### Safety Pharmacology

A total of 3 safety pharmacology studies were performed in accordance with Good Laboratory Practice (GLP) and guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (Studies Project E-PT-0001, Project E-PT-0002 and Project E-PT-0004). All dose levels and concentrations are expressed as PROJECT E free form.

### Effects on CNS in Rats

PROJECT E solid dispersion was orally administered once to female rats under fasted conditions at dose levels of 100, 300 and 600 mg/kg (Study Project E-PT-0001). The observational parameters of the general activity and behavior were set by the modified Irwin's method. PROJECT E did not affect the general activity and behavior at dose levels up to 600 mg/kg. PROJECT E did cause diarrhea and soft stool at 100 mg/kg or more, and miosis at 600 mg/kg.

### Effects on hERG Current

The effects of PROJECT E on the hERG current were studied in hERG-transfected human embryonic kidney (HEK)293 cells by the whole-cell patch-clamp technique (Study Project E-PT- 0002). The suppression rates of PROJECT E at calculated exposure concentrations of 0.202,

2.55 and 8.99 µmol/L were 0.7%, 16.6% and 34.9%, respectively; a statistically significant difference from the control was noted at 2.55 and 8.99 µmol/L. An IC50 for PROJECT E could not be established for the inhibition of hERG current, as only 34.9% inhibition was observed at the highest concentration tested (8.99 μmol/L; 5562 ng/mL).

### 4.1.3.3 Effects on Cardiovascular and Respiratory Systems in Cynomolgus Monkeys

PROJECT E solid dispersion was orally administered under fasted conditions to 4 male conscious cynomolgus monkeys implanted with transmitters of a telemetry system at dose levels of 20, 60 and 300 mg/kg with a dose-escalation regimen and an interval of 7 days (Study Project E-PT-0004). At 20 and 60 mg/kg, PROJECT E did not affect body temperature, blood pressure, heart rate, ECG, respiration rate, blood gases or blood electrolyte concentrations. At 300 mg/kg, heart rate increased or tended to increase in 2 animals (the individual maximum increases were 44% and 66% from the predose values). Vomiting (including retching) was observed at 60 and 300 mg/kg, and soft stool was observed at 300 mg/kg. All of the findings disappeared 24 h after dosing. Therefore, PROJECT E is

unlikely to affect the cardiovascular system at pharmacologically relevant plasma exposure levels.

## Toxicology

A total of 16 toxicity and toxicokinetic studies were conducted in rats, cynomolgus monkeys and rabbits and 1 toxicity study in cynomolgus monkeys is ongoing. All pivotal studies were performed in accordance with GLP and guidelines of ICH or Organisation for Economic Cooperation and Development. All dose levels and concentrations are expressed as PROJECT E free form. An overview of toxicology studies of PROJECT E can be found in

[End-of-Text Table 3.1].

Since incurred sample reanalysis (ISR) using toxicokinetic samples in the 4-week oral dose toxicity study in cynomolgus monkeys (Study Project E-TX-0004) failed, toxicokinetic data on days 1, 14 and 28 of dosing using a new bioanalytical method with successful ISR results from the ongoing 13-week oral-dose toxicity study in cynomolgus monkeys (Study

Project E-TX-0014) was used to evaluate drug exposure findings noted in the pivotal safety pharmacology and toxicity studies in cynomolgus monkeys.

### Single-dose Toxicity

### Single-dose Oral Toxicity in Rats

PROJECT E solid dispersion was orally administered once to 5 male and 5 female rats per group under fasted conditions at dose levels of 600 and 1200 mg/kg (Study Project E-TX-0001). No death occurred in any groups. At 600 mg/kg, abnormal stool (diarrhea, mucus stool and soft stool) with soiled perineal region (stool) was observed between 30 min and 8 h after dosing, and miosis was observed between 6 and 7 h after dosing. Suppression of body weight gain and low food consumption were noted in 1 male until 2 days after dosing. At 1200 mg/kg, abnormal stool (diarrhea, mucus stool and/or soft stool) with soiled perineal region (stool) was observed between 30 min and 8 h after dosing. Miosis in both sexes, salivation in males and lacrimation in females were observed between 4 and 7 h after dosing. A decrease in stool volume was observed on day 1, and body weight loss or suppression of body weight gain and low food consumption were noted until day 2. Erosion or regeneration in the mucosa of the glandular stomach was observed in 1 male. All findings except the stomach lesion disappeared or recovered in the early phase of the 14-day observation period.

### Single-dose Oral Toxicity in Cynomolgus Monkeys

PROJECT E solid dispersion was orally administered once to 1 male and 1 female cynomolgus monkey per group under fasted conditions at dose levels of 300 and 450 mg/kg (Study

Project E-TX-0002). The observation period after dosing was set at 22 days. No death occurred

in any groups. At 300 mg/kg, vomiting was observed at 4 or 5 h after dosing. Increased aspartate transaminase was noted in the male on day 1 (the day following the dosing day) and decreased erythrocyte count and hemoglobin concentration and increased reticulocyte ratio were noted in the female on day 7. At 450 mg/kg, vomiting was observed between 0.5 and 24 h after dosing. Soft stool or diarrhea was observed between 2 and 5 h after dosing.

Increased total bilirubin (direct and indirect bilirubin) on day 1, and increased counts of leukocyte consisting of increased lymphocyte, neutrophil, eosinophil, basophil and large unstained cell were noted in the female on day 7. Histopathology examination revealed no test article-related findings in any animal at any dose level for the heart, sternal/femoral bone marrow, spleen, lungs, liver, kidneys, thyroid glands, adrenals and testes. All findings recovered by day 20.

### Repeat-dose Toxicity

Four preliminary 1-week oral toxicity or toxicokinetic studies in rats, 2 preliminary 1-week oral toxicity studies in cynomolgus monkeys and pivotal 4-week oral toxicity studies in rats and cynomolgus monkeys were conducted. The 13-week oral-dose toxicity study in cynomolgus monkeys is ongoing and toxicokinetic data on days 1, 14 and 28 of dosing was evaluated. Tabulated results of repeat-dose toxicity studies can be found in [End-of-Text Tables 3.2 and 3.3].

### 4-Week Repeat-dose Oral Toxicity in Rats

Four preliminary 1-week oral toxicity or toxicokinetic studies in rats (Studies Project E-TX-3001, Project E-TX-3002, Project E-TX-3005 and Project E-TX-3006) were done by using chloride, free form or solid dispersion of PROJECT E [End-of-Text Table 3.2]. Based on the results of the toxicokinetic studies, the dose levels of PROJECT E solid dispersion in the 4-week oral repeated-dose toxicity study in male and female rats were set at 0, 10, 30, 100 and 300 mg/kg once daily (Study Project E-TX-0003). In the pivotal 4-week toxicity study

(Study Project E-TX-0003), no test article-related changes were observed in any observation or examination at 10 or 30 mg/kg per day. At 100 mg/kg per day, high liver weight was observed in females. At 300 mg/kg per day, 2 females died at week 4 of dosing. Salivation, soft stool, soiled fur, marked body weight loss and marked low food consumption were observed in both animals before death, and lateral position, a decrease in spontaneous activity, hypothermia and a decrease in stool volume were also observed in 1 animal at moribundity.

Decreased zymogen granules in the pancreas and diffuse vacuolation of the hepatocytes (stained positive for Oil red O staining) with pale discoloration in gross pathology were observed in both animals. Erosion in the glandular stomach, which was found as black focus in gross pathology, was observed in 1 animal. The cause of death was considered to be deteriorated general condition (mainly body weight loss with decreased food consumption). In the surviving animals at 300 mg/kg per day, salivation, low body weight and food consumption, high serum total cholesterol, urea nitrogen and inorganic phosphorus, low serum glucose, erosion in the glandular stomach, which was found as black focus in gross pathology, and high liver weights were observed. In males, high urine volume with low specific gravity, high serum creatinine, low serum triglycerides, and hemorrhage and edema

in the lamina propria and submucosa of the glandular stomach were observed. In females, high leukocyte count (high lymphocyte and large unstained cell counts) and vacuolation of the myocardium (stained negative for Oil red O staining) were observed. During the recovery period at 100 and 300 mg/kg per day, the low glucose in females showed a tendency toward recovery. The other toxic findings observed during the dosing period showed recovery. It was concluded that, under the conditions of this study, the NOAEL was

100 mg/kg per day for males and 30 mg/kg per day for females.

### 4-Week Repeat-dose Oral Toxicity in Cynomolgus Monkeys

Two preliminary 1-week oral toxicity studies in cynomolgus monkeys (Studies

Project E-TX-3003 and Project E-TX-3004) were done by using free form or solid dispersion of PROJECT E [End-of-Text Table 3.2]. Based on the results of the preliminary study using PROJECT E solid dispersion, the dose levels of PROJECT E solid dispersion in the pivotal 4-week oral repeated-dose toxicity study in male and female cynomolgus monkeys were set at 0, 6, 20, 60 and 150 mg/kg once daily (Study Project E-TX-0004). Four males and 4 females were assigned to each group for toxicity evaluation during a 4-week treatment period. Three additional males and females per group were assigned to the 60 and 150 mg/kg per day groups for reversibility evaluation during a subsequent 4-week recovery period.

In the preliminary study using the free form of PROJECT E (50, 200 and 1000 mg/kg per day, 1 male and 1 female per dose level), myocardial necrosis was observed in 1 male at the dose of 1000 mg/kg per day. Therefore, in the pivotal 4-week toxicity study (Study

Project E-TX-0004), total and isoenzymes of CK and lactate dehydrogenase (LDH) and cardiac troponin T and I were measured on day 10 and day 22 of dosing and on day 22 of recovery. In the pivotal 4-week toxicity study (Study Project E-TX-0004), no test article-related changes were noted at 6 mg/kg per day. At 20 mg/kg per day, soft stool and/or diarrhea were observed in 2 males. Increased total serum CK and CK-MM values were noted in 1 male at day 10 but neither increase in other cardiac markers nor increases on day 22 were observed. At 60 mg/kg per day, soft stool and/or diarrhea were observed in all males and 6 females.

Vomiting was observed in 4 males and 4 females most frequently during week 1. Salivation was observed in 6 males and 5 females for 1 to 18 days from day 7 of dosing. Transiently decreased food consumption was noted in 2 females at week 1. Increased liver weight was noted in 1 male and 1 female. Hypocellularity was observed in the sternal bone marrow in 1 male. At 150 mg/kg per day, soft stool and/or diarrhea were observed in all animals.

Vomiting was observed in all animals most frequently during week 1. Salivation was observed in 6 males and all females for 1 to 16 days from day 9 of dosing. Transiently decreased food consumption was noted in 2 females at week 1. Decreased serum albumin and total cholesterol and increased serum triglycerides were noted in 1 male at weeks 2 and/or 4. Increased serum CK-MB value in 1 male, increased total serum CK, CK-MB and CK-MM values in another male, and increased levels of serum troponin T and troponin I in another male were noted at day 10, but not at day 22 of dosing. Increased liver weight was noted in 2 males and 3 females. During the recovery period, no test article-related changes were noted at 60 or 150 mg/kg per day in any examination, except for the hypocellularity observed in the sternal and femoral bone marrow in 1 male at 150 mg/kg per day. It was concluded that, under the conditions of this study, the NOAEL was 6 mg/kg per day for males and 20 mg/kg per day for females. Since ISR using toxicokinetic samples failed, toxicokinetic data on days 1, 14 and 28 of dosing using a new bioanalytical method with successful ISR results from the ongoing 13-week oral-dose toxicity study in cynomolgus monkeys (Study Project E-TX-0014) has been used for drug exposure evaluation of findings noted in the pivotal safety pharmacology and toxicity studies in cynomolgus monkeys.

### 13-Week Repeat-dose Oral Toxicity in Cynomolgus Monkeys

A 13-week repeated-dose toxicity study in cynomolgus monkeys is ongoing. PROJECT E solid dispersion is orally administered once daily for 13 weeks at dose levels of 0, 6, 20 and

150 mg/kg per day (Study Project E-TX-0014). An interim toxicokinetic analysis was performed on day 1, 14 and 28 of dosing. These toxicokinetic results are shown in [[Table 3](#_bookmark53)].

### Table 3 Summary of Toxicokinetic Parameters in Cynomolgus Monkeys

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Dose Level (mg/kg/day)** | | **6** | | **20** | | **150** | |
| **Males** | **Females** | **Males** | **Females** | **Males** | **Females** |
| tmax (h) | Day 1 | 2.8 | 2.3 | 3.5 | 2.8 | 4.6 | 4.3 |
| Day 14 | 3.0 | 2.5 | 4.0 | 2.0 | 4.3 | 4.6 |
| Day 28 | 2.5 | 3.0 | 3.3 | 2.5 | 4.6 | 4.6 |
| Cmax (ng/mL) | Day 1 | 799.7 | 681.3 | 3861.0 | 3892.3 | 10134.5 | 9653.8 |
| Day 14 | 686.8 | 580.1 | 2492.3 | 4151.7 | 12382.6 | 13768.6 |
| Day 28 | 670.8 | 579.8 | 1465.1 | 1860.2 | 11961.5 | 12310.7 |
| AUC24  (ng·h/mL) | Day 1 | 3067.7 | 2897.1 | 21967.6 | 20275.8 | 104075.3 | 89177.7 |
| Day 14 | 3201.1 | 2221.9 | 13749.7 | 17242.3 | 119390.0 | 123693.5 |
| Day 28 | 2736.7 | 2182.3 | 12349.1 | 11523.5 | 121850.5 | 121249.1 |

Source: Study Project E-TX-0014

### Genotoxicity

* + - 1. **In Vitro Reverse Mutation**

A reverse mutation test was performed with Salmonella typhimurium (TA100, TA1535, TA98, and TA1537) and Escherichia coli (WP2uvrA), using the preincubation method with and without metabolic activation (Study Project E-TX-0005). Based on the results of a

dose-finding test up to 5000 µg/plate, in which cell death was observed at 500 µg/plate or more, the main test was performed at 9.77, 19.5, 39.1, 78.1, 156 and 313 µg/plate without

metabolic activation and at 78.1, 156, 313, 625, 1250 and 2500 µg/plate with metabolic

activation in TA1537, and 156, 313, 625, 1250, 2500 and 5000 µg/plate with or without metabolic activation in the other strains. In comparison with the negative control, no 2-fold or greater increase in the number of revertant colonies was observed in any test strain with or without metabolic activation. It was concluded that PROJECT E has no potential to induce gene mutation in bacteria.

### In Vitro Chromosomal Aberration

A chromosomal aberration test was performed with cultured mammalian (Chinese hamster lung cell line [CHL/IU]) cells in short-term treatments with the test article for 6 h with and without metabolic activation and continuous treatment with the test article for 24 h without metabolic activation (Study Project E‑TX‑0006). Chromosomal aberrations were analyzed at the following doses: 70, 150, 190 and 270 µg/mL in short-term treatment without metabolic activation; 30, 70, 110 and 190 µg/mL in short-term treatment with metabolic activation, and 20, 60, 80 and 100 µg/mL in continuous treatment for 24 h. No statistically significant increase in the number of cells with structural or numerical chromosomal aberrations was noted in any treatment group when compared with the negative control group. It was concluded that PROJECT E has no potential to induce chromosomal aberrations in

CHL/IU cells.

### Carcinogenicity

No studies have been performed with PROJECT E.

### Reproductive and Developmental Toxicity

One dose range finding embryo-fetal development study in rabbits and 1 pivotal embryo-fetal development study in rats were conducted (Studies Project E-TX-0009 and Project E-TX-0008, respectively). No pivotal study has been conducted in rabbits.

### Effects on Embryo-fetal Development

* + - * 1. **Effects on Embryo-fetal Development in Rats**

PROJECT E solid dispersion was orally administered once daily to 19 to 20 pregnant rats per dose group (0, 30, 100 and 300 mg/kg from day 7 to day 17 of gestation)

(Study Project E-TX-0008). In dams, no test article-related changes were noted at 30 mg/kg per day. At 100 and 300 mg/kg per day decreased body weight or low body weight gain and food consumption were noted. At 300 mg/kg per day, 2 of 20 dams died on day 13 or 17 of gestation. In the 2 dams that died, soft stool, trace of reddish rhinorrhea, soiled perineal region (urine) and decreased body weight and food consumption were observed before death. Black focus in the stomach, discoloration of the liver and/or soiled perineal region were observed at gross pathology. Trace of reddish rhinorrhea was also observed in 1 surviving dam in this group. In fetuses, no test article-related changes were noted at dose levels up to 300 mg/kg per day. It was concluded that the NOAEL was 30 mg/kg per day for dams and 300 mg/kg per day for embryo-fetal development.

### Effects on Embryo-fetal Development in Rabbits

A dose range finding study was conducted (Study Project E-TX-0009). To set the dose levels for pregnant animals, PROJECT E solid dispersion was orally administered for 5 days to

3 nonpregnant female rabbits per group at 100 and 300 mg/kg per day (once daily) and

900 mg/kg per day (450 mg/kg/dose, bid). At 300 mg/kg per day, a decrease in spontaneous activity and decreases in body weight and food consumption were noted, and all animals died or were moribund sacrificed on day 3 of dosing. At gross pathology, dirty around anus was observed as external appearance in all animals and no abnormalities were observed in any organ. At 900 mg/kg per day, all animals died in the morning on day 2 of dosing. Soft stool was observed. In gross pathology, dirty around anus was observed as external appearance in all animals and no abnormalities were observed in any organ.

According to the results in nonpregnant rabbits, PROJECT E solid dispersion was administered orally to 6 pregnant rabbits per group at 0, 10, 30, and 100 mg/kg per day from day 6 to day 18 of gestation. In dams or fetuses, no test article-related changes were noted at dose levels up to 100 mg/kg per day.

### Local Tolerance

No studies have been performed with PROJECT E.

### Other Toxicity Studies

### In Vitro Phototoxicity

A phototoxicity study was performed with cultured mammalian cells (Balb/c 3T3 cells) at 4.74, 6.64, 9.30, 13.0, 18.2, 25.5, 35.7 and 50 µg/mL in the presence and absence of ultraviolet (UV)-A irradiation (Study Project E-TX-0011). The IC50, values for cell viability in the presence and absence of irradiation were determined to be 19.4 and 23.5 µg/mL, respectively, and the photo irritation factor (actual value: 1.21) was less than 2. Therefore, PROJECT E was categorized as having no phototoxicity.

### 1-Week Cardiac Toxicity Study in Cynomolgus Monkeys

As one of the pivotal studies, PROJECT E solid dispersion was orally administered once daily to 4 male and 4 female cynomolgus monkeys per group at 0 and 300 mg/kg for 1 week in order to investigate whether PROJECT E induces cardiac toxicity (Study Project E-TX-0012). Clinical sign observations, body weight measurement, food consumption measurement, CK and LDH (total and isoenzymes) and cardiac troponin I and T measurement, gross pathology, organ weight measurement (heart) and histopathology (heart and gross lesion) were performed. At 300 mg/kg per day, there were no test article-related findings suggesting cardiac toxicity.

Vomiting and soft stool were observed. Transiently decreased food consumption was noted in females on day 2 or 3. Gross lesions were observed in the stomach of 2 female cynomolgus monkeys. At histopathological evaluation mucosal erosions accompanied by edema was observed in the stomach. It was concluded that PROJECT E did not induce cardiac toxicity in cynomolgus monkeys treated with 300 mg/kg per day PROJECT E for 1 week.

## 4.4 Integrated Nonclinical Overview and Conclusion: Potential Clinical Relevance

DU has been defined by the ICS as a contraction of reduced strength and/or duration, resulting in prolonged bladder emptying and/or failure to achieve complete bladder emptying, within a normal time span (Abrams et al, 2002) and can only be diagnosed by invasive urodynamically PFS. UAB has been proposed as the clinical syndrome associated with DU, diagnosed by urodynamic PFS.

Voiding is a process that requires afferent input to the pontine micturition center to switch from storage to a voiding mode of the bladder. The efferent drive of bladder voiding is via parasympathetic pelvic nerves ending in synapses releasing ACh as neurotransmitter. In this process, ACh is released following activation of the pelvic nerve, thereby activating postsynaptic muscarinic M3 receptors located at the detrusor muscle to contract the bladder.

Therefore positive allosteric modulation of the M3 receptors is thought to be a reasonable mechanism to improve bladder contraction and impaired bladder emptying.

PROJECT E is a PAM for M3 receptors, which was shown by a shift of CCh CRCs to the lower-concentration side in cells expressing human and rat muscarinic M3 receptors. PROJECT E enhanced EFS-induced contractions of rat isolated bladder strips and potentiated PNS-induced elevation of intravesical pressure in rats. PROJECT E also inhibited the increase in residual urine volume and prevented the reduction of voiding efficiency in a rat model of voiding dysfunction induced by midodrine and atropine. PROJECT E slightly increased mean basal tone at isolated rat bladder at higher doses (10 µmol/L). However, PROJECT E had no significant effects on any micturition parameters related to urine storage in conscious rats at doses ≤ 10 mg/kg iv (Study Project E-PH-9002), suggesting a low risk for adverse effects on bladder capacity at doses where improvement in bladder contraction and bladder emptying are shown. This implies proof of principle for the applicability of the positive allosteric modulation effect for PROJECT E to improve urinary voiding efficiency.

Based on its pharmacological profile, PROJECT E is expected to facilitate bladder emptying by increasing the force of bladder contraction. PROJECT E is thereby envisaged to alleviate symptoms of a weak bladder contraction such as prolonged and/or incomplete bladder emptying and thereby can contribute to the treatment of patients with UAB.

PROJECT E absorption was rapid and the drug was extensively distributed following oral administration. Terminal t1/2 after a single iv administration in rats and monkeys were 2.43 and 4.98 h, respectively. Bioavailability increased with dose in rats (10% to 28%) and monkeys (21% to 30%). PROJECT E highly binds to plasma proteins (99.70% to 99.96%) in rats, monkeys, and humans. Tissue distribution data in rats showed negligible passage of PROJECT E and its metabolites across the blood-brain barrier. No human-specific metabolites were formed by liver microsomes or hepatocytes. Based on in vitro data, PROJECT E may potentially inhibit the metabolism of substrates for CYP1A2, CYP3A4, and the transport of substrates for P-gp and BCRP. CYP3A4 is the principal isoform for the CYP-mediated metabolism of PROJECT E.

PROJECT E showed PAM activity on the M5 receptor with a similar potency to that on the M3 receptor. Although functional implications of M5 receptors have not been fully explored, studies using M5 knockout mice suggest its role in the facilitation of dopamine release in striatum (Wess et al, 2007) and dilation of cerebral arteries and arterioles (Araya et al, 2006). However it is not likely that the PAM activity of PROJECT E on M5 aggravates these risks because of its low brain permeability.

PROJECT E (10 µmol/L) had no potent affinity for the binding sites of various other receptors, ion channels and transporters and no potent inhibitory effect for the enzyme reactions, except for a partial agonist response to mGluR3. PROJECT E (10 µmol/L) had no potent PAM activities on various GPCRs. Muscarinic M3 receptors are located on many other places in the body and most of these are innervated by parasympathetic nerve activity to modulate activity of e.g., the eyes, secretory glands, bronchi, bowel and other organs. PROJECT E did not affect the number of stools in conscious rats (tested up to 10 mg/kg iv), nor affect airway pressure up to 10 mg/kg iv in anesthetized rats, while effects on bladder were observed at lower doses (3 mg/kg iv). This tissue selectivity is considered to be brought about at least in part by the difference in the relative functional contribution of muscarinic receptor subtypes (M3 and M2) in each organ (Matsui et al, 2002; Stengel et al, 2002) and selective positive allosteric modulation effect of PROJECT E on M3 receptors over M2 receptors.

In the in vitro safety pharmacology studies, an IC50 for PROJECT E could not be established for inhibition of hERG current, as only 34.9% inhibition was observed at the highest concentration tested (8.99 μmol/L; 5562 ng/mL). This concentration is more than

400000-fold higher than calculated human Ceff,u (0.0132 ng/mL). In the cardiovascular safety pharmacology study in cynomolgus monkeys, no effects on ECG were noted at dose levels up to 300 mg/kg. Increased heart rate was observed at the highest dose level (300 mg/kg).

Therefore, PROJECT E is unlikely to affect the cardiovascular system at pharmacologically relevant plasma exposure levels. No effects on the CNS were shown in the safety pharmacology study in rats, at dose levels up to 600 mg/kg.

The adverse events (AEs), that have been observed in the safety pharmacology, single-dose and 4-week repeated-dose toxicity studies in rats and cynomolgus monkeys, such as salivation (in rats at 300 mg/kg per day and cynomolgus monkeys at 60 mg/kg per day or more), diarrhea, soft stool (in rats after a single-dose of 100 mg/kg or more and in cynomolgus monkeys during repeated-dose of 20 mg/kg per day or more), vomiting (in cynomolgus monkeys at 60 mg/kg or more), miosis (in rats after a single-dose of 600 mg/kg or more) and lacrimation (in rats after a single-dose of 1200 mg/kg) are considered to be related to the pharmacology of PROJECT E, as these AEs are known to occur with chronic stimulation of muscarinic ACh receptors, in organs and muscles innervated by the parasympathetic nervous system.

In addition to the findings related to pharmacology, the 4-week toxicity study in rats showed high liver weight, without histopathology or clinical pathology findings, in females at

100 mg/kg per day. At 300 mg/kg per day, 2 females died at week 4 of dosing. The cause of death was considered to be deteriorated general condition (mainly body weight loss with decreased food consumption). In the surviving animals at 300 mg/kg per day, high blood urea nitrogen (possibly related to protein catabolism, due to low body weight and food consumption), high serum total cholesterol, and inorganic phosphorus, low serum glucose, erosion in the glandular stomach and high liver weights were observed. In males high serum creatinine, low serum triglycerides, and hemorrhage and edema in the lamina propria and submucosa of the glandular stomach were observed. In females, high leukocyte count (high lymphocyte and large unstained cell counts) and vacuolation of the myocardium were observed. During the recovery period at 100 and 300 mg/kg per day, the low glucose in females showed a tendency toward recovery. The other toxic findings observed during the dosing period showed recovery. It was concluded that, under the conditions of this study, the NOAEL was 100 mg/kg per day for males and 30 mg/kg per day for females.

In male cynomolgus monkeys soft stool/diarrhea was observed at 20 mg/kg per day. Soft stool/diarrhea, vomiting, salivation and increased liver weight were found at 60 mg/kg or more. At 60 mg/kg also very slight hypocellularity in the sternal bone marrow was observed in 1 male and decreased food consumption was found in female cynomolgus monkeys. At 150 mg/kg per day, decreased serum albumin and total cholesterol and increased serum triglycerides were noted in 1 male at weeks 2 and/or 4. During the recovery period, no test article-related changes were noted at 60 or 150 mg/kg per day in any examination, except for the hypocellularity observed in the sternal (very slight) and femoral (slight) bone marrow in 1 male at 150 mg/kg per day. The toxicological significance of bone marrow hypocellularity was considered to be low because it was very slight or slight and no relevant hematological changes were noted. It was concluded that, under the conditions of this study, the NOAEL was 6 mg/kg per day for males and 20 mg/kg per day for females.

In the preliminary 1-week toxicity study in cynomolgus monkeys, myocardial necrosis was observed in 1 male out of 1 male and 1 female at 1000 mg/kg per day (PROJECT E free form). Therefore, in the pivotal 4-week toxicity and 1-week cardiac toxicity studies in cynomolgus monkeys, cardiac biomarkers such as cardiac troponin I and T, CK and LDH isoenzymes were measured in both sexes before dosing, on days 10 and 22 of dosing and on day 22 of recovery. At 20 mg/kg increased total serum CK and CK-MM values were noted on day 10 (1 male out of 4 males and 4 females), but neither increase in other cardiac markers nor increases on day 22 were observed. At 60 mg/kg none of the animals showed an increase in any of the cardiac biomarkers. At 150 mg/kg increases in cardiac biomarkers were only observed at day 10 (total 3 males out of 7 males and 7 females); Increased serum CK-MB value in 1 male, increased total serum CK, CK-MB and CK-MM values in another male, and increased levels of serum troponin T and troponin I in another male were noted, with no histopathological changes in the heart (exposure margin for the biomarker findings at

150 mg/kg over the calculated human Ceff,u (0.0132 ng/mL), at least 1900 times and at least 800 times over the AUCeff,u, 0.307 ng.h/mL). Therefore the risk for myocardial necrosis is considered to be low. In the 4-week toxicity study in rats, vacuolation of myocardium was noted in 2 out of 9 females after the 4-week treatment period and in none of 4 recovery animals after the 4-week recovery period. This lesion was observed only at the lethal dose level and therefore, considered not to be clinically relevant. In the 2 dead female rats at 300 mg/kg per day, no histopathological changes in the heart were noted.

PROJECT E revealed no genotoxicity potential in the in vitro reverse mutation and chromosome aberration tests. In addition, PROJECT E did not induce phototoxicity in the in vitro 3T3 NRU assay.

In the embryo-fetal development study in rats, PROJECT E did not show embryo-fetal toxicity, but did show maternal toxicity (e.g., low body weight, decreased body weight gain and food consumption). The NOAEL for dams was 30 mg/kg per day and for embryo-fetal development the NOAEL was 300 mg/kg per day. A dose range finding study in rabbits up to 100 mg/kg did not show any embryo-fetal toxicity.

Overall, several toxicological findings were identified in the safety pharmacology and

toxicity studies. The toxicological targets are considered to be the heart, gastrointestinal tract, liver, kidney and hematopoietic system. The exposure margins for most of these findings are large [[Table 4](#_bookmark65)]. In addition the findings at the LOAEL are of mild severity, reversible and can be monitored in phase 1 clinical studies [[Table 5](#_bookmark68)].

### Table 4 Exposures and Exposure Margin Between Toxicokinetic Values and the Anticipated Effective Human Exposure at NOAEL and LOAEL

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Species/ Dosing Duration**  **/Route/Study No.** | **Dose** | **Sex** | **Cmax (last dose)** | | **AUC24 (last dose)** | | **Findings on which NOAEL was based** |
| **Cmax, total**  **(ng/mL)** | **Exposure margin†** | **AUC24, total**  **(ng.h/mL)** | **Exposure margin†** |
| Rat/4 weeks/po/ Project E-TX-0003 | 100 mg/kg  (NOAEL) | M | 4664 | 252 | 60146 | 139 | High liver weight (F) and several changes (such as low body weight and food consumption, high liver weight, and erosion in the glandular  stomach) (M) |
| 30 mg/kg  (NOAEL) | F | 2703 | 146 | 16646 | 39 |
| 300 mg/kg  (LOAEL) | M | 13040 | 703 | 215301 | 499 |
| 100 mg/kg (LOAEL) | F | 10544 | 569 | 91296 | 212 |
| Cynomolgus monkey/4 weeks/po/ Project E-TX-0004  Project E-TX-0014‡ | 6 mg/kg  (NOAEL) | M | 671 | 111 | 2737 | 19 | Soft stool/diarrhea and increased CK total and CK-MM (M)  and soft stool/ diarrhea, vomiting, decreased food consumption  and increased liver weight (F) |
| 20 mg/kg  (NOAEL) | F | 1860 | 308 | 11524 | 82 |
| 20 mg/kg  (LOAEL) | M | 1465 | 242 | 12349 | 88 |
| 60 mg/kg (LOAEL) | F | ND | ND | ND | ND |

† calculated by dividing the Cmax,u or AUC24,u values on day 28 of dosing by 0.0132 ng/mL as the calculated human estimated effective plasma unbound concentration Ceff,u and 0.307 ng.h/mL as the calculated human AUCeff,u, respectively. Cmax,u or AUC24,u were calculated from Cmax, total or AUC24, total based on the plasma concentration at an effective dose in the animal model (Studies Project E-PH-0007 and Project E-PH-0008) taking into consideration the rat fp of 0.000712 or cynomolgus monkey fp of 0.002184 (Study Project E-ME-0006), respectively.

‡ Toxicokinetic values originated from Study Project E-TX-0014.

CK: creatine kinase; CK-MM: isoenzyme of creatine kinase with muscle subunit; LOAEL: lowest observed adverse-effect level; ND: not determined; NOAEL: no observed adverse-effect level.

#### List of References

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, et al. The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. Neurourol Urodyn. 2002;21:167-78.

Araya R, Noguchi T, Yuhki M, Kitamura N, Higuchi M, Saido TC, et al. Loss of M5 muscarinic acetylcholine receptors leads to cerebrovascular and neuronal abnormalities and cognitive deficits in mice. Neurobiol Dis. 2006;24:334-44.

Matsui M, Motomura D, Fujikawa T, Jiang J, Takahashi S, Manabe T, et al. Mice lacking M2 and M3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. J Neurosci. 2002;22:10627-32.

Stengel PW, Yamada M, Wess J, Cohen ML. M(3)-receptor knockout mice: muscarinic receptor function in atria, stomach fundus, urinary bladder, and trachea. Am J Physiol Regul Integr Comp Physiol. 2002;282:R1443-9.

Wess J, Eglen RM, Gautam D. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov. 2007;6:721-33.