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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;

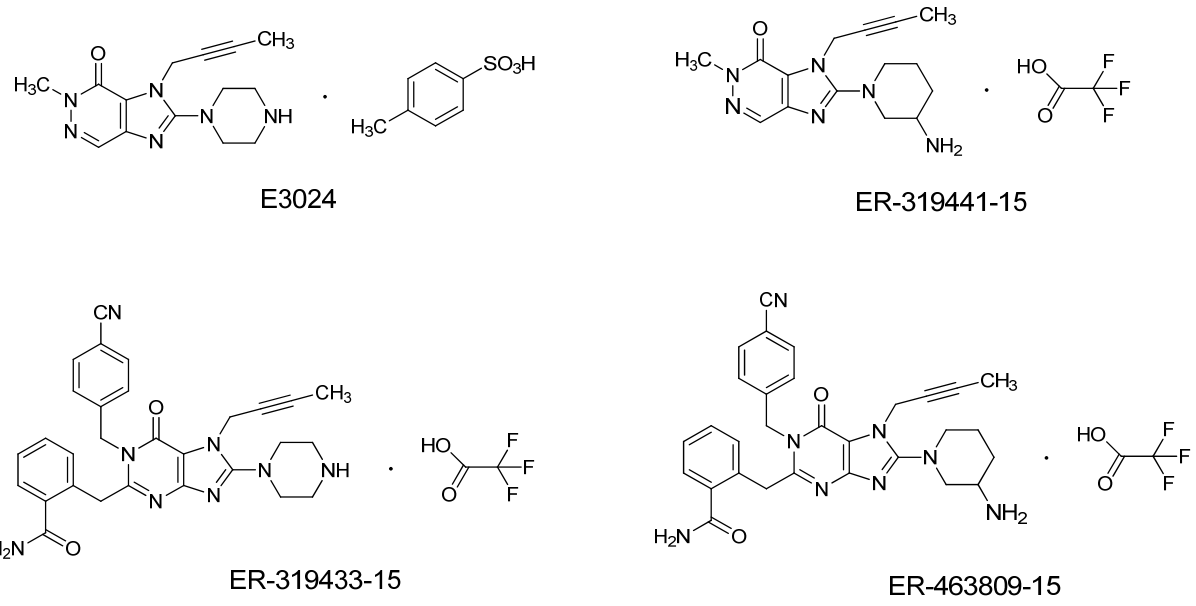

Figure 1. Chemical structures of E3024, ER-319441-15, ER-319433-15, and ER-463809-15.

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:

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

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.

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