GLP-1 (Fig. 4B). Both GLP-1 alone and glybenclamide alone caused significant reduction of the AUC, compared with the control. The AUC values of the GLP-1 plus glybenclamide treatment were significantly lower than those of treatment with either GLP-1 or glybenclamide alone. Likewise, we also observed an additive effect of improvement of glucose tolerance by the combination of glybenclamide and GIP from results of two-way ANOVA (Fig. 5, A and B).

We examined the effects of the combination of glybenclamide and nateglinide on glucose tolerance in the OGTT (Figs. 6, A and B). Two-way ANOVA shows significant main effects of both glybenclamide and nateglinide, and significant interaction between their effects (Fig. 6B). The AUC values were almost the same for the groups treated with glybenclamide alone, nateglinide alone, or with both.

Figure 7 shows the changes of fasting blood glucose levels in normal rats that were treated with E3024 (3 mg/kg) and/or glybenclamide (1 mg/kg) (Fig. 7A). Two-way ANOVA indicated that there is a significant main effect of glybenclamide on delta blood glucose values at 2 h, whereas the main effect of E3024 was not significant (Fig. 7B). There was no significant interaction. In the case of the combination of E3024 and nateglinide, a clear reduction in blood glucose levels was detected in both the nateglinide alone, and the E3024 plus nateglinide groups (Fig. 8A). As for the combination of E3024 and glybenclamide, two-way ANOVA demonstrated a significant main effect of nateglinide on delta blood glucose values at 1 h, whereas the main effect of E3024 was not significant (Fig. 8B). Neither was there a significant interaction.

**Discussion**

Takasaki et al. (2004) reported that a long-acting DPP-IV inhibitor, K579, significantly suppressed blood glucose elevation in glybenclamide-pretreated rats, but they did not measure insulin concentrations. The insulinotropic effect of glybenclamide was further amplified by intravenous infusion of GLP-1 in perfused rat pancreas and in noninsulin-dependent diabetes mellitus patients (Gutniak et al., 1996). Our study demonstrated that the combination of glybenclamide and GLP-1 additively improved glucose tolerance in the OGTT in mice fed a high-fat diet, and Yasuda et al. (2006) previously reported that E3024 elevated plasma active GLP-1 levels in the OGTT using Zucker fa/fa rats. Taken together, these findings suggest that DPP-IV inhibition may augment the glucose-lowering effects of glybenclamide through the pres-
ence of active GLP-1, which has escaped from inactivation by DPP-IV.

Glybenclamide binds to the sulfonylurea receptor type 1 of ATP-sensitive potassium channels in pancreatic β-cell membranes and closes this channel. The resultant reduction in K⁺ permeability generates membrane depolarization, opening of Ca²⁺ channels, elevation of [Ca²⁺]ᵢ, and eventually initiation of Ca²⁺-dependent exocytosis of insulin-containing granules (Aguilar-Bryan et al., 1995). Nateglimide also binds to sulfonylurea receptor type 1, but another binding site, specific to nateglimide, has been proposed (Fujita et al., 1996). In contrast, GLP-1 binds to the GLP-1 receptor, a guanine nucleotide-binding protein-coupled receptor, in pancreatic β-cell membranes, and activates adenyl cyclase, leading to an increase in cAMP production and activation of the cAMP-dependent protein kinase (protein kinase A) pathway. This pathway is thought to potentiate glucose-induced insulin release. In addition, the cAMP-cAMP-regulated guanine nucleotide exchange factor II Rab3-interacting molecule pathway has been reported to be a protein kinase A-independent mechanism for the potentiation of insulin secretion by GLP-1 (Kashima et al., 2001; Gromada et al., 2004). Therefore, because the mechanisms of insulin secretion are different for these insulin secretagogues and GLP-1, synergistic or additive insulin release is triggered with the combination of E3024 and glybenclamide or nateglimide.

GIP is the other incretin, released from K cells in the upper portion of the small intestine after ingestion of glucose and fat (Yip and Wolfe, 2000). Like GLP-1, GIP-(1-42), the active form of GIP, potentiates glucose-dependent insulin secretion, and GIP-(1-42) is degraded by DPP-IV to inactive GIP-(3-42). Hansotia et al. (2004) showed that DPP-IV inhibitors lowered glucose and increased plasma insulin in OGTT in both GLP-1 receptor knockout mice and GIP receptor knockout mice. In contrast, the DPP-IV inhibitor actions were eliminated in double incretin receptor knockout mice. Thus, both GLP-1 and GIP contribute to the improvement of glucose tolerance elicited by DPP-IV inhibitors. Interestingly, both GLP-1 and GIP simulate insulin secretion via GPCR activation of adenyl cyclase, coupled with the production of cAMP (Ehses et al., 2002). The present study suggested that GIP is also be involved in the combination effect of a DPP-IV inhibitor and an insulin secretagogue. Using the incretin receptor deficient mice and double knockout mice, we could document more clearly and precisely the involvement of GLP-1 and/or GIP in the combination effect.

The combination of sulfonylureas and nateglimide is not recommended, because the “fast on-fast off” mode of action of nateglimide is negated by the sustained action of sulfonylureas (Van Gaal and De Leeuw, 2003). In this study, the combination of glybenclamide and nateglimide did not show efficacy on glucose excursions in mice. Also in in vitro study,
simultaneous exposure of isolated rat islets to nateglinide and glybenclamide did not cause a further increase of insulin release in comparison with that produced by either agent alone (Ikenoue et al., 1997). Thus, the combination of glybenclamide and nateglinide may not be of use. However, E3024 could be combined with either agent: the combination of E3024 and a sulfonylurea could reduce both fasting and postprandial glucose levels, whereas that of E3024 and a rapid-acting insulin secretagogue could enhance postprandial glucose-lowering effects.

We examined the combination of E3024 and glybenclamide or nateglinide on fasting blood glucose levels in normal rats. E3024 alone did not reduce fasting blood glucose levels, probably because E3024 induces insulin secretion in a glucose-dependent manner. Moreover, E3024 did not affect the blood-lowering effect of either glybenclamide or nateglinide under fasting conditions. Therefore, the risk of hypoglycemia may not be increased by combining E3024 and glybenclamide or nateglinide, compared with treatment using either glybenclamide or nateglinide alone, despite the greater hypoglycemic effects seen for the combination compared with the single treatment.

This is the first report on the statistically evident effectiveness of the combination of E3024, a DPP-IV inhibitor, and glybenclamide, and the combination of E3024 and nateglinide, presented with data for the accompanying changes in insulin levels. E3024 plus glybenclamide improved glucose tolerance additively, with insulin levels increased synergistically in the OGGT in mice. Likewise, the combination of E3024 and nateglinide ameliorated glucose tolerance additively with additively elevated insulin levels. E3024 did not affect the glucose-lowering effect of glybenclamide or nateglinide in fasting normal rats. These findings of our study suggest the combination of a DPP-IV inhibitor and glybenclamide or nateglinide may be a promising option for the treatment of type 2 diabetes, and especially for controlling postprandial hyperglycemia.

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