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Safety, Tolerability, Pharmacokinetics and Pharmacodynamics of E3024, a Novel and Selective Dipeptidyl Peptidase-IV Inhibitor, in Healthy Japanese Male Subjects: Rash Development in Men and Its Possible Mechanism*

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ABSTRACT

E3024 (3-but-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4*H*-imidazo[4,5-*d*]pyridazin-4-one tosylate) is a dipeptidyl peptidase-IV (DPP-IV) inhibitor that was expected to be an antidiabetic agent. Its safety, tolerability, pharmacokinetics (PK), and pharmacodynamics (PD) were investigated in a randomized, double-blind, placebo-controlled, ascending single-dose study in 48 healthy Japanese male subjects. Fasted subjects were orally administered E3024 (5, 10, 20, 40, or 80 mg) or placebo. E3024 was rapidly absorbed, with t_{max} values ranging between 0.33 and 3 h after dosing. The mean $t_{1/2}$ ranged from 5.34 to 11.68 h. $AUC_{0\text{-inf}}$ and C_{max} increased dose-proportionately. PK-PD relationship of E3024 was evaluated by using an I_{max} model, indicating that plasma E3024 concentrations and inhibitory effects of plasma DPP-IV activity were well correlated. The IC_{50} value was calculated as 33.7 ng/mL, which was consistent with *in vitro* data. Thus, E3024 showed a good PK profile and inhibited DPP-IV dose-dependently. Of 30 subjects administered E3024, 12 (40%) experienced adverse events (AEs). Dose escalation to 160 mg was abandoned owing to undesired subjective/objective findings in 4 of 6 subjects receiving 40 mg and 5 of 6 subjects receiving 80 mg. The most prominent AE was rash, but there were no serious AEs or deaths. The maximum tolerated dose was considered to be 20 mg. We hypothesized that histamine was a cause of the rash induction, and examined blood histamine levels of normal Fischer rats treated with E3024. Blood histamine levels were increased significantly by E3024 at 500 mg/kg ($p < 0.001$), but not by vildagliptin or valine-pyrrolidide (DPP-IV inhibitors) at the same dose. No blood histamine increases were observed in genetically mast cell-deficient *Ws*/*Ws* rats treated with E3024 at 500 mg/kg. In *in vitro* assays, E3024 induced histamine release from normal rat peritoneal mast cells in a concentration-dependent manner, but not from basophils. The structure-activity relationship study suggested that a piperazine group *N*-linked to the 2-position of the 5,6-membered fused heterocyclic rings was a key structural element for triggering histamine release.

Keywords: Dipeptidyl Peptidase-IV Inhibitor; Rash; Histamine; Structure-Activity Relationship

1. Introduction

Dipeptidyl peptidase-IV (DPP-IV) inhibitors have been considered highly attractive for the treatment of type 2 diabetes, as the inhibition of DPP-IV results in an increase of the endogenous active glucagon-like peptide-1 (GLP-1) levels [1-5]. Of this class of drugs, sitagliptin (MK-0431) [6], vildagliptin (LAF237) [7], saxagliptin (BMS-477118) [8], alogliptin (SYR-322) [9], linagliptin

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(BI-1356) [10] and anagliptin [11] have all been launched into the market for the treatment of type 2 diabetes.

E3024 (3-but-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4*H*-imidazo[4,5-*d*]pyridazin-4-one tosylate) (**Figure 1**) with a molecular weight of 282.91 is a novel, highly selective and competitive DPP-IV inhibitor synthesized by Eisai Co., Ltd. [12-14]. E3024 inhibited the DPP-IV activity in human, mouse, rat and canine plasma with IC_{50} (concentration required for 50% of the maximum inhibition) values of 0.14, 0.28, 0.40 and 0.36 μmol/L, respectively. In an oral glucose tolerance test using Zucker *fa/fa* rats, E3024 dose-dependently increased plasma insulin levels and reduced the area under the curve (AUC) of delta blood glucose at doses of 1 and 3 mg/kg. E3024 had no effect on fasting blood glucose levels in normal rats at doses of 1 or 10 mg/kg. These non-clinical data had suggested that E3024 would be a novel antidiabetic agent in the treatment of postprandial hyperglycemia with a low risk of causing hypoglycemia.

The objectives of the present studies were: 1) to evaluate the safety, tolerability, pharmacokinetics (PK) and pharmacodynamics (PD) after single oral dose of E3024 in healthy Japanese male subjects, and 2) to examine possible mechanisms of rash development observed in this clinical trial, using normal and genetically mast cell-deficient rats.

2. Materials and Methods

2.1. Clinical Study

This study was conducted at Sekino Clinical Pharmacology Clinic, Tokyo, Japan, in accordance with the ethical principles of the Declaration of Helsinki, good clinical practice in Japan, and International Conference on Harmonization guidelines. The clinical study protocol and informed consent documents were approved by the institutional review board of Sekino Clinical Pharmacology Clinic. Informed consent was obtained from all subjects in writing before implementation of any study-related procedures.

2.1.1. Study Design

This study was performed as a randomized, double-blind, placebo-controlled, escalating single-dose study. Different groups of eight subjects each were orally administered single doses of E3024 (5, 10, 20, 40, or 80 mg, n = 6) or placebo (n = 2 for 5, 10, 80 mg; n = 6 for 20, 40 mg) after an overnight fast of 10 h.

E3024 was supplied by Eisai Co., Ltd. E3024 was administered in film-coated tablets containing 1, 10, or 40 mg of E3024. Placebo was administered in visually matching tablets.

2.1.2. Subjects

Healthy Japanese male subjects between 20 and 39 years of age and with body mass index (BMI) of 18.5 to 25.0 kg/m^2 were eligible for participation in this study. Subjects were excluded if they had a known history of any significant drug or food allergy, a significant organ dysfunction, or any clinically significant deviation from normal in medical history, physical examination findings, vital signs, electrocardiogram, or laboratory test results. Subjects with gastrointestinal, hepatic, renal, respiratory, or cardiovascular diseases; congenital metabolic disorder;

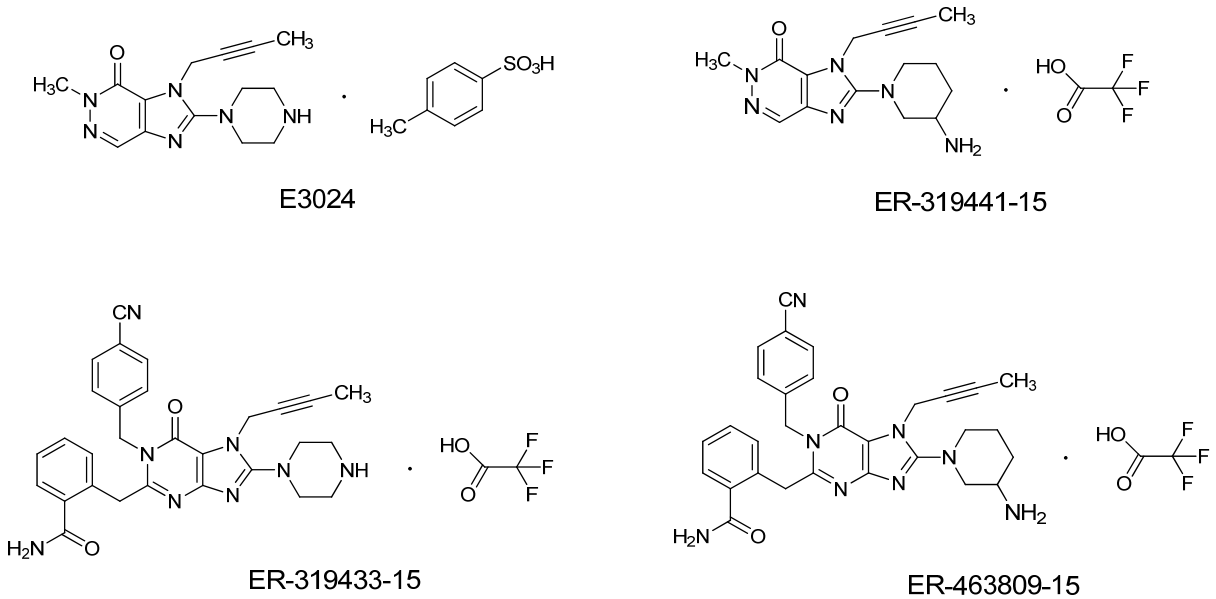


Figure 1. Chemical structures of E3024, ER-319441-15, ER-319433-15, and ER-463809-15.

a positive test result for hepatitis B surface antigen, hepatitis C antibody or human immunodeficiency virus; or alcohol or drug abuse (or a positive urine drug test result at screening) were excluded from participation. Subjects were excluded if they had a known history of any gastrointestinal surgery that could impact upon absorption of the study drug. Subjects were also excluded if they had experienced a weight change >10% from screening to baseline. Furthermore, any subject was excluded who had received blood within three months or donated blood (400 mL within three months or 200 mL within 30 days of study start), or ingested any investigational medication within four months before study start. Subjects were prohibited from any prescription drugs and over-the-counter (OTC) acid controllers within 30 days prior to and during the study, and other OTC medications within seven days prior to and during the study.

2.1.3. Procedures

Screening procedures, including medical history taking, physical examination, 12-lead electrocardiography (ECG), clinical laboratory evaluations, vital signs measurement, and urine drug screening, were performed from 30 days before study drug administration along with the assessment of inclusion/exclusion criteria. Eligible subjects were admitted to the study site on the day prior to dosing for base line evaluations. Subjects were required to abstain from food and beverages, except water, for at least 10 h prior to check-in. After the check-in evaluation was completed, subjects were provided with an appropriate meal(s); thereafter, they were required to fast (abstain from food and fluids, except water) overnight for at least 10 h prior to drug administration on the following day. Subjects took the study drug with 200 mL of water in a fasted state. Water was allowed *ad libitum*, except from 2 h before dosing to 1 h after dosing. Subjects were required to abstain from food up to 4.5 h after dosing. Subjects received a standardized meal at 4.5 (lunch) and 10.5 (dinner) h after dosing to assess the pharmacodynamic effects of E3024 on GLP-1, insulin, C-peptide, glucagon, and glucose. The total energy of each meal was 800 kcal, with a nutrient breakdown of 25% fat, 15% protein, and 60% carbohydrate. Subsequent meals were provided as per the regular meal schedule at the site. Subjects were to maintain an upright (seated or standing) position for at least 4.5 h following administration of the study drug.

2.1.4. Pharmacokinetic Assessments

Blood samples were collected at 0 (pre-dose), 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h after administration of the study drug for determination of E3024 in human plasma. Blood samples (3 mL each) were collected from a cutaneous vein in the forearm into a sodium-heparinized tube. Samples were centrifuged (4˚C at 1500× *g* for 15 min) to obtain plasma. Urine samples were collected before dosing and at the following intervals: 0 to 6 h, 6 to 12 h, 12 to 24 h, 24 to 48 h, 48 to 72 h, and 72 to 96 h after dosing, for determination of E3024 in human urine. Plasma and urine samples were stored at −20˚C until sample analysis.

Analysis was performed by Bioanalysis Section, Clinical Research Center at Eisai Co., Ltd. (Tokyo, Japan). For quantitative determination of E3024, plasma and urine samples were analyzed by a validated liquid chromatographic-tandem mass spectrometry (LC/MS/MS) method. This method was based on solid-phase extraction using Empore extraction disk plates (3M, St. Paul, MN) in a 96-well format, with 0.02 mL (plasma) or 0.005 mL (urine) eluent samples injected into the LC/MS/MS.

Pharmacokinetic parameters were calculated from plasma and urine concentrations of E3024 by model-independent analysis using WinNonlin Professional version 4.1 (Pharsight Corp., Mountain View, CA). The dose-proportionality of maximum observed concentration (C_{max}) and area under the plasma concentration-time curve from 0 to infinity ($AUC_{0\text{-}inf}$) obtained from model-independent analysis was assessed both visually and using a power model ($Y = \alpha X^{\beta}$; X, dose; Y, C_{max} or $AUC_{0\text{-}inf}$). Dose proportionality was assessed based on whether 95% confidence intervals (CIs) of β lay within the range from 0.7 to 1.3 [15].

2.1.5. Pharmacodynamic Assessments

For DPP-IV activity assay, blood samples were collected before dosing and at 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after dosing. For active GLP-1 and glucagon, blood collection was performed before the meals provided at 4.5 and 10.5 h after dosing (lunch and dinner, respectively); and at 0.33, 0.67, 1, 1.5, 2, and 3 h after the meals provided at 4.5 and 10.5 h after dosing. Blood (2 mL) was withdrawn into tubes containing ethylenediaminetetraacetic acid (EDTA) alone (plasma DPP-IV activity assay and plasma active GLP-1) or EDTA plus aprotinin (plasma glucagon). For serum insulin, C-peptide and glucose, blood (3 mL) was withdrawn into serum separator tubes. For active GLP-1 samples, 50 µL of DPP-IV inhibitor solution (Linco Research, Inc., St. Charles, MO) was added to each tube within 30 sec after collection, and the tubes were gently mixed and placed on ice water immediately. After centrifugation, plasma and serum samples were stored at −20˚C or below until assayed.

Pharmacodynamic parameters were measured at Mitsubishi Kagaku Bio-clinical Laboratories, Inc. (now,

Mitsubishi Chemical Medience Corp., Tokyo, Japan). DPP-IV enzyme activity was determined via incubation of 20-µL EDTA-treated human plasma (5-fold dilution in assay) with the substrate, glycyl-L-proline 7-amido-4-methyl-coumarin hydrobromide (H-Gly-Pro-AMC·HBr; 0.08 mmol/L in assay) at room temperature for 10 min by measurement of the release of 7-amino-4-methyl-coumarin with a multifunctional microplate reader (excitation 360 nm; emission 465 nm). Enzyme activity (1 mU/mL) was defined as the amount of enzyme required to degrade 1 nmole of substrate per min in 1 mL of reaction solution (mU/mL = nmol/mL·min). The range of reliable quantitation was 0.05 to 20.0 mU/mL. Active GLP-1 (GLP-1-[7-36]amide and GLP-1-[7-37]) was assayed with an enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, Inc.). The lower limit of reliable quantitation was estimated to be 5.00 pmol/L. If concentrations could be calculated from measured fluorescence intensity, the values were used in the analysis even if less than 5.00 pmol/L. If concentrations could not be calculated, the measured values were defined as zero. Insulin, C-peptide, glucagon, and glucose concentrations were measured by standard methods in the laboratory, *i.e.* immunoradiometric assay for insulin, radioimmunoassay for glucagon and C-peptide, and enzymatic assay for glucose.

For pharmacodynamic parameters, the values measured and changes from baseline at each time point were summarized using descriptive statistics by dose. Percent inhibition of plasma DPP-IV activity for each subject was plotted against plasma E3024 concentration, and an I_{max} model (effect = $I_{max} \cdot C/(IC_{50} + C)$; where C is plasma E3024 concentration) was used to determine the IC_{50} values.

2.1.6. Safety Assessments

The following data were collected during the study to assess safety: physical examination findings, vital signs (blood pressure, pulse rate, respiratory rate, and body temperature), body weight, 12-lead ECGs, and clinical laboratory parameters (hematology, biochemistry and urinalysis). In the case of a clinically significant abnormal value, the evaluation was to be repeated until the value was within an acceptable or normal range. AEs were to be followed to resolution.

From subjects who had rash in the 40-mg group, blood samples were collected for measurement of non-specific immunoglobulin E (IgE) at 24 and 96 h, and drug-induced lymphocyte stimulation test (DLST) at 96 h after dosing. In the same way, from subjects who had rash in the 80-mg group, blood samples were collected for measurement of IgE, serotonin, histamine, and substance P at onset of rash (the nearest pharmacokinetic time point), 24 and 96 h after dosing. Blood samples, which were collected for clinical laboratory tests the day before dosing, were also used to obtain baseline data for IgE, histamine and substance P. These additional assays were performed at the study site for IgE, and at Mitsubishi Kagaku Bio-clinical Laboratories, Inc. for DLST, serotonin, histamine and substance P.

The numbers of subjects with AEs were tabulated. For clinical laboratory parameters (except urinalysis), vital signs, body weight, and 12-lead ECG parameters, the values measured and changes from baseline at each time point were summarized using descriptive statistics by dose. For urinalysis, cross tables were prepared.

2.2. Non-Clinical *in Vivo* and *in Vitro* Studies

2.2.1. Chemicals

E3024, vildagliptin, valine-pyrrolidide (a DPP-IV inhibitor [16]), ER-319441-15 (trifluoroacetate salt form of ER-319441 (2-(3-amino-piperidin-1-yl)-3-but-2-ynyl-5-methyl-3,5-dihydro-4*H*-imidazo[4,5-*d*]pyridazin-4-one)), ER-319433-15 (trifluoroacetate salt form of ER-319433 (2-{[7-(but-2-yn-1-yl)-1-[(4-cyanophenyl)methyl]-6-oxo-8-(piperazin-1-yl)-6,7-dihydro-1*H*-purin-2-yl]methyl} benzamide)), and ER-463809-15 (trifluoroacetate salt form of ER-463809 (2-({8-(3-aminopiperidin-1-yl)-7-(but-2-yn-1-yl)-1-[(4-cyanophenyl)methyl]-6-oxo-6,7-dihydro-1*H*-purin-2-yl}methyl)benzamide)) were synthesized in our laboratories. Chemical structures of ER-319441-15, ER-319433-15 and ER-463809-15 are indicated in **Figure 1**. A23187 (a calcium ionophore) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Methylcellulose (MC) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2.2. Animals

Five-week-old normal male Fischer (F344/Jcl) rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). Five-week-old male Slc:WsRC-*Ws*/*Ws* (*Ws*/*Ws*) and Slc:WsRC-+/+ (+/+; wild-type homozygous) rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The 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-hr light/dark cycle with lights on at 07:00 a.m.). All procedures were conducted according to the Eisai Animal Care Committee's guideline.

2.2.3. Determination of Plasma Compound Concentrations in Rats

Compounds were suspended in 0.5% MC, and administered to Fischer rats aged eight weeks orally (10 mL/kg).

After 30 min later, blood samples were taken from the tail vein (~200 μL). Plasma was obtained after centrifugation. The concentrations of compounds were determined by the LC/MS/MS method.

2.2.4. Effects of Compounds on Blood Histamine Levels

Compounds or vehicle (0.5% MC, 10 mL/kg) were orally administered to seven-week-old Fischer rats. Blood (20 μL) was taken from the tail vein at 0, 0.5 or 1 h after administration, and was mixed with saline containing 50 mg/mL EDTA (20 μL). Blood histamine levels were determined using a Histamine ELISA kit (Immunotech; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

2.2.5. Effects of Compounds on Histamine Release from Rat Peritoneal Mast Cells and Basophils

Peritoneal exudate cells (PECs) were used for studies on histamine release from rat mast cells [17]. Seven-week-old Fischer rats were sacrificed by exsanguination from the carotid artery under deep diethyl ether anesthesia. PECs were recovered by washing the peritoneal cavity with the injection of 10 mL of Ca^{2+}-free Dulbecco's phosphate-buffered saline (D-PBS(-)) containing 5 units/mL of heparin and 0.1% bovine serum albumin, followed by gentle massage for 90 sec. The peritoneal fluids were pooled and spun down at 1200 rpm for 5 min at 4˚C. Cells were washed in D-PBS(-) three times, and resuspended in D-PBS(-).

We used whole blood cells to investigate histamine release from basophils according to the method of Kowal *et al*. [18]. Heparinized whole blood was obtained from the posterior vena cava of seven-week-old Fischer rats under deep diethyl ether anesthesia, and whole blood was diluted to 1/25 with D-PBS(-).

Mast cells (10^4 cells/180 μL/tube) or whole blood cells (180 μL of the diluted whole blood/tube) were incubated with a test compound or vehicle (DMSO) for 30 min at 37˚C. A23187 was used as a control compound to release histamine. Addition of 0.1% Triton X-100 to the tubes was performed to obtain total histamine content of cells (Hc). Blank tubes containing only cells and buffers were used for non-specific spontaneous release (Hs) during the reaction. Histamine levels were determined using an ELISA kit. The percentage of histamine release induced by a compound was calculated according to the following formula:

$$\{(\mathrm{Ht}-\mathrm{Hs})/(\mathrm{Hc}-\mathrm{Hs})\}\times 100$$

where Ht = test release caused by a compound, Hs = spontaneous release, and Hc = total cellular histamine content [17].

2.2.6. Statistical Analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). A probability (p) value < 0.05 (two-sided) was considered statistically significant. In the comparison of data, we performed two-way repeated measures analysis of variance followed by Bonferroni's test as a post hoc test, or one-way analysis of variance followed by Dunnett's test as a post hoc test, using GraphPad Prism Version 6 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Subject Demographics

A total of 48 healthy Japanese male subjects were enrolled. Subject demographics were similar across dose groups (**Table 1**). The mean age of enrolled subjects was 24.3 ± 3.4 years (mean ± standard deviation); range, 20 - 35 years), with an average BMI of 21.47 ± 1.48 kg/m^2 (range, 19.2 - 24.2 kg/m^2). All subjects completed the study.

Table 1. Demographic characteristics of the study population.

E3024 dose	No. of subjects	Age (years)	Height (cm)	Body weight (kg)	BMI (kg/m^2)
5 mg	6	22.5 ± 2.0	169.80 ± 4.07	62.02 ± 6.01	21.48 ± 1.30
10 mg	6	23.7 ± 3.8	175.95 ± 3.69	68.87 ± 4.22	22.27 ± 1.50
20 mg	6	26.7 ± 5.4	170.05 ± 4.90	59.77 ± 5.14	20.65 ± 1.18
40 mg	6	23.5 ± 1.5	170.12 ± 7.76	59.48 ± 4.72	20.55 ± 0.94
80 mg	6	26.2 ± 4.9	169.68 ± 6.62	62.48 ± 5.26	21.70 ± 1.45
All E3024-treated	30	24.5 ± 3.9	171.12 ± 5.77	62.52 ± 5.86	21.33 ± 1.37
All placebo-treated	18	23.9 ± 2.4	171.34 ± 6.38	63.78 ± 6.63	21.69 ± 1.67
All subjects	48	24.3 ± 3.4	171.20 ± 5.94	63.00 ± 6.12	21.47 ± 1.48

BMI, body mass index. Mean ± standard deviation.

3.2. Pharmacokinetic Profiles

Pharmacokinetic profiles of E3024 were assessed in 30 healthy adult male subjects each receiving a single oral dose of E3024 (5, 10, 20, 40, or 80 mg) under fasted conditions. Mean pharmacokinetic profiles after the single dose are presented in **Figure 2(a)**, and E3024 single-dose pharmacokinetic parameters are provided in **Table 2**. E3024 was rapidly absorbed after dosing, with a median t_{max} of 0.83 - 1.50 h; thereafter, concentrations declined with a mean $t_{1/2}$ of 5.34 - 11.68 h. After administration of 5 to 80 mg E3024, mean C_{max} increased from 37 to 819 ng/mL, and mean $AUC_{0\text{-inf}}$ increased from 223 to 3571 ng·h/mL. Mean CL/F ranged between 23.0 and 25.4 L/h, remaining nearly constant over the studied dose range. Mean V_z/F showed slightly higher values at doses of 40 and 80 mg.

Dose-proportionality for C_{max} and $AUC_{0\text{-inf}}$ obtained from model-independent analysis was assessed both visually and using a power model ($Y = \alpha X^{\beta}$). Plots of individual C_{max} and $AUC_{0\text{-inf}}$ values against dose are presented in **Figures 2(b)** and **(c)**, respectively. Point estimates of β in C_{max} and $AUC_{0\text{-inf}}$ were 1.077 and 0.993, respectively, indicating that both values were approximately 1. The 95% CIs of β for C_{max} and $AUC_{0\text{-inf}}$ were 1.000 - 1.153 and 0.925 - 1.061, respectively, showing that both C_{max} and $AUC_{0\text{-inf}}$ following a single oral dose of E3024 (5 - 80 mg) increased dose-proportionately.

Urinary pharmacokinetic parameters of E3024 are provided in **Table 3**. Mean cumulative excretion rate (fraction of drug excreted unchanged in urine; fe) reached a plateau within 96 h after dosing and ranged between 52.3% and 63.2%. Mean cumulative excretion rate and renal clearance (CL_R) remained nearly constant over the studied dose range.

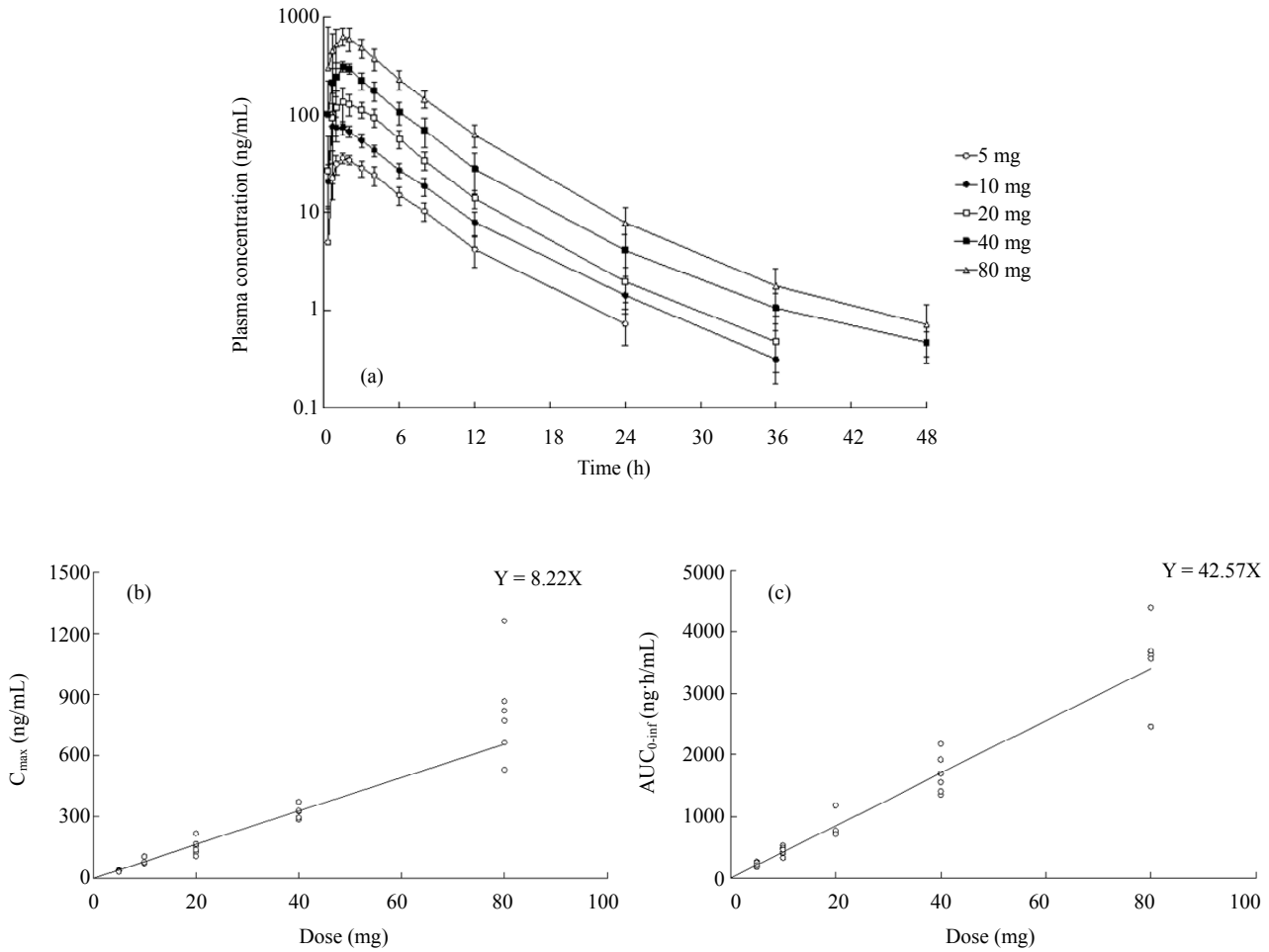


Figure 2. (a) Mean plasma E3024 concentrations (semilogarithmic plotting) after single oral doses of E3024 (5 - 80 mg) in healthy adult male subjects under fasted conditions. Each plotted point represents mean value and standard deviation (six subjects per group). Relationship between dose and C_{max} (b) or $AUC_{0\text{-inf}}$ (c) after single oral doses of E3024 (5 - 80 mg) in healthy adult male subjects under fasted conditions. Each point represents an individual value. Solid lines are the results of application of a linear regression model from a method of least squares.

Table 2. Summary of pharmacokinetic parameters for plasma E3024 obtained by model-independent analysis.

Parameter	E3024 dose				
	5 mg	10 mg	20 mg	40 mg	80 mg
C_{max} (ng/mL)	36.7 ± 3.2	87.6 ± 15.5	152.4 ± 37.9	323.9 ± 30.7	819.3 ± 247.5
t_{max} (h)	1.50 (1.00 - 2.00)	0.83 (0.67 - 2.00)	1.50 (0.67 - 3.00)	1.50 (1.00 - 2.00)	1.25 (0.33 - 2.00)
$AUC_{0\text{-inf}}$ (ng·h/mL)	223.0 ± 38.1	440.4 ± 71.9	812.6 ± 183.8	1684.9 ± 314.9	3571.1 ± 623.2
$t_{1/2}$ (h)	5.34 ± 1.43	5.75 ± 1.68	6.08 ± 1.21	11.68 ± 5.20	9.43 ± 2.16
CL/F (L/h)	23.0 ± 4.0	23.3 ± 4.1	25.4 ± 4.3	24.4 ± 4.3	23.1 ± 4.9
V_z/F (L)	175.1 ± 47.7	186.5 ± 30.0	217.9 ± 31.0	416.8 ± 194.9	318.6 ± 108.6

C_{max}, maximum observed concentration; t_{max}, time to C_{max}; $AUC_{0\text{-inf}}$, area under the plasma concentration-time curve from 0 to infinity; $t_{1/2}$, terminal half-life; CL, clearance; F, bioavailability; CL/F, apparent clearance; V_z, volume of distribution during the terminal phase; V_z/F, apparent volume of distribution during the terminal phase. Mean ± standard deviation, except t_{max}. t_{max}, median (minimum - maximum). Six subjects per group.

Table 3. Summary of pharmacokinetic parameters for urine E3024 obtained by model-independent analysis.

Parameter	E3024 dose				
	5 mg	10 mg	20 mg	40 mg	80 mg
Ae (mg)	3.16 ± 0.56	5.58 ± 0.93	11.01 ± 2.03	20.93 ± 3.14	47.23 ± 14.05
fe (%)	63.2 ± 11.1	55.8 ± 9.3	55.0 ± 10.1	52.3 ± 7.9	59.0 ± 17.6
CL_R (mL/min)	236.6 ± 14.2	211.2 ± 6.3	227.2 ± 13.6	208.4 ± 14.7	215.7 ± 42.3

Ae, amount of unchanged drug excreted in urine; fe, fraction of drug excreted unchanged in urine; CL_R, renal clearance. Mean ± standard deviation. Six subjects per group.

3.3. Pharmacodynamic Profiles

The DPP-IV inhibitory activity of E3024 was measured up to 24 h after administration of the study drug to assess the pharmacodynamic profile. **Figure 3(a)** shows the time course of plasma DPP-IV activity inhibition after a single oral dose (5 - 80 mg) of E3024 or placebo. The inhibition of DPP-IV activity in the E3024 groups increased immediately after drug administration, reached peak levels 1 to 2 h after administration, then decreased to the pre-dose levels at 24 h after administration. On the other hand, DPP-IV inhibitory activity was not observed in the placebo group. The DPP-IV inhibitory activity of E3024 increased dose-dependently. The relationships between plasma concentration of E3024 and inhibition of DPP-IV activity after single oral administration of E3024 (5 - 80 mg) were analyzed using the I_{max} model. As shown in **Figure 3(b)**, the relationship between plasma concentration of E3024 and inhibition of DPP-IV activity was well-adapted to the I_{max} model, with an IC_{50} value of 33.7 ng/mL.

Following administration of the study drug, measurement of active GLP-1, insulin, C-peptide, glucagon, and glucose concentrations was performed within 3 h after lunch and dinner, which were started at 4.5 and 10.5 h after dosing, respectively. The time course of active GLP-1 concentrations after single oral doses of E3024 (5 - 80 mg) or placebo is shown in **Figure 4**. The levels of active GLP-1 increased immediately after food intake and reached maximum concentrations 20 min after food intake, then decreased. In the E3024 groups receiving doses of 20 mg or more, although there were large variations in measured values, the increase in active GLP-1 after food intake was larger than that in the placebo group. In addition, the increase in active GLP-1 tended to be larger after lunch compared with after dinner. An increase in insulin and C-peptide, and a decrease in glucagon and glucose were observed after food intake in all groups, but a dose-dependent change was not observed (data not shown).

3.4. Safety and Tolerability

No deaths or serious AEs were reported following single oral doses of 5 to 80 mg E3024. AEs observed in this study are listed in **Table 4**. Twenty-three events of subjective symptoms or objective findings (erythema, rash, pruritus, diarrhea, feeling hot, conjunctival hyperemia, and headache) occurred in ten subjects and three events of abnormal changes in laboratory values (blood amylase increased, alanine aminotransferase (ALT) increased, and lipase increased) in three subjects. All of these AEs occurred in E3024 groups. No abnormal changes were observed in 12-lead ECG parameters, vital signs, or body weight. The number of AEs in each of the 5 mg, 10 mg, and 20 mg E3024 groups was one event in one (16.7%) of six subjects, while that in the 40 mg group was five events in four (66.7%) of six subjects and that in the 80 mg group was 18 events in five of six subjects (83.3%). Thus, the incidence of AEs increased when 40 mg or more of E3024 was administered.

Rash developed in four subjects each of the 40 mg and

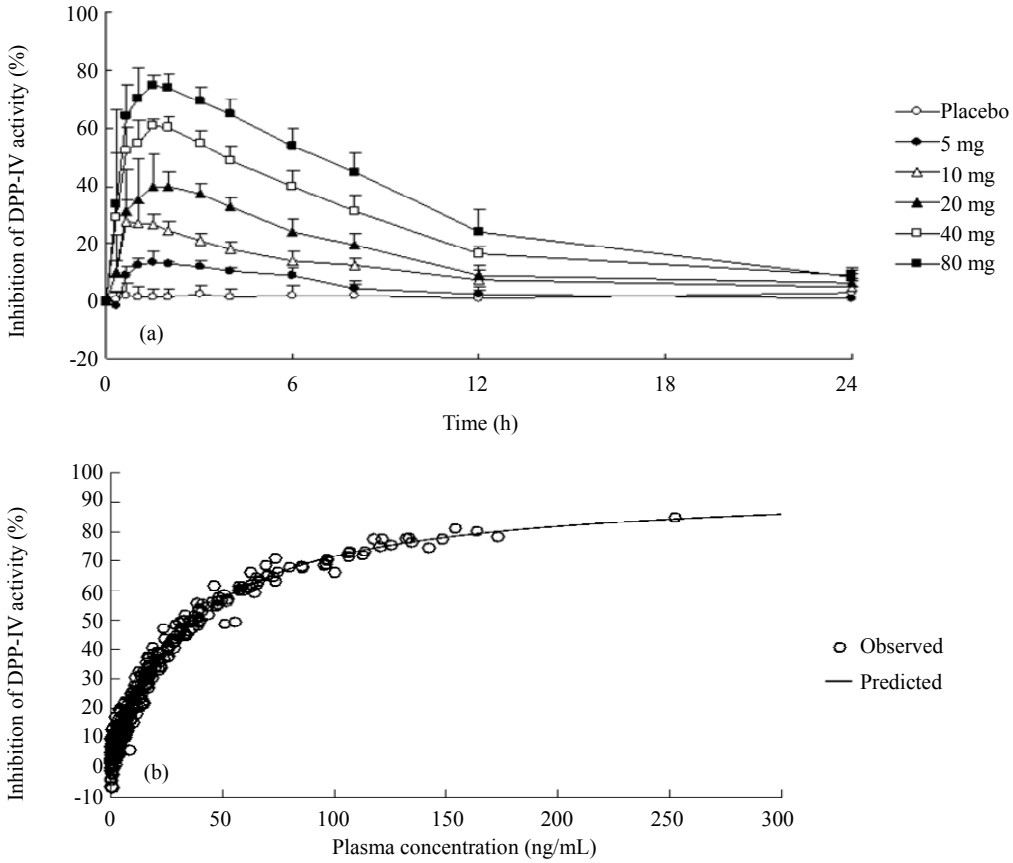


Figure 3. (a) Time course of inhibition of plasma dipeptidyl peptidase-IV (DPP-IV) activity after single oral doses of E3024 (5 - 80 mg) or placebo in healthy adult male subjects. Each plot represents mean values and standard deviations (six subjects for each E3024 group, 18 subjects for placebo); (b) Relationship between plasma E3024 concentration and inhibition of DPP-IV activity. Observed: Plots of 1/5 of actual plasma E3024 concentrations and inhibition of DPP-IV activity at each time point (plasma was diluted to 1/5 in assay for DPP-IV activity). Predicted: I_{max} model prediction of plasma E3024 concentrations and inhibition of DPP-IV activity.

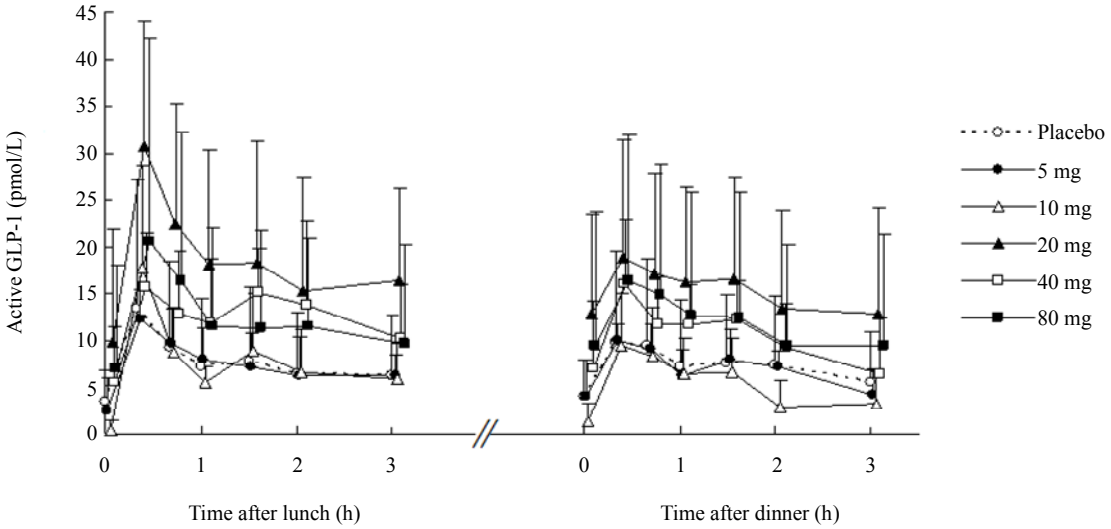


Figure 4. Time course of active glucagon-like peptide-1 (GLP-1; GLP-1-[7-36]amide and GLP-1-[7-37]) concentrations after single oral doses of E3024 (5 - 80 mg) or placebo in healthy adult male subjects. Each plot represents mean values and standard deviations (six subjects for each E3024 group, 18 subjects for placebo).

80 mg groups. The onset of rash was 31 - 83 and 4 - 28 min after dosing in the 40 mg and 80 mg groups, respectively. Additional tests were conducted for the subjects with rash. In the four subjects receiving 40 mg, no changes were observed in eosinophil fraction or non-specific IgE, and DLST was also negative. The results of additional tests of the four subjects receiving 80 mg are shown in **Table 5**. No changes were observed in eosinophil fraction, IgE, or serotonin, but three of the four subjects (subjects A, C, and D) showed a trend to increased histamine levels immediately after the onset of rash following study drug administration. Substance P showed an upward tendency in two subjects (subjects B and D).

Although all AEs occurring in this study were mild

Table 4. Summary of adverse events after single oral dose of E3024.

	E3024 dose					
	Placebo (n = 18)	5 mg (n = 6)	10 mg (n = 6)	20 mg (n = 6)	40 mg (n = 6)	80 mg (n = 6)
Number (%) of subjects experiencing any adverse events	0 (0.0%)	1 (16.7%)	1 (16.7%)	1 (16.7%)	4 (66.7%)	5 (83.3%)
Headache	0	0	0	0	0	1
Conjunctival hyeremia	0	0	0	0	0	4
Diarrhea	0	0	0	0	0	3
Erythema	0	0	1	0	0	0
Pruritus	0	0	0	0	1	1
Rash	0	0	0	0	4	4
Feeling hot	0	0	0	0	0	4
ALT increased	0	0	0	1	0	0
Amylase increased	0	1	0	0	0	0
Lipase increased	0	0	0	0	0	1

ALT, alamine aminotransferase.

Table 5. Individual data for additional tests in subjects having rash in the 80 mg group.

Subject	Time of treatment	Eosinophil (%)	Non-specific IgE (U/mL)	Serotonine (ng/mL)	Histamine (ng/mL)	Substance P (pg/mL)
A	Day -1	10.6	106	-	1.09	Not tested
	Rash onset (0.33 h after dose)	8.0	144	141	1.60	
	24 h after dose	7.2	157	166	0.76	
	96 h after dose	5.4	187	159	1.28	
B	Day -1	2.3	112	-	0.41	105.24
	Rash onset (1 h after dose)	2.1	154	198	0.42	176.09
	24 h after dose	2.4	162	153	0.29	120.70
	96 h after dose	1.4	195	189	0.28	100.74
C	Day -1	0.8	112	-	0.24	115.71
	Rash onset (1.5 h after dose)	0.6	154	114	0.43	116.39
	24 h after dose	0.5	162	131	0.26	114.58
	96 h after dose	0.7	195	149	0.19	105.35
D	Day -1	2.0	59	-	0.42	83.74
	Rash onset (0.67 h after dose)	1.3	57	165	0.57	127.67
	24 h after dose	1.4	65	159	0.92	80.83
	96 h after dose	2.3	84	190	0.73	80.75

IgE, immunoglobulin E.

and non-serious, the incidence of AEs tended to increase when 40 mg or more of E3024 was administered, and their manifestation including rash also suggested tolerability-related problems. Consequently, it was concluded that for single dose administration of E3024, the maximum permissible dose that would not induce tolerability problems was 20 mg.

3.5. Effects of E3024 on Blood Histamine Levels of Normal Fischer Rats

We speculated that histamine was involved in rash development in men treated with E3024. Therefore, we treated Fischer rats with E3024 to examine if this increased blood histamine levels. Blood histamine levels were about 0.5 μmol/L in pre-treatment of E3024 in this rat strain. Thirty min after oral administration of E3024, significant increases in blood histamine levels were observed in the 500 ($p < 0.001$) and 750 mg/kg groups ($p < 0.001$) (**Figure 5(a)**). Next, we examined if the well-known DPP-IV inhibitors, vildagliptin and valine-pyrrolidide, caused increases in blood histamine levels. Oral administration of vildagliptin at 500 mg/kg induced no elevation of blood histamine levels 0.5 h after treatment (**Figure 5(b)**). Similarly, neither did valine-pyrrolidide increase blood histamine levels (data not shown). Plasma concentrations of E3024, vildagliptin and valine-pyrrolidide were 52, 54 and 221 μmol/L, respectively. Regarding E3024, in rat blood the concentration was about 18-fold higher than the C_{max} for the 80-mg group of the E3024 clinical trial which was 819 ng/mL corresponding to 2.89 μmol/L.

3.6. Effects of E3024 on Blood Histamine Levels of Mast Cell-Deficient (*Ws*/*Ws*) Rats and Wild-Type Homozygous (+/+) Rats

We investigated whether E3024 increased blood histamine levels in genetically mast cell-deficient rats, comparing their wild-type homozygous rats. In +/+ rats, E3024 treatment increased blood histamine concentrations in a dose-dependent manner (**Figure 6(a)**). A significant increase in blood histamine was found at 1 h in 500 mg/kg treatment ($p < 0.001$). On the other hand, no effects were detected in *Ws*/*Ws* rats treated with 500 mg/kg E3024 (**Figures 6(a)** and **(b)**), although treatment with the same dose caused increases in blood histamine to >12 μmol/L in +/+ rats.

3.7. *In Vitro* Histamine Release from Rat Peritoneal Mast Cells and Basophils, and Structure-Activity Relationship (SAR) Study

To examine which cells, mast cells or basophiles, were involved in E3024-induced histamine release, we collected these cells from normal Fischer rats and subjected them to *in vitro* assays. At first, we studied the effects of vildagliptin and our DPP-IV inhibitors (E3024, ER-319441-15, ER-319433-15 and ER-463809-15) on histamine release from rat mast cells. Concerning the chemical structures, the piperazin-1-yl group of E3024 and ER-319433-15 was replaced with a 3-amino-

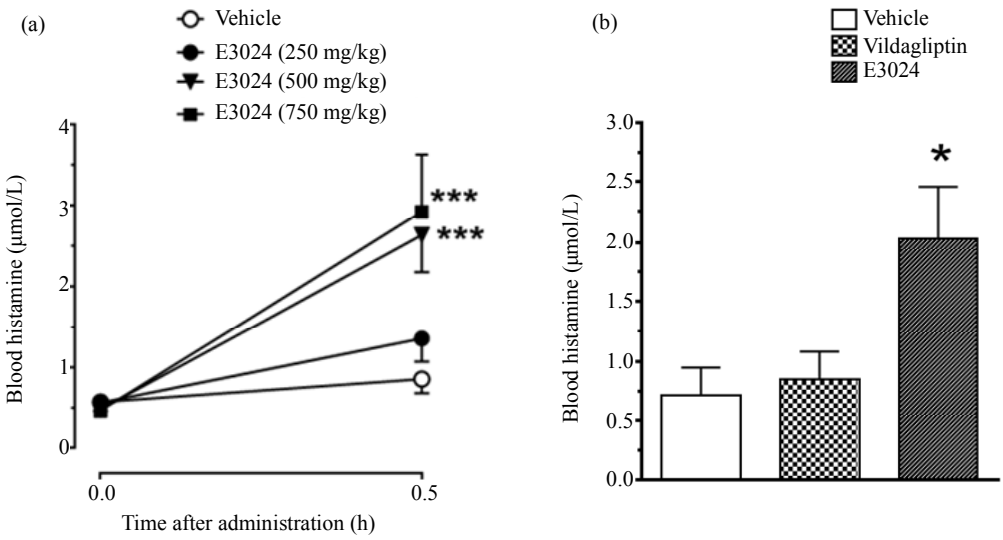


Figure 5. (a) Changes in blood histamine levels in Fischer rats treated with vehicle (0.5% methylcellulose, 10 mL/kg) or E3024 (250, 500 or 750 mg/kg) (n = 8). Blood histamine was determined before and 0.5 h after administration. The data were analyzed by two-way repeated measures analysis of variance followed by Bonferroni's test as a post hoc test. ***, $p < 0.001$; (b) Blood histamine levels 0.5 h after vehicle, vildagliptin (500 mg/kg) or E3024 (500 mg/kg) in Fischer rats (n = 8). *, $p < 0.05$. Values are expressed as the mean ± standard error of the mean.

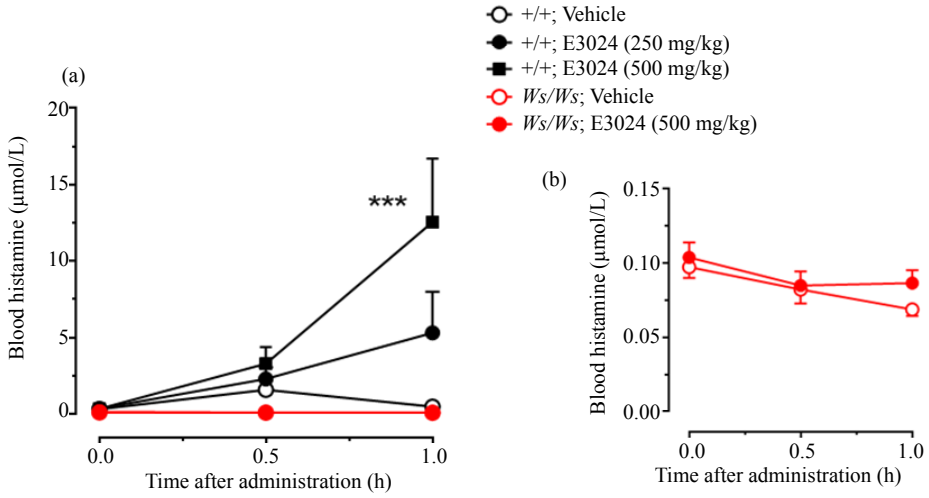


Figure 6. (a) Changes in blood histamine levels in genetically mast cell-deficient (*Ws*/*Ws*) and wild-type normal (+/+) rats. We treated +/+ rats with vehicle (0.5% methylcellulose, 10 mL/kg) or E3024 (250 or 500 mg/kg), and *Ws*/*Ws* rats with vehicle or E3024 (500 mg/kg) (n = 7). The data were analyzed by two-way repeated measures analysis of variance followed by Bonferroni's test as a post hoc test. *, *p* < 0.001; (b) Changes in blood histamine levels in *Ws*/*Ws* rats are extracted from (a). Values are expressed as the mean ± standard error of the mean.**

piperidin-1-yl group to give ER-319441-15 and ER-463809-15, respectively (**Figure 1**). E3024 and ER-319433-15 treatment triggered a significant histamine release in a concentration-dependent fashion (**Figure 7(a)**). On the other hand, neither ER-319441-15 nor ER-463809-15 caused histamine release like vildagliptin. In the case of whole blood, no histamine release was found until 300 μmol/L of E3024, but it was detected in the treatment of A23187 at 1 μmol/L (**Figure 7(b)**). To confirm if this *in vitro* observation was reproduced in *in vivo*, we administered ER-319441-15 orally to Fischer rats. Although blood histamine was significantly increased 0.5 h after E3024 treatment at 500 mg/kg ($p < 0.01$) again, ER-319441-15 caused no increases in blood histamine at 500 mg/kg (**Figure 7(c)**).

4. Discussion

Pharmacokinetic profiles following administration of single oral doses of E3024 were assessed in healthy adult male volunteers. E3024 was absorbed immediately after administration and eliminated at a mean $t_{1/2}$ of 5.34 to 11.68 h. The CL/F was almost constant regardless of dose level, while V_z/F levels showed an upward tendency in high-dose (40 mg and 80 mg) groups. Inappropriate evaluation of the terminal elimination phase in low-dose groups was considered to have influenced the above results. Urinary profiles of E3024 revealed that E3024 was excreted in the urine at a mean cumulative excretion rate of 52.3% to 63.2%. Therefore, it was estimated that at least 52.3% to 63.2% of the orally administered dose would be absorbed. In addition, the CL_R was greater than the glomerular filtration rate of approximately 125 mL/min in healthy adults (70 kg) [19], demonstrating active secretion of E3024 from glomeruli into the urine.

To assess the pharmacodynamic profile of E3024 on single oral administration to healthy male adult volunteers, DPP-IV activity was measured up to 24 h after dosing, and active GLP-1, insulin, C-peptide, glucagon and glucose levels were measured up to 3 h after lunch and dinner, which started at 4.5 and 10.5 h after dosing, respectively. E3024 inhibited plasma DPP-IV activity dose-dependently. A good correlation was found between plasma E3024 concentration and inhibition of DPP-IV activity, and the IC_{50} value was calculated at 33.7 ng/mL from the I_{max} model. It was reported that when E3024 was added to human plasma, the IC_{50} value was 0.14 μmol/L [12], which was equivalent to 39.61 ng/mL. The IC_{50} value from this clinical study was similar to that previously obtained from the *in vitro* study. Thus, the pharmacological effect could be explained by the plasma drug concentration.

GLP-1 is an incretin that is released from L-cells in the intestine postprandially as active GLP-1, and then is rapidly degraded to inactive GLP-1 in the body by DPP-IV, a type of serine proteases. Therefore, it was expected that endogenous active GLP-1 would be increased by inhibition of DPP-IV [1-5]. In the placebo group in this study, the level of active GLP-1 increased immediately after food intake, reached a maximum concentration 20 min after eating, and then decreased. In the E3024 groups administered with doses of 20 mg or more, although there were large variations in measured values, the increase in active GLP-1 after eating was larger than that in

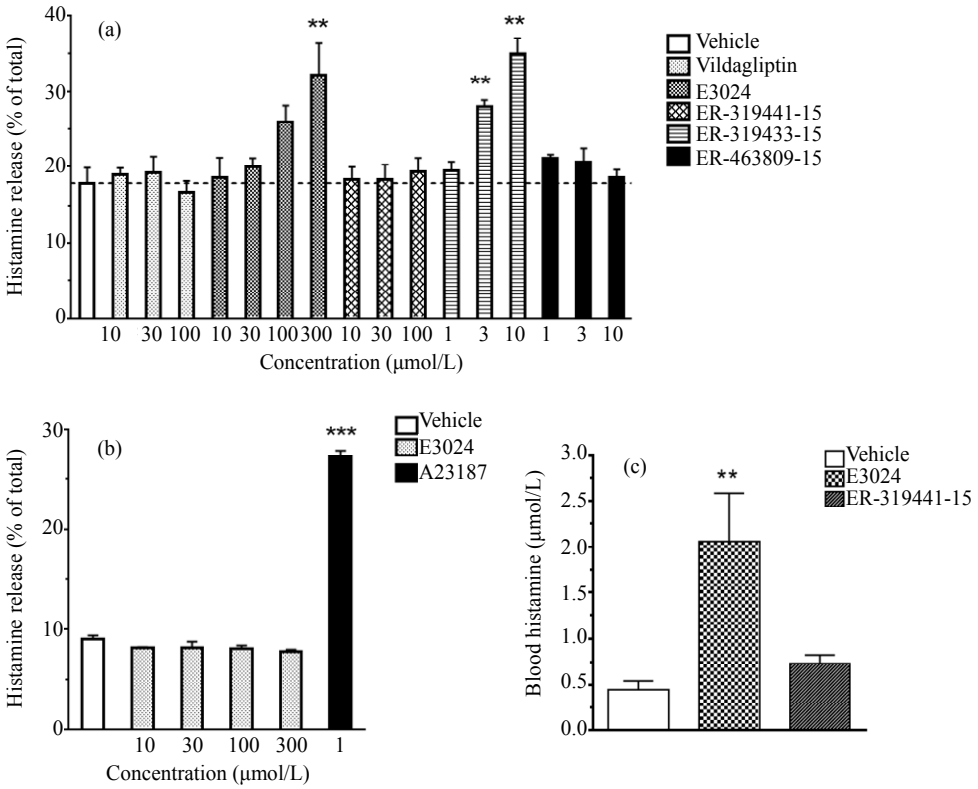


Figure 7. *In vitro* histamine release from peritoneal mast cells (a) and whole blood (b) of Fischer rats by several dipeptidyl peptidase-IV inhibitors. (a) Peritoneal exudate cells were treated with vehicle (dimethyl sulfoxide; DMSO), vildagliptin (10, 30 or 100 μmol/L), E3024 (10, 30, 100 or 300 μmol/L), ER-319441-15 (10, 30 or 100 μmol/L), ER-319433-15 (1, 3 or 10 μmol/L) or ER-463809-15 (1, 3 or 10 μmol/L) (n = 3). A broken line indicates vehicle's value; (b) Whole blood was treated with vehicle (DMSO), E3024 (10, 30, 100 or 300 μmol/L) or A23187 (1 μmol/L) (n = 3); (c) Blood histamine levels 0.5 h after vehicle (0.5% methylcellulose, 10 mL/kg), E3024 (500 mg/kg) or ER-319441-15 (500 mg/kg) in Fischer rats (n = 8). The data were analyzed by one-way analysis of variance followed by Dunnett's test as a post hoc test. **, $p < 0.01$; ***, $p < 0.001$. Values are expressed as the mean ± standard error of the mean.

the placebo group, suggesting that E3024 could enhance the increase of active GLP-1 after eating. If the study drug was administered just before eating, it is expected that a greater enhancement of active GLP-1 would have been obtained, and there may have also been obvious changes in insulin, C-peptide, glucagon or glucose. Although this study was originally designed to examine the postprandial change in active GLP-1 levels when E3024 was administered just prior to food intake in a later portion of the study, we could not conduct this portion because the study was discontinued before reaching this point.

In this study, E3024 was administered to healthy adults, but the drug had been administered to patients with type 2 diabetes, and a greater enhancing effect may have been obtained. Active GLP-1 can enhance glucose-dependent insulin secretion from islet β cells as well as have an inhibitory action on glucagon secretion [1-5]. However, E3024 showed no dose-dependent effects on the postprandial (after-lunch or -dinner) insulin, C-peptide, glucagon, or glucose change. Because healthy adults, the target subjects in this study, had normal insulin secretion and did not show postprandial hyperglycemia, it might be difficult to detect the changes in these parameters in these subjects. On the other hand, if the study drug is administered to patients with type 2 diabetes, the changes in these endpoints might be more evident.

Subjective symptoms and objective findings after single oral doses of 5, 10, 20, 40, and 80 mg of E3024 in 48 subjects were as follows: one event (redness) in one subject in the 10 mg group; five events (rash, pruritus) in four subjects in the 40 mg group; 18 events (rash, feeling hot, conjunctival hyperemia, headache, diarrhea, pruritus) in five subjects in the 80 mg group. Although all these events were mild, a greater variety of symptoms and a larger number of the events were observed in the 80 mg group compared with the 40 mg group. These events suggested that drug administration at greater than 80 mg might be capable of eliciting more numerous and more severe events. Thus, administration of 160 and 320 mg of

E3024 was not conducted.

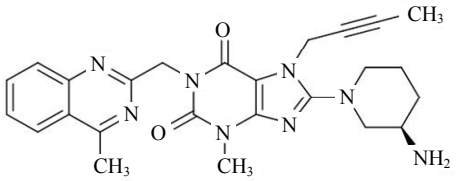
In eight subjects reporting rash, no changes in eosinophil fraction or non-specific IgE, the parameters for type 1 (immediate) allergy, were observed. DLST was performed on four subjects to examine for the possibility of drug hypersensitivity due to delayed allergy, also with negative results. In three subjects from the 80 mg group, an increasing tendency in histamine level was observed immediately after the onset of rash following study-drug administration. In view of the extremely early onset of rash, the dose-dependency in the rash development profile, and the increase in blood histamine levels, histamine release due to the direct action of E3024 on mast cells was considered most likely to have caused the rash. Because there has been no report of such a frequent development of rash associated with other DPP-IV inhibitors, it is considered unlikely that the rash following E3024 administration had been induced by DPP-IV inhibition. It has been suggested that assessment of the selectivity of DPP-IV inhibition over dipeptidyl peptidase-8 (DPP-8) and dipeptidyl peptidase-9 (DPP-9) is important for obtaining an optimal safety profile of DPP-IV inhibitors in the treatment of type 2 diabetes [20]. The Food and Drug Administration (FDA) requested conduct of skin lesion assessments in monkeys for all DPP-IV inhibitors, based on findings of necrotizing skin lesions due to some DPP-IV inhibitors. The FDA considers the skin lesion a result of off-target inhibition of DPP-8 or DPP-9. Because E3024 was shown to be a highly selective DPP-IV inhibitor which did not inhibit DPP-8 or DPP-9 activity [12], we speculate that inhibition of DPP-8 or DPP-9 is not the cause of the rashes observed with E3024.

We inferred that rash in the clinical trial was related to histamine release which was caused by E3024 directly. Then, we examined if E3024 increased histamine levels in rats. The reasons why we chose rats were: 1) we had pharmacokinetic and pharmacodynamics data of E3024 in rats, and 2) there is a mutant rat strain in which mast cells are deficient. We found that E3024 increased blood histamine levels in normal rats. On the other hand, valine-pyrrolidide and vildagliptin had no effects on blood histamine levels, even in the presence of sufficient plasma concentrations. However, there is a large difference in the concentrations of E3024 causing rash in men and increasing blood histamine in the rat. This may be due to different sensitivity between the species: men may be more sensitive to E3024 than rats.

Ws/*Ws* rats are deficient in both mucosal-type and connective tissue-type mast cells [21]. The defected gene of *Ws* is c-*kit*, receptor-type tyrosine kinase [22]. c-*kit* is a receptor of stem cell factor essential for migration, differentiation, and proliferation of cells, such as hematopoietic stem cells, neural crest-derived melanocytes. Thus, *Ws*/*Ws* mutant rats manifest depigmentation, anemia, and mast cell deficiency [21]. Histamine is produced in mast cells, basophils, and entero-chromaffin-like (ECL) cells. Basophils of *Ws*/*Ws* rats are not different from wild-type rats in number, and produce histamine [23]. In addition, histamine is synthesized and stored in ECL cells of *Ws*/*Ws* rats [24]. In our study, E3024 increased blood histamine in wild-type homozygous (+/+) rats, while no response was observed in *Ws*/*Ws* rats. In view of the difference in histamine-producing cells between *Ws*/*Ws* and wild-type rats, we concluded that E3024 acted specifically on mast cells to release histamine. The cell-specific effect was also confirmed by the observation that histamine was released from normal rat mast cells, but not from basophils *in vitro*.

From the *in vitro* study, we obtained the following SAR: comparing between E3024 and ER-319441-15, the presence of a piperazin-1-yl group on position 2 of the imidazo[4,5-*d*]pyridazine may be a causal structure for induction of histamine release in rats. Similarly, comparison of ER-319433-15 and ER-463809-15 revealed that the piperazin-1-yl group on position 8 of purine is important in determining whether histamine release occurs. These results showed that a key structure causing histamine release is piperazine linked to a 5,6-membered fused heterocyclic core, namely, either a pyrimidine ring fused to an imidazole ring, or a pyridazine ring fused to an imidazole ring. More interestingly, this piperazine-associated histamine release can be avoided by substitution with 3-amino-piperidine, while maintaining DPP-IV inhibitory activity (data not shown).

Unfortunately, we cannot demonstrate whether or not the 3-amino-piperidin-1-yl compounds do not induce rash clinically. However, among marketed DPP-IV inhibitors, linagliptin (8-[(3*R*)-3-amino-piperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl) methyl]-3,7-dihydro-1*H*-purine-2,6-dione) [10] has a structure very similar to our compounds and contains a 3-amino-piperidin-1-yl group, not a piperazin-1-yl group, on position 8 of the purine (**Figure 8**). High incidence of rash as observed in the Phase I trial of E3024 has not been reported in clinical trials of linagliptin: the first-in-man study was performed as a randomized, doubled-blind, placebo-controlled Phase I trial in which 63 healthy, male, Caucasian volunteers received the treatment (47 received linagliptin; 16 received placebo) [25]. Once-daily oral doses of linagliptin were 2.5, 5, 25, 50, 100, 200, 400 and 600 mg. No rash was observed in this trial. There was a report of a randomized, double-blind, placebo-controlled Phase I trial enrolling eight healthy Japanese male subjects (six received linagliptin and two received placebo per group). Linagliptin was administered as single escalating doses of 1, 2.5, 5 and 10 mg, or



Linagliptin

Figure 8. Chemical structure of linagliptin.

multiple escalating doses of 2.5, 5 and 10 mg once daily for 12 days [26]. One subject in the 5 mg group showed an increase in histamine concentration. No clinical signs or symptoms attributed to this event were observed, and then they considered unrelated to linagliptin. In addition, no rash was reported in 2523 patients receiving linagliptin 5 mg once daily in eight randomed, double-blind, placebo-controlled Phase III trials lasting 12 to 24 weeks [27]. These facts may support that our speculation that rash could be avoided by the combination of 5- and 6-membered fused heterocyclic rings and 3-amino-piperidine.

In conclusion, we found that E3024 was a compound with favorable pharmacokinetic profiles that inhibited plasma DPP-IV activity dose-dependently with a good correlation between its plasma concentrations and inhibition of DPP-IV activity. Because AEs including rash occurred frequently when 40 mg or more of E3024 was administered, the maximum permissible single dose of E3024 that would not induce tolerability problems was considered to be 20 mg. Since superior safety is required for antidiabetic therapy, we decided not to continue further development of E3024. The results of *in vivo* and *in vitro* pre-clinical examinations using rats indicated that histamine release from mast cells by E3024 was involved in rash development in men.

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REFERENCES

[1] J. J. Holst and C. F. Deacon, "Inhibition of the Activity of Dipeptidyl-Peptidase IV as a Treatment for Type 2 Diabetes," *Diabetes*, Vol. 47, No. 11, 1998, pp. 1663-1670. http://dx.doi.org/10.2337/diabetes.47.11.1663

[2] D. J. Drucker, "Enhancing Incretin Action for the Treatment of Type 2 Diabetes," *Diabetes Care*, Vol. 26, No. 10, 2003, pp. 2929-2940. http://dx.doi.org/10.2337/diacare.26.10.2929

[3] C. F. Deacon, "Therapeutic Strategies Based on Glucagon-Like Peptide 1," *Diabetes*, Vol. 53, No. 9, 2004, pp. 2181-2189. http://dx.doi.org/10.2337/diabetes.53.9.2181

[4] J. J. Holst and C. Ørskov, "The Incretin Approach for Diabetes Treatment. Modulation of Islet Hormone Release by GLP-1 Agonism," *Diabetes*, Vol. 53, Suppl. 3, 2004, pp. S197-S204. http://dx.doi.org/10.2337/diabetes.53.suppl_3.S197

[5] B. Ahrén, "Dipeptidyl Peptidase-4 Inhibitors. Clinical Data and Clinical Implications," *Diabetes Care*, Vol. 30, No. 6, 2007, pp. 1344-1350. http://dx.doi.org/10.2337/dc07-0233

[6] D. Kim, L. Wang, M. Beconi, G. J. Eiermann, M. H. Fisher, H. He, G. J. Hickey, J. E. Kowalchick, B. Leiting, K. Lyons, F. Marsilio, M. E. McCann, R. A. Patel, A. Petrov, G. Scapin, S. B. Patel, R. S. Roy, J. K. Wu, M. J. Wyvratt, B. B. Zhang, L. Zhu, N. A. Thornberry and A. E. Weber, "(2*R*)-4-Oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine: A Potent, Orally Active Dipeptidyl Peptidase IV Inhibitor for the Treatment of Type 2 Diabetes," *Journal of Medicinal Chemistry*, Vol. 48, No. 1, 2005, pp. 141-151. http://dx.doi.org/10.1021/jm0493156

[7] E. B. Villhauer, J. A. Brinkman, G. B. Naderi, B. F. Burkey, B. E. Dunning, K. Prasad, B. L. Mangold, M. E. Russell and T. E. Hughes, "1-[[(3-Hydroxy-1-adamantyl) amino]acetyl]-2-cyano-(*S*)-pyrrolidine: A Potent, Selective, and Orally Bioavailable Dipeptidyl Peptidase IV Inhibitor with Antihyperglycemic Properties," *Journal of Medicinal Chemistry*, Vol. 46, No. 13, 2003, pp. 2774-2789. http://dx.doi.org/10.1021/jm0300911

[8] D. J. Augeri, J. A. Robl, D. A. Betebenner, D. R. Magnin, A. Khanna, J. G. Robertson, A. Wang, L. M. Simpkins, P. Taunk, Q. Huang, S. P. Han, B. Abboa-Offei, M. Cap, L. Xin, L. Tao, E. Tozzo, G. E. Welzel, D. M. Egan, J. Marcinkeviciene, S. Y. Chang, S. A. Biller, M. S. Kirby, R. A. Parker and L. G. Hamann, "Discovery and Preclinical Profile of Saxagliptin (BMS-477118): A Highly Potent, Long-Acting, Orally Active Dipeptidyl Peptidase IV Inhibitor for the Treatment of Type 2 Diabetes," *Journal of Medicinal Chemistry*, Vol. 48, No. 15, 2005, pp. 5025-5037. http://dx.doi.org/10.1021/jm050261p

[9] J. Feng, Z. Zhang, M. B. Wallace, J. A. Stafford, S. W. Kaldor, D. B. Kassel, M. Navre, L. Shi, R. J. Skene, T. Asakawa, K. Takeuchi, R. Xu, D. R. Webb and S. L.

Gwaltney, II, "Discovery of Alogliptin: A Potent, Selective, Bioavailable, and Efficacious Inhibitor of Dipeptidyl Peptidase IV," *Journal of Medicinal Chemistry*, Vol. 50, No. 10, 2007, pp. 2297-2300. http://dx.doi.org/10.1021/jm070104l

[10] M. Eckhardt, E. Langkopf, M. Mark, M. Tadayyon, L. Thomas, H. Nar, W. Pfrengle, B. Guth, R. Lotz, P. Sieger, H. Fuchs and F. Himmelsbach, "8-(3-(*R*)-Aminopiperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methyl-quinazolin-2-ylmethyl)-3,7-dihydropurine-2,6-dione (BI 1356), a Highly Potent, Selective, Long-Acting, and Orally Bioavailable DPP-4 Inhibitor for the Treatment of Type 2 Diabetes," *Journal of Medicinal Chemistry*, Vol. 50, No. 26, 2007, pp. 6450-6453. http://dx.doi.org/10.1021/jm701280z

[11] N. Kato, M. Oka, T. Murase, M. Yoshida, M. Sakairi, S. Yamashita, Y. Yasuda, A. Yoshikawa, Y. Hayashi, M. Makino, M. Takeda, Y. Mirensha and T. Kakigami, "Discovery and Pharmacological Characterization of *N*-[2-({2-[(2*S*)-2-cyanopyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl-2-methylpyrazolo[1,5-*a*]pyrimidine-6-car-boxamide Hydrochloride (Anagliptin Hydrochloride Salt) as a Potent and Selective DPP-IV Inhibitor," *Bioorganicand Medicinal Chemistry*, Vol. 19, No. 23, 2011, pp. 7221-7227. http://dx.doi.org/10.1016/j.bmc.2011.09.043

[12] N. Yasuda, T. Nagakura, T. Inoue, K. Yamazaki, N. Katsutani, O. Takenaka, R. Clark, F. Matsuura, E. Emori, S. Yoshikawa, K. Kira, H. Ikuta, T. Okada, T. Saeki, O. Asano and I. Tanaka, "E3024, 3-But-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4*H*-imidazo[4,5-*d*]pyridazin-4-one Tosylate, Is a Novel, Selective and Competitive Dipeptidyl Peptidase-IV Inhibitor," *European Journal of Pharmacology*, Vol. 548, No. 1-3, 2006, pp. 181-187. http://dx.doi.org/10.1016/j.ejphar.2006.08.011

[13] K. Yamazaki, T. Inoue, N. Yasuda, Y. Sato, T. Nagakura, O. Takenaka, R. Clark, T. Saeki and I. Tanaka, "Comparison of Efficacies of a Dipeptidyl Peptidase IV Inhibitor and α-Glucosidase Inhibitors in Oral Carbohydrate and Meal Tolerance Tests and the Effects of Their Combination in Mice," *Journal of Pharmacological Sciences*, Vol. 104, No. 1, 2007, pp. 29-38. http://dx.doi.org/10.1254/jphs.FP0061376

[14] K. Yamazaki, N. Yasuda, T. Inoue, E. Yamamoto, Y. Sugaya, T. Nagakura, M. Shinoda, R. Clark, T. Saeki and I. Tanaka, "Effects of the Combination of a Dipeptidyl Peptidase IV Inhibitor and an Insulin Secretagogue on Glucose and Insulin Levels in Mice and Rats," *The Journal of Pharmacological and Experimental Therapeutics*, Vol. 320, No. 2, 2007, pp. 738-746. http://dx.doi.org/10.1124/jpet.106.112011

[15] T. Hashimoto, H. Kasai, M. Yamada, M. Yamada, H. Sakaki, J. Handa, T. Takizawa and A. Hirata, "Statistical Assessment of Linear Pharmacokinetics in Clinical Pharmacological Studies," *Xenobiotic Metabolism and Disposition*, Vol. 16, No. 3, 2001, pp. 244-252.

[16] C. F. Deacon, T. E. Hughes and J. J. Holst, "Dipeptidyl Peptidase IV Inhibition Potentiates the Insulinotropic Effect of Glucagon-Like Peptide 1 in the Anesthetized Pig," *Diabetes*, Vol. 47, No. 5, 1998, pp. 764-769. http://dx.doi.org/10.2337/diabetes.47.5.764

[17] S. N. Pramod, Y. P. Venkatesh and P. A. Mahesh, "Potato Lectin Activates Basophils and Mast Cells of Atopic Subjects by Its Interaction with Core Chitobiose of Cell-Bound Non-Specific Immunoglobulin E," *Clinical and Experimental Immunology*, Vol. 148, No. 3, 2007, pp. 391-401. http://dx.doi.org/10.1111/j.1365-2249.2007.03368.x

[18] K. Kowal, H. Nolte, P. S. Skov and L. M. DuBuske, "Effect of Allergen-Specific Immunotherapy on Recombinant Human Interleukin 3-Mediated Amplification of Allergen-Induced Basophil Histamine Release," *Allergy and Asthma Proceedings*, Vol. 26, No. 6, 2005, pp. 456-462.

[19] B. Davies and T. Morris, "Physiological Parameters in Laboratory Animals and Humans," *Pharmaceutical Research*, Vol. 10, No. 7, 1993, pp. 1093-1095. http://dx.doi.org/10.1023/A:1018943613122

[20] G. R. Lankas, B. Leiting, R. S. Roy, G. J. Eiermann, M. G. Beconi, T. Biftu, C. C. Chan, S. Edmondson, W. P. Feeney, H. He, D. E. Ippolito, D. Kim, K. A. Lyons, H. O. Ok, R. A. Patel, A. N. Petrov, K. A. Pryor, X. Qian, L. Reigle, A. Woods, J. K. Wu, D. Zaller, X. Zhang, L. Zhu, A. E. Weber and N. A. Thornberry, "Dipeptidyl Peptidase IV Inhibition for the Treatment of Type 2 Diabetes: Potential Importance of Selectivity over Dipeptidyl Peptidases 8 and 9," *Diabetes*, Vol. 54, No. 10, 2005, pp. 2988-2994. http://dx.doi.org/10.2337/diabetes.54.10.2988

[21] Y. Niwa, T. Kasugai, K. Ohno, M. Morimoto, M. Yamazaki, K. Dohmae, Y. Nishimune, K. Kondo and Y. Kitamura, "Anemia and Mast Cell Depletion in Mutant Rats that Are Homozygous at 'White Spotting (*Ws*)' Locus," *Blood*, Vol. 78, No. 8, 1991, pp. 1936-1941.

[22] T. Tsujimura, S. Hirota, S. Nomura, Y. Niwa, M. Yamazaki, T. Tono, E. Morii, H.-M. Kim, K. Kondo, Y. Nishimune and Y. Kitamura, "Characterization of *Ws* Mutant Allele of Rats: A 12-Base Deletion in Tyrosine Kinase Domain of c-*kit* Gene," *Blood*, Vol. 78, No. 8, 1991, pp. 1942-1946.

[23] T. Kasugai, M. Okada, M. Morimoto, N. Arizono, K. Maeyama, M. Yamada, H. Tei, K. Dohmae, H. Onoue, G. F. Newlands, T. Watanabe, Y. Nishimune, H. R. P. Miller and Y. Kitamura, "Infection of *Nippostrongylus brasiliensis* Induces Normal Increase of Basophils in Mast Cell-Deficient *Ws*/*Ws* Rats with a Small Deletion at the Kinase Domain of c-*kit*," *Blood*, Vol. 81, No. 10, 1993, pp. 2521-2529.

[24] S. Nakamura, H. Watanabe, T. Yokota, H. Matsui, M. Onji and K. Maeyama, "Effect of Rabeprazole on Histamine Synthesis in Enterochromaffin-Like Cells of Mast Cell-Deficient (*Ws/Ws*) Rats," *European Journal of Pharmacology*, Vol. 394, No. 1, 2000, pp. 9-16. http://dx.doi.org/10.1016/S0014-2999(00)00080-7

[25] S. Hüttner, E. U. Graefe-Mody, B. Withopf, A. Ring and K. A. Dugi, "Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of Single Oral Doses of BI 1356, an Inhibitor of Dipeptidyl Peptidase 4, in Healthy Male Volunteers," *The Journal of Clinical Pharmacology*, Vol. 48, No. 10, 2008, pp. 1171-1178. http://dx.doi.org/10.1177/0091270008323753

[26] A. Sarashina, S. Sesoko, M. Nakashima, N. Hayashi, A. Taniguchi, Y. Horie, E. U. Graefe-Mody, H.-J. Woerle and K. A. Dugi, "Linagliptin, a Dipeptidyl Peptidase-4 Inhibitor in Development for the Treatment of Type 2 Diabetes Mellitus: A Phase I, Randomized, Double-Blind, Placebo-Controlled Trial of Single and Multiple Escalating Doses in Healthy Adult Male Japanese Subjects," *Clinical Therapeutics*, Vol. 32, No. 6, 2010, pp. 1188-1204. http://dx.doi.org/10.1016/j.clinthera.2010.06.004

[27] G. Schernthaner, A. H. Barnett, A. Emser, S. Patel, J. Troost, H.-J. Woerle and M. von Eynatten, "Safety and Tolerability of Linagliptin: A Pooled Analysis of Data from Randomized Controlled Trials in 3572 Patients with Type 2 Diabetes Mellitus," *Diabetes*, *Obesity and Metabolism*, Vol. 14, No. 5, 2012, pp. 470-478. http://dx.doi.org/10.1111/j.1463-1326.2012.01565.x

Abbreviations

Ae, amount of unchanged drug excreted in urine; AE, adverse event; ALT, alanine aminotransferase; AUC, area under the curve; $AUC_{0\text{-inf}}$, area under the plasma concentration-time curve from 0 to infinity; BMI, body mass index; CI, confidence interval; CL, clearance; CL/F, apparent clearance; CL_R, renal clearance; C_{max}, maximum observed concentration; DLST, drug-induced lymphocyte stimulation test; DMSO, dimethyl sulfoxide; D-PBS, Dulbecco's phosphate-buffered saline; DPP-IV, dipeptidyl peptidase-IV; DPP-8, dipeptidyl peptidase-8; DPP-9, dipeptidyl peptidase-9; ECG, electrocardiography; ECL, entero-chromaffin-like; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; F, bioavailability; FDA, Food and Drug Administration; fe, fraction of drug excreted unchanged in urine; GLP-1, glucagon-like peptide-1; IC_{50}, concentration required for 50% of the maximum inhibition; IgE, immunoglobulin E; LC/MS/MS, liquid chromatographic-tandem mass spectrometry; MC, methycellulose; OTC, over-the-counter; PD, pharmacodynamics; PEC, peritoneal exudate cell; PK, pharmacokinetics; SAR, structure-activity relationship; $t_{1/2}$, terminal half-life; t_{max}, time to C_{max}; V_z, volume of distribution during the terminal phase; V_z/F, apparent volume of distribution during the terminal phase