Title: Uterine Adenocarcinoma in the rat induced by Afidopyropen. An analysis of the lesion’s induction, progression and its relevance to humans.

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Supplemental Data File

This supplemental data file contains summaries of the following studies with Afidopyropen

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<td>90-Day Rat</td>
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</tr>
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<td>2.1.2</td>
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<td></td>
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<td>2.1.2</td>
<td>Rat chronic (high Dose)</td>
<td></td>
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<td>2.1.3</td>
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<td></td>
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<td>2.1.3</td>
<td>Rat Cancer (High Dose)</td>
<td></td>
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<td>2.2.2</td>
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<td></td>
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<td></td>
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<td>2.2.5</td>
<td>Pharmacokinetics</td>
<td></td>
</tr>
</tbody>
</table>

1 In these summaries, Afidopyropen is referred to as ME5343 or BAS 440 I.
Study 1  Repeated dose 90-day oral toxicity study of ME5343 Technical in rats

Executive Summary
Groups of 10 male and 10 female Fischer rats (F344/DuCrIrlj) rats were administered BAS 4401 (ME5343) in feed at doses of 0, 150, 300, 1000 or 3000 ppm (equal to 0, 8.9, 18.3, 61.0 and 182.0 mg/kg bw/d for males and 0, 10.2, 20.4, 68.2 and 197.0 mg/kg bw/d for females) for 13 weeks.

No treatment-related effect on mortality, clinical signs, final body weight or ophthalmoscopy was noted.

At 3000 ppm there was a transient decrease in female bodyweight in week 2 and the average food consumption of this group was reduced over the 13 week period. There was no treatment-related effects on the food consumption of other groups.

The hind limb grip strength of males in the 3000 ppm group was reduced; there were no other changes in the functional observation battery.

There were some statistically significant but minor decreases in a number of hematology parameters at 1000 and 3000 ppm in both sexes. These included Red Blood Cells, Hemoglobin, Hematocrit, Mean Corpuscular Hemoglobin, and Mean Corpuscular Volume. There was also a statistically significant rise in Platelets (PLT) in males given 3000 ppm and females given 1000 ppm.

In females given 1000 or 3000 ppm there was a decrease in plasma globulin resulting in an increase in the albumin/globulin ratio; at 3000 ppm total protein was also slightly decreased. In males this change was restricted to a rise in the albumin/globulin ratio at 3000 ppm only. In the 3000 ppm group in both sexes there was a fall in total bilirubin and an increase in blood urea nitrogen and alkaline phosphate activity; these changes were also apparent to a lesser extent in females given 1000 ppm. In addition, females given 1000 ppm or 3000 ppm showed an increase in aspartate aminotransferase and alanine aminotransferase activity. There was also a disturbance in plasma electrolyte levels (decrease in calcium and increase in potassium) in females given 3000 ppm and to a lesser extent females given 1000 ppm (increase in potassium). Females given 3000 ppm also showed a rise in plasma glucose levels.

Urinalysis revealed a rise in urobilinogen in males given 3000 ppm, and to a lesser extent females in this dose group.

There were a number of treatment-related changes in organ weights. The weight of the liver and spleen was increased in both sexes at 3000 ppm and the weight of the heart was decreased in females only; there were also pathological changes in these organs. Females given 1000 ppm also showed increased liver weight. In addition, at 3000 ppm, there were increases in the weight of the male kidneys and female thymus and the ovaries and uterus were notably reduced in weight; there were no histopathological changes in any of these organs.

At necropsy the livers of both sexes given 3000 ppm appeared cloudy and histopathological examination revealed lipid deposits in the peripheral hepatocytes; the pathological changes were also apparent in females at 1000 ppm. Vacuolar changes (lipid deposits) were also observed in
the myocardium of both sexes at 3000 ppm and females at 1000 ppm. Slight congestion was observed in the spleen of both sexes given 3000 ppm.

Based on changes in food consumption, hematology, blood chemistry, organs weights and pathology at 3000 ppm and 1000 ppm the NOAEL is set at 300 ppm (equal to 18.3 and 20.4 mg/kg bw/d for males and females, respectively).

II. MATERIALS AND METHODS

Test Type: subacute
Limit Test: No
Test Guideline: According to:
JMAFF No 12 Nosan No 8147; EPA 870.3100; OECD 408
Deviations from Guideline: No
Principles of Method if other Than Guideline: None
GLP Compliance: Yes

Test Material:
Test Material Equivalent to Submission Substance Identity: Yes
Test Material Identity: ME5343 technical (BAS 440 I)

Test Animals:
Species: Rat
Strain: Fischer rats (F344/DuCrI Crj)
Sex: Male/female

Details on Test Animals and Environmental Conditions:

Test Animals: Source: Atsugi Breeding Center, Charles River Japan, Inc. (Kanagawa, Japan)
Age at study initiation: 6 weeks at dosing
Weight at dosing: 102-114 g (males) and 85-95 g (females)
Fasting period before study: Not reported
Housing: housed in wire-mesh stainless steel cages (width 210 mm x depth 350 mm x height 200 mm) sustained in movable stainless steel racks. Each cage accommodated 2 animals of the same sex before grouping. After grouping, 2 animals of the same sex and the same group were housed in each cage.
Diet: Certified diet MF powdered (Oriental Yeast Co., Ltd., Azusawa, Itabashi-ku, Tokyo), ad libitum
Water: Sterilized tap water in bottles, ad libitum
Acclimation period: 11 days

Environmental Conditions: Temperature (°C): 21-25°C
Humidity (%): 34 - 60%;
Air changes (per hr): 10 or more changes per hour
Photoperiod (hrs dark/hrs light): 12 hours/day (light on at 07:00 and off at 19:00)
Experimental Dates: 01-Dec-2008 to 08-June-2009

Administration/Exposure:

Route of Administration: Oral: feed

Vehicle (and/or positive control): Unchanged (no vehicle)

Details on Exposure:
Preparation of dosing solutions: None

Diet Preparation

For each dose level, a specified amount of test substance was mixed with a small part of basal feed and this pre-mix was mixed with the remaining part of the basal feed to obtain test diets with the target concentrations one week before the start of treatment and 4 times during the test period (approximately every four weeks). Weekly aliquots of diet were stored under light-protected and refrigerated conditions (3 – 6 °C) until moved to the animal room for the allocated week of use (light protected and 21 – 25 °C).

Analytical Verification of Doses or Concentrations: Yes

Details on Analytical Verification of Doses or Concentrations:
The coefficient of variation for homogeneity was determined to be 11.3 % for week 1 and 3.4 % for week 4. Test substance concentrations were tested at the beginning and the end of the study and were found to be within 89-101% of target concentrations directly after preparation. The test substance was also determined to be stable in the diet for the duration of use.

<table>
<thead>
<tr>
<th>Nominal Dose level [ppm]</th>
<th>Mean % of nominal concentration week 1</th>
<th>Mean % of nominal concentration week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt; detection limit</td>
<td>&lt; detection limit</td>
</tr>
<tr>
<td>150</td>
<td>99.7</td>
<td>89.7</td>
</tr>
<tr>
<td>300</td>
<td>100.3</td>
<td>91.0</td>
</tr>
<tr>
<td>1000</td>
<td>100.3</td>
<td>92.0</td>
</tr>
<tr>
<td>3000</td>
<td>96.3</td>
<td>91.7</td>
</tr>
</tbody>
</table>

Duration of Treatment/Exposure: About 13 weeks (91 days for males and 92 days for females)

Frequency of Treatment: Daily in the diet.

Doses/Concentrations: Target dose levels of 0, 150, 300, 1000 and 3000 ppm

Basis: Nominal in diet
No. of Animals per Dose Group: For all dose groups, 10 males and 10 females were assigned to each group.

Control Animals: Yes; concurrent no treatment

Details on Study Design: Rationale for animal assignment (if not random): All animals in a good healthy condition were weighed and those at the extremities of the range discarded. The remaining animals were allocated to groups through a stratified randomization procedure based on body weight.

Positive Control: No

Examinations:

Observations and Examinations Performed and Frequency:

Cage Side Observations: Yes
Cage-side observations for mortality and moribundity were performed on all animals at least twice a day (except for Saturdays, Sundays, and holidays when animals were observed at least once a day). General clinical observations were performed at least once a day usually in the morning.

Detailed Clinical Observations: Yes
Detailed clinical observations were performed on all animals once prior to initiation of treatment and once weekly during the treatment period basically at the same time in the afternoon. Animals were observed on the following signs, and the findings were recorded using a scoring system.

- **Home cage:** Posture, Respiration, Grooming, Stereotyped behaviour, Tremor, Twitch, and Convulsion
- **Handling:** Positional passivity, Salivation, Lacrimation, Discharge, Dermal and mucosal color, Pupil size, Abdominal tone, Righting reflex
- **Open field:** Ease of removal from cage, Palpebral closure, Exophthalmos, Fur appearance, Piloerection, Urination (including numbers), Defecations (including numbers), Diarrhea, Alertness, Rears, Abnormal gait, Vocalization, Pinna response, Corneal response, Touch response, and Tail-pinch

Body Weight: Yes
Body weights were recorded on the day of initiation of treatment (test day 0), weekly thereafter and on the day of necropsy.
Food consumption and compound intake (if feeding study):
Food consumption by each cage was measured for a period of 3 or 4 consecutive days once in test week one and then twice weekly on test weeks 2 to 12. In test week 13 food consumption was measured on 3 occasions to accommodate urinalysis investigations. The obtained value was divided by the number of animals alive in the cage and by the number of days for measurement to obtain mean daily food consumption per animal in the cage. Group mean food consumption (g/rat/day) of each sex was calculated from these individual cage values at each week. An overall average of the group mean food consumption throughout the treatment period was also calculated for each sex as the mean of these data. Group mean test substance intake (mg/kg/day) was calculated at each week for each sex according to the following formula:

\[
\text{Group mean test substance intake} = \frac{\text{Group mean food consumption} \times \text{Nominal concentration}}{\text{Group mean body weight}}
\]

An overall average of the group mean test substance intake throughout the treatment period was also calculated for each sex as the mean of these data.

Food Efficiency:
Group mean food efficiency was not calculated.

Water Consumption:
No water consumption data were recorded.

Ophthalmoscopic Examination: Yes
Ophthalmological examinations including observation with an indirect ophthalmoscope (All Pupil Indirect, Konan-Keeler Ltd., Tokyo, Japan) and a 28-diopter aspheric lens (Nikon Corp, Tokyo, Japan) were performed on all animals during the acclimatization period and all animals in the 0 and 3000 ppm groups at 13 weeks of treatment.

The following parts of both eyes were examined: External (Eyeball, Eyelid, Conjunctiva and Cornea), Anterior chamber, optic media and Fundus.

Since no treatment-related ophthalmological abnormalities were observed in the 3000 ppm group at week 13, the animals in other treated groups were not examined at this time point.
**Hematology:** Yes
Time schedule for collection of blood: On the day of necropsy
Collection site: abdominal aorta
Anticoagulant: 3.2 % sodium citrate for PT and APTT, EDTA-2K for remaining parameters.
Animals fasted: Yes (approximately 17 hours)
How many animals: All surviving animals
Analysis: automated hematology analyzer (SF-3000/ SFVU-1, Sysmex Co., Hyogo, Japan). And coagulation analyzer (ACL100, Mitsubishi Kagakulatron, Inc., Tokyo, Japan). Parameters in the table below were examined.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell (RBC)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (HGB)</td>
<td></td>
</tr>
<tr>
<td>Hematocrit (HCT)</td>
<td></td>
</tr>
<tr>
<td>Mean corpuscular volume (MCV)</td>
<td></td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (MCH)</td>
<td></td>
</tr>
<tr>
<td>Total leukocyte count (WBC)</td>
<td></td>
</tr>
<tr>
<td>Differential leukocyte count</td>
<td></td>
</tr>
<tr>
<td>Banded neutrophil (Band)</td>
<td></td>
</tr>
<tr>
<td>Segmented neutrophil (Seg)</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte (Lympho)</td>
<td></td>
</tr>
<tr>
<td>Monocyte (Mono)</td>
<td></td>
</tr>
<tr>
<td>Eosinophil (Esino)</td>
<td></td>
</tr>
<tr>
<td>Basophil (Baso)</td>
<td></td>
</tr>
<tr>
<td>Unclassified cell (Others)</td>
<td></td>
</tr>
<tr>
<td>Prothrombin time (PT)</td>
<td></td>
</tr>
<tr>
<td>Activated partial thromboplastin time</td>
<td></td>
</tr>
<tr>
<td>(APTT)</td>
<td></td>
</tr>
<tr>
<td>Platelet count (PLT)</td>
<td></td>
</tr>
<tr>
<td>Reticulocyte</td>
<td></td>
</tr>
<tr>
<td>Sample taken for differential myelocyte count (stored)</td>
<td></td>
</tr>
</tbody>
</table>

**Clinical Chemistry:** Yes
Time schedule for collection of blood: On the day of necropsy
Collection site: abdominal aorta
Anticoagulant: Heparinized plasma samples from hematology collection
Animals fasted: Yes (approximately 17 hours)
How many animals: All surviving animals
Analysis: auto-analyzer for electrolytes (EA07, ATWiLL Corp., Kanagawa, Japan) and reaming on an auto-analyzer (Accute TBA-40FR, Toshiba Medical Systems, Corp., Tochigi, Japan) Parameters in the table below were examined.

Page 7
Aspartate aminotransferase (AST) | Glucose (GLU)  
--- | ---  
Alanine aminotransferase (ALT) | Total bilirubin (TB)  
Alkaline phosphatase (ALP) | Total protein (TP)  
Creatine kinase (CK) | Albumin (ALB)  
Blood urea nitrogen (BUN) | Albumin/globulin ratio (A/G)  
Creatinine (CRE) | Calcium (Ca)  
Total cholesterol (T.Chol) | Inorganic phosphorus (P)  
Free cholesterol (FCHO) | Sodium (Na)  
Ester ratio (E/T) | Potassium (K)  
Triglyceride (TGL) | Chloride (Cl)  
Globulin (Glob) | Chloride (Cl)  
Albumin/globulin ratio (A/G ratio)  

**Urinalysis:** Yes  
Urinalysis was performed on all animals at 13 weeks of treatment. Animals were individually placed in clean individual metabolic cages and a 24 hour urine sample collected.  
Analysis: Visual, microscopy and Clinitek status (Bayer Medical Co., Ltd., Tokyo, Japan) and urinalysis testing paper (N-Multistixs SG, Bayer Medical Co., Ltd.).  
Parameters in the table below were examined.

<table>
<thead>
<tr>
<th>Turbidity</th>
<th>Urobilinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Bilirubin</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>Sediments</td>
</tr>
<tr>
<td>pH</td>
<td>leukocytes</td>
</tr>
<tr>
<td>Ketone</td>
<td>erythrocytes</td>
</tr>
<tr>
<td>Protein</td>
<td>casts</td>
</tr>
<tr>
<td>Glucose</td>
<td>epithelium</td>
</tr>
<tr>
<td>Occult blood</td>
<td>24-hour urine volume</td>
</tr>
</tbody>
</table>

**Neurobehavioral Examination:** Yes  
**Functional observation battery (FOB):**  
Functional observations were performed on all animals at 11 weeks of treatment. The following parameters were examined:

Sensorimotor response (response to noise of Galton’s whistle, visual placing response and proprioceptive sense); these observations were scored as normal, no response or increased response.

Forelimb and hind limb grip strength, using a grip strength meter, (Muromachi Kikai Co., Ltd., Tokyo, Japan).
Motor activity (Locomotor activity meter, Muromachi Kikai Co., Ltd.), animals were not acclimatised to the cages prior to monitoring.

**Sacrifice and Pathology:**
The animals were sacrificed under diethyl ether anesthesia and exsanguination (after blood collection) from the abdominal aorta. The following organs were sampled, weighed and examined histopathologically. Where indicated, all dose groups were analyzed. Otherwise, only the control and the 3000 ppm dose group organs were analyzed histopathologically.

**Pathology:**
The following organs were collected, weighed (W) and examined histopathologically (H, ✓: all groups, #: control and top dose).

<table>
<thead>
<tr>
<th>W</th>
<th>Groups Analyzed</th>
<th>Organ</th>
<th>W</th>
<th>Groups Analyzed</th>
<th>Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓</td>
<td>#</td>
<td>Brain (cerebrum, cerebellum, pons, and medulla)</td>
<td>#</td>
<td>Rectum</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Spinal cord with vertebrae (cervical, thoracic, and lumbar regions)</td>
<td>#</td>
<td>Nasal cavity (taken with oral mucosa and tympanum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Sciatic nerve (unilateral)</td>
<td>#</td>
<td>Pharynx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Pituitary</td>
<td>#</td>
<td>Larynx</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Thymus</td>
<td>#</td>
<td>Trachea</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Thyroids with parathyroids</td>
<td>✓</td>
<td>Lung (including bronchi)</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Adrenals</td>
<td>✓</td>
<td>Kidneys</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Spleen</td>
<td>#</td>
<td>Urinary bladder</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Bone with bone marrow (sternum, right femur and knee joint)</td>
<td>✓</td>
<td>Testes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Knee joint (unilateral)</td>
<td>✓</td>
<td>Epididymides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Lymph nodes (cervical and mesenteric)</td>
<td>#</td>
<td>Prostate,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>✓</td>
<td>Heart</td>
<td>#</td>
<td>Seminal vesicles</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Aorta</td>
<td>#</td>
<td>Coagulating glands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Salivary glands (submaxillary and parotid)</td>
<td>✓</td>
<td>Ovaries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Esophagus</td>
<td>✓</td>
<td>Uterus (including horns and cervix)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Stomach (forestomach and glandular stomach)</td>
<td>#</td>
<td>Vagina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>✓</td>
<td>Liver</td>
<td>#</td>
<td>Eyes,</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Pancreas</td>
<td>#</td>
<td>Harderian glands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Duodenum</td>
<td>#</td>
<td>Skeletal muscle (right femur)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Jejunum</td>
<td>#</td>
<td>Skin (dorsal region)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Ileum</td>
<td>#</td>
<td>Mammary gland (abdominal region)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Cecum</td>
<td>✓</td>
<td>All gross lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The organs or tissues were fixed in 10% neutral-buffered formalin. The eyes and testes were fixed in Bouin solution.

**Statistics:**
Statistical significance of the difference between the control and the treated groups was estimated at 5 and 1% levels of probability.
The data of quantitative clinical observations (numbers of urination, defecations and rears, grip strength, locomotor activity), body weights, food consumption, volume of urine, hematology, blood biochemistry, and organ weights were evaluated by Bartlett's test for equality of variance. When group variances were homogeneous, a parametric analysis of variance of a one way layout type was conducted to determine if any statistical differences existed among the groups. When the analysis of variance was significant, Dunnett's multiple comparison test was applied. When the group variances were heterogeneous, the data were evaluated by Kruskal-Wallis non-parametric analysis of variance. When significant, Dunnett-type multiple comparison test was applied to determine if any statistical differences existed in mean rank scores among the groups.

The data of detailed clinical observation, (posture, respiration, grooming, stereotyped behaviour, tremor, twitch, convulsion, ease of removal from cage, palpebral closure, exophthalmos, fur-appearance, piloerection, urination, diarrhoea, alertness, abnormal gait, vocalization, pinna response, conneal response, ease of removal from cage, palpebral closure, exophthalmos, fur-appearance, piloerection, urination, diarrhoea, alertness, abnormal gait, vocalization, pinna response, corneal response, touch response, tail-pin, positional passivity, salivation, lacrimation, discharge, dermal and mucosal color, pupil size, abdominal tone, righting reflex) and functional examinations (response to noise, visual placing response, proprioceptive sense) were evaluated by Dunnett-type multiple comparison test to determine if any statistical differences existed in mean rank scores among the groups.

Fisher's exact probability test (one-tail analysis) was used to analyze the data of ophthalmology, necropsy, and histopathology.

Any Other Information on Materials and Methods Incl. Tables: None

III. RESULTS AND DISCUSSION

Effect Levels:

Endpoint: NOAEL
Effect Level: 300 ppm (males 18.3 mg/kg bw/day and females 20.4 mg/kg bw/day)
Sex: Male/female
Basis for Effect Level/Remarks:

Food consumption, Hematology, Clinical chemistry; organ weights and histopathology

Observations:

Clinical Signs and Mortality: No
Body Weight and Weight Gain: Yes
Food Consumption and Compound Intake (if feeding study): Yes
Food Efficiency: Not measured
Water Consumption and Compound Intake: Not measured
Ophthalmoscopic examination: Yes
Hematology: Yes
Clinical Chemistry: Yes
Urinalysis: Yes
Neurobehavior: Yes
Organ Weights: Yes
Gross Pathology: Yes
Histopathology: Yes

Details on Results:

Clinical Signs and Mortality:
No treatment related clinical signs of toxicity were observed throughout the study. No mortality was observed in this study.

Detailed clinical observation:
No test-substance effects were observed during the weekly detailed clinical observations.
There was a statistically significant decrease in the number of rears in females at 1000 and 3000 ppm in test weeks 4 and 5 and an increase in week 13. The changes were not dose-related and considered not to be an effect of treatment.

Water Consumption:
Water Consumption was not evaluated in this study.

Neurobehavior: Functional observation
In the 3000 ppm male group there was a statistically significant (p<0.05) decrease in hind limb grip strength. The control value was 2.29 and the 3000 ppm group was 1.87. No other parameters were affected.
There were no test-substance effects on female functional observations.

Ophthalmoscopy
Ophthalmological examinations performed on the animals in the 0 and 3000 ppm groups at 13 weeks of treatment demonstrated no abnormalities in either sex.
**Body weight and weight gain:**
Body weight development was not affected by treatment.

The mean weight of females given 3000 ppm was statistically reduced (p<0.01) in week 2 only.

### Table 1: Mean body weight of rats administered BAS 440 I for 13 weeks

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>0</th>
<th>150</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
<th>0</th>
<th>150</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight [g]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Day 0</td>
<td>108</td>
<td>108</td>
<td>109</td>
<td>108</td>
<td>88</td>
<td>89</td>
<td>89</td>
<td>89</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>- Week 2</td>
<td>139</td>
<td>142</td>
<td>141</td>
<td>134</td>
<td>108</td>
<td>108</td>
<td>107</td>
<td>106</td>
<td>106</td>
<td>103**</td>
</tr>
<tr>
<td>- Week 13</td>
<td>331</td>
<td>335</td>
<td>332</td>
<td>333</td>
<td>322</td>
<td>188</td>
<td>184</td>
<td>188</td>
<td>183</td>
<td>186</td>
</tr>
<tr>
<td>Overall body weight gain [g]</td>
<td>223</td>
<td>227</td>
<td>224</td>
<td>224</td>
<td>214</td>
<td>100</td>
<td>95</td>
<td>99</td>
<td>94</td>
<td>97</td>
</tr>
<tr>
<td>Change (% of Control)</td>
<td>-</td>
<td>101</td>
<td>101</td>
<td>97</td>
<td>-</td>
<td>98</td>
<td>100</td>
<td>97</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01
Figure 1: Body weight development of rats administered BAS 440 I for 13 weeks

Male

Female
Food Consumption and Compound Intake
The average food consumption of females given 3000 ppm, over weeks 1 – 13, was significantly reduced (p<0.05); the reduction was statistically significant (p<0.01) in weeks 1, 6 and 9.

The food consumption of males given 3000 ppm was reduced in week 1, and of females given 1000 ppm in week 6, only; these observations are considered to be incidental.

Table 2: Average weekly food consumption of rats administered BAS 440 I for 13 weeks

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>Week 1</td>
<td>13.1</td>
<td>13.4</td>
</tr>
<tr>
<td>Weeks 1-13 Average</td>
<td>15.3</td>
<td>15.2</td>
</tr>
<tr>
<td>% of Control</td>
<td>-</td>
<td>99</td>
</tr>
</tbody>
</table>

An actual mean daily test substance intake in mg/kg bw/d is shown below

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Male (mg/kg/bw/day)</th>
<th>Female (mg/kg/bw/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>8.9</td>
<td>10.2</td>
</tr>
<tr>
<td>300</td>
<td>18.3</td>
<td>20.4</td>
</tr>
<tr>
<td>1000</td>
<td>61.0</td>
<td>68.2</td>
</tr>
<tr>
<td>3000</td>
<td>182.0</td>
<td>197.0</td>
</tr>
</tbody>
</table>

Hematological findings
In the 3000 ppm group a number of parameters were statistically significantly (p<0.01) decreased compared to the controls, these included RBC, HGB, HCT, MCV and MCH. PLT was increased in males only.

In the 1000 ppm group, RBC, HGB and HCT were decreased in males (p<0.01) and HCT was decreased in females (p<0.01). In addition, PLT was increased in females only.

In the 300 and 150 ppm groups, no statistically significant changes were observed in either sex that were considered to be related to test-substance.
### Table 3: Selected hematology findings in rats administered BAS 440 I for 13 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group mean (% of the control value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Dose level</td>
<td>150 300 1000 3000</td>
</tr>
<tr>
<td>Red Blood Cells (RBC)</td>
<td>98 99 97** 95**</td>
</tr>
<tr>
<td>Hemoglobin (HGB)</td>
<td>98* 98 96** 93**</td>
</tr>
<tr>
<td>Hematocrit (HCT)</td>
<td>98 99 97** 94**</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin (MCH)</td>
<td>100 99 99 98*</td>
</tr>
<tr>
<td>Mean Corpuscular Volume (MCV)</td>
<td>100 100 99 99</td>
</tr>
<tr>
<td>Platelets (PLT)</td>
<td>103 105 106 111**</td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01

**Clinical chemistry:**

In the 3000 ppm group, females showed a statistically significant rise in a number of parameters which can be associated with liver function (UN, AST, ALT and ALP). There was a fall in total protein and globulin and a rise in the A/G ratio. Calcium was decreased and potassium increased. In addition glucose was increased. In males given 3000 ppm there were similar changes in A/G ratio, TGL, UN, and ALP. ALT, however was decreased. In addition there was a fall in TB levels; these were also decreased in females but without statistical significance.

In the 1000 ppm group, females exhibited a statistically significant increase in UN, AST, ALT and potassium levels. There were no statistically significant changes in males.

In the 300 and 150 ppm groups, no statistically significant changes were observed in either sex.
### Table 4: Selected clinical chemistry findings in rats administered BAS 440 I for 13 weeks (group means)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group mean (% of the control value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>150</td>
</tr>
<tr>
<td>Globulin (Glob)</td>
<td>99</td>
</tr>
<tr>
<td>Albumin / globulin ratio</td>
<td>101</td>
</tr>
<tr>
<td>Glucose (GLU)</td>
<td>102</td>
</tr>
<tr>
<td>Total Protein (TP)</td>
<td>101</td>
</tr>
<tr>
<td>Albumin/globulin ratio (A/G)</td>
<td>95</td>
</tr>
<tr>
<td>Triglycerides (TGL)</td>
<td>88</td>
</tr>
<tr>
<td>Total Bilirubin (TB)</td>
<td>103</td>
</tr>
<tr>
<td>Blood Urea Nitrogen (UN)</td>
<td>97</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST)</td>
<td>96</td>
</tr>
<tr>
<td>Alanine Aminotransferase (ALT)</td>
<td>100</td>
</tr>
<tr>
<td>Alkaline Phosphate (ALP)</td>
<td>103</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>100</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>99</td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01

**Urinalysis:**

In the 3000 ppm group (males) there was a statistically significant (p<0.05) rise in urinary urobilinogen levels; a similar rise was observed in females but this was not statistically significant.

There was a statistically significant decrease in urine volume in females at 3000 ppm and also a decrease in urinary sediment/casts in males at this dose; these are variable parameters and considered not to be of biological significance.
Table 1: Selected urinalysis findings in rats administered BAS 440 I for 13 weeks (group means)

<table>
<thead>
<tr>
<th>Items</th>
<th>Group mean (% of the control value)</th>
<th>Male</th>
<th></th>
<th></th>
<th></th>
<th>Female</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>150</td>
<td>300</td>
<td>1000</td>
<td>3000</td>
<td>150</td>
<td>300</td>
<td>1000</td>
</tr>
<tr>
<td>Dose (ppm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine Volume</td>
<td></td>
<td>86</td>
<td>100</td>
<td>100</td>
<td>86</td>
<td>100</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td></td>
<td>145</td>
<td>136</td>
<td>155*</td>
<td>155*</td>
<td>100</td>
<td>108</td>
<td>117</td>
</tr>
<tr>
<td>Urinary sediment/Casts</td>
<td></td>
<td>70</td>
<td>80</td>
<td>65</td>
<td>55*</td>
<td>118</td>
<td>65</td>
<td>82</td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01

Organ Weights:

In the 3000 ppm group there was a statistically significant increase (p<0.05, 0.01) in the absolute and relative weight of the liver and kidneys in both sexes and the thymus of females. In addition, in females only, there was a statistically significant (p<0.01) decrease in the relative and absolute weight of the heart, ovaries and uterus.

In the 1000 ppm group there was a significant (p<0.01) rise in absolute and relative liver weight in females. There was also an increase (p<0.05) in relative spleen and thymus weight.

In males given 3000 ppm there was a statistically significant (p<0.01, 0.05) increase in relative kidney and thymus weight but in the absence of any corresponding pathology these were considered not to be related to test-substance administration.
Table 2: Selected mean absolute and relative organ weights of rats administered BAS 440 I for 13 weeks

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dose [ppm]</th>
<th>Males</th>
<th></th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute weight</td>
<td>% of Control</td>
<td>Relative weight [% of b.w.]</td>
<td>% of Control</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>150</td>
<td>0.97 101</td>
<td>0.301 99</td>
<td>0.60 97</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.95 99</td>
<td>0.298 98</td>
<td>0.59 95</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.96 100</td>
<td>0.302 100</td>
<td>0.58 94*</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>0.92 96</td>
<td>0.300 99</td>
<td><strong>0.56 90</strong></td>
</tr>
<tr>
<td>Liver (g)</td>
<td>0</td>
<td>6.8 -</td>
<td>2.15 -</td>
<td>3.9 -</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>7.0 103</td>
<td>2.16 100</td>
<td>3.7 95</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6.9 101</td>
<td>2.17 101</td>
<td>3.8 97</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>7.2 106</td>
<td>2.25* 105*</td>
<td><strong>4.3</strong></td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>7.8** 115**</td>
<td>2.56** 119**</td>
<td><strong>4.7</strong></td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>0</td>
<td>1.94 -</td>
<td>0.612 -</td>
<td>1.16 -</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>1.99 103</td>
<td>0.619 101</td>
<td>1.14 98</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>2.00 103</td>
<td>0.628 103</td>
<td>1.15 99</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>2.06 106</td>
<td>0.645* 105*</td>
<td>1.18 102</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>2.05 106*</td>
<td>0.671** 110**</td>
<td>1.19 103</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>0</td>
<td>635 -</td>
<td>0.200 -</td>
<td>411 -</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>643 101</td>
<td>0.200 100</td>
<td>399 97</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>637 100</td>
<td>0.200 100</td>
<td>407 99</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>667 105</td>
<td>0.209 105</td>
<td>427 104</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>690* 109*</td>
<td>0.226** 113*</td>
<td><strong>461</strong></td>
</tr>
<tr>
<td>Thymus (mg)</td>
<td>0</td>
<td>206 -</td>
<td>0.065 -</td>
<td>166 -</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>204 99</td>
<td>0.063 97</td>
<td>165 99</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>237 115*</td>
<td>0.075 115</td>
<td>174 105</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>225 109</td>
<td>0.071 109</td>
<td>181 109</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>235 114</td>
<td>0.077 118*</td>
<td><strong>205</strong></td>
</tr>
<tr>
<td>Ovaries (mg)</td>
<td>0</td>
<td>n/a -</td>
<td>n/a -</td>
<td>61.6 -</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td>58.8 95</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td>58.7 95</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td>59.3 96</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td><strong>49.9</strong></td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>0</td>
<td>n/a -</td>
<td>n/a -</td>
<td>511 -</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td>546 107</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td>549 107</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td>463 91</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>n/a n/a</td>
<td>n/a n/a</td>
<td><strong>289</strong></td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01

Gross and histopathology

At necropsy, cloudiness of the liver was observed in males and females in the 3000 ppm group.
In the 3000 ppm group, vacuolar changes in the hepatocytes at the peripheral portion of the liver were observed in both sexes; these were identified as lipid deposits by oil red O stain. In females vacuolar changes were also seen in the myocardium, these were also lipid deposits. Congestion of the spleen was observed in both sexes.

In the 1000 ppm group similar changes were observed in the heart and liver of females.

There were no histopathological chances considered to be test-substance related in any other tissues.

Table 3: Summary of histopathology of rats administered BAS 440 I for 90 days

<table>
<thead>
<tr>
<th>Organ Findings</th>
<th>Incidence (Number of animals with the lesion/ Number of animals examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male 0 150 300 1000 3000</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Vacuolar change, hepatocyte, periphery - slight</td>
<td>0/10 0/10 0/10 0/10 4/10*</td>
</tr>
<tr>
<td>Vacuolar change, hepatocyte, periphery - moderate ......</td>
<td>0/10 0/10 0/10 0/10</td>
</tr>
<tr>
<td>Vacuolar change, hepatocyte, periphery TOTAL</td>
<td>0/10 0/10 0/10 0/10 4/10**</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td>Vacuolar Change myocardium - slight</td>
<td>0/10 0/10 0/10 0/10 3/10*</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>congestion - slight</td>
<td>0 - - - - 4/10*</td>
</tr>
</tbody>
</table>

* p<0.05  
** p<0.01  
- = not examined
Any Other Information on Results Incl. Tables: None

IV. OVERALL REMARKS, ATTACHMENTS
None

V. SUMMARY AND CONCLUSION

Conclusion:

BAS 440 I elicited effects in food consumption, hematology, blood chemistry, organ weights and histopathology in both males and females at 3000ppm. At 1000ppm changes in hematology in males and in blood chemistry, organ weights and histopathological examinations in females were considered to be treatment related. The no-observed-adverse-effect level (NOAEL) of BAS 440 I in SPF Fischer rats (F344/DuCrlCrj) was determined to be 300 ppm in both sexes (males, 18.3 mg/kg/day; females, 20.4 mg/kg/day).
Study 2  Repeated Dose 1-year Oral Toxicity Study of ME5343 Technical in Rats

Executive Summary

Groups of 24 male and 24 female Fischer rats were administered BAS 440 I (ME5343 Technical) in the diet at levels of 0, 75, 150, 300 or 1000 ppm for a period of 1 year.

There were no treatment-related effects reported for mortality, detailed and general observations, organ weights, functional examination, ophthalmology, feed efficiency, urinalysis, or body weight ratio in any of the treated groups.

At the 1000 ppm dietary level, there were treatment-related decreases in body weights and food consumption of females, and alterations in hematology and biochemistry in both sexes, gross changes in the liver of one female and an increase in the incidence of fatty vacuolar changes in both the liver (15/24) and the heart (6/24) of females. At 300 ppm, vacuolar changes were reported in the liver of two females which were also considered to be treatment related.

Based on the results of this study, the no adverse effect level (NOAEL) was 300 ppm (14.6 mg/kg/day) in males and 150 ppm (8.9 mg/kg/day) in females.
II. MATERIALS AND METHODS

Test Type: Chronic
Limit Test: No
Test Guideline: According to:
MAFF No 12 Nousan No 8147; EPA 870.4100; OECD 452
Deviations from Guideline: No
Principles of Method if other Than Guideline: None
GLP Compliance: Yes

Test Material:
Test Material Equivalent to Submission Substance Identity: Yes
Test Material Identity: ME5343 technical (BAS 440 I)

Test Animals:
Species: Rats
Breed: Fischer 344 (F344/DuCrICrIj)
Sex: Male/female

Details on Test Animals and Environmental Conditions:
Test Animals: Source: Charles River, Japan, Inc. Kanagawa, Japan
Age at study initiation: 6 weeks at dosing
Weight at dosing: 121-140 g (males); 95-105 g (females)
Housing: 2 of the same sex per cage in stainless steel, wire mesh cages; 21 cm (W) x 35 cm (D) x 20 cm (H)
Diet: Powdered basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan)
Water: Tap water (well water), ad libitum. Water was sterilized with sodium hypochlorite.
Acclimation period: 14 days

Environmental Conditions: Temperature (°C): 20-25 °C
Humidity (%): 30 - 70%;
Air changes (per hr.): 10 or more changes per hour
Photoperiod (hrs. dark/hrs. light): 12 hours/day (light on at 07:00 and off at 19:00)

Experimental Dates: Sept 2009 to Sept. 2010 (in-life)

Route of Administration: Oral, in the diet
Vehicle (and/or positive control): Basal diet without test material

Details on Exposure:
Preparation of dosing solutions:

Diet Preparation: Appropriate amounts of test substance for the 75 ppm, 150 ppm, 300 ppm and 1000 ppm groups were measured accurately and were mixed with 1.997 kg, 1.994 kg, 1.988 kg and 1.960 kg of basal diets, respectively. The test substance was smashed and mixed gradually with small amount of the basal diet, and subsequently a total 2 kg of the preliminary mixed diet was shaken and mixed in a vinyl bag for approximately 5 minutes. The preliminary mixed diets in each group were mixed with 38 kg of basal diets, to make 40 kg of test diet for each group, using a mixer Mighty 120 (Aicohsha Manufacturing Co., Ltd., Saitama, Japan), for which wing rotation was set at 140 times/minute, for 20 minutes.

The test diet was prepared before the first treatment and 14 times during the study (every 4 weeks).

The mean test substance concentrations in the test substance mixed diets at the 1st, 8th and 14th preparations were analyzed by using 3 out of 5 samples in each dose level. In results, the mean dose levels of the 75 ppm, 150 ppm, 300 ppm and 1000 ppm groups were 66.5 ppm, 134.4 ppm, 265.4 ppm and 925.6 ppm at the 1st preparation, 73.0 ppm, 146.9 ppm, 289.6 ppm and 979.7 ppm at the 8th preparation and 73.6 ppm, 142.7 ppm, 288.3 ppm and 988.0 ppm at the 14th preparation, respectively. Each analyzed concentration ranged from 87% to 93% of the selected dose levels at the 1st preparation, 94% to 99% at the 8th preparation and from 93% to 99% at the 14th preparation, being within the allowable range (± 15%).

Duration of Treatment/Exposure: About 1 year (52 weeks)

Frequency of Treatment: Daily, 7 days /week.

Doses: 0, 75, 150, 300 and 1000 ppm

Basis: Nominal in diet

No. of Animals per Dose Group: 24

Control Animals: 24

Details on Study Design:
Examinations:
**Observations and Examinations Performed and Frequency:**

**Cage Side Observations:** Yes
All animals were observed from cage-side for morbidity and mortality at least twice a day (in the morning and afternoon) on weekdays and once a day (in the morning) on Saturdays, Sundays, and holidays during the treatment period. All animals were observed clinically once a day (in the morning) during the treatment period. Any abnormal signs including nature, date of onset, recovery and death were recorded.

**Detailed Clinical Observations:** Yes
Detailed clinical observations were performed in all animals once before the initiation of treatment (test week -1) and weekly during the treatment period. The observations were performed in the afternoon. The following items were observed and findings were recorded using a scoring system.

**Home cage:** Posture, Respiration, Grooming, Stereotypy behavior, Tremor, Twitch, Convulsion

**Open field:** Ease of removal from cage, Palpebral closure, Exophthalmos, Fur appearance, Piloerection, Urination (including numbers), Defecations (including numbers), Diarrhea, Alertness, Rears, Abnormal gait, Vocalization, Pinna response, Corneal response, Touch response, Tail pinch

**Handling:** Positional passivity, Salivation, Lacrimation, Discharge, Dermal and mucosal color, Pupil size, abdominal tone, righting reflex.

**Functional Examinations:**
Functional examinations were performed in 10 males and 10 females during test week 49. Sensor-motor response (response to noise of Galton’s whistle, visual placing response and proprioceptive sense), grip strength (forelimb and hind limb, Grip strength meter, Muromachi Kikai Co., Ltd., Tokyo, Japan) and locomotor activity (Locomotor activity meter, Muromachi Kikai Co., Ltd.) were examined. Acclimatization of rats to the equipment cages for locomotor activity was not done.

**Body Weight:** Yes

Body weights of all animals were measured on the day of receipt and on the grouping day. After grouping, body weights were measured in the morning once a week from test week 1 to 13 and once per 4 weeks from test week 16 to the termination of treatment. Final body weights of the animals were measured before euthanasia on each necropsy day.
Food consumption: Yes

Food consumption (3-day or 4-day total amount) in each cage was measured once at test week 1 and twice a week during test week 2 to 13 and twice per 4 weeks from test week 16 to the termination of treatment. The total amount of food consumption was converted to a daily amount of one animal in each group. The weekly mean food consumption (g/rat/day) of males and females in each group was calculated based on the mean food consumption of each cage. The total mean food consumption of males and females in each group during the treatment period was calculated by averaging the weekly mean food consumption.

Food Efficiency: Yes

The mean group body weight gain of all dose groups in each treatment week was divided by the mean group food consumption from the initiation of treatment to test week 13 and then the mean group feed efficiency percentage (%) was calculated. In addition, the total mean feed efficiency of males or females in each dose group during the first 13 treatment weeks was calculated by averaging the mean group feed efficiency in each week.

Water Consumption:
No water consumption data were recorded.

Test Substance Intake: Yes

The mean test substance intake (mg/kg/day) of males and females in each dose-group was calculated in each measuring week according to the following formula:

\[
\text{Mean test substance intake} = \frac{\text{mean food consumption} \times \text{selected dose level}}{\text{mean body weight}}.
\]

In addition, total mean test substance intake during the treatment period in males and females in each group was calculated by averaging the weekly mean test substance intake.
Ophthalmoscopic Examination: Yes

Ophthalmology was performed for all animals 2 weeks before initiation of treatment (test week -2) and also for the animals in the control and high dose groups at test week 52. In the examination, external, anterior segment, optic media and fundus of both eyes were observed externally and with an indirect ophthalmoscope (All Pupil Indirect, Konan-Keeler Ltd., Tokyo, Japan) and a 28-diopter aspheric lens (Nikon Corp, Tokyo, Japan). No animals in other treatment groups were examined because no abnormal findings related to the treatment with the test substance were observed in any animals in the high dose group at test week 52.

Hematology: Yes

At test weeks 14 and 27, blood samples (2 ml) were collected from 10 males and 10 females (the same animals as those used for urinalysis) in each group via the cervical vein after an overnight fast. Heparin sodium was used for anticoagulant at the collection. The blood samples collected were examined on the items shown in the following table, except for reticulocyte, prothrombin time (PT) and activated partial thromboplastin time (APTT).

At test week 53, blood samples were collected from 10 males and 10 females in each group, which were used for urinalysis in principle, via the abdominal aorta. Animals were under deep anesthetization with diethyl ether inhalation. The items examined are shown in the following table. EDTA-2K was used as anticoagulant in measurement of the items except for PT and APTT, and 3.2% sodium citrate solution in measurement of PT and APTT.

Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) and platelet (PLT) were measured by using an automated hematology analyzer (SF-3000/SFVU-1, Sysmex Co., Hyogo, Japan). PT and APTT were measured by using a coagulation analyzer (ACL100, Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan).
<table>
<thead>
<tr>
<th>Red blood cell (RBC)</th>
<th>Prothrombin time (PT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (HGB)</td>
<td>Activated partial thromboplastin time (APTT)</td>
</tr>
<tr>
<td>Hematocrit (HCT)</td>
<td>Total leukocyte count (WBC)</td>
</tr>
<tr>
<td>Mean corpuscular volume (MCV)*</td>
<td>Differential leukocyte count</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (MCH)*</td>
<td>Platelet (PLT)</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (MCHC)</td>
<td>Differential myelocyte count</td>
</tr>
<tr>
<td>Reticulocyte</td>
<td></td>
</tr>
</tbody>
</table>

*calculated using values for RBC, HGB and HCT

**Clinical Chemistry:** Yes

Blood biochemical examinations were carried out on 10 males and 10 females in each group (the same animals as those used for hematology) at test weeks 14, 27 and 53. The items examined are shown in the following table. Heparinized plasma samples separated from the hematology sample were used. The items were measured by using an auto-analyzer (Accute TBA-40FR, Toshiba Medical Systems, Corp., Tochigi, Japan) except for sodium (Na), potassium (K) and chloride (Cl), which were measured by using an auto-analyzer for electrolyte (EA07, ATWILL Corp.).

<table>
<thead>
<tr>
<th>Aspartate aminotransferase (AST)</th>
<th>Glucose (GLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine aminotransferase (ALT)</td>
<td>Total bilirubin (TB)</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>Total protein (TP)</td>
</tr>
<tr>
<td>Creatine kinase (CK)</td>
<td>Albumin (ALB)</td>
</tr>
<tr>
<td>Blood urea nitrogen (UN)</td>
<td>Albumin/globulin ratio (A/G ratio)*</td>
</tr>
<tr>
<td>Creatinine (Creat)</td>
<td>Calcium (Ca)</td>
</tr>
<tr>
<td>Total cholesterol (TCHO)</td>
<td>Inorganic phosphorus (IP)</td>
</tr>
<tr>
<td>Triglyceride (TGL)</td>
<td>Sodium (Na)</td>
</tr>
<tr>
<td>Free cholesterol (FCHO)</td>
<td>Potassium (K)</td>
</tr>
<tr>
<td>Ester ratio (E/T)*</td>
<td>Chloride (Cl)</td>
</tr>
</tbody>
</table>

*calculated values

**Urinalysis:** Yes

Urinalysis was carried out on 10 males and 10 females in each group at test weeks 13, 26 and 51. Fresh urine was collected from the animals which were housed individually in a metabolic cage for urine sampling. The items examined are shown in the following table. Specific gravity, pH, ketones, protein, glucose, occult blood, urobilinogen and bilirubin were measured using Clinitek status (Bayer Medical Co., Ltd., Tokyo, Japan) with a urinalysis testing paper (N-Multistixs SG, Bayer Medical Co., Ltd.). Subsequently, urine volume for approximately 24 hours were measured.
Sacrifice and Pathology: Yes
The animals were anesthetized by diethyl ether inhalation. The animals killed without blood sampling were euthanized by exsanguination from the neck artery, the sampled animals were exsanguinated form the abdominal artery. Body weights of all animals were measured before necropsy. Weights (absolute organ weights) of the organs shown in the following table were measured before fixation in 10 males and 10 females killed in each group (same animals as those used for hematology at test week 53). Relative organ weights were calculated as ratios of organ weights to final body weights.

The following organs and tissues were removed from all animals at necropsy and fixed in 10% neutral-buffered formalin except for the eyes and testes, which were fixed in Bouin solution. The lungs were infused with applied volume of 10% neutral-buffered formalin from the trachea and then fixed in the fixative.

Histopathological examination was conducted on the following designated organs from all animals in the control and high dose (1000 ppm) groups and one male (No. 311) in the 150 ppm group that was found dead. Gross lesions observed in any animals in other groups were also examined. In addition, the heart and liver in females in other treated groups were examined because vacuolar change of the heart and liver were observed in females in the 1000 ppm group.

<table>
<thead>
<tr>
<th>Turbidity</th>
<th>Urobilinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occult blood</td>
<td>Bilirubin</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>Sediments (WBC, RBC, casts, epithelium)</td>
</tr>
<tr>
<td>pH</td>
<td>Volume (24hr)</td>
</tr>
<tr>
<td>Ketone</td>
<td>Protein</td>
</tr>
<tr>
<td>Glucose</td>
<td>Color</td>
</tr>
</tbody>
</table>
Pathology:
The following organs were collected, weighed (W) and examined \( \checkmark \) histopathologically for control and high dose animal, *examined for all females

<table>
<thead>
<tr>
<th>W</th>
<th>Groups Analyzed</th>
<th>Organ</th>
<th>W</th>
<th>Groups Analyzed</th>
<th>Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓</td>
<td>#</td>
<td>Brain (cerebrum, cerebellum, pons, and medulla oblongata)</td>
<td>#</td>
<td>Colon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>#</td>
<td>Spinal cord with vertebrae (cervical, thoracic, and lumbar regions)</td>
<td>#</td>
<td>Rectum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>#</td>
<td>Sciatic nerve (right)</td>
<td>#</td>
<td>Nasal cavity</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Pituitary</td>
<td>#</td>
<td>Laryngopharynx</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Thymus</td>
<td>#</td>
<td>Trachea</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Thyroids with parathyroids</td>
<td>#</td>
<td>Lung (including bronchi)</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Adrenals</td>
<td>✓</td>
<td>Kidneys</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>Spleen</td>
<td>#</td>
<td>Urinary bladder</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Bone with bone marrow (sternum, femur, knee joint)</td>
<td>✓</td>
<td>Testes</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Harderian gland</td>
<td>✓</td>
<td>Epididymides</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Lymph nodes (mandibular and mesenteric)</td>
<td>#</td>
<td>Prostate</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#*</td>
<td>Heart</td>
<td>#</td>
<td>Seminal vesicle and coagulation gland</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Aorta</td>
<td>#</td>
<td>Mammary gland (2nd and 3rd glands)</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Salivary glands (mandibular and sublingual)</td>
<td>✓</td>
<td>Ovaries</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Esophagus,</td>
<td>✓</td>
<td>Uterus (including horns, and cervix)</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Stomach (foregut and glandular)</td>
<td>#</td>
<td>Vagina</td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>#*</td>
<td>Liver</td>
<td>#</td>
<td>Eyes, with optic nerve</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Pancreas</td>
<td></td>
<td>Lacrimal glands</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Duodenum</td>
<td>#</td>
<td>Skeletal muscle (M. triceps surae)</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Jejunum</td>
<td>#</td>
<td>Skin (dorsal region)</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Ileum (Peyer’s patches)</td>
<td>#</td>
<td>All gross lesions (any animals)</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>#</td>
<td>Cecum</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Page 29
Statistics:
The data on detailed clinical observations (numbers of defecations, urination and rears), grip strength, locomotor activity, body weight, food consumption, feed efficiency, urine volume, hematological findings, blood biochemistry findings, organ weights and organ weight to body weight ratio were assessed by Bartlett’s test (significance level: 5%) at first. As the results, if the variance was homogeneous, the data were assessed by one-way layout analysis of variance (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett's multiple comparison test (two-tailed, significance level: 5% and 1%). Subsequently, if the variance was heterogeneous, the data were assessed by Kruskal-Wallis rank test (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett type joint-ranking test (two-tailed, significance level: 5% and 1%).

The data on mortality, general clinical observations, ophthalmological findings, gross pathological findings and histopathological findings were assessed between the control group and the treated groups by Fisher's exact probability test (one-tailed, significance level: 5% and 1%).

The data on urinalysis except for urine volume were assessed between the control group and the treated groups by Dunnett type joint-ranking test (two-tailed, significance level: 5% and 1%).

SAS and EXSUS statistical evaluation software was used (SAS Institute Japan Ltd., Tokyo, Japan and CAC Corp., Tokyo, Japan)

Any Other Information on Materials and Methods Incl. Tables: None
III. RESULTS AND DISCUSSION

Effect Levels:

Endpoint: NOAEL
Effect Level: 300 ppm in females and 1000 ppm in males
Sex: Male/female

Basis for Effect Level/Remarks: bodyweights, gross and histopathological findings (F) and food consumption, hematological findings biochemical findings (both sexes),

Observations:

Clinical Signs and Mortality: No effect
Body Weight and Weight Gain: Yes
Food Consumption: Yes
Food Efficiency: No
Water Consumption: Not measured
Ophthalmoscopic examination: No effect
Hematology: Yes
Clinical Chemistry: Yes
Urinalysis: No effect
Organ Weights: No effect
Gross Pathology: Yes
Histopathology: Yes

Details on Results:

Clinical Signs and Mortality
One male (No. 311) in the 150 ppm group died at test week 30. No females died in any treated groups throughout the treatment period. This death was not characterized as being related to treatment.

Detailed clinical observation:
There were no clinical observations that were considered to be related to treatment.

In the 1000 ppm group, the number of rears of males increased significantly at test weeks 2 and 8. In females, the number of rears increased significantly at test weeks 5, 7, 38 and 39. In the 300 ppm group, the number of rears of females increased significantly at test week 8. In the 75 ppm group, the number of rears of males increased significantly at test week 46. The differences in the number of rears were sporadic and unrelated to the dose levels, and not likely to be related to treatment with the test substance.
**Functional examination:**
In the 1000 ppm group, locomotor activity in females decreased significantly, but this change was unrelated to the dose levels. Locomotor activity in males in the 1000 ppm group increased but was not significantly different from that in the control group, and was considered to be unrelated to the treatment with the test substance. No statistically significant differences in any items examined were noted in males or females of other treated groups.

**Water Consumption:**
Water Consumption was not evaluated in this study.

**Ophthalmoscopy:**
There were no treatment-related abnormalities in ophthalmology.

**Body weight and weight gain:**
There was a slight reduction in female body weight at 1000 ppm; the mean bodyweight was significantly lower at test weeks 20 and 48. No significant changes were noted in males during the treatment period. No statistically significant differences in the body weight were noted in females of other treated groups or in any treated groups of males.

### Table 1: Mean body weight of rats administered BAS 440 I for 1 year (main group)

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>Body weight [g]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>131</td>
<td>131</td>
</tr>
<tr>
<td>Week 8</td>
<td>300</td>
<td>305</td>
</tr>
<tr>
<td>Week 20</td>
<td>377</td>
<td>381</td>
</tr>
<tr>
<td>Week 48</td>
<td>446</td>
<td>451</td>
</tr>
<tr>
<td>Week 52</td>
<td>452</td>
<td>457</td>
</tr>
<tr>
<td>BW (% of control) at week 52</td>
<td>-</td>
<td>101</td>
</tr>
</tbody>
</table>

*p<0.05, ** p<0.01
Figure 1: Body weight in male rats

Figure 2: Body weight in female rats
Food Consumption and Compound Intake:
In the 1000 ppm group there was a consistent decrease in female food consumption throughout the treatment period, the majority of results being statistically significantly lower than the control value. In the remaining groups there were sporadic statistically significant differences, but these were neither consistent or dose related and were not considered to be related to treatment.

Table 2: Mean daily food consumption in rats administered BAS 440 l for 1-year

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>Mean consumption (g/rat/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>14.2</td>
<td>14.8*</td>
</tr>
<tr>
<td>Week 52</td>
<td>14.9</td>
<td>14.9</td>
</tr>
<tr>
<td>- Average weeks 1 - 52</td>
<td>15.4</td>
<td>15.7</td>
</tr>
<tr>
<td>Mean Consumption (% of Control)</td>
<td>102</td>
<td>102</td>
</tr>
</tbody>
</table>

*p<0.05, ** p<0.01

No statistically significant and treatment-related effects on food efficiency were noted.

The overall average of test substance intake is shown in the following table.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>mg/kg bw/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>75</td>
<td>3.7</td>
</tr>
<tr>
<td>150</td>
<td>7.3</td>
</tr>
<tr>
<td>300</td>
<td>14.6</td>
</tr>
<tr>
<td>1000</td>
<td>47.6</td>
</tr>
</tbody>
</table>

Hematological findings:
Significant decreases of red blood cell (RBC), hemoglobin (HGB) and hematocrit (HCT) at test week 53 in males at 1000 ppm were considered to be related to treatment. In females HGB and HCT at test weeks 27 and 53, mean corpuscular volume (MCV) at test weeks 14, 27 and 53 and mean corpuscular hemoglobin (MCH) at test week 53 in females in the 1000 ppm group were noted in the high dose group and also considered to be test substance related.
The following statistically significant changes were considered in the report not to be related to treatment because they were not dose related, occurred only transiently or changes in associated parameters were not observed.

In the 1000 ppm group, platelets (PLT) at test week 27 and prothrombin time (PT) at test week 53 increased significantly in males. In females, PLT at test weeks 14 and 27 increased significantly. No significant changes were noted in any items in males at test week 14.

In the 300 ppm group, PT at test week 53 increased significantly in males. In females, PLT at test week 14 increased significantly and HGB, HCT and differential monocyte counts (Mono) at test week 27 decreased significantly. No significant changes were noted in any items in males at test weeks 14 and 27 and in females at test week 53.

In the 150 ppm group, HGB and HCT at test week 27 decreased significantly in females. No significant changes were noted in any items in males at test weeks 14, 27 and 53 and in females at test weeks 14 and 53. In the 75 ppm group, no significant changes were noted in any items in males and females at test weeks 14, 27 and 53.
<table>
<thead>
<tr>
<th>Dose</th>
<th>Male Control</th>
<th>Male 75 ppm</th>
<th>Male 150 ppm</th>
<th>Male 300 ppm</th>
<th>Male 1000 ppm</th>
<th>Female Control</th>
<th>Female 75 ppm</th>
<th>Female 150 ppm</th>
<th>Female 300 ppm</th>
<th>Female 1000 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[10^6/µL]</td>
<td>[g/dL]</td>
<td>[%]</td>
<td>[fl]</td>
<td>[pg]</td>
<td>[g/dL]</td>
<td>[10^4/µL]</td>
<td>[sec]</td>
<td>[10^4/µL]</td>
<td>[sec]</td>
</tr>
<tr>
<td></td>
<td>RBC</td>
<td>HGB</td>
<td>HCT</td>
<td>MCV</td>
<td>MCH</td>
<td>MCHC</td>
<td>PT</td>
<td></td>
<td>PT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>945</td>
<td>16.5</td>
<td>50.2</td>
<td>53.1</td>
<td>17.1</td>
<td>32.8</td>
<td>57.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>974</td>
<td>16.8</td>
<td>51.5</td>
<td>53.0</td>
<td>17.2</td>
<td>32.5</td>
<td>68.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>944</td>
<td>15.7</td>
<td>49.1</td>
<td>52.0</td>
<td>16.6</td>
<td>31.9</td>
<td>66.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>75 ppm</td>
<td>14</td>
<td>941</td>
<td>16.2</td>
<td>49.7</td>
<td>52.9</td>
<td>17.2</td>
<td>32.6</td>
<td>56.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>1023</td>
<td>17.4</td>
<td>54.2</td>
<td>53.0</td>
<td>17.1</td>
<td>32.2</td>
<td>69.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>953</td>
<td>15.8</td>
<td>49.6</td>
<td>52.0</td>
<td>16.6</td>
<td>31.9</td>
<td>67.5</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>150 ppm</td>
<td>14</td>
<td>946</td>
<td>16.3</td>
<td>50.1</td>
<td>53.0</td>
<td>17.2</td>
<td>32.4</td>
<td>57.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>970</td>
<td>16.7</td>
<td>51.4</td>
<td>52.9</td>
<td>17.2</td>
<td>32.5</td>
<td>72.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>948</td>
<td>15.8</td>
<td>49.4</td>
<td>52.2</td>
<td>16.7</td>
<td>32.0</td>
<td>69.0</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>300 ppm</td>
<td>14</td>
<td>942</td>
<td>16.3</td>
<td>49.8</td>
<td>52.9</td>
<td>17.3</td>
<td>32.7</td>
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</tr>
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</tbody>
</table>

* p < 0.05, ** p < 0.01 (Dunnett's multiple comparison test, Dunnett's joint-ranking test)

**Clinical chemistry findings:**

Significant increases of blood urea nitrogen (UN) in males at test week 53 and in females at test weeks 14 and 27 in the 1000 ppm group and alkaline phosphatase (ALP) in females at test weeks 14 and 53 in the 1000 ppm group and significant decrease of triglyceride (TGL) in males at test weeks 14 and 53 in the 1000 ppm group and total cholesterol (TCHO) in males at test week 53 in the 1000 ppm group were either related to the dose levels or remarkable changes in the high dose level, suggesting to be related to the treatment with the test substance.
Significant decreases of total bilirubin (TB) in males at test week 14 and in females at test week 27 in the 1000 ppm group and alanine aminotransferase (ALT) in males at test weeks 14 and 53 in the 1000 ppm group and in males at test week 14 in the 300 ppm group were decreasing changes, which were considered to be of no toxicological significance. Other significant changes were considered to be unrelated to the treatment with the test substance because those changes were not related to the dose levels or not continuous until test week 53 such as the end of treatment.

Table 4: Selected clinical chemistry findings in rats administered BAS 440 I for 1 year (group means)

<table>
<thead>
<tr>
<th>Dose</th>
<th>After Study Week</th>
<th>ALP (IU/L)</th>
<th>ALT (IU/L)</th>
<th>UN (mg/dL)</th>
<th>TB (mg/dL)</th>
<th>TGL (mg/dL)</th>
<th>TCHO (mg/dL)</th>
<th>CL (mg/dL)</th>
<th>Ca (mg/dL)</th>
<th>E/T</th>
<th>A/G</th>
<th>TP (g/dL)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>14</td>
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<td>0.066</td>
<td>108.4</td>
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<td>64.4</td>
<td>112</td>
<td>10.09</td>
<td>0.79</td>
<td>2.80</td>
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<td>53</td>
<td>396</td>
<td>105.4</td>
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<td>0.056</td>
<td>102.8</td>
<td>90.0</td>
<td>107</td>
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<td>10.46</td>
<td>0.74</td>
<td>3.56</td>
<td>7.01</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01 (Dunnett’s multiple comparison test, Dunnett’s type joint-ranking test)
Urinalysis
There were no treatment related observations in urinalysis for males or females.

Organ weight:
No significant changes were noted in any organ weight and any body weight ratio in males and females in all treated groups.

Gross and histopathology:
Histopathologically, vacuolar change of hepatocytes in the peripheral portion of the liver (slight degree) was observed in 15 of 24 females in the 1000 ppm group and in two of 24 females in the 300 ppm group,. Vacuolar change of myocardium of the heart (slight degree) was observed in six of 24 females in the 1000 ppm group, and the incidence of the lesion was significantly higher than that in the control group.

Protein casts of the kidney (slight degree) was observed in 16 of 24 males in the 1000 ppm group and the incidence of the lesion was significantly higher than that in the control group. The lesion was slight in severity and was not related to changes in urinalysis and blood biochemistry. It was considered to be spontaneous and unrelated to the treatment with the test substance.

In one male in the 150 ppm group that died, fibrosis of myocardium (slight degree) in the heart, fibrosis of capsule (slight degree) and extramedullary hematopoiesis (moderate degree) in the spleen, increased hematopoiesis (moderate degree) in the bone marrow, dilatation of sinus (slight degree) in the renal lymph node, brown pigment deposit in the tubular epithelium (slight degree), protein casts (slight degree) and nephroblastoma in the kidney and diffuse hypertrophy of the cortex (slight degree) in the adrenal gland were observed. These findings were not associated with treatment.

Other lesions observed were spontaneously occurring lesions and not related to the test treatment.

Table 5: Incidence of selected gross pathological findings in rats administered BAS 440 I for 1 year

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 75</td>
<td>150 150</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of animals</td>
<td>24</td>
<td>24 24</td>
</tr>
<tr>
<td>Vacuolar change, myocardium, +</td>
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<td>-- --</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacuolar change, hepatocyte, periphery, +</td>
<td>0 -</td>
<td>- -</td>
</tr>
</tbody>
</table>

+: Slight. -: Not examined.
*: Significantly different (p<0.05) from 0 ppm group (Fisher's exact probability test).
**: Significantly different (p<0.01) from 0 ppm group (Fisher's exact probability test).
Any Other Information on Results Including Tables:
None

IV. OVERALL REMARKS, ATTACHMENTS
No additional remarks or attachments.

V. APPLICANT’S SUMMARY AND CONCLUSION

Conclusion:
In this study, the 1000 ppm dose level, was associated with decreased body weight and gross and histopathological findings of the liver and the heart in females and decreased food consumption, hematological findings, and blood biochemical findings in males and females. At the level of 300 ppm, histopathological findings in females were suspected to be related to treatment with the test substance, whereas no adverse effects were noted in males. No adverse effects were observed in either sex at levels of 150 ppm and 75 ppm. Based on the results of this study, the no-observed-adverse-effect level (NOAEL) was 300 ppm (14.6 mg/kg/day) in male Fischer 344 rats and 150 ppm (8.9 mg/kg/day) in female Fischer 344 rats.
Study 3: Repeated Dose 1-Year Oral Toxicity Study of BAS 440 I (Reg. No. 5599022, ME5343 technical) in Rats Administration via the Diet

Executive Summary
This study was conducted to support a previous chronic study (2011/8000141) and characterize the effects found at a higher dose. Rats were given BAS 440 I at doses in the feed of 0, 1000 or 3000 ppm for 52 weeks.

There were no treatment-related clinical signs or neurobehavioral effects. Toxicity was observed in both sexes at both doses resulting in effects on bodyweight, food consumption, hematology, clinical chemistry, urinalysis, organ weights and gross pathology. Histopathological changes were observed in the liver, heart and pituitary of females.

Administration of BAS 440 I to Fischer rats in the diet at dose levels of 1000 ppm (males 48.2 mg/kg/day, females 57.1 mg/kg/day) and 3000 ppm (males 143.3 mg/kg/day, females 160.9 mg/kg/day) was associated with a number of changes in hematology, clinical chemistry and histopathology. The NOAEL has been identified in other studies.

II. MATERIALS AND METHODS

Limit Test: No
Guidelines: According to: JMAFF No 12 Nousan No 8147; EPA 870.4100; OECD 452
Deviations from Guideline: No
GLP Compliance: Yes

Test Materials
Test Material Equivalent to Submission Substance Identity: Yes
Test Material Identity: BAS 440 I (ME5343 technical)

Test Animals
Species: Rat
Strain: SPF/VAF Fischer rats (F344/DuCrI Crj)
Sex: Male/female

Details on Test Animals and Environmental Conditions:
Test Animals:

Source: Atsugi Breeding Center, Charles River Japan, Inc. (Kanagawa, Japan)
Age at study initiation: 5 – 6 weeks at dosing
Weight at dosing (Day 0): 116-137 g for males and 93-105 g for females
Fasting period before study: Not required.
Housing: Animals (in pairs) were housed in wire-mesh stainless steel cages (width 210 mm x depth 350 mm x height 200 mm) sustained in movable stainless steel racks. Cage rotation as performed every 4 weeks to equalize lighting conditions.
Diet: Powdered basal diet (MF, Oriental Yeast CO., Ltd. Tokyo, Japan), ad libitum
Water: well water (filtered and sterilised with sodium hypochlorite and UV-light), *ad libitum*

Acclimation period: 14 days

Environmental Conditions:
- Temperature (°C): 20 – 25 °C
- Humidity (%): 30 – 70 %
- Air changes (per hr): ≥10 times/hour
- Photoperiod (hrs dark/hrs light): 12 hours/day (light on at 07:00 and off at 19:00)

Experimental Dates: 14-Nov-2011 (males) and 21-Nov-2011 (females) to 26-Apr-2013 (termination of histopathology)

Administration/Exposure
- Route of Administration: Oral: feed
- Vehicle: Unchanged (no vehicle)

Details on Exposure:
Preparation of Dosing Solutions:

**Diet Preparation**
Test diets fed to animals were prepared once prior to initiation of treatment and once every 4 weeks during the treatment period.

At preparation, a required amount of the test substance was mixed with a part of the basal diet in a mortar to provide a premix of each dose level. The premix was then added to the rest of the basal diet, and they were mixed using a Mighty 120 (Aicohsha Manufacturing Co., Ltd., Saitamia, Japan), to obtain good homogeneity.

No adjustment for purity was made when calculating the amount of the test substance needed. The basal diet was used as a control diet.

The formulated test diets were sealed in 4 vinyl bags in a dark and cold (about 2 – 7 °C) storage room until use. The diets were brought to room temperature on the day of feeding and kept at room temperature.

**Analytical Verification of Doses or Concentrations:** Yes

Details on Analytical Verification of Doses or Concentrations:
Chemical analyses for homogeneity and concentration of the test substance in test diets were performed on samples (approximately 10 g each) taken from the top, middle, and bottom portions of the mixer at the first, 8th and 14th (final) preparations. The same amount of sample was also taken from the control diet and analyzed to verify that the diet was not contaminated by the test substance. In addition, samples from the same batch were collected from the animal room at the end of the feeding period to check for stability.
Overall mean concentration of test substance in the test diet immediately after preparation

<table>
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<th>First mix Mean Concentration (ppm)</th>
<th>CV</th>
<th>mean % recovery</th>
<th>Eighth mix Mean Concentration (ppm)</th>
<th>CV</th>
<th>mean % recovery</th>
<th>Fourteenth mix Mean Concentration (ppm)</th>
<th>CV</th>
<th>mean % recovery</th>
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<td>1000</td>
<td>970.6</td>
<td>0.8</td>
<td>97.3</td>
<td>983.6</td>
<td>0.5</td>
<td>98.7</td>
<td>967.1</td>
<td>0.1</td>
<td>97.0</td>
</tr>
<tr>
<td>3000</td>
<td>2932.0</td>
<td>0.8</td>
<td>98.0</td>
<td>2941.1</td>
<td>0.9</td>
<td>98.0</td>
<td>2871.2</td>
<td>2.8</td>
<td>95.7</td>
</tr>
</tbody>
</table>

The homogeneity of the test substance in the diet was confirmed by the coefficient of variation (CV) from the multiple analyses of the samples. The overall range of CV was 0.1 – 2.6%, indicating well homogenized samples.

The measured concentration in the prepared test diets ranged from 95.7 to 98.7% of the nominal concentration.

Duration of Treatment/Exposure: 12 months
Frequency of Treatment: Daily for 12 months
Post Exposure Period: None specified
Doses/Concentration: 0, 1000 (middle dose) and 3000 ppm (high-dose)
Basis: Nominal in diet
No. of Animals per Dose Group:
BAS 440 I was administered to groups of 24 male and 24 female SPF Fischer rats at dietary concentrations of 0, 1000 and 3000 ppm. The animals were assigned to the treatment groups by means of a computer generated randomization list based on body weights. At the end of the administration period the animals were sacrificed after fasting overnight.

Control Animals: Yes, plain diet
Dose selection rationale: A previous rat carcinogenicity study used does levels of 0, 100, 300 and 1000 ppm. A review by the Health Effects Division of the US Environmental Protection Agency considered that a dose level of 3000 ppm was required to achieve the MTD. Hence an additional 1 year study was also required using the MTF dose of 3000 ppm and a dose of 1000 ppm to allow comparison to the original study.

Rationale for selecting satellite groups: For hematology, clinical chemistry and pathology.

Examinations
Observations and examinations performed and frequency:

Cage Side Observations: Yes
Cage-side observations for mortality and moribundity were performed on all animals at least twice a day (except for Saturdays, Sundays, and holidays when animals were observed at least once
a day). General clinical observations were performed on all animals in the morning.

**Detailed Clinical Observations:** Yes
Detailed clinical observations were performed on all animals once prior to initiation of treatment and once weekly during the treatment period basically at the same time in the afternoon. The following parameters were examined:

- **Home cage:** Posture, Respiration, Grooming, Stereotypy behavior, Tremor, Twitch, Convulsion
- **Open field:** Ease of removal from cage, Palpebral closure, Exophthalmos, Fur-appearance, Piloerection, Urination (including numbers), Defecations (including numbers), Diarrhea, Alertness, Rears, Abnormal gait, Vocalization, Pinna response, Conneal response, Touch response, Tail-pinch
- **Handling:** Positional passivity, Salivation, Lacrimation, Discharge, Dermal and mucosal color, Pupil size, Abdominal tone, Righting reflex

**Functional Observation:** Yes
At 49 weeks of treatment, functional observations were performed on 10 animals/sex/group selected in the ascending order of animal number.

Motor activity of each animal was monitored using an automated activity recording system (Locomotor activity meter, Muromachi Kikai Co., Ltd.). Forelimb and hindlimb grip strengths were measured using a strain gauge (forelimb and hindlimb, Grip strength meter, Muromachi Kikai Co., Ltd., Tokyo, Japan). Sensorimotor responses were assessed by scoring reactions to the sound of Galton’s whistle, visual placing response and proprioceptive sense.

The following parameters were examined:

<table>
<thead>
<tr>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motor activity</td>
</tr>
<tr>
<td>Grip strength (forelimb, hindlimb)</td>
</tr>
<tr>
<td>Sensorimotor responses (auditory response, visual placing response and proprioceptive sense)</td>
</tr>
</tbody>
</table>

**Body Weight:** Yes
Body weights were recorded for all animals on receipt and on the day of group allocation. Body weight was measured during
treatment weekly for weeks 1 to 13 and then once every 4 weeks from week 16 onwards.

Group mean body weight was calculated at each measurement for each sex. Final body weights were recorded for all animals before euthanasia or when found dead and used for calculation of organ/body weight ratios except for those of dead animals.

**Food Consumption and Compound Intake:**

Food consumption by each cage in the main group was measured for a period of 3 or 4 consecutive days once weekly in test week one, twice weekly from week 2 to 13 and twice every 4 weeks from week 16 onward. The obtained value was divided by the number of animals alive in the cage and by the number of days for measurement to obtain mean daily food consumption per animal in the cage. Group mean food consumption (g/rat/day) of each sex was calculated from these individual cage values at each measurement. An overall average of the group mean food consumption throughout the treatment period was also calculated for each sex as the weighted mean of these values. Group mean test substance intake (mg/kg/day) was calculated at each measurement for each sex according to the following formula:

\[
\text{Group mean test substance intake} = \frac{\text{Group mean food consumption} \times \text{Nominal concentration}}{\text{Group mean body weight}}
\]

An overall average of the group mean test substance intake throughout the treatment period was also calculated for each sex as the weighted mean of these values.

**Food Efficiency:** Yes

Group mean food efficiency was calculated for each dose group of each sex weekly during the first 13 weeks of treatment as a ratio (%) of group mean body weight gain to group mean food consumption at the week concerned. An overall average of the group mean food efficiency during the 13 weeks of treatment was also calculated for each sex as the mean of these data.

**Water Consumption and Compound Intake (if drinking water study):** No

Water consumption was observed daily by visual inspection of the water bottles for any apparent changes in volume. No water consumption values were recorded.

**Ophthalmoscopic Examination:** Yes

Ophthalmological examinations including the observation with an indirect ophthalmoscope (All Pupil Indirect, Konan-Keeler Ltd., Tokyo, Japan) and a 28 diopter aspheric lens (Nikon Corp., Tokyo, Japan) were performed on all animals during the acclimatization period and all surviving animals in the 0 and 3000
ppm groups at 52 weeks of treatment. The following parts of the eye were examined:

<table>
<thead>
<tr>
<th>Eye part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyeball</td>
</tr>
<tr>
<td>Pupil</td>
</tr>
<tr>
<td>Eyelid</td>
</tr>
<tr>
<td>Iris</td>
</tr>
<tr>
<td>Conjunctiva</td>
</tr>
<tr>
<td>Lens/Vitreous body</td>
</tr>
<tr>
<td>Cornea</td>
</tr>
<tr>
<td>Fundus</td>
</tr>
<tr>
<td>Anterior chamber</td>
</tr>
</tbody>
</table>

**Hematology and Clinical Chemistry:** Yes

**Hematology**
Hematological examinations were performed on 10 animals / sex / group after 14, 27 and 53 weeks of treatment. The same animals as those selected for urinalysis were subjected to the examinations, where possible.

Animals were fasted overnight (approximately 17 hours) before blood sampling from the cervical vein (weeks 14 and 27) or the abdominal vena cava (week 53, terminal sample). Note that for females, a separate sample was also taken in week 30 for analysis of prothrombin time (PT) and activated partial thromboplastin time (APTT) as this was omitted in week 27. EDTA-2K was used as the anticoagulant except for PT and APTT, which required 3.2% sodium citrate solution.

<table>
<thead>
<tr>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (Ht)</td>
</tr>
<tr>
<td>Hemoglobin concentration (Hb)</td>
</tr>
<tr>
<td>Erythrocyte count (RBC)</td>
</tr>
<tr>
<td>Mean corpuscular volume (MCV)</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (MCH)</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (MCHC)</td>
</tr>
<tr>
<td>Platelet count (PLT)</td>
</tr>
<tr>
<td>Total leukocyte count (WBC)</td>
</tr>
<tr>
<td>Differential leukocyte count [Lymphocyte (L), Neutrophil (N), Stab form neutrophil (St), Segmented form neutrophil (Seg), Monocyte (M), Eosinophil (E), Basophil (B), and Unclassified cell (UC)]</td>
</tr>
<tr>
<td>Reticulocyte count (Retics)</td>
</tr>
<tr>
<td>Prothrombin time (PT)</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (APTT)</td>
</tr>
<tr>
<td>Differential myelocyte count</td>
</tr>
</tbody>
</table>

At termination, blood smears for reticulocyte count measurement were prepared by the Brecher method and examined. Myelocyte smears of the bone marrow were also prepared, but these were not examined as there were not notable changes in the hematopoietic system.

**Clinical chemistry:** Yes
Blood biochemical examinations were performed on the animals subjected to the hematological examinations after 14, 27 and 53 weeks of treatment. A part of the blood collected as mentioned above (Hematology) was heparinized and plasma samples obtained were used for the examinations of the following parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase (ALP)</td>
</tr>
<tr>
<td>Glucose (Glue)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST)</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT)</td>
</tr>
<tr>
<td>Creatinine (CRE)</td>
</tr>
<tr>
<td>Blood urea nitrogen (BUN)</td>
</tr>
<tr>
<td>Total protein (TP)</td>
</tr>
<tr>
<td>Albumin (Alb)</td>
</tr>
<tr>
<td>Globulin (Glob)</td>
</tr>
<tr>
<td>Albumin/globulin ratio (A/G ratio)</td>
</tr>
</tbody>
</table>

**Urinalysis:**  Yes

Urinalysis was performed on 10 animals/sex/group after 13, 26 and 51 weeks of treatment.

Animals were individually placed in metabolic cages. Fresh urine samples were used for the analysis of the following parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbidity</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Color</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Specific gravity</td>
</tr>
<tr>
<td>Urobilinogen</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Bilirubin</td>
</tr>
<tr>
<td>Ketones</td>
</tr>
<tr>
<td>Sediments (leukocytes, erythrocytes, casts, and epithelium)</td>
</tr>
<tr>
<td>Occult blood</td>
</tr>
<tr>
<td>Volume (over 24 hours)</td>
</tr>
</tbody>
</table>

**Sacrifice and pathology:**

Necropsy was performed on all animals including those found dead or killed during the treatment period. Animals were euthanized by exsanguination from the abdominal aorta and posterior vena cava under deep isoflurane anesthesia and necropsied.

All surviving animals were killed after 52 weeks of treatment after an overnight fast. Animals found dead during the treatment period were necropsied immediately after discovery to minimize autolysis and to avoid cannibalism. At necropsy, the whole body of each animal was examined carefully and all gross findings were recorded.
The following organs were collected, weighed and examined histopathologically. Tissues were preserved in all animals and fixed in 10% neutral-buffered formalin; the lung was filled with 10% neutral-buffered formalin through the trachea before fixation. The eyes and testes were fixed in Bouins solution.

Paraffin-embedded and hematoxylin/eosin-stained sections were prepared by a routine method and examined microscopically.

<table>
<thead>
<tr>
<th>Pathology:</th>
<th>The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: control and top dose females).</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>W</td>
</tr>
<tr>
<td>✓ ✓ Brain¹</td>
<td>✓ ✓ Liver</td>
</tr>
<tr>
<td>✓ # Spinal cord²</td>
<td>✓ Pancreas</td>
</tr>
<tr>
<td>✓ # Sciatic nerve (right)</td>
<td>✓ # Duodenum</td>
</tr>
<tr>
<td>✓ Pituitary</td>
<td>✓ # Jejunum</td>
</tr>
<tr>
<td>✓ # Thymus</td>
<td>✓ # Ileum</td>
</tr>
<tr>
<td>✓ ✓ Thyrdois with parathyroids</td>
<td>✓ # Cecum</td>
</tr>
<tr>
<td>✓ Adrenals</td>
<td>✓ # Colon</td>
</tr>
<tr>
<td>✓ # Spleen</td>
<td>✓ # Rectum</td>
</tr>
<tr>
<td>✓ # Bone with bone marrow⁴</td>
<td>✓ Head⁷</td>
</tr>
<tr>
<td>✓ # Knee joint (unilateral)</td>
<td>✓ # Tongue</td>
</tr>
<tr>
<td>✓ # Lymph nodes⁵</td>
<td>✓ # Pharynx</td>
</tr>
<tr>
<td>✓ # Heart</td>
<td>✓ # Larynx</td>
</tr>
<tr>
<td>✓ # Aorta</td>
<td>✓ # Trachea</td>
</tr>
<tr>
<td>✓ # Salivary glands⁶</td>
<td>✓ # Lung, including bronchi</td>
</tr>
<tr>
<td>✓ # Esophagus</td>
<td>✓ # Kidneys</td>
</tr>
<tr>
<td>✓ # Stomach⁸</td>
<td>✓ # Urinary bladder</td>
</tr>
</tbody>
</table>

¹ cerebrum, cerebellum, pons, and medulla
² cervical, thoracic, and lumbar regions
³ sternum; femur
⁴ mandibular and mesenteric
⁵ submandibular and sublingual
⁶ forestomach and glandular stomach
⁷ including nasal cavity, paranasal sinuses, oral mucosa, and middle ears
⁸ including foregut, glandular stomach
Statistics: The data on detailed clinical observations (numbers of defecations, urination and rears), grip strength, locomotor activity, body weight, food consumption, feed efficiency, urine volume, hematological findings, blood biochemistry findings, organ weights and organ weight to body weight ratio were assessed by Bartlett's test (significance level: 5%) at first. As the results, if the variance was homogeneous, the data were assessed by one-way layout analysis of variance (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett's multiple comparison test (two-tailed, significance level: 5% and 1%). Subsequently, if the variance was heterogeneous, the data were assessed by Kruskal-Wallis rank test (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett type joint-ranking test (two-tailed, significance level: 5% and 1%).

The data on mortality, general clinical observations, ophthalmological findings, gross pathological findings and histopathological findings were assessed between the control group and the treated groups by Fisher's exact probability test (one-tailed, significance level: 5% and 1%).

The data on urinalysis except for urine volume were assessed between the control group and the treated groups by Dunnett type joint-ranking test (two-tailed, significance level: 5% and 1%).

Software for statistical evaluation used were SAS (SAS Institute Japan Ltd., Tokyo, Japan) and EXSUS (CAC EXICARE Corp., Tokyo, Japan).

Any Other Information on Material and Methods Including Tables: None
III. RESULTS AND DISCUSSION

Effect Levels: 1000 ppm for both male and female
Endpoint: NOAEL not identified
Effect Type: Chronic NOAEL < 1000 ppm
Sex: Male/female

Basis For Effect Level/Remarks: Body weight, clinical chemistry, and non-neoplastic changes

Observations

- **Clinical Signs and Mortality:** Yes, mortality only
- **Body Weight and Weight Gain:** Yes
- **Food Consumption and Compound Intake:** Yes
- **Food Efficiency:** No effect
- **Water Consumption and Compound Intake:** No effect
- **Ophthalmoscopic examination:** No effect
- **Hematology:** Yes
- **Clinical Chemistry:** Yes
- **Urinalysis:** Yes
- **Neurobehaviour:** No effect
- **Organ Weights:** Yes
- **Gross Pathology:** Yes
- **Histopathology: Non-neoplastic:** Yes
- **Histopathology: Neoplastic:** No

Details on Results

**Clinical Signs and Mortality:**

**Mortality:**

One male (No. 211) in the 1000 ppm group died at test week 27.
One female (No. 257) in the 1000 ppm group was moribund at test week 41.
One female (No. 373) in the 3000 ppm group died at test week 51.

Mortality in the control, 1000 ppm and 3000 ppm groups at the end of the treatment period were 0.0%, 4.2% and 0.0%, respectively, in males and 0.0%, 4.2% and 4.5%, respectively, in females.

**Clinical signs of toxicity:** There were no treatment related adverse effects.

**Detailed clinical observations and functional examination:**

There were no treatment-related clinical observations.

Statistically significant changes in the number of rears in 1000 and 3000 ppm females, compared to controls were sporadic and considered to be incidental.

**Ophthalmoscopy:** There were no treatment related adverse effects at the high dose.
**Body Weight and Body Weight Gain:** There was a slight decrease in male body weight at 3000 ppm (95 – 98 % of control value); this was statistically significant on several occasions.

Slight increases in bodyweight in all treated females and males given 1000 ppm were observed occasionally and considered not to be an adverse finding.

**Figure 2: Body weight development of rats administered BAS 440 I for 1 year**

![Graph showing body weight development](image)
Table 1: Mean body weight of rats administered BAS 440 I for 1 year

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>Body weight [g]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Day 0</td>
<td>127</td>
<td>127</td>
</tr>
<tr>
<td>- Week 2</td>
<td>161</td>
<td>163</td>
</tr>
<tr>
<td>- Week 13</td>
<td>334</td>
<td>340</td>
</tr>
<tr>
<td>BW (% of control at wk 13)</td>
<td>102</td>
<td>99</td>
</tr>
<tr>
<td>- Week 52</td>
<td>437</td>
<td>448</td>
</tr>
<tr>
<td>BW (% of control at wk 52)</td>
<td>103</td>
<td>98</td>
</tr>
</tbody>
</table>

*p<0.05, ** p<0.01

Food & Water Consumption and Compound Intake:
Overall there was a slight, and often statistically significant, reduction in food consumption in both sexes given 3000 ppm, compared to controls. The overall food consumption of females given 1000 ppm was also slight reduced.
Table 2: Mean daily food consumption in rats administered BAS 440 I for 1-year

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>Mean consumption (g/rat/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Week 1</td>
<td>13.9</td>
<td>13.8</td>
</tr>
<tr>
<td>- Average weeks 1 - 52</td>
<td>15.1</td>
<td>15.1</td>
</tr>
<tr>
<td>Mean Consumption (% of Control)</td>
<td>100</td>
<td>96</td>
</tr>
</tbody>
</table>

*p<0.05, ** p<0.01

No statistically significant and treatment-related effects on food efficiency were noted.

The overall average of test substance intake is shown in the following table.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>mg/kg bw/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>1000</td>
<td>48.2</td>
</tr>
<tr>
<td>3000</td>
<td>143.3</td>
</tr>
</tbody>
</table>

**Hematological findings:**

In hematology there was a small, but statistically significant decrease in a number of red blood cell parameters in males given 3000 ppm (number of red blood cells hemoglobin concentration, and hematocrit); these changes were also seen at in 1000 ppm males, but only at week 27. There were some similar changes in females. All changes were very small in magnitude and their toxicological significance is unclear. The number of platelets was increased in both sexes given 3000 ppm and there was a notable increase in the percent (%) of reticulocytes at 27 weeks in 3000 ppm males.
Clinical chemistry findings: In blood biochemistry, significant increases of alkaline phosphatase (ALP) were apparent in males and females at 3000 ppm throughout the treatment period; similar increases were seen in males at 1000 ppm from week 27 onwards. In females given 3000 ppm this was accompanied by a consistent rise in blood urea nitrogen which was only apparent in 1000 ppm females at week 27. In both males and females there were slight changes in electrolytes, which was also statistically significant on some occasions. These changes were small but electrolyte levels are usually maintained within strict boundaries; potassium tended to increase whilst calcium and inorganic phosphorus were lowered, especially in the latter half of treatment. In addition, triglyceride levels were notably reduced in males given 3000 ppm throughout treatment and, although not statistically significant, a similar trend was seen at 1000 ppm in the first half of the treatment period. 3000 ppm females also had decreased triglycerides in the latter half of treatment and reduced glucose levels.
Table 4: Selected clinical chemistry findings in rats administered BAS 440 I for 1 year (group means)

<table>
<thead>
<tr>
<th>Dose</th>
<th>After Study Week</th>
<th>ALP (IU/L)</th>
<th>UN (mg/dL)</th>
<th>K (mEq/L)</th>
<th>GLU (mg/dL)</th>
<th>TGL (mg/dL)</th>
<th>IP (mg/dL)</th>
<th>Ca (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MALE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14</td>
<td>462</td>
<td>15.7</td>
<td>3.99</td>
<td>121</td>
<td>92.2</td>
<td>6.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>401</td>
<td>16.7</td>
<td>4.26</td>
<td>130</td>
<td>111</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>365</td>
<td>17.9</td>
<td>3.48</td>
<td>182</td>
<td>57.4</td>
<td>4.22</td>
</tr>
<tr>
<td></td>
<td>1000 ppm</td>
<td>14</td>
<td>449</td>
<td>16.3</td>
<td>4.07</td>
<td>124</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>128</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>187</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>3000 ppm</td>
<td>14</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>110</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>FEMALE</td>
<td></td>
<td>14</td>
<td>338</td>
<td>17.2</td>
<td>4.12</td>
<td>93</td>
<td>7.8</td>
<td>6.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>258</td>
<td>15.5</td>
<td>3.78</td>
<td>103</td>
<td>27.4</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>195</td>
<td>18.0</td>
<td>3.22</td>
<td>142</td>
<td>21.7</td>
<td>3.89</td>
</tr>
<tr>
<td></td>
<td>1000 ppm</td>
<td>14</td>
<td>370</td>
<td>19.0</td>
<td>4.24</td>
<td>96</td>
<td>8.4</td>
<td>6.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>296</td>
<td>17.0*</td>
<td>4.12*</td>
<td>107</td>
<td>19.9</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>202</td>
<td>19.7</td>
<td>3.43</td>
<td>147</td>
<td>24.5</td>
<td>3.59</td>
</tr>
<tr>
<td></td>
<td>3000 ppm</td>
<td>14</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>83</td>
<td>8.9</td>
<td>7.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>110</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01

Statistically significant changes observed in other parameters were not considered to be treatment-related because they were not correlated with the dose levels or they had no toxicological significance.

**Urinalysis**

A significant decrease in urine volume at test week 51 and significant increases of protein at test weeks 13 and 26 were noted in females given 3000 ppm. These changes were suggested to be related to the treatment with test substance.

Significant increases of urinary ketone levels in females at test weeks 13, 26 and 51 in the 3000 ppm group was slight in degree and was not considered to be an obvious toxicological effect. Significant increases of bilirubin in males at test week 51 and in females at test week 26 in the 3000 ppm group were considered to be of no toxicological significance because increase of bilirubin was not noted in the blood biochemistry examination. The relation of this change and treatment with the test substance was unknown.

Other changes (specific gravity, pH, urobilinogen, and urinary sediments of WBC and cast) increased or decreased significantly in the treated groups compared to control group were slight in degree or not related to the dose levels. Therefore, these changes were considered to be unrelated to the treatment with the test substance.
Organ weight:
In organ weight measurement, significant increases of absolute and relative weights of liver, spleen and kidneys in both sexes, pituitary in females in the 3000 ppm and 1000 ppm groups and adrenal glands and thyroid in males in the 3000 ppm group were observed. Significant decreases of absolute and relative weights of heart in females in the 3000 ppm and 1000 ppm groups and ovaries and uterus in females in the 3000 ppm group were observed.

Statistical differences were also noted in the brain, testis and epididymides but these were not accompanied by any pathological changes.

Table 5: Selected relative organ weight findings in rats administered BAS 440 I for 1 year (group means); percentage of bodyweight

<table>
<thead>
<tr>
<th>Dose</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidneys</th>
<th>Adrenals</th>
<th>Pituitary</th>
<th>Thyroid</th>
<th>Ovaries</th>
<th>Uterus</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MALE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.03</td>
<td>0.161</td>
<td>0.581</td>
<td>0.0095</td>
<td>0.0023</td>
<td>0.0066</td>
<td>-</td>
<td>-</td>
<td>0.271</td>
</tr>
<tr>
<td>1000 ppm</td>
<td><strong>2.16</strong></td>
<td><strong>0.168</strong></td>
<td><strong>0.610</strong></td>
<td>0.0102</td>
<td>0.0023</td>
<td>0.0070</td>
<td>-</td>
<td>-</td>
<td>0.275</td>
</tr>
<tr>
<td>3000 ppm</td>
<td><strong>2.39</strong></td>
<td><strong>0.186</strong></td>
<td><strong>0.633</strong></td>
<td><strong>0.0108</strong></td>
<td><strong>0.0025</strong></td>
<td><strong>0.0077</strong></td>
<td>-</td>
<td>-</td>
<td>0.276</td>
</tr>
<tr>
<td><strong>FEMALE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.14</td>
<td>0.197</td>
<td>0.678</td>
<td>0.0219</td>
<td>0.0054</td>
<td>0.0083</td>
<td>0.0253</td>
<td>0.434</td>
<td>0.335</td>
</tr>
<tr>
<td>1000 ppm</td>
<td><strong>2.39</strong></td>
<td><strong>0.215</strong></td>
<td><strong>0.713</strong></td>
<td><strong>0.0232</strong></td>
<td><strong>0.0061</strong></td>
<td>0.0083</td>
<td>0.0245</td>
<td>0.386</td>
<td><strong>0.322</strong></td>
</tr>
<tr>
<td>3000 ppm</td>
<td><strong>2.57</strong></td>
<td><strong>0.231</strong></td>
<td><strong>0.721</strong></td>
<td><strong>0.0216</strong></td>
<td><strong>0.0066</strong></td>
<td>0.0113</td>
<td><strong>0.0233</strong></td>
<td><strong>0.241</strong></td>
<td><strong>0.313</strong></td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01

Gross and histopathology:
In the 3000 ppm group, discoloration of the liver was observed in 20 of 21 females, and the incidence of the lesion was significantly higher than that in the control group. Swelling of the liver was observed in 2 of 21 females.

Other findings in the treated groups were not significantly different from those of the control group.

Neoplastic Lesions
There were no differences in the incidences of neoplasms between the treated groups and the control group in either sex.
Non-neoplastic Lesions
The heart was a target organ in females and there was vacuolar change in the myocardium at 1000 and 3000 ppm; at 3000 ppm this was accompanied by a decrease in the incidence of cellular infiltration.

There were a number of changes in the liver of treated females including: vacuolar change of hepatocyte in the peripheral portion of the liver, hyperplasia of bile duct and foci of altered cell of the liver. There were also decreased Zymogen granules of acinar cell of the pancreas.

Focal hyperplasia of anterior lobe of the pituitary was significantly higher in females given 3000 ppm.

None of the above lesions were apparent in the 3000 ppm males at an increased incidence.

Vacuolar change of the hepatocyte in the peripheral portion of the liver and vacuolar change of myocardium of the heart in females have been observed in other studies with the test material.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Dose (ppm)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1000</td>
<td>3000</td>
</tr>
<tr>
<td>Heart</td>
<td>0</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacular change, myocardium, +</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Vacular change, myocardium, ++</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Vacular change, myocardium, ++</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Vacular change, myocardium, ++</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Cellular infiltration, +</td>
<td>15</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>Cellular infiltration, ++</td>
<td>0</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cellular infiltration</td>
<td>15</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>3000</td>
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<td>23</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacular change, hepatocyte, periphery, +</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vacular change, hepatocyte, periphery, ++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vacular change, hepatocyte, periphery, ++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vacular change, hepatocyte, periphery, ++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hyperplasia, bile duct, +</td>
<td>23</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>Hyperplasia, bile duct, ++</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Hyperplasia, bile duct</td>
<td>24</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Foci, altered cell, basophilic, tigroid, +</td>
<td>16</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Microgranuloma, +</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Microgranuloma, ++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Microgranuloma</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Pancreas</td>
<td>24</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>Decreased Zymogen granules, acinar cell, +</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>0</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>Hyperplasia, focal, anterior lobe, +</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Adenoma, anterior lobe</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

+: Slight. ++: Moderate. -: Not examined.
*: Significantly different (p<0.05) from 0 ppm group (Fisher's exact probability test).
**: Significantly different (p<0.01) from 0 ppm group (Fisher's exact probability test).
Any Other Information on Results Including Tables:
None

IV. OVERALL REMARKS, ATTACHMENTS
No additional remarks or attachments.

V. APPLICANT’S SUMMARY AND CONCLUSION

Conclusion:
Administration of BAS 440 I to Fischer rats in the diet at dose levels of 1000 ppm (males 48.2 mg/kg/day, females 57.1 mg/kg/day) and 3000 ppm (males 143.3 mg/kg/day, females 160.9 mg/kg/day) was associated with a number of changes in hematology, clinical chemistry and histopathology. The NOAEL has been identified in other studies.
Study 4: Carcinogenicity study of ME5343 technical in rats

Executive Summary
Afidopyropen was given in the feed to F344 rats (50/sex/dose) at doses of 0, 100, 300 or 1000 ppm (equal to 0, 4.4, 12.9 and 42.7 mg/kg bw/d for males and 0, 5.3, 15.5 and 50.8 mg/kg bw/d for females) for 104 weeks.

No treatment-related abnormalities were noted in mortality, general clinical observation, hematology, organ weight and body weight ratio or gross pathology in any treated groups.

Body weights and food consumption in the 1000 ppm group (females) were significantly decreased for multiple weeks over the course of the study.

Adenocarcinoma of the uterus in females killed by design increased significantly in the 1000ppm group. However, the incidences in the treatment groups were unrelated to the dose levels and no significant differences of the incidences of the tumor were noted in all females in any treatment groups. Due to the described increased incidence of spontaneous adenocarcinoma in the uterus in the F344 rat, the occurrence of adenocarcinoma in the uterus in females was considered to be unrelated to the treatment with the test substance.

A NOAEL for the study was not reported, but a NOAEL of 300 ppm (12.9 for males and 15.5 mg/kg bw/d for females) is consistent with the reported data.
II. MATERIALS AND METHODS

Limit Test: No
Guidelines: According to: JMAFF No 12 Nosan No 8147; EPA 870.4200; OECD 451
Deviations from Guideline: No
GLP Compliance: Yes

Test Materials
Test Material Equivalent to Submission Substance Identity: Yes
Test Material Identity: Afidopyropen (BAS 440 I; ME5343)

Test Animals
Species: Rat
Strain: F344/DuCrlCrlj rats (SPF/VAF)
Sex: Male/female

Details on Test Animals and Environmental Conditions:
Test Animals:
Source: Atsugi Breeding Center, Charles River Japan, Inc. (Kanagawa, Japan)
Age at study initiation: 5 – 6 weeks at dosing
Weight at dosing (Day 0): 101-119 g for males and 87-99 g for females
Fasting period before study: Not specified in the report
Housing: Two animals of the same sex were housed in a stainless steel wire-meshed cage (width 21cm x depth 35 cm x height 20 cm) during the quarantine and acclimatization period before grouping, and two animals of the same sex in each group were housed in the same cage during the treatment period after grouping. Cages and racks were exchanged once per 3 weeks. Cage rotation was performed once per 4 weeks in order to equalize lighting condition in each animal.
Diet: A powdered basal diet for rats, Oriental Yeast Co., Ltd., Tokyo, Japan ad libitum
Water: Tap water sterilized with sodium hypochlorite, ad libitum
Acclimation period: 12 Days
Environmental Conditions:
Temperature (°C): 20-25°C
Humidity (%): 30-70%
Air changes (per hr): ≥10 times/hour
Photoperiod (hrs dark/hrs light): 12 hours/day (light on at 07:00 and off at 19:00).

In-life Dates: 26-May-2009 - 06-Feb-2012 (termination of histopathology)

Administration/Exposure
Route of Administration: Oral: feed
Vehicle: Unchanged (no vehicle)

Details on Exposure:
Preparation of Dosing Solutions:

Diet Preparation

At first, 4 g, 12 g and 40 g of test substance for the 100ppm, 300ppm and 1000ppm groups were measured accurately and were mixed with 1.996 kg, 1.988 kg and 1.960 kg of basal diets, respectively. And then the test substance was smashed and mixed gradually with small amount of the basal diet, and subsequently a total 2 kg of the preliminary mixed diet was shaken and mixed in a vinyl bag for approximately 5 minutes. Two lots of the preliminary mixed diet (2 kg) were prepared for each dose group.

Each test substance mixed diet was prepared one week and 2 weeks before the initiation of treatment in males and in females, respectively, and then at a 3 or 4-week-interval.

The preliminary mixed diets in each group were mixed with 38 kg of basal diets, and finally two lots of 40 kg of mixed diets were prepared for each dose group, using a mixer Mighty 120 (Aicohsha Manufacturing Co., Ltd., Saitama, Japan).

The test substance mixed diet in each group prepared was subdivided to 8 lots, approximately 10 kg in each, and tightly packed in a vinyl bag with a label indicating dose groups, preparation date and treatment weeks, put in a container and stored in the refrigerated stock room (No. 1 or No. 2) for diets and beddings under light-protected and refrigerated condition (actual temperature: 2-8°C) until feeding.

For feeding, the test substance mixed diets, kept in the airtight containers, were brought from the refrigerated stock room (No. 1 or 2) for diets and beddings and then were kept in the animal room under light-protected and room temperature condition
The test substance mixed diets were prepared one week before the treatment and 27 times during the test period.

**Analytical Verification of Doses or Concentrations:** Yes

**Details on Analytical Verification of Doses or Concentrations:**

Three out of 5 samples (approximately 10 g each) collected from the test substance mixed diet in each treated group at the 1st, 7th, 14th, 21st and 27th (Final) preparations were analyzed for concentration and homogeneity of test substance. One sample from the basal diet in the control group was analyzed in the same manner. A part (approximately 10 g) of the residual mixed diets (the 1st, 7th, 14th, 21st and 27th prepared diets) in each group was collected after feeding and analyzed for stability of the test substance. The allowable range of the concentration of test substance in the test substance mixed diets was defined to be within ±15% of selected dose levels. On the day of each measurement of test substance concentrations, the medium concentration (ST3: 0.5 mg/L) of the calibration curve was measured (n = 1) following the measurement of the test substance concentration for the quality control of measurement system. The allowable range of the measured values was defined to be within ±5% of the theoretical value (0.5 mg/L).

<table>
<thead>
<tr>
<th>Nominal Concentration (ppm)</th>
<th>1(^{st}) Mixture</th>
<th>7(^{th}) Mixture</th>
<th>14(^{th}) Mixture</th>
<th>21(^{st}) Mixture</th>
<th>27(^{th}) Mixture</th>
<th>Overall Mean Concentration (ppm)</th>
<th>Percentage of Nominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>90.0</td>
<td>96.5</td>
<td>99.2</td>
<td>95.8</td>
<td>96.6</td>
<td>96.4</td>
<td>96%</td>
</tr>
<tr>
<td>300</td>
<td>270.7</td>
<td>287.2</td>
<td>293.8</td>
<td>288.7</td>
<td>292.3</td>
<td>288.8</td>
<td>96%</td>
</tr>
<tr>
<td>1000</td>
<td>920.9</td>
<td>978.0</td>
<td>980.1</td>
<td>971.3</td>
<td>973.7</td>
<td>970.7</td>
<td>97%</td>
</tr>
</tbody>
</table>

Homogeneity of test substance in the test substance mixed diets at the 1st, 7th, 14th, 21st and 27th preparations was also analyzed by using 3 out of 5 samples in each dose level. In results, the coefficient of variation in all samples from each dose level was within 2.9% at the 1st preparation, within 2.0% at the 7th preparation, within 1.5% at the 14th preparation, within 0.7% at the 21st preparation and within 0.5% at the 27th preparation. Consequently, homogeneity of test substance in the test substance mixed diets in all dose groups was adequate.
Stability of the test substance in the test substance mixed diets was analyzed by using a part (approximately 10 g) of the residual mixed diets (the 1st, 7th, 14th, 21st and 27th prepared diets) in each dose level. Those actual levels ranged from 85% to 90% of the selected dose levels at the 1st preparation, from 101% to 103% at the 7th preparation, from 94% to 97% at the 14th preparation, from 91% to 96% at the 21st preparation and 96% at the 27th preparation being within the allowable range (± 15%). The test substance was stable in the diet during the treatment period and the storage method of the test substance mixed diets was acceptable.

Duration of Treatment/Exposure: 2 years  
Frequency of Treatment: Daily for 2 years  
Post Exposure Period: None specified  
Doses/Concentration: 100 (low dose), 300 (mid dose) and 1000 ppm (top-dose)  
Basis: Nominal in diet  
No. of Animals per Dose Group: 50 animals  
Control Animals: Yes, plain diet  
Dose selection rationale: Previously, the 90-day feeding study was performed in the testing facility. In the study, the test substance was mixed in the diet at 0ppm (control), 150ppm, 300ppm, 1000ppm and 3000ppm with F344 rats. In the results, at the dose level of 3000ppm, a temporal decrease of food consumption, a trend of anemia, increase of blood urea nitrogen, alkaline phosphatase and albumin/globulin ratio, a decreases of triglyceride, and increases of absolute and relative weights of the liver and spleen were noted in males and females, and a temporal decrease of body weight gain, a decrease of urine volume, increases of urine urobinogen, platelets, glucose, aspartate aminotransferase, alanine aminotransferase and potassium, and decreases of total protein and calcium were noted in males or females.
In the 1000ppm group, a temporal decrease of food consumption, a trend of anemia, increases of urine urobilinogen, platelets, blood urea nitrogen, aspartate aminotransferase, alanine aminotransferase and potassium, and increases of absolute and relative weights of the liver were noted in males or females.

No adverse effects were noted in any of the animals in the 300ppm and lower dose.

A dose level of 1000ppm was selected as the high dose level considering the longer period of the present study, and then 300ppm was selected as the middle dose level, in accordance with a common ratio of 3. In addition, 100ppm was selected as the low dose level, expecting to be a no-observed-adverse-effect level of the test substance.

**Rationale for selecting satellite groups:** Not applicable.

**Examinations**

**Observations and examinations performed and frequency:**

**Cage Side Observations:** Yes

All animals were observed clinically once a day (in the morning) during the treatment period. Any abnormal signs including nature, date of onset, recover and death were recorded.

When animals showed marked debility and an unfavorable prognosis was predicted, they were euthanized by exsanguination under deep ether anesthesia and necropsied. Dead animals were removed from the cage immediately after discovery and necropsied to minimize autolysis and to avoid cannibalism. Mortality of each dose group was calculated weekly for up to 104 weeks as a ratio of cumulative number of animals found dead or killed *in extremis* to the group size.

General clinical observations were performed on all animals in the main and satellite groups at least once a day basically in the morning. In addition, careful examinations including palpation of masses were conducted at least once a week. The time of onset, nature, severity, and duration of the signs were recorded when any abnormalities were detected.

**Detailed Clinical Observations:** Yes

Detailed clinical observations were performed on all animals in the main group once prior to initiation of treatment and once weekly during the treatment period basically at the same time in the afternoon. The following parameters were examined:
Home Cage

- Excitement, Sedation, Abnormal posture (prone position, lateral position, etc.), Abnormal behavior (moving backward, stereotypy, self-biting, etc.)

### Handling

<table>
<thead>
<tr>
<th>Positional passively (including response changes to stimulation)</th>
<th>Exophthalmos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changes of muscle tone (spasticity, flaccidity)</td>
<td>Changes of body temperature (hyperthermia, hypothermia)</td>
</tr>
<tr>
<td>Tremor</td>
<td>Abnormal breathing noise</td>
</tr>
<tr>
<td>Palpebral closure</td>
<td>Fur-appearance</td>
</tr>
<tr>
<td>Salivation</td>
<td>Dermal and mucosal color</td>
</tr>
<tr>
<td>Lacrimation,</td>
<td></td>
</tr>
<tr>
<td>Discharge (auditory, nasal, vaginal, etc.)</td>
<td></td>
</tr>
</tbody>
</table>

### Open Field

<table>
<thead>
<tr>
<th>Jumping</th>
<th>Respiration (rapid, slow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circling</td>
<td>Vocalization</td>
</tr>
<tr>
<td>Convulsions</td>
<td>Piloerection</td>
</tr>
<tr>
<td>Abnormal gait (including ataxia, e.g., staggering gait, dragging gait, paralysis of hind limbs)</td>
<td>Urination (counts)</td>
</tr>
<tr>
<td>Spontaneous motor activity (increase, decrease)</td>
<td>Defecation (counts)</td>
</tr>
<tr>
<td>Grooming (frequency)</td>
<td>Abnormal posture (e.g., flattened, lying on side)</td>
</tr>
<tr>
<td>Rearing (frequency), Abnormal behavior (e.g., backward moving, stereotypies, self-destructive biting)</td>
<td></td>
</tr>
</tbody>
</table>

### Functional Observation

No. A concurrent chronic rat study conducted an FOB

### Body Weight

Yes

Body weights of all animals were measured on the day of animal receipt and on the grouping day. After grouping, body weights of the animals used were measured in the morning once a week from test week 1 to 13 and once per 4 weeks from test week 16 to the termination of treatment. Final body weights of the animals were measured before euthanasia on each necropsy day and on the day at death. Individual values of the final body weights were described in the report.
Food Consumption and Compound Intake:
Food consumption (3-day or 4-day total amount) in each cage was measured once at test week 1 and twice a week during test week 2 to 13 and twice per 4 weeks from test week 16 to the termination of treatment. The total amount of food consumption was converted to a daily amount of one animal in each group. The weekly mean food consumption (g/rat/day) of males and females in each group was calculated based on the mean food consumption of each cage. The total mean food consumption of males and females in each group during the treatment period was calculated by averaging the weekly mean food consumption.

The mean test substance intake (mg/kg/day) of males and females in each dose-group was calculated in each measuring week according to the following formula:

\[
\text{Group mean test substance intake} = \frac{\text{Group mean food consumption} \times \text{Nominal concentration}}{\text{Group mean body weight}}
\]

In addition, total mean test substance intake during the treatment period in males and females in each group was calculated by averaging the weekly mean test substance intake.

Food Efficiency: Not reported

Water Consumption and Compound Intake (if drinking water study):
Water consumption was observed daily by visual inspection of the water bottles for any apparent changes in volume. No water consumption values were recorded.

Ophthalmoscopic Examination: Not reported.

Hematology and Clinical Chemistry: Yes

Hematology

Hematological examinations were performed on all animals killed as designed at each necropsy day (after the termination of 104-week treatment). The animals were anesthetized by diethyl ether inhalation and euthanized by blood collection from the abdominal artery. Simultaneously, blood smear slides of each animal were prepared and stained with May-Grünwald Giemsa. EDTA-2K was used as anticoagulant. The number of white blood cells in all animals killed as designed was measured by using an automated hematology analyzer (SF-3000/SVFU-1, Sysmex Co., Hyogo, Japan). Differential leukocyte count in all animals killed as designed in the control and high dose groups was measured microscopically. However, differential leukocyte count in other dose groups was not examined because hematopoietic tumors related to the treatment with the test substance were not observed in the high dose groups.
At the termination of test week 52 and 78 (test week 53 and 79, respectively), small amounts of blood samples in all surviving animals were collected by cutting the tip of tail under anesthesia with diethyl ether inhalation, and blood smear slides were prepared and stained with May-Grünwald Giemsa. However, differential leukocyte count was not measured because hematopoietic tumors were not noted in relation with the test substance treatment in any examinations after the termination of treatment.

Blood smear slides stained with May-Grünwald Giemsa were prepared from the blood of any animals in moribund state by cutting the tip of tail under anesthesia with diethyl ether inhalation during the treatment period. Differential leukocyte count was measured microscopically using the slides in moribund animals which were suspected to have hematopoietic tumors at necropsy. Individual values of differential leukocyte counts in those animals were only described in the final report and were not calculated.

**Clinical chemistry:** No

A concurrent chronic rat study measured clinical chemistry endpoints

**Urinalysis:** No

A concurrent chronic rat study conducted urinalysis.

**Sacrifice and pathology:**

Necropsy was conducted on all dead animals, all moribund animals and all remaining animals killed as designed. The moribund animals and animals killed as designed were anesthetized by diethyl ether inhalation. The moribund animals were euthanized by bleeding from the neck artery, and the animals killed as designed with blood sampling were euthanized by blood collection from the abdominal artery.

Body weight of all animals was measured before necropsy. Weights (absolute organ weights) of the organs shown in Table 4 were measured before fixation in 10 males and 10 females killed as designed in order of identification numbers in each group. Relative organ weights were calculated as ratios of organ weights to final body weight.

Organs and tissues as listed in Table 4 were removed from all animals at necropsy and fixed in 10% neutral-buffered formalin except for the eyes and testes, which were fixed in Bouin solution.
The lungs were infused with applied volume of 10% neutral-buffered formalin from the trachea and then fixed in the fixative.

Table 4: Pathology

<table>
<thead>
<tr>
<th>C</th>
<th>W</th>
<th>H</th>
<th>C</th>
<th>W</th>
<th>H</th>
<th>C</th>
<th>W</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓ ✓</td>
<td>#</td>
<td>□</td>
<td>✓ ✓</td>
<td>□</td>
<td>□</td>
<td>✓ ✓</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

All gross lesions

1. cerebrum, cerebellum, pons, and medulla
2. cervical, thoracic, and lumbar regions
3. sternum; femur and knee joint
4. mandibular and mesenteric
5. submandibular and sublingual
6. forestomach and glandular stomach
7. including nasal tissue

*Any animal found dead or killed in extremis had the listed organs examined, no matter the dose group
Statistics:

The data on body weight, food consumption, number of white blood cells, organ weights and organ weight to body weight ratio were assessed by Bartlett’s test (significance level: 5%) at first. As the results, if the variance was homogeneous, the data were assessed by one-way layout analysis of variance (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett’s multiple comparison test (two-tailed, significance level: 5% and 1%). Subsequently, if the variance was heterogeneous, the data were assessed by Kruskal-Wallis rank test (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett type joint-ranking test (two-tailed, significance level: 5% and 1%).

The data on mortality, general clinical observations, gross pathological findings (except for animals killed by moribund and died during the test) and histopathological findings (except for animals killed by moribund and died during the test) were assessed between the control group and the treated groups by Fisher’s exact probability test (one-tailed, significance level: 5% and 1%). However, the statistical evaluation of the histopathological findings was carried out between the control group and the high dose (1000ppm) group in the total number of all animals and animals killed by design. Those of the adrenal gland in male and the uterus in female were carried out between the control group and the treated groups.

The data on differential leukocyte count were assessed by F test (two-tailed, significance level: 5%) between the control group and the high dose group. As the results, if the variance was homogeneous, the data were assessed by Student’s t-test (two-tailed, significance level: 5% and 1%). If the result was significant, the data were assessed by Wilcoxon test (two-tailed, significance level: 5% and 1%).

Mortality was assessed by the life table test. In the life table test, survival curves in each group were estimated by Kaplan-Meier method and assessed by Log-Rank test (significance level: 5% and 1%) between the control group and each treated group. Software used for statistical evaluation was SAS (SAS Institute Japan Ltd., Tokyo, Japan) and EXSUS (CAC Corp., Tokyo, Japan).

Any Other Information on Materials and Methods Including Tables: None
III. RESULTS AND DISCUSSION

Effect Levels: A NOAEL for the study was not reported, but a NOAEL of 300 ppm (12.9 for males and 15.5 mg/kg bw/d for females) is consistent with the reported data.

Endpoint: NOAEL
Effect Type: Chronic
Sex: Male/female

Basis For Effect Level/Remarks: Body weight, food consumption, hematology, organ weight chemistry, non-neoplastic changes

Observations
Clinical Signs and Mortality: No
Body Weight and Weight Gain: Yes
Food Consumption and Compound Intake: Yes
Food Efficiency: No
Water Consumption and Compound Intake: No
Ophthalmoscopy examination: Not applicable
Hematology: Not applicable
Clinical Chemistry: Not applicable
Urinalysis: Not applicable
Neurobehaviour: Not applicable
Organ Weights: Yes
Gross Pathology: No
Histopathology: Non-neoplastic: Yes
Histopathology: Neoplastic: No

Details on Results
Clinical Signs and Mortality: There were no treatment related adverse effects.
Clinical signs of toxicity: There was no treatment related mortality.

Mortality: There was no treatment related mortality.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.0%</td>
<td>26.0%</td>
</tr>
<tr>
<td>100</td>
<td>20.0%</td>
<td>24.0%</td>
</tr>
<tr>
<td>300</td>
<td>28.0%</td>
<td>16.0%</td>
</tr>
<tr>
<td>1000</td>
<td>20.0%</td>
<td>20.0%</td>
</tr>
</tbody>
</table>

Ophthalmoscopy: Not Measured

Body Weight and Body Weight Gain: Slight alterations in body weight were noted in males (slight increase) and females (slight decrease) over several weeks of treatment.
Figure 3: Body weight development of rats administered Afidopyropen for 2 years

Males

Females
Table 5: Mean body weight and body weight gain of rats administered Afidopyropen for 2 years

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><strong>Body weight [g]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>109</td>
<td>109</td>
</tr>
<tr>
<td>Week 13</td>
<td>333</td>
<td>337</td>
</tr>
<tr>
<td>Week 52</td>
<td>449</td>
<td>454</td>
</tr>
<tr>
<td>Week 104</td>
<td>461</td>
<td>461</td>
</tr>
<tr>
<td>Overall body weight (% of Control)</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td><strong>Overall body weight gain [g]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1 to Week 104 bw gain</td>
<td>352</td>
<td>352</td>
</tr>
<tr>
<td>0 – 104 Gain (% of control)</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

Food & Water Consumption and Compound Intake:

There were several transient and sporadic changes in food consumption. These changes were not related to the dose level and were not accompanied by body weight effects and were therefore considered to be incidental.

Table 6: Mean daily food consumption in rats administered Afidopyropen for 2-years

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><strong>Mean consumption (g/rat/day)</strong></td>
<td>14.7</td>
<td>15.1</td>
</tr>
<tr>
<td><strong>Mean Consumption (% of Control)</strong></td>
<td>103%</td>
<td>101%</td>
</tr>
</tbody>
</table>

No differences of water consumption were noted in treated groups.

The overall average of test substance intake is shown in the following table.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>mg/kg bw/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>100</td>
<td>4.4</td>
</tr>
<tr>
<td>300</td>
<td>12.9</td>
</tr>
<tr>
<td>1000</td>
<td>42.7</td>
</tr>
</tbody>
</table>
Hematological findings:

In the 1000ppm group, differential monocyte counts (Mono) in males decreased significantly. However, the decrease was considered to be of little toxicological significance because the difference from the control group was small and there was no difference in white blood cell counts. No significant changes were noted in any other items of both sexes.

Clinical chemistry findings: Not measured

Urinalysis: Not analyzed

Organ weight:

In the 1000ppm group, absolute and relative weights of the kidneys and relative weight of the liver increased significantly in males. However, a significant change which suggests an increase in organ weight was not observed in the histopathological examination. Therefore, the liver weight changes were considered to be of no toxicological significance in this study. No significant changes were found in any organs in females.

In the 300ppm and 100ppm group, no significant changes were noted in any organ weight or any body weight ratio of either sex.

Table 7: Selected organ weight findings in rats administered Afidopyropen for 2 years (group means)

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Parameter</th>
<th>Kidney (total)</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weight (g)</td>
<td>% of Cntl</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2.76</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>2.69</td>
<td>97</td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>2.82</td>
<td>102</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>3.03*</td>
<td>110</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>1.82</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.88</td>
<td>103</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>1.87</td>
<td>103</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>1.89</td>
<td>104</td>
</tr>
</tbody>
</table>

*, P<0.05; **, P<0.01
Gross and histopathology:

In the males and females there was no treatment related increase in gross lesions.

In females, there was a statistically significant increase in cyst of the pituitary. Because the incidences were unrelated to the dose levels the lesions were considered to be not related to treatment.

Table 8: Incidence of selected gross findings in all animals.

<table>
<thead>
<tr>
<th>Organ / Lesion</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 ppm</td>
</tr>
<tr>
<td>Pituitary Cyst</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/50</td>
</tr>
<tr>
<td></td>
<td>(2%)</td>
</tr>
<tr>
<td>Pituitary Nodule</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27/50</td>
</tr>
<tr>
<td></td>
<td>(54%)</td>
</tr>
</tbody>
</table>

*, P<0.05, **, P<0.01 Fisher’s exact probability test

Neoplastic Lesions (all animals)

In males, no significant differences of the incidences in any neoplastic lesions, with the exception of pheochromocytoma in the adrenal gland, were detected in the 1000ppm group. The incidences of pheochromocytoma in the adrenal gland were 8.0% (4/50), 4.0% (2/50), 8.0% (4/50) and 20.0% (10/50) in the 0ppm (control), 100ppm, 300ppm and 1000ppm groups, respectively.

In females, no significant differences of the incidences in any neoplastic lesions, with the exception of adenocarcinoma in the uterus, were detected in the 1000ppm group. The incidences of adenocarcinoma in the uterus were 8.0% (4/50), 2.0% (1/50), 4.0% (2/50) and 20.0% (10/50) in the 0ppm (control), 100ppm, 300ppm and 1000ppm groups, respectively.

No noticeable differences of the incidences in any neoplastic lesions were detected in the 300ppm and 100ppm groups of both sexes.

Incidences where neoplastic lesions were increased, or where lesion incidence is of particular interest are shown in Table 9.
Table 9: Select incidence of microscopic neoplastic lesions in male and female rats administered Afidopyropen for 2-years. All animals examined

<table>
<thead>
<tr>
<th>Site and Lesion</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals examined</td>
<td>Dose (ppm)</td>
</tr>
<tr>
<td></td>
<td>50  50  50  50  50  50</td>
<td>0  100  300  1000  0  100  300  1000</td>
</tr>
<tr>
<td>Pancreas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>50</td>
<td>18&lt;sup&gt;a&lt;/sup&gt;  19&lt;sup&gt;a&lt;/sup&gt;  50</td>
</tr>
<tr>
<td>Acinar cell adenoma [Be]</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Islet cell adenoma [Be]</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Islet cell carcinoma [Ma]</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Pituitary</td>
<td>N</td>
<td>50</td>
</tr>
<tr>
<td>Adenoma, anterior lobe [Be]</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Carcinoma, anterior lobe [Ma]</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Thyroid</td>
<td>N</td>
<td>50</td>
</tr>
<tr>
<td>C-cell adenoma [Be]</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Follicular cell adenoma</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C-cell carcinoma [Ma]</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Adrenal</td>
<td>N</td>
<td>50</td>
</tr>
<tr>
<td>Cortical adenoma [Be]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pheochromocytoma [Be]</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Malignant pheochromocytoma [Ma]</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Testis</td>
<td>N</td>
<td>50</td>
</tr>
<tr>
<td>Interstitial cell tumor [Be]</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>Ovary</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Adenoma [Be]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uterus</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Adenoma [Be]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leiomyoma</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Endometrial stromal sarcoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma [Ma]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Malignant schwannoma [Ma]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mammary</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Adenoma [Be]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma [Ma]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibroadenoma [Be]</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>N</sup> Number of animals examined at the designated site
[Be]: Benign neoplasm; [Ma]: Malignant neoplasm.
<sup>a</sup>: Examined on the animals that showed macroscopic lesions. Not subjected to statistical analysis
*<sup>;</sup> <i>p</i> 0.05; **<sup>;</sup> <i>p</i> 0.01 Fisher's exact probability test
Non-neoplastic Lesions (all animals)
In males in the 1000ppm group, the incidences of atrophy of seminiferous tubule (+++) and that of atrophy of seminiferous tubule (++) in the testis was significantly lower and higher than that in the control group, respectively, whereas the total incidence of the lesion was not significantly different from that in the control group. In the same group, the incidence of hyperplasia of interstitial cells in the testis was significantly lower than that in the control group. Those significant changes were unrelated to the dose levels or decreasing of the incidences, being considered to be not related to the treatment with the test substance.

In females in the 1000ppm group, the incidences of calcification of blood vessels (+) in the lung with bronchus, osseous metaplasia of sclera (+) in the eye and cellular infiltration (+) in the Harderian gland were significantly lower than those in the control group. Those significant changes were unrelated to the dose levels or decreasing of the incidences, being considered to be not related to the treatment with the test substance. The incidence of hyperplasia of bile duct (a total of all degrees) in the liver (70.0%, 35/50) was significantly higher than that in the control group (50.0%, 25/50).

No noticeable differences of the incidences in any non-neoplastic lesions were detected in the 300ppm and 100ppm groups of both sexes.

Incidences where neoplastic lesions were increased, or where lesion incidence is of particular interest are shown in Table 10.
Table 10: Incidence of selected histopathological findings in rat administered Afidopyropen for 2-years: All animals examined

<table>
<thead>
<tr>
<th>Organ / Lesion</th>
<th>Dose Group (ppm)</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>300</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>N</td>
<td>50</td>
<td>28</td>
<td>31</td>
<td>50</td>
</tr>
<tr>
<td>Hyperplasia, bile duct +</td>
<td>45</td>
<td>28</td>
<td>31</td>
<td>46</td>
<td>25</td>
</tr>
<tr>
<td>Hyperplasia, bile duct ++</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Hyperplasia, bile duct +</td>
<td>50</td>
<td>30</td>
<td>31</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Pancreas</td>
<td>N</td>
<td>50</td>
<td>18</td>
<td>19</td>
<td>50</td>
</tr>
<tr>
<td>Atrophy, acinar cell +</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Hyperplasia, acinar cell +</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Hyperplasia, islet cell +</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pituitary</td>
<td>N</td>
<td>50</td>
<td>26</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Hypertrophy, focal, anterior lobe +</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Hyperplasia, focal, anterior lobe ++</td>
<td>8</td>
<td>6</td>
<td>7</td>
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<tr>
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<tr>
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<td>9</td>
<td>6</td>
<td>8</td>
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<td>Thyroid</td>
<td>N</td>
<td>50</td>
<td>23</td>
<td>28</td>
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<tr>
<td>Hyperplasia, focal, C cell +</td>
<td>14</td>
<td>5</td>
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<td>9</td>
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<tr>
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<td>16</td>
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<td>Hyperplasia, diffuse, C cell +</td>
<td>7</td>
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<td>Adrenal</td>
<td>N</td>
<td>50</td>
<td>50</td>
<td>50</td>
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<tr>
<td>Fatty change, focal, cortex +</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>7</td>
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</tr>
<tr>
<td>Fatty change, diffuse, cortex +</td>
<td>6</td>
<td>4</td>
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</tr>
<tr>
<td>Hypertrophy, focal, cortex +</td>
<td>5</td>
<td>2</td>
<td>3</td>
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<td>2</td>
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<td>12</td>
<td>16</td>
<td>13</td>
<td>15</td>
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<tr>
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<td>13</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Atrophy, seminiferous tubule +</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>-</td>
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<tr>
<td>Atrophy, seminiferous tubule ++</td>
<td>9</td>
<td>9</td>
<td>11</td>
<td>19*</td>
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<td>36</td>
<td>37</td>
<td>33</td>
<td>25*</td>
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<td>48</td>
<td>47</td>
<td>46</td>
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</tr>
<tr>
<td>Arteritis +</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Hyperplasia, interstitial cell +</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>4*</td>
<td>-</td>
</tr>
<tr>
<td>Ovary</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Atrophy +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Cyst +</td>
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<td>Uterus</td>
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<tr>
<td>Hyperplasia, endometrium+</td>
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<td>Hyperplasia, endometrium+++</td>
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<td>Hyperplasia, endometrium+++</td>
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<tr>
<td>Hyperplasia, endometrium</td>
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<tr>
<td>Organ / Lesion</td>
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<td>100</td>
<td>300</td>
<td>1000</td>
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<tr>
<td>Hyperplasia, squamous epithelium+</td>
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<tr>
<td>Hyperplasia, stromal cell+</td>
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<tr>
<td>Hyperplasia, stromal cell+++</td>
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<tr>
<td>Hyperplasia, stromal cell</td>
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</tr>
<tr>
<td>Cyst, endometrium +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Metaplasia, squamous epithelium+</td>
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<td>1</td>
</tr>
<tr>
<td>Cellular infiltration +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Pyometra +++</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Fisher's exact probability test: **P<0.01; * P<0.05.
N Number of animals examined at the designated site.

Any Other Information on Results Including Tables:
None

IV. OVERALL REMARKS, ATTACHMENTS
An additional rat cancer study was conducted at dose levels of 0, 1000 and 3000 ppm (2014/1215781).

V. APPLICANT’S SUMMARY AND CONCLUSION

Conclusion:

No treatment related effects were observed in relation to mortality, general clinical observation, hematology, organ weight and body weight ratio or gross pathology in any treated groups.

Slight body weight suppression was noted in females at 1000 ppm and this effect was considered to be related to treatment. No treatment related effects were noted in the males.

In histopathology, the incidence of hyperplasia of bile duct (+) in the liver in females increased significantly in the 1000ppm group. Bile duct hyperplasia is a common spontaneous lesions in aging F344 rats. The degrees of hyperplasia of bile duct in this study were also slight. Further, neoplastic lesions of bile duct, oval cell hyperplasia in the liver and hepatic injury did not increase in 1000ppm group. Consequently, increased incidence of the hyperplasia of bile duct in the liver was considered to be unrelated to the treatment with the test substance.

The incidence of pheochromocytoma in the adrenal gland in males killed by design increased significantly in the 1000ppm group (Incidence: 22.5%), whereas no significant differences were observed in the incidences of pheochromocytoma in all males (Incidence: 20.0%). In addition, no significant differences were noted in the incidences of hyperplasia of medulla (pheochromocytes) in males and females and pheochromocytoma in females in any treatment groups. Moreover, the incidence of pheochromocytoma in male in the present study was within the ranges of incidence in historical background data of F344 rats (from 14% to 63%). Consequently, occurrence of pheochromocytoma in the adrenal gland in males was considered to be unrelated to the treatment with the test substance.
The incidence of adenocarcinoma in the uterus in females killed by design increased significantly in the 1000 ppm group, whereas the incidences in the treatment groups were unrelated to the dose levels and no significant differences were observed in the incidences of the tumor in all females. In addition, no significant differences were noted in the incidences of endometrial hyperplasia and adenoma of the uterus in any treatment groups. Otherwise, there were no other tumors with significantly high incidences including endocrine and reproductive organs. It has been described in published literature that the incidence of spontaneous adenocarcinoma in the uterus in the F344 rat has been increasing recently. Consequently, the study report considered the occurrence of adenocarcinoma in the uterus in females at 1000 ppm to be unrelated to the treatment with the test substance.

The study report did not state a NOAEL for the study. However, a NOAEL of 300 ppm (12.9 for males and 15.5 mg/kg bw/d for females) is consistent with the reported data.
Study 5 Carcinogenicity study of BAS 440I (ME5343 technical) in Rats Administration via the Diet

Executive Summary

Prior to this study, the Dose Adequacy Review Team (DART) of the Health Effects Division of the US Environmental Protection Agency reviewed the dose levels of an ongoing 2-year rat cancer study (2014/8000287) and concluded that an additional dose of 3000 ppm was required to meet the maximum tolerated dose (MTD) requirements. The agency also requested that the 1000 ppm dose be repeated to bridge the results to the ongoing rat cancer study that was being conducted at dose levels of 100, 300 and 1000 ppm (Reference Memorandum dated November 18, 2010; PC Code 036605; Decision No. 440705, TXR No. 0055537, DP Barcode D382921).

Afidopyropen was given in the feed to F344 rats (50/sex/dose) at doses of 0, 1000 or 3000 ppm (equal to 0, 41.6 and 128.2 mg/kg bw/d for males and 0, 50.4 and 146.9 mg/kg bw/d for females) for 104 weeks.

There was a significant increase in the mortality of the 3000 ppm female group. No treatment-related abnormalities were noted in mortality with the males. There were no treatment related clinical observations.

Food consumption was decreased in all of the groups treated with Afidopyropen. Body weights for males at 3000 ppm were statistically significantly reduced, but other treatment groups were not significantly different than control.

In the 3000 ppm group, treatment related organ weight changes included an increased absolute and relative weights of the liver, spleen and kidneys in males and females. In males, absolute and relative weights of the epididymides and relative weight of the brain increased significantly. In females, relative weights of the heart increased significantly and absolute weights of the heart and ovaries decreased significantly. At 1000 ppm, absolute and relative weights of the epididymides increased significantly in males. In females, relative weight of the liver increased significantly and absolute weight of the heart decreased significantly.

Gross histopathology of note included a decrease in the incidence of testis atrophy at 3000 ppm in the males as well as a decrease in thyroid nodules in the females. Pituitary cysts were increased in the females at 3000 ppm.

Non neoplastic histopathologic findings included liver effects (increased bile duct hyperplasia and microgranuloma in all treatment groups and increased altered foci (male at 3000 ppm). In the kidney an increase in chronic progressive nephrosis (female, 3000 ppm) was noted. The testis showed decreased atrophy as well as decreased hyperplasia at 3000 ppm. In the uterus there was an increase in endometrium hyperplasia at 3000 ppm. Also at 3000 ppm, there was a decrease in the dilation of the mammary gland duct.

Neoplastic lesions present included an increased incidence of uterine adenocarcinoma at both 1000 and 3000 ppm in the females. There was a decreased incidence of testes interstitial tumors
in the males at 3000 ppm as well as a decrease in thyroid C-cell carcinoma in both the males and females at 3000 ppm.

NOAELs for all findings in this study were established in a prior rat cancer study conducted at lower doses (2014/8000287).

II. MATERIALS AND METHODS

Limit Test: No
Guidelines: According to: JMAFF No 12 Nosan No 8147; EPA 870.4200; OECD 451
Deviations from Guideline: No
GLP Compliance: Yes

Test Materials
Test Material Equivalent to Submission Substance Identity: Yes
Test Material Identity: Afidopyropen (BAS 440 I; ME5343)

Test Animals
Species: Rat
Strain: F344/DuCrI Crj rats (SPF/VAF)
Sex: Male/female

Details on Test Animals and Environmental Conditions:
Test Animals:
Source: Atsugi Breeding Center, Charles River Japan, Inc. (Kanagawa, Japan)

Age at study initiation: 5 – 6 weeks at dosing
Weight at dosing (Day 0): 107-115 g for males and 89-97 g for females
Fasting period before study: Not specified in the report
Housing: Two or one animals of the same sex were housed in a stainless steel wire-meshed cage (width 21 cm x depth 35 cm x height 20 cm) during the quarantine and acclimatization period before grouping, and two animals of the same sex in each group were housed in the same cage during the treatment period after grouping. Cages and racks were exchanged once every 3 weeks.
Diet: A powdered basal diet for rats, Oriental Yeast Co., Ltd., Tokyo, Japan ad libitum
Water: Tap water sterilized with sodium hypochlorite, ad libitum
Acclimation period: 12 Days

Environmental Conditions:
Temperature (°C): 20-25°C
Humidity (%): 30-70%
Air changes (per hr): ≥10 times/hour
Photoperiod (hrs dark/hrs light): 12 hours/day (light on at 07:00 and off at 19:00).
In-life Dates: 19-July-2011 - 26-Dec-2013 (termination of histopathology)

Administration/Exposure
Route of Administration: Oral: feed
Vehicle: Unchanged (no vehicle)

Details on Exposure:
Preparation of Dosing Solutions:

Diet Preparation

To prepare the treated diets, 40 g and 120 g of test substance for the 1000ppm and 3000ppm groups were mixed with 1.960 kg and 1.880 kg of basal diets, respectively. Then the test substance was smashed and mixed gradually with small amount of the basal diet, and subsequently a total 2 kg of the preliminary mixed diet was shaken and mixed in a vinyl bag for approximately 5 minutes. Two lots of the preliminary mixed diet (2 kg) were prepared for each dose group.

Each test substance mixed diet was prepared one week and 2 weeks before the initiation of treatment in males and in females, respectively, and then at a 3 or 4-week-interval.

The preliminary mixed diets in each group were mixed with 38 kg of basal diets, and finally two lots of 40 kg of mixed diets were prepared for each dose group, using a mixer Mighty 120 (Aicohsha Manufacturing Co., Ltd., Saitama, Japan).

The test substance mixed diet in each group prepared was subdivided to 8 lots, approximately 10 kg in each, and tightly packed in a vinyl bag with a label indicating dose groups, preparation date and treatment weeks, put in a container and stored in the refrigerated stock room (No. 1 or No. 2) for diets and beddings under light-protected and refrigerated condition (actual temperature: 2-8°C) until feeding.

For feeding, the test substance mixed diets, kept in the airtight containers, were brought from the refrigerated stock room (No. 1 or 2) for diets and beddings and then were kept in the animal room under light-protected and room temperature condition

The test substance mixed diets were prepared one week before the treatment and 27 times during the test period.

Analytical Verification of Doses or Concentrations: Yes

Details on Analytical Verification of Doses or Concentrations:
Three out of 5 samples (approximately 10 g each) collected from the test substance mixed diet in each treated group at the 1st, 7th, 14th, 21st and 27th (Final) preparations were analyzed for concentration and homogeneity of test substance. One sample from the basal diet in the control group was analyzed in the same manner. A part (approximately 10 g) of the residual mixed diets (the 1st, 7th, 14th, 21st and 27th prepared diets) in each group was collected after feeding and analyzed for stability of the test substance. The allowable range of the concentration of test substance in the test substance mixed diets was defined to be within ±15% of selected dose levels. On the day of each measurement of test substance concentrations, the medium concentration (ST3: 0.5 mg/L) of the calibration curve was measured (n = 1) following the measurement of the test substance concentration for the quality control of measurement system. The allowable range of the measured values was defined to be within ±5% of the theoretical value (0.5 mg/L).

| Overall mean concentration of test substance in the test diet immediately post prep |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| Nominal Concentration (ppm) | 1st Mixture | 7th Mixture | 14th Mixture | 21st Mixture | 27th Mixture | Overall Mean Concentration (ppm) | Percentage of Nominal |
| 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | - |
| 1000 | 980.3 | 961.1 | 964.6 | 968.2 | 991.1 | 973.1 | 97% |
| 3000 | 2926.7 | 2965.1 | 2890.9 | 2934.7 | 2996.4 | 2942.8 | 98% |

Homogeneity of test substance in the test substance mixed diets at the 1st, 7th, 14th, 21st and 27th preparations was also analyzed by using 3 out of 5 samples in each dose level. In results, the coefficient of variation in all samples from each dose level was within 2.0% at the 1st preparation, within 0.8% at the 7th preparation, within 0.5% at the 14th preparation, within 1.4% at the 21st preparation and within 1.1% at the 27th preparation. Consequently, homogeneity of test substance in the test substance mixed diets in all dose groups was adequate.

<table>
<thead>
<tr>
<th>Overall mean concentration of test substance; Stability Samples</th>
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</thead>
<tbody>
<tr>
<td>Nominal Concentration (ppm)</td>
</tr>
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</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>3000</td>
</tr>
</tbody>
</table>

Stability of the test substance in the test substance mixed diets was analyzed by using a part (approximately 10 g) of the residual mixed diets (the 1st, 7th, 14th, 21st and 27th prepared diets) in each dose level. Those actual levels ranged from 97% to 95% of the selected dose levels at the 1st preparation, from 89% to 93% at the 7th preparation, from 96% to 97% at the 14th preparation, from 96% to 97% at the 21st preparation and 97 to 98% at the 27th preparation being within the allowable range (±15%). The test substance was
stable in the diet during the treatment period and the storage method of the test substance mixed diets was acceptable.

**Duration of Treatment/Exposure:** 2 years  
**Frequency of Treatment:** Daily for 2 years  
**Post Exposure Period:** None specified  
**Doses/Concentration:** 1000 (low dose), 3000 ppm (high dose)  
**Basis:** Nominal in diet  
**No. of Animals per Dose Group:** 50 animals  

**Control Animals:** Yes, plain diet  
**Dose selection rationale:**

The 2-year feeding study entitled “Carcinogenicity study of ME5343 technical (BASF synonym BAS 440 l (Reg. No. 5599022, ME5343 technical)) in Rats (NISSEIKEN Testing No. C-32)”2) of the test substance has been performed in the testing facility. In the study, the test substance was mixed in the diet at 0ppm (control), 100ppm, 300ppm and 1000ppm and fed to 50 males and 50 females Fischer strain (F344/DuCrlCrlj) of rats in each dose group. The Dose Adequacy Review Team (DART) of the Health Effects Division of the US Environmental Protection Agency reviewed the dose levels in an ongoing 2-year rat cancer study and concluded that an additional dose of 3000ppm was required to meet the maximum tolerated dose (MTD) requirements. The agency also requested that the 1000 ppm dose be repeated to bridge the results to the ongoing rat cancer study that was being conducted at dose levels of 100, 300 and 1000 ppm (Reference Memorandum dated November 18, 2010; PC Code 036605; Decision No. 440705, TXR No. 0055537, DP Barcode D382921).

Rationale for selecting satellite groups: Not applicable. A concurrent rat chronic study was conducted.

**Examinations**  
**Observations and examinations performed and frequency:**

**Cage Side Observations:** Yes

All animals were observed clinically once a day (in the morning) during the treatment period. Any abnormal signs including nature, date of onset, recover and death were recorded.

When animals showed marked debility and an unfavorable prognosis was predicted, they were euthanized by exsanguination under deep ether anesthesia and necropsied. Dead animals were removed from the cage immediately after discovery and necropsied to minimize autolysis and to avoid cannibalism. Mortality of each dose group was calculated weekly for up to 104 weeks as a ratio of cumulative number of animals found dead or killed in extremis to the group size.
General clinical observations were performed on all animals in the main and satellite groups at least once a day basically in the morning. In addition, careful examinations including palpation of masses were conducted at least once a week. The time of onset, nature, severity, and duration of the signs were recorded when any abnormalities were detected.

**Detailed Clinical Observations:** Yes

Detailed clinical observations were performed on all animals in the main group once prior to initiation of treatment and once weekly during the treatment period basically at the same time in the afternoon. The following parameters were examined:

<table>
<thead>
<tr>
<th>Home Cage</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Excitement, Sedation, Abnormal posture (prone position, lateral position, etc.), Abnormal behavior (moving backward, stereotypy, self-biting, etc.)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Handling</th>
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</thead>
<tbody>
<tr>
<td>Positional passively (including response changes to stimulation)</td>
<td>Exophthalmos</td>
</tr>
<tr>
<td>Changes of muscle tone (spasticity, flaccidity)</td>
<td>Changes of body temperature (hyperthermia, hypothermia)</td>
</tr>
<tr>
<td>Tremor</td>
<td>Abnormal breathing noise</td>
</tr>
<tr>
<td>Palpebral closure</td>
<td>Fur-appearance</td>
</tr>
<tr>
<td>Salivation</td>
<td>Dermal and mucosal color</td>
</tr>
<tr>
<td>Lacrimation, Discharge (auditory, nasal, vaginal, etc.)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Open Field</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Jumping</td>
<td>Respiration (rapid, slow)</td>
</tr>
<tr>
<td>Circling</td>
<td>Vocalization</td>
</tr>
<tr>
<td>Convulsions</td>
<td>Paloection</td>
</tr>
<tr>
<td>Abnormal gait (including ataxia, e.g., staggering gait, dragging gait, paralysis of hind limbs)</td>
<td>Urination (counts)</td>
</tr>
<tr>
<td>Spontaneous motor activity (increase, decrease)</td>
<td>Defecation (counts)</td>
</tr>
<tr>
<td>Grooming (frequency)</td>
<td>Abnormal posture (e.g., flattened, lying on side)</td>
</tr>
<tr>
<td>Rearing (frequency),</td>
<td>Abnormal behavior (e.g., backward moving, stereotypies, self-destructive biting)</td>
</tr>
</tbody>
</table>
**Functional Observation:** No. A concurrent chronic rat study conducted an FOB

**Body Weight:** Yes

Body weights of all animals were measured on the day of animal receipt and on the grouping day. After grouping, body weights of the animals used were measured in the morning once a week from test week 1 to 13 and once per 4 weeks from test week 16 to the termination of treatment. Final body weights of the animals were measured before euthanasia on each necropsy day and on the day at death. Individual values of the final body weights were described in the report.

**Food Consumption and Compound Intake:**
Food consumption (3-day or 4-day total amount) in each cage was measured once at test week 1 and twice a week during test week 2 to 13 and twice per 4 weeks from test week 16 to the termination of treatment. The total amount of food consumption was converted to a daily amount of one animal in each group. The weekly mean food consumption (g/rat/day) of males and females in each group was calculated based on the mean food consumption of each cage. The total mean food consumption of males and females in each group during the treatment period was calculated by averaging the weekly mean food consumption.

The mean test substance intake (mg/kg/day) of males and females in each dose-group was calculated in each measuring week according to the following formula:

\[
\text{Group mean test substance intake} = \frac{\text{Group mean food consumption} \times \text{Nominal concentration}}{\text{Group mean body weight}}
\]

In addition, total mean test substance intake during the treatment period in males and females in each group was calculated by averaging the weekly mean test substance intake.

**Food Efficiency:** Not reported

**Water Consumption and Compound Intake (if drinking water study):**
Water consumption was observed daily by visual inspection of the water bottles for any apparent changes in volume. No water consumption values were recorded.

**Ophthalmoscopic Examination:** Not reported.

**Hematology and Clinical Chemistry:** Yes
Hematology

Hematological examinations were performed on all animals killed as designed at each necropsy day (after the termination of 104-week treatment). The animals were anesthetized by diethyl ether inhalation and euthanized by blood collection from the abdominal artery. Simultaneously, blood smear slides of each animal were prepared and stained with May-Grünwald Giemsa. EDTA-2K was used as anticoagulant. The number of white blood cells in all animals killed as designed was measured by using an automated hematology analyzer (SF-3000/SFVU-1, Sysmex Co., Hyogo, Japan). Differential leukocyte count in all animals killed as designed in the control and high dose groups was measured microscopically. However, differential leukocyte count in other dose groups was not examined because hematopoietic tumors related to the treatment with the test substance were not observed in the high dose groups.

At the termination of test week 52 and 78 (test week 53 and 79, respectively), small amounts of blood samples in all surviving animals were collected by cutting the tip of tail under anesthesia with diethyl ether inhalation, and blood smear slides were prepared and stained with May-Grünwald Giemsa. However, differential leukocyte count was not measured because hematopoietic tumors were not noted in relation with the test substance treatment in the any examinations after the termination of treatment.

Blood smear slides stained with May-Grünwald Giemsa were prepared from the blood of any animals in moribund state by cutting the tip of tail under anesthesia with diethyl ether inhalation during the treatment period. Differential leukocyte count was measured microscopically using the slides in moribund animals which were suspected to have hematopoietic tumors at necropsy. Individual values of differential leukocyte counts in those animals were only described in the final report and were not calculated.

Clinical chemistry:  No

A concurrent chronic rat study measured clinical chemistry endpoints

Urinalysis:  No

A concurrent chronic rat study conducted urinalysis.
Sacrifice and pathology:

Necropsy was conducted on all dead animals, all moribund animals and all remaining animals killed as designed. The moribund animals and animals killed as designed were anesthetized by diethyl ether inhalation. The moribund animals were euthanized by bleeding from the neck artery, and the animals killed as designed with blood sampling were euthanized by blood collection from the abdominal artery.

Body weight of all animals was measured before necropsy. Weights (absolute organ weights) of the organs shown in Table 4 were measured before fixation in 10 males and 10 females killed as designed in order of identification numbers in each group. Relative organ weights were calculated as ratios of organ weights to final body weight.

Organs and tissues as listed in Table 4 were removed from all animals at necropsy and fixed in 10% neutral-buffered formalin except for the eyes and testes, which were fixed in Bouin solution. The lungs were infused with applied volume of 10% neutral-buffered formalin from the trachea and then fixed in the fixative.
Table 11: Pathology

The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: control and top dose).*

<table>
<thead>
<tr>
<th>C</th>
<th>W</th>
<th>H</th>
<th>C</th>
<th>W</th>
<th>H</th>
<th>C</th>
<th>W</th>
<th>H</th>
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<tr>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>#</td>
<td>Brain1</td>
<td>✓</td>
<td>✓</td>
<td>#</td>
<td>Liver</td>
<td>✓</td>
<td>✓</td>
<td>#</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>✓</td>
<td>#</td>
<td>✓</td>
<td>#</td>
<td>Pancreas</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Ischiadic nerve</td>
<td>✓</td>
<td>#</td>
<td>Duodenum</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Pituitary</td>
<td>✓</td>
<td>#</td>
<td>Jejunum</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Thymus</td>
<td>✓</td>
<td>#</td>
<td>Ileum</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Thyroids with parathyroids</td>
<td>✓</td>
<td>#</td>
<td>Cecum</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>Adrenals</td>
<td>✓</td>
<td>#</td>
<td>Colon</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Spleen</td>
<td>✓</td>
<td>#</td>
<td>Rectum</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Bone with bone marrow3</td>
<td>✓</td>
<td>#</td>
<td>Head7</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Skeletal muscle (right femur)</td>
<td>✓</td>
<td>#</td>
<td>Tongue</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Lymph nodes4</td>
<td>✓</td>
<td>#</td>
<td>Laryngopharynx</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Heart</td>
<td>✓</td>
<td>#</td>
<td>Larynx</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Aorta</td>
<td>✓</td>
<td>#</td>
<td>Trachea</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Salivary glands5</td>
<td>✓</td>
<td>#</td>
<td>Lung, including bronchi</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>✓</td>
<td>Esophagus</td>
<td>✓</td>
<td>✓</td>
<td>Kidneys</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>✓</td>
<td>#</td>
<td>#</td>
<td>Stomach6</td>
<td>✓</td>
<td>#</td>
<td>Urinary bladder</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

1 cerebrum, cerebellum, pons, and medulla
2 cervical, thoracic, and lumbar regions
3 sternum; femur and knee joint
4 mandibular and mesenteric
5 submandibular and sublingual
6 forestomach and glandular stomach
7 including nasal tissue

*Any animal found dead or killed in extremis had the listed organs examined, no matter the dose group.
Statistics:

The data on body weight, food consumption, number of white blood cells, organ weights and organ weight to body weight ratio were assessed by Bartlett’s test (significance level: 5%) at first. As the results, if the variance was homogeneous, the data were assessed by one-way layout analysis of variance (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett's multiple comparison test (two-tailed, significance level: 5% and 1%). Subsequently, if the variance was heterogeneous, the data were assessed by Kruskal-Wallis rank test (significance level: 5%). If the result was significant, the data were assessed between the control group and the treated groups by Dunnett type joint-ranking test (two-tailed, significance level: 5% and 1%).

The data on mortality, general clinical observations, gross pathological findings (except for animals killed by moribund and died during the test) and histopathological findings (except for animals killed by moribund and died during the test) were assessed between the control group and the treated groups by Fisher’s exact probability test (one-tailed, significance level: 5% and 1%). However, the statistical evaluation of the histopathological findings was carried out between the control group and the high dose (1000ppm) group in the total number of all animals and animals killed by design. Those of the adrenal gland in male and the uterus in female were carried out between the control group and the treated groups.

The data on differential leukocyte count were assessed by F test (two-tailed, significance level: 5%) between the control group and the high dose group. As the results, if the variance was homogeneous, the data were assessed by Student’s t-test (two-tailed, significance level: 5% and 1%). If the result was significant, the data were assessed by Wilcoxon test (two-tailed, significance level: 5% and 1%).

Mortality was assessed by the life table test. In the life table test, survival curves in each group were estimated by Kaplan-Meier method and assessed by Log-Rank test (significance level: 5% and 1%) between the control group and each treated group. Software used for statistical evaluation was SAS (SAS Institute Japan Ltd., Tokyo, Japan) and EXSUS (CAC Corp., Tokyo, Japan).

Any Other Information on Materials and Methods Including Tables:  None
III. RESULTS AND DISCUSSION

Effect Levels: A NOAEL for the study was not reported.
Endpoint: Not applicable
Effect Type: Chronic
Sex: Male/female

Basis for Effect Level/Remarks: Not applicable

<table>
<thead>
<tr>
<th>Observations</th>
<th>Clinical Signs and Mortality: Yes</th>
<th>Body Weight and Weight Gain: Yes</th>
<th>Food Consumption and Compound Intake: Yes</th>
<th>Food Efficiency: No</th>
<th>Water Consumption and Compound Intake: No</th>
<th>Ophthalmoscopy: Not Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Signs and Mortality:</td>
<td>Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight and Weight Gain:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Consumption and Compound Intake:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Efficiency:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water Consumption and Compound Intake:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ophthalmoscopy:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematology:</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical Chemistry:</td>
<td>Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinalysis:</td>
<td>Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurobehaviour:</td>
<td>Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ Weights:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gross Pathology:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histopathology: Non-neoplastic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histopathology: Neoplastic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Details on Results

Clinical Signs and Mortality:
Clinical signs of toxicity: There were no treatment related adverse effects.

Mortality: There was a significant increase in the mortality of the 3000ppm female group when compared to that of the control females.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.0%</td>
<td>18.0%</td>
</tr>
<tr>
<td>1000</td>
<td>18.0%</td>
<td>20.0%</td>
</tr>
<tr>
<td>3000</td>
<td>14.0%</td>
<td>36.0%</td>
</tr>
</tbody>
</table>

Ophthalmoscopy: Not Measured

Body Weight and Body Weight Gain: Body weights of males and females in the 3000ppm group and of females in the 1000ppm group were significantly lower during the treatment period.
Figure 4: Body weight development of rats administered Afidopyropen for 2 years

Males

Females
Table 12: Mean body weight of rats administered Afidopyropen for 2 years

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td><strong>Body weight [g]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>111</td>
<td>111</td>
</tr>
<tr>
<td>Week 13</td>
<td>330</td>
<td>328</td>
</tr>
<tr>
<td>Week 52</td>
<td>443</td>
<td>441</td>
</tr>
<tr>
<td>Week 104</td>
<td>449</td>
<td>449</td>
</tr>
<tr>
<td><strong>Overall body weight gain [g]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1 to Week 104 bw gain</td>
<td>338</td>
<td>338</td>
</tr>
<tr>
<td>0 – 104 Gain (% of control)</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

*: P<0.05; **: P<0.01

Food & Water Consumption and Compound Intake:

Food consumptions of males and females in the 3000ppm group were significantly lower during the treatment period. The changes in food consumption in males in females were considered to be treatment related since they were accompanied by changes in body weight.

Table 13: Mean daily food consumption in rats administered Afidopyropen for 2-years

<table>
<thead>
<tr>
<th>Dose level [ppm]</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td><strong>Mean consumption (g/rat/day)</strong></td>
<td>14.5</td>
<td>14.0*</td>
</tr>
<tr>
<td><strong>Mean Consumption (% of Control)</strong></td>
<td>-</td>
<td>97%</td>
</tr>
</tbody>
</table>

*: P<0.05; **: P<0.01

No differences of water consumption were noted in treated groups.

The overall average of test substance intake is shown in the following table.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>mg/kg bw/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>1000</td>
<td>41.6</td>
</tr>
<tr>
<td>3000</td>
<td>128.2</td>
</tr>
</tbody>
</table>
Hematological findings:

No significant changes in the 1000ppm and 3000ppm groups were noted compared with the control group. In the 3000ppm group, however, WBC of 3 males and 2 females were apparently increased because of large granular lymphocytic leukemia.

Clinical chemistry findings: Not measured

Urinalysis: Not analyzed

Organ weight:

In the 3000ppm group, absolute and relative weights of the liver, spleen and kidneys increased significantly in males and females. In males, absolute and relative weights of the epididymides and relative weight of the brain increased significantly. In females, relative weights of the heart, adrenal glands and brain increased significantly and absolute weights of the heart and ovaries decreased significantly. Significant increases of relative weights of the brain in males and females and of the heart and adrenal glands in females were considered to be related to the low body weight. In the 1000ppm group, absolute and relative weights of the epididymides increased significantly in males. In females, relative weight of the liver increased significantly and absolute weight of the heart decreased significantly.

Table 14: Selected organ weight findings in rats administered Afidopyropen for 2 years (group means)

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Parameter</th>
<th>Kidney (total)</th>
<th></th>
<th>Liver</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight (g)</td>
<td>% of Cntl</td>
<td>Relative Wt</td>
<td>% of Cntl</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.75</td>
<td>-</td>
<td>0.628</td>
<td>-</td>
<td>12.1</td>
</tr>
<tr>
<td>1000</td>
<td>2.86</td>
<td>104</td>
<td>0.643</td>
<td>102</td>
<td>12.7</td>
</tr>
<tr>
<td>3000</td>
<td>2.94</td>
<td>107</td>
<td>0.688**</td>
<td>110</td>
<td>14.3**</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.98</td>
<td>-</td>
<td>0.673</td>
<td>-</td>
<td>8.6</td>
</tr>
<tr>
<td>1000</td>
<td>2.04</td>
<td>103</td>
<td>0.714</td>
<td>106</td>
<td>9.0</td>
</tr>
<tr>
<td>3000</td>
<td>2.11**</td>
<td>103</td>
<td>0.813**</td>
<td>121</td>
<td>9.9**</td>
</tr>
</tbody>
</table>

*, P<0.05; **, P<0.01
Gross and histopathology:

In the 3000ppm group, the incidences of cyst in the mandibular lymph node and cyst in the pituitary were significantly higher than those in the control group in males. The incidences of emaciation, nodule in the chest, callus (bilateral), nodule in the pancreas, atrophy of the testis (unilateral and a total of unilateral and bilateral) and nodule in the testis (unilateral, bilateral and a total of unilateral and bilateral) were significantly lower than those in the control group. In females, the incidence of cyst in the pituitary was significantly higher than that in the control group. The incidences of gray foci of the heart and nodule in the thyroid (unilateral) were significantly lower than those in the control group. Cyst in the pituitary in males and females with significant increased incidences was considered to be not related to the treatment with the test substance because the incidences of corresponding lesion, cyst of the pituitary, were not significantly different from those in the control group in histopathology. Emaciation, nodule in the chest, callus (bilateral) and nodule in the pancreas in males and gray foci of the heart and nodule in the thyroid (unilateral) in females with significant decreased incidences were considered to be not related to the treatment with the test substance because decreased changes of the incidence of lesions may be not regarded with toxic effects of the test substance.

Table 15: Incidence of selected gross findings in all animals.

<table>
<thead>
<tr>
<th>Organ / Lesion</th>
<th>0 ppm</th>
<th>1000 ppm</th>
<th>3000 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest Nodule</td>
<td>9/50</td>
<td>4/50</td>
<td>1/50**</td>
</tr>
<tr>
<td></td>
<td>(18%)</td>
<td>8.0%</td>
<td>(2.0%)</td>
</tr>
<tr>
<td>Callus, bilateral</td>
<td>6/50</td>
<td>3/50</td>
<td>0/50*</td>
</tr>
<tr>
<td></td>
<td>(12%)</td>
<td>(6%)</td>
<td>(0%)</td>
</tr>
<tr>
<td>Mandibular LN Cyst</td>
<td>0/50</td>
<td>2/50</td>
<td>6/50*</td>
</tr>
<tr>
<td></td>
<td>(0%)</td>
<td>(4%)</td>
<td>(12%)</td>
</tr>
<tr>
<td>Pancreas Nodule</td>
<td>18/50</td>
<td>12/50</td>
<td>3/49**</td>
</tr>
<tr>
<td></td>
<td>(36%)</td>
<td>(24%)</td>
<td>(6.1%)</td>
</tr>
<tr>
<td>Testis Atrophy</td>
<td>13/50</td>
<td>4/50</td>
<td>3/50**</td>
</tr>
<tr>
<td></td>
<td>(26%)</td>
<td>(8%)</td>
<td>(6%)</td>
</tr>
<tr>
<td>Testis Nodule</td>
<td>39/50</td>
<td>35/50</td>
<td>14/50**</td>
</tr>
<tr>
<td></td>
<td>(78%)</td>
<td>(70%)</td>
<td>(28%)</td>
</tr>
<tr>
<td>Pituitary Cyst</td>
<td>1/50</td>
<td>1/50</td>
<td>7/50*</td>
</tr>
<tr>
<td></td>
<td>(2%)</td>
<td>(2%)</td>
<td>(14%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart, Gray Foci</td>
<td>7/50</td>
<td>4/50</td>
<td>1/50*</td>
</tr>
<tr>
<td></td>
<td>(14%)</td>
<td>(8%)</td>
<td>(2%)</td>
</tr>
</tbody>
</table>
Neoplastic Lesions (all animals)

In females, the incidence of adenocarcinoma in the uterus was significantly higher than that in the control group.

Incidences where neoplastic lesions were increased, or where lesion incidence is of particular interest are shown in the following table.

Table 16: Select incidence of microscopic neoplastic lesions in male and female rats administered Afidopyropen for 2-years. All animals examined

<table>
<thead>
<tr>
<th>Site and Lesion</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 ppm</td>
<td>1000 ppm</td>
</tr>
<tr>
<td>Pituitary Cyst</td>
<td>5/50 (10%)</td>
<td>8/50 (16%)</td>
</tr>
<tr>
<td>Thyroid Nodule, Unilateral</td>
<td>9/50 (18%)</td>
<td>6/50 (12%)</td>
</tr>
</tbody>
</table>

*, P<0.05; **, P<0.01 Fisher’s exact probability test

N Number of animals examined at the designated site
[Be]: Benign neoplasm; [Ma]: Malignant neoplasm.
*, P<0.05; **, P<0.01 Fisher’s exact probability test
Non-neoplastic Lesions (all animals)

In the 3000ppm group, the incidences of dilatation of sinus (slight degree) in the mandibular lymph node, atrophy of seminiferous tubule (slight degree) in the testis and osseous metaplasia of sclera (slight degree) in the eye were significantly higher than those in the control group in males. The incidences of atrophy of seminiferous tubule (severe degree and total number) in the testis, hyperplasia of interstitial cell (slight degree) in the testis, decreased number of sperm (moderate and severe degrees and total number) in the epididymis were significantly lower than those in the control group. In females, the incidences of hyperplasia of bile duct (slight degree and total number) in the liver, decreased Zymogen granules of acinar cell (slight degree) in the pancreas, chronic progressive nephrosis (moderate degree and total number) in the kidney and hyperplasia of endometrium (total number) in the uterus were significantly higher than those in the control group.

Incidences where neoplastic lesions were increased, or where lesion incidence is of particular interest are shown in Table 10.

Table 17: Incidence of selected histopathological findings in rat administered Afidopyropen for 2-years: All animals examined

<table>
<thead>
<tr>
<th>Organ /Lesion</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1000 3000</td>
<td>0 1000 3000</td>
</tr>
<tr>
<td>Mandibular LN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>49 50 50</td>
<td>3 2 1</td>
</tr>
<tr>
<td>Dilation, sinus +</td>
<td>11 15 23*</td>
<td>3 2 1</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>50 28 31</td>
<td>50 38 44</td>
</tr>
<tr>
<td>Hyperplasia, bile duct +</td>
<td>46 46 41</td>
<td>21 39** 43**</td>
</tr>
<tr>
<td>Hyperplasia, bile duct ++</td>
<td>4 4 9</td>
<td>1 3 6</td>
</tr>
<tr>
<td>Hyperplasia, bile duct ++</td>
<td>50 50 50</td>
<td>22 42** 49**</td>
</tr>
<tr>
<td>Microgranuloma +</td>
<td>12 10 3*</td>
<td>23 23 14*</td>
</tr>
<tr>
<td>Microgranuloma ++</td>
<td>0 0 0</td>
<td>8 4 2*</td>
</tr>
<tr>
<td>Microgranuloma ++</td>
<td>12 10 3*</td>
<td>31 27 16**</td>
</tr>
<tr>
<td>Foci, altered cell, clear cell +</td>
<td>2 4 10*</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Foci, altered cell, clear cell ++</td>
<td>1 1 2</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Foci, altered cell, clear cell</td>
<td>3 5 12*</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Pancreas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>50 50 49</td>
<td>50 50 50</td>
</tr>
<tr>
<td>Decreased zymogen, granules, acinar cell +</td>
<td>4 6 2</td>
<td>2 5 10*</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>50 50 50</td>
<td>50 50 50</td>
</tr>
<tr>
<td>Basophilic tubular epithelium +</td>
<td>11 10 12</td>
<td>31 23 19*</td>
</tr>
<tr>
<td>Basophilic tubular epithelium +</td>
<td>0 1 0</td>
<td>1 1 0</td>
</tr>
<tr>
<td>Basophilic tubular epithelium</td>
<td>11 11 12</td>
<td>32 24 19*</td>
</tr>
<tr>
<td>Brown pigment deposit, tubular epithelium +</td>
<td>13 13 14</td>
<td>35 28 23**</td>
</tr>
<tr>
<td>Brown pigment deposit, tubular epithelium ++</td>
<td>0 0 0</td>
<td>1 1 2</td>
</tr>
</tbody>
</table>

Page 96
<table>
<thead>
<tr>
<th>Organ /Lesion</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>Brown pigment deposit, tubular epithelium</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Protein casts</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Chronic progressive nephrosis +</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>Chronic progressive nephrosis ++</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Chronic progressive nephrosis +++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic progressive nephrosis</td>
<td>36</td>
<td>36</td>
</tr>
</tbody>
</table>

**Testis**

| Atrophy, seminiferous tubule +       | 1  | 12** | 9**   |
| Atrophy, seminiferous tubule ++      | 10 | 10   | 7     |
| Atrophy, seminiferous tubule +++     | 37 | 23** | 7**   |
| Atrophy, seminiferous tubule         | 48 | 45   | 23**  |
| Hyperplasia, interstitial cell +      | 41 | 34   | 11**  |

**Epididymis**

| Decreased number, sperm +            | 3  | 9   | 8     |
| Decreased number, sperm ++           | 14 | 10  | 3**   |
| Decreased number, sperm +++          | 31 | 26  | 11**  |
| Decreased number, sperm              | 48 | 45  | 22**  |

**Uterus**

| Hyperplasia, endometrium+            | -  | -   | -     |
| Hyperplasia, endometrium++           | -  | -   | -     |
| Hyperplasia, endometrium+++          | -  | -   | -     |
| Cyst, endometrium +                  | -  | -   | -     |

**Eye**

| Osseous metaplasia, sclera +         | 25 | 34  | 35*   |

**Mammary gland**

| Dilatation, duct +                   | 3  | 0   | 0     | 22 | 15  | 11*  |
| Dilatation, duct ++                  | 1  | 0   | 0     | 0  | 1   | 0    |
| Dilatation, duct +++                 | 0  | 0   | 0     | 1  | 1   | 0    |
| Dilatation, duct                      | 4  | 0*  | 0     | 23 | 17  | 11** |

*, P<0.05; **, P<0.01 Fisher's exact probability test

Any Other Information on Results Including Tables:

None

IV. OVERALL REMARKS, ATTACHMENTS

None.
V. APPLICANT'S SUMMARY AND CONCLUSION

Conclusion:

Afidopyropen was given in the feed to F344 rats (50/sex/dose) at doses of 0, 1000 or 3000 ppm (equal to 0, 41.6 and 128.2 mg/kg bw/d for males and 0, 50.4 and 146.9 mg/kg bw/d for females) for 104 weeks.

There was a significant increase in the mortality of the 3000 ppm female group. No treatment-related abnormalities were noted in mortality with the males. There were no treatment related clinical observations.

Food consumption was decreased in all of the groups treated with Afidopyropen. Body weights for males at 3000 ppm were statistically significantly reduced, but other treatment groups were not significantly different than control.

In the 3000 ppm group, treatment related organ weight changes included an increased absolute and relative weights of the liver, spleen and kidneys in males and females. In males, absolute and relative weights of the epididymides and relative weight of the brain increased significantly. In females, relative weights of the heart increased significantly and absolute weights of the heart and ovaries decreased significantly. At 1000 ppm, absolute and relative weights of the epididymides increased significantly in males. In females, relative weight of the liver increased significantly and absolute weight of the heart decreased significantly.

Gross histopathology of note included a decrease in the incidence of testis atrophy at 3000 ppm in the males as well as a decrease in thyroid nodules in the females. Pituitary cysts were increased in the females at 3000 ppm.

Non neoplastic histopathologic findings included liver effects (increased bile duct hyperplasia and microgranuloma in all treatment groups and increased altered foci (male at 3000 ppm). In the kidney an increase in chronic progressive nephrosis (female, 3000 ppm) was noted. The testis showed decreased atrophy of the seminiferous tubule as well as decreased hyperplasia of interstitial cell at 3000 ppm. In the uterus there was an increase in endometrium hyperplasia at 3000 ppm. Also at 3000 ppm, there was a decrease in the dilation of the mammary gland duct.

Neoplastic lesions present included an increased incidence of uterine adenocarcinoma at both 1000 and 3000 ppm in the females. There was a decreased incidence of testes interstitial tumors in the males at 3000 ppm as well as a decrease in thyroid C-cell carcinoma in both the males and females at 3000 ppm.

NOAELs for all findings in this study were established in a prior rat cancer study conducted at lower doses (2014/8000287).
Study 6  Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC)

Executive Summary

The purpose of this study was to determine the potential for Afidopyropen and two Afidopyropen metabolites (Reg. No. 5741532 (M440I002) and Reg. No. 5741530 (M440I001)) to interact with estrogen receptors isolated from rat uteri, through measurement of its displacement of radiolabeled ligand ([3H]17β-estradiol), using the EPA recommended methodology. The reference standard (non-radiolabeled estradiol) as well as a weak positive control and a negative control (19-norethindrone and octyltriethoxysilane, respectively), were used to verify the performance of the assay and to compare the effects of the test compounds.

The estradiol receptor binding assay using rat uterine cytosol is part of the Tier 1 screening battery of tests and is intended to identify chemicals that may interact with the hormone-binding domain of the estradiol receptor. This assay is part of a Tier 1 battery which is intended for screening purposes only and is not intended to be used for endocrine classification or risk assessment.

The final concentrations of BAS 440 I, Reg. No. M440I002 and Reg. No. M440I001 tested in the ER binding assays were 10-10, 10-9, 10-8, 10-7, 10-6, 10-5, 10-4 and 10-3 M for all three valid independent runs. The reference standard and the positive and negative control compounds gave results that were consistent with the EPA criteria for assay performance.

Based on the results of three assay runs, Reg. No. M440I002 and Reg. No. M440I001 have a final classification of “non-interacting” with the estrogen receptor. Afidopyropen was classified of “equivocal.” A result of “equivocal” is not a positive or a potential positive classification, but rather, it means the result is ambiguous, or cannot be properly identified due to limitations of the assay.
II. MATERIAL AND METHODS

A. MATERIALS

1. Test materials
   Test Substance Name: BAS 440 I (Afidopyropen)
   Synonym: BAS 440 I (ME5343 Technical)
   BASF Substance Number: 09/0676-1
   CAS Number: 915972-17-7

   Test Substance Name: Reg. No. 5741532 (M440I002; Metabolite of BAS 440 I, Afidopyropen)
   Test Substance Manufacturer: BASF SE
   BASF Substance Number: 15/0197-1

   Test Substance Name: Reg. No. 5741530 (M440I001 Metabolite of BAS 440 I, Afidopyropen)
   Test Substance Manufacturer: BASF SE
   BASF Substance Number: 15/0196-1

2. Materials

   Preparation of the multiple solutions required for the assays are listed in the study report. Materials used to prepare solutions are listed below.

<table>
<thead>
<tr>
<th>Control Substance</th>
<th>CAS Number</th>
<th>Molecular Weight (g/mol)</th>
<th>Catalog Number</th>
<th>Lot Number</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-Estradiol [Strong Positive Control]</td>
<td>50-28-2</td>
<td>272.4</td>
<td>E8875</td>
<td>SLBL7310V</td>
<td>100%</td>
</tr>
<tr>
<td>19-Norethindrone [Weak Positive Control]</td>
<td>68-22-4</td>
<td>298.42</td>
<td>N4128</td>
<td>SLBF1672V</td>
<td>100%</td>
</tr>
<tr>
<td>Octyltriethoxysilane [Negative Control]</td>
<td>2943-75-1</td>
<td>276.49</td>
<td>440213</td>
<td>SHBD3756V</td>
<td>98.90%</td>
</tr>
</tbody>
</table>
3. **Rat Uteri Source**

   Charles River

**B. Study design and methods**

1. **Dates of work:** 31 Aug 2015 to 08 Sep 2015

2. **Preparation of rat uterine cytosol**

   Uteri from 100 female Sprague-Dawley rats (85 to 100 days of age at time of kill) ovariectomized seven to ten days prior to being humanely killed were purchased from Charles River and were used to prepare the cytosol. Cytosol was prepared and validated at Cyprotex per OPPTS guideline and Cyprotex SOP-2057 for use on this study.

3. **Solubility/Precipitation Assay**

   The limit of test substance solubility was determined visually.

4. **Rat Uterine Cytosol**

   Uteri from 100 female Sprague-Dawley rats (85 to 100 days of age at time of kill) ovariectomized seven to ten days prior to being humanely killed were purchased from Charles River and were used to prepare the cytosol. Cytosol was prepared and validated at Cyprotex per OPPTS guideline and Cyprotex SOP-2057 for use on this study.

5. **Stock Solution Preparation**

   Stock solutions of EDTA, Tris buffer, 2X TEG Buffer, 60% hydroxyapatitie (HAP) slurry, Working Assay Buffer were prepared as detailed in the study report (section 11).
6. **Summary Table of Assay Conditions**

<table>
<thead>
<tr>
<th>Source of receptor</th>
<th>Rat uterine cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of radioligand</td>
<td>1 nM</td>
</tr>
<tr>
<td>Concentration of receptor</td>
<td>~0.10 mg/mL (sufficient to bind 10-15% of radioligand)</td>
</tr>
<tr>
<td>Concentration of test substances (as serial dilutions)</td>
<td>100 pM to 1 mM</td>
</tr>
<tr>
<td>Temperature</td>
<td>~4°C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>16-20 hours</td>
</tr>
<tr>
<td>Composition of assay buffer</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>10 mM (pH ~7.4)</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Protease Inhibitor</td>
<td>0.5% (v/v)</td>
</tr>
<tr>
<td>DTT</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

7. **Assay Preparations**

Twelve (12) x 75 mm siliconized tubes were used for the assay. A master mixture of radioligand and buffer was prepared. Trace tubes were also required (tubes containing buffer and [3H]-17β-estradiol but no protein to determine total activity). The following table describes the preparation of the BAS 440 I, Reg. No. 5741532 and Reg. No. 5741530 samples:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Target Volume/Tube (µL)</th>
<th># of Tubes</th>
<th>Total Volume Needed (mL)</th>
<th>Master Mix Volumes (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEDG+PI</td>
<td>Assay Tubes = 370</td>
<td>Trace Tubes = 48.72</td>
<td>Assay Tubes = 155</td>
<td>Trace Tubes = 6</td>
</tr>
<tr>
<td>Diluted [3H]-17β-estradiol (50 nM)</td>
<td>Assay Tubes = 10</td>
<td>Trace Tubes = 1.28</td>
<td>Assay Tubes = 155</td>
<td>Trace Tubes = 6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
8. Competitive Binding Assay Additions

<table>
<thead>
<tr>
<th>Volume (µL)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>17β-estradiol, 19-norethindrone, octyltriethoxysilane, or test substances</td>
</tr>
<tr>
<td>380</td>
<td>Master mixture (TEDG+PI assay buffer + [³H]-17β-estradiol)</td>
</tr>
<tr>
<td>100</td>
<td>Uterine cytosol (diluted to appropriate protein concentration)</td>
</tr>
<tr>
<td>500</td>
<td>Total volume in each assay tube</td>
</tr>
</tbody>
</table>

9. Separation of Bound [³H]-17β-Estradiol from Free [³H]-17β-Estradiol

The ER assay tubes were removed from the rotator and placed in an ice-water bath. A repeating pipette was used to add approximately 250 µL of ice cold HAP slurry (60% in TEDG+PI) to each assay tube. The tubes were vortexed for approximately 10 seconds at approximately 5 minute intervals for a total of approximately 15 minutes with tubes remaining in the ice-water bath between vortexing. Following the vortexing step, approximately 2 mL of the cold (~4°C) TEDG+PI buffer was added, quickly vortexed, and centrifuged at ~4°C for approximately 10 minutes at 1000 x g. After centrifugation, the supernatant containing the free [³H]-17β-estradiol was immediately decanted and discarded. The HAP pellet contained the estrogen receptor bound [³H]-17β-estradiol. Approximately 2 mL of ice-cold TEDG+PI buffer was added to each tube and vortexed to resuspend the pellet. The tubes were centrifuged again at ~4°C for approximately 10 minutes at approximately 1000 x g. The supernatant was quickly decanted and discarded. The wash and centrifugation steps were repeated once more. After the final wash, the supernatant was decanted. The assay tubes were allowed to drain briefly.

Extraction and Quantification of [³H]-17β-Estradiol Bound to ER

Approximately 1.5 mL of absolute ethanol was added to each assay tube. The tubes were allowed to sit at room temperature for approximately 15 to 20 minutes, vortexing for approximately 10 seconds at approximately 5-minute intervals. The assay tubes were centrifuged for approximately 10 minutes at approximately 1000 x g. An approximately 1 mL aliquot was pipetted, taking care to avoid the centrifuged pellet, into a 20 mL scintillation vial containing approximately 10 mL of scintillation cocktail. The vials were assayed by LSC.
Competitive Binding Data Analysis and Interpretation

Analysis and Considerations

The competitive binding assay was functioning correctly if all of the following criteria had been met, according to OPPTS 890.1250:

- Increasing concentrations of unlabeled 17β-estradiol should displace [3H]-17β-estradiol from the receptor in a manner consistent with one-site competitive binding. Specifically, the curve fitted to the unlabeled estradiol data points using nonlinear regression should descend from 90% to 10% over approximately an 81-fold increase in the concentration of unlabeled 17β-estradiol.

- Ligand depletion should be minimal. Specifically, the ratio of total binding in the absence of competitor, 17β-estradiol, to the total amount of [3H]-17β-estradiol added per assay tube should be no greater than 15%.

- The parameter values (top, bottom, and slope) for 17β-estradiol and the concurrent weak positive control, 19-norethindrone, should be within the tolerance bounds outlined in the OPPTS guideline, which are also provided in the table that follows.

- The reference control vehicle should not alter the sensitivity or reliability of the assay. The final DMSO concentration in the reference control assay tubes was held ≤10%, per the test guidelines. All reference control tubes contained equal amounts of solvent. The negative control, octyltriethoxysilane, should not displace more than 25% of the radioligand from the ER on average across all concentrations.

- The test substance should be tested over a concentration range that fully defines the top of the curve (i.e. a range that would show that a top plateau was achieved), and the top should be within 25 percentage points of either the vehicle control or the value for the lowest concentration of the 17β-estradiol positive control for that run.

Upper and Lower Limits for Parameters in Competitive Binding Assay Curves for the Standards (17β-Estradiol and 19-Norethindrone)

<table>
<thead>
<tr>
<th></th>
<th>17 β -Estradiol</th>
<th>19-Norethindrone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower Limit</td>
<td>Upper Limit</td>
</tr>
<tr>
<td>Loge(Syx)</td>
<td>--</td>
<td>2.35</td>
</tr>
<tr>
<td>Bottom plateau level</td>
<td>% binding</td>
<td>-4</td>
</tr>
<tr>
<td>Top plateau level</td>
<td>% binding</td>
<td>94</td>
</tr>
<tr>
<td>Hill Slope</td>
<td>Log10(M)-1</td>
<td>-1.1</td>
</tr>
</tbody>
</table>
Classification

The classification of a test substance as a binder or non-binder is made on the basis of the average results of three non-concurrent runs, each of which meet the performance criteria and taken together, are consistent with each other, as per OPPTS guideline 890.1250. Each run is classified as “interacting,” “not interacting,” “equivocal,” or “equivocal up to the limit of the concentrations tested.”

A run was classified as “interactive” with the ERs if the lowest point on the fitted response curve within the range of the data is less than 50%. “Percent” refers to binding of the radiolabeled [3H]-17β-estradiol. Thus, “less than 50%” means that less than 50% of the radiolabeled estradiol is bound, or equivalently, that more than 50% of the radiolabeled [3H]-17β-estradiol is displaced from the receptor. In other words, a run was classified as “interactive” if a Log(IC50) is obtained.

A run was classified as “equivocal up to the limit of concentrations tested” if there are no data points at or above a test substance concentration of 10-6 M and one of the two following conditions occur:

• A binding curve could be fit but 50% or less of the radiolabeled [3H]-17β-estradiol is displaced by concentration of 10-6 M.

OR

• A binding curve could not be fit and the lowest average percent binding among the concentration groups in the data is above 50%.

A run was classified as “not interactive” if there are usable data points at or above 10-6 M and either:

• The lowest point on the fitted response curve within the range of the data is above 75%.

OR

• A binding curve could not be fitted and the lowest average percent binding among the concentration groups in the data is above 75%.

A run is classified as “equivocal” if it falls in none of the above categories.

After each run is classified, the test substance is classified by assigning the following values to each run and averaging across runs:

Interactive: 2
Equivocal: 1
Not Interactive: 0
Equivocal up to the limit of concentrations tested: (“missing”)
Test substance classification, based on the average of all the runs performed for a test substance:

- Interactive: average $\geq 1.5$
- Equivocal: $0.5 \leq$ average $< 1.5$
- Not Interactive: average $< 0.5$
- Equivocal up to the limit of concentrations tested: (“missing”)

III. RESULTS AND DISCUSSION

Concentration Range for the Test Substances

In order to identify a suitable top concentration for use in the binding assays, preliminary assessments of precipitation were conducted. Precipitation was observed for $10^{-3}$ M BAS 440 I in the first run, but was not observed in runs 2 or 3. Therefore, the suitable top concentration of BAS 440 I, Reg. No. 5741532 and Reg. No. 5741530 for use in the binding assays was $10^{-3}$ M.

The final concentrations of BAS 440 I, Reg. No. 5741532 and Reg. No. 5741530 tested in the binding assays were: $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M for all three valid independent runs (02-September-2015, 03-September-2015 and 04-September-2015).

Binding Assay Acceptance Criteria

In all three independent runs of the assay, increasing concentrations of unlabeled 17β-estradiol displaced [3H]-17β-estradiol from the receptor in a manner consistent with one site competitive binding. Therefore, all three independent runs of the assay were considered to have met the assay acceptance criteria and were considered to be valid:

Binding Assay Results

First valid independent run (02-September-2015)

- The mean specific binding for BAS 440 I was $>93\%$ at every concentration tested with the exception of $10^{-3}$ M where the binding was 58.1%, classifying BAS 440 I as “equivocal” for this run.

- The mean specific binding for Reg. No. 5741532 (M440I002) was $>92\%$ at every concentration tested, classifying Reg. No. 5741532 (M440I002) as “non-interacting” for this run.

- The mean specific binding for Reg. No. 5741530 (M440I001) was $>92\%$ at every concentration tested, classifying Reg. No. 5741530 (M440I001) as “non-interacting” for this run.
• The weak positive control 19-norethindrone had a LogIC$_{50}$ of –5.4 M, and the LogIC$_{50}$ of 17β-estradiol was -9.2 M, resulting in an RBA for 19-norethindrone of 0.0158% and a log(RBA) of -1.8.

Second valid independent run (03-September-2015)
• The mean specific binding for BAS 440 I was >97% at every concentration tested with the exception of 10-3 M where the binding was 78.6%, classifying BAS 440 I as “non-interacting” for this run.

• The mean specific binding for Reg. No. 5741532 (M440I002) was >96% at every concentration tested, classifying Reg. No. 5741532 (M440I002) as “non-interacting” for this run.

• The mean specific binding for Reg. No. 5741530 (M440I001) was >95% at every concentration tested, classifying Reg. No. 5741530 (M440I001) as “non-interacting” for this run.

• The weak positive control 19-norethindrone had a LogIC$_{50}$ of –5.4 M, and the LogIC$_{50}$ of 17β-estradiol was -9.2 M, resulting in an RBA for 19-norethindrone of 0.0160% and a log(RBA) of -1.8.

Third valid independent run (04-September-2015)
• The mean specific binding for BAS 440 I was >95% at every concentration tested with the exception of 10-3 M where the binding was 72.7%, classifying BAS 440 I as “equivocal” for this run.

• The mean specific binding for Reg. No. 5741532 (M440I002) was >94% at every concentration tested, classifying Reg. No. 5741532 (M440I002) as “non-interacting” for this run.

• The mean specific binding for Reg. No. 5741530 (M440I001) was >97% at every concentration tested, classifying Reg. No. 5741530 as “non-interacting” for this run.

• The weak positive control 19-norethindrone had a LogIC$_{50}$ of –5.4 M, and the LogIC$_{50}$ of 17β-estradiol was -9.2 M, resulting in an RBA for 19-norethindrone of 0.0076% and a log(RBA) of -2.1.

The mean relative binding affinity, or RBA (calculated by dividing the LogIC$_{50}$ of the control/test substance by the LogIC$_{50}$ of the positive control 17β-estradiol) was 0.0131% for 19-norethindrone (or a log(RBA) of -1.9).

IV. OVERALL REMARKS, ATTACHMENTS
None
V. APPLICANT’S SUMMARY AND CONCLUSION

Reg. No. 5741532 (M440I002) was classified as “non-interacting” in all three valid independent runs and thus has a final classification of “non-interacting” with the estrogen receptor.

Reg. No. 5741530 (M440I001) was classified as “non-interacting” in all three valid independent runs and thus has a final classification of “non-interacting” with the estrogen receptor.

BAS 440 I was classified as “equivocal” in the first and third valid independent runs, and as “non-interacting” in the second valid independent run and thus has a final classification of “equivocal.” A result of “equivocal” should not be taken as a positive or a potential positive result, rather, it means the result is ambiguous, or cannot be properly identified due to limitations of the assay.
Study 7  Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-0-9903))

Executive Summary

The objective of this study was to evaluate the ability of Afidopyropen to act as an agonist of human estrogen receptor alpha (hERα) using the hERα-HeLa-9903 cell line.

The suitable top concentration of BAS 440 I for use in the transcriptional activation assays was 10^{-6} M, as higher concentrations exhibited problems with solubility (test substance precipitation observed) and cytotoxicity. There was no cytotoxicity (≥20% reduction in cell viability) observed with the test substance or the controls in any of the valid independent runs.

The final concentrations of BAS 440 I tested in the transcriptional activation assays were: 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M for both valid independent runs (03-August-2015 and 06-August-2015). In addition, for each concentration, 2 replicates/plate were prepared that incorporated the hERα antagonist ICI 182,780. Replicates incorporating the hERα antagonist allow for the identification of non-specific (i.e., non-hERα-mediated) induction of the luciferase gene. The duration of exposure was 24 hours.

The positive controls were 17β-estradiol and 17α-estradiol, the weak positive control was 17α-methyltestosterone and the negative control compound was corticosterone. For each transcriptional assay performed an acceptable concentration response curve was run for each of the reference substances.

In two valid and independent runs of the transcriptional activation assay, BAS 440 I did not increase luciferase activity at any of the viable concentrations tested (RPC_{max}<10%). These data suggest BAS 440 I is not an agonist of human estrogen receptor alpha (hERα) in the HeLa-9903 model system.
II. MATERIAL AND METHODS

A. MATERIALS

1. Test materials

<table>
<thead>
<tr>
<th>Test Substance Name</th>
<th>BAS 440 I (Afidopyropen)</th>
<th>Synonym</th>
<th>BAS 440 I (ME5343 Technical)</th>
<th>BASF Substance Number</th>
</tr>
</thead>
</table>

2. Materials

Preparation of the multiple solutions required for the assays are listed in the study report. Materials used to prepare solutions are listed below.

<table>
<thead>
<tr>
<th>Control Substance</th>
<th>CAS Number</th>
<th>Molecular Weight (g/mol)</th>
<th>Catalog Number</th>
<th>Lot Number</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-Estradiol [Strong Positive Control]</td>
<td>50-28-2</td>
<td>272.4</td>
<td>E8875</td>
<td>SLBL7310V</td>
<td>100%</td>
</tr>
<tr>
<td>17α-Estradiol [Strong Positive Control]</td>
<td>57-91-0</td>
<td>272.4</td>
<td>E8750</td>
<td>113M4057V</td>
<td>99.50%</td>
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<tr>
<td>17α-methyltestosterone [Weak Positive Control]</td>
<td>58-18-4</td>
<td>302.5</td>
<td>M7252</td>
<td>051M1143V</td>
<td>99%</td>
</tr>
<tr>
<td>Corticosterone [Negative Control]</td>
<td>50-22-6</td>
<td>346.5</td>
<td>27840</td>
<td>BCBN2622V</td>
<td>99.5%</td>
</tr>
</tbody>
</table>

3. Source and stability of the cell line

The stably transfected hERα-HeLa-9903 cell line was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, 7-6-8 Asagi Saito, Ibaraki-shi, Osaka 567-0085, Japan. The cell line was certified to be free of mycoplasma and were passage 24 (rangefinder 28-July-2015) and passages 26 and 27 (03-August-2015 and 06-August-2015 runs, respectively) prior to seeding into plates. The stability of the cell line was monitored by the use of the reference chemicals listed in section 2; a complete concentration response curve for each reference compound was run for each assay and confirmed calculated Hill slope values were within the acceptable range.

B. Study design and methods

2. Dates of work: 28 July 2015 to 07 August 2015

2. Cell culture and plating conditions

Cells were maintained in Eagle’s Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of Kanamycin (antibiotic) and 10% dextran-coated-charcoal-
treated fetal bovine serum (DCC-FBS), in a 5% CO₂ incubator at approximately 37°C. When the cells reached 75-90% confluency, they were subcultured at 10 mL of 0.4 X 10⁵ – 1 X 10⁵ cells/mL. The cells were suspended with 10% DCC-FBS in EMEM and plated into wells of a 96- well cell culture plate at a density of ~1 X 10⁴ cells/100 µL/well. The cells were then placed into a 5% CO₂ incubator at approximately 37°C for at least 3 hours prior to chemical exposure.

3. Chemical exposure and assay plate organization

The reference chemicals and BAS 440 I were dissolved in DMSO and then serially diluted in DMSO and medium to prepare 2 x concentration stock solutions (the final concentration of DMSO in the medium was always 0.1 % v/v). When added to the cell culture plates (prepared as described in section 2), 8 serial concentrations for the reference standards were obtained over the following ranges:

- 17β-Estradiol – 10⁻¹⁵ to 10⁻⁶ M
- 17α-Estradiol – 10⁻¹³ to 10⁻₅ M
- Corticosterone - 10⁻¹¹ to 10⁻₄ M
- 17α-Methyltestosterone - 10⁻¹² to 10⁻⁵ M

After the three hour post-seeding incubation (section 2), the media was aspirated and 75 µL of fresh media together with 75 µL of the 2x concentration stock solutions were added to wells containing ~1 x 10⁴ cells/well to give a final volume of 150 µL/well.

All test substance and reference control assay plates were organized as detailed below:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Blank*</td>
<td>E2 (1 nM)</td>
<td>VC**</td>
<td>VC</td>
<td>Conc. 1</td>
<td>Conc. 2</td>
<td>Conc. 3</td>
<td>Conc. 4</td>
<td>Conc. 5</td>
<td>Conc. 6</td>
<td>Con. c.</td>
<td>Con. c.</td>
</tr>
<tr>
<td>B</td>
<td>↓***</td>
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<tr>
<td>G</td>
<td>-----------------</td>
<td>As above + antagonist (1 µM ICI 182,780) ------------------</td>
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</tr>
</tbody>
</table>

E2 = 17β-estradiol
*Blank wells contain media only (no cells)
**Vehicle control (VC) wells contain cells and media + 0.1% (v/v) DMSO
***↓ Indicates the composition of the well is identical to the well directly above it

After adding the reference /test substance, the plates were incubated in a 5% CO₂ incubator at approximately 37°C for 20 – 24 hours.

All concentrations were tested in replicates of 6/plate. In addition, for each concentration, 2 replicates/plate were prepared that incorporated the hERα antagonist ICI 182,780. Replicates incorporating a hERα antagonist allow for the identification of non-specific (i.e.,
non-hERα-mediated) induction of the luciferase gene as true hERα-mediated induction is inhibited by addition of an antagonist whereas non-specific induction is not.

In view of the short-term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration(s) were carried out.

4. Cytotoxicity assay and assessment of solubility

Cell viability was monitored by propidium iodide (PI) uptake under low light conditions. PI is a dye that can only cross weak or damaged cell membranes and then intercalates into DNA/RNA and yields a fluorescent signal.

Cells were seeded as described in Section 2, with the exception that a black-walled 96-well cell culture plate was used. The cells were exposed to the test substance in replicates of 6 (rows A-F) while the last 2 rows (G and H) received 125 μM digitonin as a positive control for cell death. Following chemical exposure, the growth medium was removed and 50 μL of a PI working solution was added to each well. Background fluorescence was evaluated (544 nm and 612 nm) and 50 μL of a 2% (v/v) triton X-100 solution was added to each well and the plate was incubated at room temperature for a minimum of 15 minutes before measuring fluorescence at the same wavelengths.

The background-corrected fluorescence was calculated for each well and the change in cell viability was determined by comparing treated wells to the vehicle control wells. A ≥20% reduction in cell viability was considered evidence of cytotoxicity.

Limit of solubility was determined by laser based light scattering. The test substance was prepared in the HeLa-9903 culture media at the final exposure concentrations and added to wells of a 96-well plate. The samples were assessed using a NEPHELOstar nephelometer (BMG LabTech, Ortenberg, Germany) and visual inspection.

5. Transcriptional activation assay

A luciferase assay was performed according to Cyprotex standard method (proprietary information)

A preliminary cytotoxicity and precipitation assay was conducted with BAS 440 I.

Data analysis and interpretation
In order to determine the relative transcriptional activity as compared to the positive control (PC), 1 nM 17β-estradiol, the luminescence data from each plate were analyzed according to the steps outlined below. Wells incorporating ICI 182,780 were analyzed in an identical fashion to wells not incorporating ICI 182,780, except that the data were normalized by subtracting the mean value for the ICI 182,780-containing vehicle control (VC) wells.

1. Any cytotoxic concentrations were excluded from data analysis.
2. The mean value for the VC wells was calculated.
3. The mean value for the VC wells was subtracted from each well to normalize the data.
4. The mean value for the normalized PC wells was calculated.
5. The normalized value for each well was divided by the mean value of the normalized PC wells (with the normalized mean of the PC wells being defined as
The final value for each well is the relative transcriptional activity for that well compared to the mean normalized PC response.

The data were then interpreted according to the following steps:

1. Where appropriate, LogPC<sub>50</sub>, LogPC<sub>10</sub>, LogEC<sub>50</sub> and Hill slope values were calculated.
2. For the test substance, the maximum response relative to the positive control (RPC<sub>max</sub>) was determined. In each individual run of the transcriptional activation assay, if RPC<sub>max</sub> was less than 10%, the test substance was considered to have given a negative response for hERα agonism.
3. For each individual run of the transcriptional activation assay, the acceptability of the data was evaluated using the following criteria:
   - The mean normalized luciferase signal of the PC (1 nM 17β-estradiol) should be at least 4-fold that of the mean VC on each plate.
   - The results of the 4 reference chemicals should be within the acceptable ranges.
4. If the acceptability criteria outlined above were met, that run of the transcriptional activation assay was considered to be definitive.
5. The test substance was considered negative if RPC<sub>Max</sub> < 10% in at least 2 definitive runs of the transcriptional activation assay. The test substance was considered positive if RPC<sub>Max</sub> ≥ 10% in at least 2 definitive runs of the transcriptional activation assay.

III. RESULTS AND DISCUSSION

Concentration Range for the Test Substances

The suitable top concentration of BAS 440 I for use in the transcriptional activation assays was 10<sup>-5</sup> M, as higher concentrations exhibited problems with solubility (test substance precipitation observed) and cytotoxicity. There was no cytotoxicity (≥20% reduction in cell viability) observed with the test substance or the controls in any valid run of the assay.

The final concentrations of BAS 440 I tested in the transcriptional activation assays were: 10<sup>-12</sup>, 10<sup>-11</sup>, 10<sup>-10</sup>, 10<sup>-9</sup>, 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> and 10<sup>-5</sup> M for both valid independent runs (03-August-2015 and 06-August-2015).

Transcriptional Activation Assay Acceptance Criteria

In both valid independent runs of the assay, the mean luciferase activity of the PC (1 nM 17β-estradiol) was greater than 4-fold that of the mean luciferase activity of the VC on each plate. In addition, in both independent runs of the assay the LogPC<sub>50</sub>, LogPC<sub>10</sub>, LogEC<sub>50</sub> and Hill slope values for the 4 reference compounds (17β-estradiol, 17α-estradiol, 17α-methyltestosterone and corticosterone) were within the acceptable ranges, with the some minor exceptions. However, these deviations from the ranges suggested in the OPPTS guideline were minor and not considered to impact the interpretation of results as the assay response with 17β-estradiol, 17α-estradiol, 17α-methyltestosterone and corticosterone were characteristic of a strong estrogen, a weak estrogen, a weak agonist, and a negative compound, respectively, and the data are within Cyprotex historic values and within values cited elsewhere (Schapaugh et al, 2015).

Therefore, both independent runs of the assay conducted on August 03, 2015 and August 06, 2015, were considered to have met the assay acceptance criteria and were considered to be valid.
Transcriptional Activation Assay Results

In the first valid independent run (03-August-2015) of the transcriptional activation assay, BAS 440 I resulted in an increase in luciferase activity of 0.3 ± 0.3% maximal induction at 10^{-8} M. In the second valid independent run (06-August-2015), BAS 440 I resulted in an increase in luciferase activity of 0.8 ± 0.9% maximal induction at 10^{-10} M.
Figure 5. BAS 440 I – Relative Transcriptional Activation (mean + standard error of mean) in absence of antagonist

Figure 6. 17β-Estradiol – Relative Transcriptional Activation (mean + standard error of mean) in absence of antagonist
Figure 7. 17α-Estradiol – Relative Transcriptional Activation (mean + standard error of mean) in absence of antagonist

Figure 8. Corticosterone – Relative Transcriptional Activation (mean + standard error of mean) in absence of antagonist
IV. OVERALL REMARKS, ATTACHMENTS
None

V. APPLICANT’S SUMMARY AND CONCLUSION

The suitable top concentration of BAS 440 I for use in the transcriptional activation assays was 10⁻⁵ M, as higher concentrations exhibited problems with solubility (test substance precipitation observed) and cytotoxicity. There was no cytotoxicity (≥20% reduction in cell viability) observed with BAS 440 I or the controls in any of the valid independent runs.

In two valid and independent runs of the transcriptional activation assay, BAS 440 I did not result in an increase in luciferase activity at any of the viable concentrations tested (RPCmax<10%).

BAS 440 I is not an agonist of human estrogen receptor alpha (hERα) in the HeLa-9903 model system.
Executive Summary

The aim of this study was to investigate a possible mode of action for uterine tumour formation by assessing the levels of hepatic and uterine CYP1A1 and CYP1B1 following 14 days BAS 440 l (Afidopyropen) administration at a concentration of 3000 ppm in RM1 powder diet to 8-9 weeks old female Fischer 344 rats.

During the course of the experiment, clinical observations were made regularly, together with measurement of food consumption and bodyweight. At termination, liver and uteri were collected and weighed, organ weights were recorded and organ-to-bodyweight ratios were calculated. Liver was processed for isolation of microsomes and the analysis of CYP1A1 and CYP1B1 enzymatic activity. Liver and uteri were processed for Taqman® analysis of CYP1A1 and CYP1B1 mRNA expression. Statistical analysis was performed using Student’s t test, comparing control (untreated) and BAS 440 l (Afidopyropen)-treated samples.

The food consumption of the BAS 440 l (Afidopyropen) treated rats was dissimilar to that of the control rats for the first experimental week, becoming comparable in the second week. For the rats administered BAS 440 l (Afidopyropen) for 14 days, there were no significant differences in either terminal bodyweights or bodyweight changes compared to the control rats.

Liver weights of treated animals were increased (1.1-fold), but not significantly, compared with control animals at 14 days administration of BAS 440 l (Afidopyropen) at 3000 ppm. Uteri weights and uterus to bodyweight ratios of treated animals were significantly decreased to 33% of the control values after 14 days administration of BAS 440 l (Afidopyropen).

In the treated rats, there was a statistically significant 1.6-fold increase in hepatic microsomal ethoxyresorufin-O-deethylation (EROD) (used as a marker for CYP1A) at day 14 following administration of BAS 440 l (Afidopyropen) at 3000 ppm in the diet. Estradiol-2-hydroxylation was similarly increased 1.7-fold. The increases in hepatic microsomal enzyme activities were accompanied by a statistically significant 4-fold increase in hepatic CYP1A1 mRNA. No increases in hepatic microsomal estradiol-4-hydroxylation or CYP1B1 expression were observed. Uterine CYP1A1 mRNA was significantly increased 57-fold in BAS 440 l (Afidopyropen) -treated rats. However, there were no changes in the expression of uterine CYP1B1.

Treatment of female F344 rats with BAS 440 l via the diet at 3000 ppm resulted in small increases in liver weight and liver/bodyweight ratio. These changes were accompanied by small increases in CYP1A1 mRNA expression, EROD activity and the 2-hydroxylation of estradiol. No effects were observed on the 4-hydroxylation of estradiol or CYP1B1 mRNA expression. In the uterus, BAS 440 l decreased uterine weight, while a moderate induction of CYP1A1 mRNA was observed. There were no increases in CYP1B1 mRNA expression.

II. MATERIALS AND METHODS
Test Type: subacute
Limit Test: No
Test Guideline: Non-guideline study
Deviations from Guideline: not applicable
Principles of Method if other Than Guideline: Not applicable
GLP Compliance: Non-glps study

Test Material:

Test Material Equivalent to Submission Substance Identity: Yes
Test Material Identity: ME5343 technical (BAS 440 I)
Details on Test Material:
Description: Pale yellow green powder

Test Animals:

Species: Rat
Strain: Fischer rats (F344)
Sex: Female

Details On Test Animals and Environmental Conditions:

Test Animals: Source: Harlan UK Limited, Shaw’s Farm, Blackthorn, Bicester, Oxon, England, OX25 1TP
Age at study initiation: 8-9 weeks at dosing
Weight at dosing: 142.94-150.71 g
Fasting period before study: Not reported
Housing: Rats were housed five per cage on sawdust in solid-bottom, polypropylene cages
Diet: powdered RM1 diet ad libitum for the duration of the study
Water: Drinking water was provided ad libitum prior to and throughout the study.
Acclimation period: 5 days

Environmental Conditions: Temperature (°C): 19-23°C
Humidity (%): 40-70%
Air changes (per hr): 14-15 or more changes per hour
Photoperiod (hrs dark/hrs light): 12 hours/day

In-life Dates: In life dates: 27 April 2015 to 11 May 2015

Administration/Exposure:

Route Of Administration: Oral: feed
Vehicle (and/or positive control): Unchanged (no vehicle)
Details on Exposure:
Preparation of dosing solutions: None

Diet Preparation
RM1 powdered diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) was used. The Medical School Resource Unit (MSRU) holds the specification of the diet. The Sponsor requested the dietary route of administration. A single batch of diet was prepared at the start of the study to last the duration of the study. The test diet containing BAS 440 I (Afidopyropen) at a nominal concentration of 3000 ppm was prepared by CXR Biosciences (in accordance with CXR Laboratory Method Sheets (LMS) VIVO-001) and samples were retained, in glass vials, at room temperature and were kept in the dark. These samples of diet were analysed for achieved concentration and homogeneity, the details of which are detailed in this report. Rats were fed RM1 powdered diet or test diet *ad libitum* throughout the study.
The BAS 440 I (Afidopyropen) powdered RM1 diet was prepared without purity correction and was stored at ambient temperature, in the dark, throughout the study.

Analytical Verification of Doses or Concentrations: Yes

Details on Analytical Verification of Doses or Concentrations:
Based on analytical results it was concluded that BAS 440 I (Afidopyropen) is stable in diet over a period of 32 days at ambient temperature. Sampling for analysis was performed as follows: Immediately after preparation, triplicate approximately 2 g aliquots were taken from the top, middle and bottom of the diet prepared at a nominal 3000 ppm BAS 440 I (Afidopyropen). Samples were retained in glass vials, stored in the dark at room temperature, and analysed for achieved concentration and homogeneity. The details of which are detailed in the Study Report. Rats were fed RM1 powdered diet or test diet *ad libitum* throughout the study.

Duration of Treatment/Exposure: About 14 days
Frequency of Treatment: Daily in the diet.
Doses/Concentrations: Target dose levels of 0, 3000 ppm
Basis: Nominal in diet
No. of Animals per Dose Group: 5 rats were assigned to each group.
Control Animals: Yes; concurrent no treatment
Details on Study Design: None
Positive Control: No

Examinations:
Observations and Examinations Performed and Frequency:

Cage Side Observations: Yes
Detailed Clinical Observations: Yes
Prior to the start of the study, all rats were observed to ensure they were physically normal and exhibited normal activity. Each rat was
observed at least once daily during the study. Daily clinical observations were recorded.

Body Weight: Yes

The bodyweight of each rat was recorded at the start of the study. The animals were weighed at least weekly. All animals were weighed prior to termination.

Food consumption and compound intake (if feeding study):

Food consumption was measured weekly.

Water Consumption: No water consumption data were recorded.

Ophthalmoscopic Examination: No

Specific ophthalmoscopic examination was not reported.

Hematology: Yes

Venous blood was taken post mortem via cardiac puncture and dispensed into lithium/heparin-coated tubes. The tubes were mixed on a roller for 10 min then cooled on ice. Red blood cells were removed by centrifugation (2,000 rpm for 10 min at 8 – 10°C), then the supernatant (plasma) was transferred to a second tube and stored at approximately -70 °C for possible future use.

Anaesthetic used for blood collection: Not applicable (post mortem)

Animals fasted: Not Applicable

How many animals: All surviving animals

Clinical Chemistry: Not Applicable

Urinalysis: Not Applicable

Sacrifice and Pathology:

On the day of termination the rats were weighed, then transferred to the post mortem room. Individual bodyweights were recorded manually, then transposed to an excel spread sheet, with a copy kept in the Study Folder. The rats were killed by exposure to a rising concentration of CO₂.

Each liver was weighed. Individual liver weights were recorded manually, and then transposed to an Excel spread sheet, with a copy kept in the Study Folder. Two pieces of liver (5 mm³) were removed from the left lobe for TaqMan® analysis. These liver pieces were placed in the same cryovial, flash frozen in liquid nitrogen and then stored at approximately -70°C until required for analysis of CYP1A1 and CYP1B1 gene expression.

Approximately 2 grams of liver were cut into chunks, snap frozen in liquid nitrogen and stored at -70°C for possible future use.
The remaining liver was weighed and scissor-minced in ice-cold 1.15% (w/v) KCl prior to processing according to CXR LMS Cent-001. Microsomes were isolated and stored at approximately -70°C until required for enzyme activity assays and protein determinations.

Uteri were removed and weighed. Individual uteri weights were recorded manually, then transposed to an Excel spread sheet, with a copy kept in the Study Folder. Four pieces of uterus (approximately 5 mm³) were removed for TaqMan® analysis. These uterus pieces were placed in the same cryovial, flash frozen in liquid nitrogen and then stored at approximately -70°C until required, for analysis of CYP1A1 and CYP1B1 gene expression.

Biochemical Measurements

Protein determination

The protein concentration of the liver microsomes was determined in aqueous solutions using a modification of the method of Lowry et al., (1951) and bovine serum albumin standards.

Cytochrome P450 activity assays

Microsomal ethoxyresorufin-O-deethylolation (EROD) was used as a marker for CYP1A activity and was measured according to LMS Fluor-0002 and EQ-001 (Burke et al., 1985).

Microsomal estradiol hydroxylation was used as a marker for CYP1A/CYP1B activity, and was measured by the formation of 2-hydroxy- and 4-hydroxy-estradiol and determined by UV-HPLC.

Taqman® analysis

RNA was extracted from rat liver according to LMS DNAI-008. cDNA was synthesized from all available RNA samples. TaqMan® analysis was performed on all available samples using primers specific for CYP1A1, and CYP1B1 (Assay-on-demand kits, Applied Biosystems). Rat B-actin was used as the internal standard (Assay-on-demand kit, Applied Biosystems). Data were analyzed by generation of generation of threshold cycle (CT) and delta CT values for all genes.

TaqMan® Genomic Assays used

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Catalogue Number</th>
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<tr>
<td>Rat CYP1A1</td>
<td>Rn01418021_g1</td>
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<tr>
<td>Rat CYP1B1</td>
<td>Rn00564055_m1</td>
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</table>
Statistics: Statistical comparisons between BAS 440 I (Afidopyropen)-treated rats and their control group were undertaken for all numerical data sets using a 2-tailed Student's t-test.

Any Other Information on Materials and Methods Incl. Tables: None

III. RESULTS AND DISCUSSION

Effect Levels:

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Effect Level</th>
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<tbody>
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</table>

Basis for Effect Level/Remarks: Not applicable

Observations:

Clinical Signs and Mortality: No effects
Body Weight and Weight Gain: Yes
Food Consumption and Compound Intake (if feeding study): Yes
Food Efficiency: No effects
Water Consumption and Compound Intake: No effects
Ophthalmoscopic examination: No effects
Hematology: Not applicable
Clinical Chemistry: Not applicable
Urinalysis: Not applicable
Neurobehaviour: Not reported
Organ Weights: Yes
Gross Pathology: Not applicable
Histopathology: Not applicable

Details on Results:

Clinical Signs and Mortality:
No treatment related clinical signs of toxicity were observed throughout the study. No mortality was observed in this study.

Water Consumption and Compound Intake:
No test-substance influence on water consumption was observed. During week 1 the control rats consumed 85.5 g/kg bodyweight/day compared to 51.2 g/kg bodyweight/day consumed by the BAS 440 I (Afidopyropen)-treated rats. During week 2 the control rats consumed 79.7 g/kg bodyweight/day compared to 81.5 g/kg bodyweight/day consumed by the BAS 440 I (Afidopyropen)-treated rats.
Neurobehaviour:
Not reported

Ophthalmoscopy
Not reported

Body weight and weight gain:
For the rats administered BAS 440 I (Afidopyropen), there were no significant differences in either terminal bodyweights or bodyweight changes at 14 days compared to the controls.

Figure 10: Bodyweight effects throughout treatment of BAS 440 I (Afidopyropen) administered to female F344 rats via the diet for 14 days

Food and Water Consumption, Food Efficiency and Compound Intake
No clear treatment-related effects on food consumption were observed.
Table 18: Average food consumption of rats administered BAS 440 I for 14 days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Female rats Control (0 ppm) 14 days</th>
<th>Female rats BAS 440 I (3000 ppm) 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet consumed g/kg bwt/day</td>
<td>85.5</td>
<td>51.2</td>
</tr>
<tr>
<td>mg/kg dosed/day</td>
<td>N/A</td>
<td>152.1</td>
</tr>
<tr>
<td>Diet consumed g/kg bwt/day</td>
<td>79.7</td>
<td>81.5</td>
</tr>
<tr>
<td>mg/kg dosed/day</td>
<td>N/A</td>
<td>242.2</td>
</tr>
<tr>
<td>Diet consumed g/kg bwt/day</td>
<td>82.6</td>
<td>66.3</td>
</tr>
<tr>
<td>mg/kg dosed/day</td>
<td>N/A</td>
<td>197.2</td>
</tr>
</tbody>
</table>

No overt changes of water consumption were noted.

An actual mean daily test substance intake for the 3000 ppm dose group was 197.2 mg/kg bw.

**Hematological findings:** Not Applicable.

**Clinical chemistry:** Not Applicable.

**Urinalysis:** Not Applicable

**Organ Weights:**

Liver weights of rats following 14 days administration of BAS 440 I (Afidopyropen) at 3000 ppm were increased, but not significantly, compared with control animals. These increased liver weights were also reflected in the liver to bodyweight ratios, which were significantly higher (1.1-fold increase, p<0.05) in the BAS 440 I (Afidopyropen)-treated group, compared to the control group, after 14 days administration.

Uteri weights and uterus to bodyweight ratios of treated animals were significantly decreased to 33% of the control values after 14 days administration of BAS 440 I (Afidopyropen).
Table 19: Selected mean absolute and relative organ weights of rats administered BAS 440 I for 14 Days

<table>
<thead>
<tr>
<th>Organ</th>
<th>Dose (ppm)</th>
<th>Absolute weight</th>
<th>% of Control</th>
<th>Relative weight</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>0</td>
<td>5.93 ± 0.58</td>
<td>-</td>
<td>3.68 ± 0.16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>6.52 ± 0.39</td>
<td>110%</td>
<td>4.10 ± 0.26*</td>
<td>111%</td>
</tr>
<tr>
<td>Uterus (g)</td>
<td>0</td>
<td>0.47 ± 0.23</td>
<td>-</td>
<td>0.29 ± 0.15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>0.15 ± 0.03*</td>
<td>33%</td>
<td>0.10 ± 0.02*</td>
<td>33%</td>
</tr>
</tbody>
</table>

Biochemical Results

Administration of BAS 440 I (Afidopyropen) at 3000 ppm for 14 days resulted in a small, 1.6-fold, increase in hepatic microsomal ethoxyresorufin-O-deethylation (EROD) activity. Similarly, hepatic microsomal estradiol-2-hydroxylation was increased 1.7-fold. The formation of 4-hydroxyestradiol was not observed in either control microsomes or microsomes isolated from BAS 440 I (Afidopyropen)-treated rats.

Table 20: Biochemical measurements for BAS 440 I (Afidopyropen) administered to female F344 rats via the diet for 3 and 7 days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Female rats Control (0 ppm) 14 days</th>
<th>Female rats BAS 440 F (3000 ppm) 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD (pmols resorufin formed/min/mg protein)</td>
<td>26.44 ± 2.34a (100.0 ± 8.86)</td>
<td>43.15 ± 3.33*** (163.20 ± 12.61)</td>
</tr>
<tr>
<td>Estradiol hydroxylation (pmols 2-hydroxyestradiol formed/min/mg protein)</td>
<td>161.45 ± 19.45 (100.00 ± 12.05)</td>
<td>280.85 ± 30.07*** (173.96 ± 18.63)</td>
</tr>
</tbody>
</table>

a Values are Mean ± SD (n=5 per group). Values in parenthesis are mean % control ± SD for the appropriate group. A Student's t-test was performed on the results; *** statistically different from control p<0.001

Taqman® analysis of rat CYP1A1 and CYP1B1 mRNA

BAS 440 I (Afidopyropen) administered for 14 days increased hepatic CYP1A1 mRNA 3.9-fold. CYP1B1 mRNA expression was not significantly increased.

In uteri, BAS 440 I (Afidopyropen) induced CYP1A1 mRNA by 57-fold but did not induce CYP1B1 mRNA levels
Table 21: Taqman® analysis of rat hepatic and uterine CYP1A1 and CYP1B1 mRNA from female F344 rats administered BAS 440 I (Afidopyropen) via the diet for 14 days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Female rats Control (0 ppm) 14 days</th>
<th>Female rats BAS 440 F (3000 ppm) 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic CYP1A1</td>
<td>1.00 ± 0.46a</td>
<td>3.93 ± 2.77**</td>
</tr>
<tr>
<td>Hepatic CYP1B1</td>
<td>1.00 ± 0.32</td>
<td>2.02 ± 0.66</td>
</tr>
<tr>
<td>Uterine CYP1A1</td>
<td>1.00 ± 0.38a</td>
<td>57.35 ± 38.00***</td>
</tr>
<tr>
<td>Uterine CYP1B1</td>
<td>1.00 ± 0.41</td>
<td>0.86 ± 0.16</td>
</tr>
</tbody>
</table>

*a Values are Mean ± SD (n=5 per group). Results are expressed as fold change relative to control, where control values were normalised to 1.00. Rat β-actin was employed as the internal control. A Student’s t-test was performed on the results; ** statistically different from control p<0.01; *** p<0.001.

Any Other Information on Results Incl. Tables: None

IV. OVERALL REMARKS, ATTACHMENTS

Treatment of female F344/DuCrIcrj rats with Afidopyropen via the diet at 3000 ppm did not induce an increase in the CYP1B1 activity and no significant increase in the level of 4-hydroxyestradiol was detected. The elevated CYP1A1 mRNA levels and as well the slightly increase EROD activity are not representative for a prototypical Aryl-Hydrocarbon Receptor (AHR)-inducer (EROD induction >100 fold (e.g. NTP reports 520, 521, 525, 529, 525, 530, 531) mRNA >1000 fold).

V. APPLICANT’S SUMMARY AND CONCLUSION

Conclusion:

Treatment of female F344 rats with BAS 440 I via the diet at 3000 ppm resulted in small increases in liver weight and liver/bodyweight ratio. These changes were accompanied by small increases in CYP1A1 mRNA expression, EROD activity and the 2-hydroxylation of estradiol. No effects were observed on the 4-hydroxylation of estradiol or CYP1B1 mRNA expression. In the uterus, BAS 440 I decreased uterine weight, while a moderate induction of CYP1A1 mRNA was observed. There were no increases in CYP1B1 mRNA expression.
Executive Summary

The purpose of this study was to test two compounds Afidopyropen (09/0676-1) and M440I002 (15/0197-1) in a field-stimulated rabbit ear artery tissue bioassay with the D2 receptor.

These compounds were tested using two different protocols
1) In a repeat of a prior bioassay experiment (2015/1117539) the two compounds (Afidopyropen (09/0676-1) and M440I002 (15/0197-1) were tested at three concentrations in duplicate followed by a treatment with the dopamine antagonist (-)sulpiride.

2) Afidopyropen (09/0676-1) and M440I002 (15/0197-1) were tested at three concentrations in duplicate after a 20-minute pre-treatment with solvent or (-)sulpiride at 3.0E-06 M.

The results of the first protocol confirmed earlier observed results: there was a concentration-dependent decrease in the twitch contraction amplitude, which was not blocked with further addition of (-)sulpiride at 3.0E-06 M. This is a repeat of the finding observed in the initial field-stimulated rabbit ear artery assay (2015/1117539) – i.e. dopamine agonism that was not reversed after addition of the dopamine antagonist (-)sulpiride.

In the second protocol: after a 20-minute pre-treatment with (-)-sulpiride at 3.0E-06 M, Afidopyropen (09/0676-1) and M440I002 (15/0197-1) induced a concentration-dependent decrease in the twitch contraction amplitude that was right shifted when compared their effect after solvent pre-treatment (see. This demonstrated a dopamine agonist response that was reduced by pre-treatment with a dopamine antagonist

Addition of (-)-sulpiride 20 minutes before treatment with Afidopyropen and M440I002 decreases the dopamine agonist effect, while addition of (-)-sulpiride after addition of the Afidopyropen and M440I002 has little effect.
In conclusion, Afidopyropen (09/0676-1) M440I002 (15/0197-1) behave as agonists at the D2 receptor in the field-stimulated rabbit ear artery tissue bioassay assay.

II. MATERIAL AND METHODS

A. MATERIALS

1. Test materials

<table>
<thead>
<tr>
<th>Test Substance Name</th>
<th>BAS 440 I (Afidopyropen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonym:</td>
<td>BAS 440 I (ME5343 Technical), 09/0676-1</td>
</tr>
<tr>
<td>BASF Substance Number</td>
<td>09/0676-1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Substance Name</th>
<th>Reg. No. 5741532 (Metabolite of BAS 440 I, Afidopyropen, M440I002)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Substance Manufacturer:</td>
<td>BASF SE</td>
</tr>
<tr>
<td>BASF Substance Number</td>
<td>15/0197-1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Client Compound ID</th>
<th>Compound ID</th>
<th>Reference Number</th>
<th>Batch Number</th>
<th>FW</th>
<th>MW</th>
<th>Purity</th>
<th>Received Form</th>
<th>Stock solution</th>
<th>Flag</th>
</tr>
</thead>
<tbody>
<tr>
<td>09/0676-1</td>
<td>100024764-1</td>
<td>-</td>
<td>60722</td>
<td>563.7</td>
<td>-</td>
<td>100.0</td>
<td>Powder</td>
<td>1 E-02 M DMSO</td>
<td>-</td>
</tr>
<tr>
<td>09/0676-1 + (-)-Subside (3.0E-08 M)</td>
<td>100024764-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Powder</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15/0197-1</td>
<td>100024764-3</td>
<td>L02-67</td>
<td>525.6</td>
<td>100.0</td>
<td>Powder</td>
<td>1 E-02 M DMSO</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>15/0197-1 + (-)-Subside (3.0E-08 M)</td>
<td>100024764-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Powder</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*FW: Formula Weight, MW: Molecular Weight*
B. METHODS

Tissue bioassay with the D2 receptor: field stimulated rabbit ear artery; agonist and antagonist activity

Source rabbit ear artery (field-stimulated)
Reference agonist quinpirole
Response inhibition of twitch contraction
Reference antagonist (-)sulpiride

Rings of rabbit ear artery were suspended in 20-ml organ baths filled with an oxygenated (95% O₂ and 5% CO₂) and pre-warmed (37°C) physiological salt solution of the following composition (in mM): NaCl 118.0, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 11.0 (pH 7.4).

Yohimbine (1 µM) and propranolol (1 µM) were also present throughout the experiments to block the α₂- and β-adrenergic receptors, respectively.

The tissues were connected to force transducers for isometric tension recordings. They were stretched to a resting tension of 1 g then allowed to equilibrate for 30 min during which time they were washed repeatedly and the tension readjusted. Thereafter, they were stimulated electrically with 10-s train pulses (minimal intensity required to trigger maximal contractions, 1 ms duration, 5 Hz), delivered at 2-min intervals by a constant current stimulator.

The experiments were carried out using semi-automated isolated organ systems possessing eight organ baths, with multichannel data acquisition.

The parameter measured is the maximum change in the amplitude of electrically-evoked twitch contractions induced by each compound concentration.

Evaluation of agonist activity
For agonist activity measurement, the tissues were exposed to a submaximal concentration of the reference agonist (-)quinpirole (0.3 µM) to verify responsiveness and to obtain a control response.

Following washings and recovery of the control twitch contractions, the tissues were exposed to increasing concentrations of the test compound or the same agonist. The concentrations were added cumulatively and each was left in contact with the tissues until a stable response was obtained or for a maximum of 20 min.

If an agonist-like response (decrease in twitch contraction amplitude) was obtained, the reference antagonist (-)sulpiride (3 µM) was tested against the highest concentration of the compound to confirm the involvement of the D₂ receptor in this response.
Evaluation of the specificity of the agonist activity

The tissues are exposed to a submaximal concentration of the reference agonist (-) quinpirole (0.3 μM) to verify responsiveness and to obtain a control response.

Following washings and recovery of twitch contractions, the tissues are exposed to (-) sulpiride (3 μM) or solvent for 20 min, then increasing concentrations of the test compound or the same agonist. The different concentrations are added cumulatively and each left in contact with the tissues until a stable response is obtained or for a maximum of 20 min.

In the case of solvent pre-treatment, the reference antagonist (-) sulpiride (3 μM) was tested at the end of the experiment against the highest concentration of the compound to confirm the involvement of the D2 receptor in this response.

C. Analysis

In Vitro pharmacology: Tissue Bioassays.

The results are expressed as a percent of the control agonist response.

When at least 6 compound concentrations are tested, the EC50 value (concentration producing a half-maximum response) or IC50 value (concentration causing a half-maximum inhibition of the response to the reference agonist) are determined by linear regression analysis of the concentration-response curves.

III. Results and Discussion

A. Results

In the field-stimulated rabbit ear artery, the D2 receptor agonist (-) quinpirole induced a concentration-dependent decrease in the twitch contraction amplitude which was inhibited by the antagonist (-) sulpiride.

Solvent or (-) Sulpiride (3.0E-06M) did not affect the twitch contraction amplitude.

After a 20-minute pre-treatment with solvent, Afidopyropen (09/0676-1) and M4401002 (15/0197-1) induced a concentration-dependent decrease in the twitch contraction amplitude which was not blocked with further addition of (-) sulpiride at 3.0E-06 M.

After a 20-minute pre-treatment with (-) sulpiride at 3.0E-06 M, Afidopyropen (09/0676-1) and M4401002 (15/0197-1) induced a concentration-dependent decrease in the twitch contraction amplitude that was right shifted when compared their effect after solvent pre-treatment.

These results confirm previous data obtained with 09/0676-1 and 15/0197-1 and the non-reversal of their effect with (-) sulpiride when added at the end of the experiment.
The results indicate the importance of the (-)sulpiride addition protocol to reveal the specificity of the Afidopyropen (09/0676-1) and M440I002 (15/0197-1) response since addition of (-)sulpiride 20 minutes before Afidopyropen (09/0676-1) and M440I002 (15/0197-1) decreases their effect.

Afidopyropen (09/0676-1) and M440I002 (15/0197-1) behave as agonists at the D2 receptor in the field-stimulated rabbit ear artery assay.

Table 22. Afidopyropen (09/0676-1) and M440I002 (15/0197-1); field stimulated rabbit ear artery tissue bioassay with the D2 receptor

<table>
<thead>
<tr>
<th>Agonist Effect</th>
<th>Test Compound ID.</th>
<th>Test Concentration (M)</th>
<th>% of control</th>
<th>1st Response</th>
<th>2nd Response</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Solvent (DMSO)</td>
<td></td>
<td></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100024784-1</td>
<td>09/0676-1</td>
<td>1.0E-07</td>
<td>15</td>
<td>18</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>100024784-1</td>
<td>09/0676-1</td>
<td>1.0E-06</td>
<td>41</td>
<td>53</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>100024784-1</td>
<td>09/0676-1</td>
<td>1.0E-05</td>
<td>81</td>
<td>80</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>+(-)Sulpiride</td>
<td></td>
<td>3.0E-06</td>
<td>90</td>
<td>82</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(-)Sulpiride</td>
<td></td>
<td>3.0E-06</td>
<td>0</td>
<td>-3</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>100024784-2</td>
<td>09/0676-1+(-)Sulpiride (3.0E-06M)</td>
<td>1.0E-07</td>
<td>7</td>
<td>4</td>
<td></td>
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</tr>
<tr>
<td>100024784-2</td>
<td>09/0676-1+(-)Sulpiride (3.0E-06M)</td>
<td>1.0E-06</td>
<td>25</td>
<td>14</td>
<td>20</td>
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<td>100024784-2</td>
<td>09/0676-1+(-)Sulpiride (3.0E-06M)</td>
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</tr>
<tr>
<td>+(-)Sulpiride</td>
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<td>3.0E-06</td>
<td>not tested</td>
<td>not tested</td>
<td>not tested</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Solvent (DMSO)</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100024784-3</td>
<td>15/0197-1</td>
<td>1.0E-07</td>
<td>12</td>
<td>7</td>
<td>10</td>
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</tr>
<tr>
<td>100024784-3</td>
<td>15/0197-1</td>
<td>1.0E-06</td>
<td>36</td>
<td>29</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>100024784-3</td>
<td>15/0197-1</td>
<td>1.0E-05</td>
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<td>58</td>
<td>61</td>
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<tr>
<td>+(-)Sulpiride</td>
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<td>3.0E-06</td>
<td>64</td>
<td>61</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(-)Sulpiride</td>
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<td>3.0E-06</td>
<td>-5</td>
<td>0</td>
<td>-3</td>
<td></td>
</tr>
<tr>
<td>100024784-4</td>
<td>15/0197-1+(-)Sulpiride (3.0E-06M)</td>
<td>1.0E-07</td>
<td>0</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100024784-4</td>
<td>15/0197-1+(-)Sulpiride (3.0E-06M)</td>
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<td>6</td>
<td>11</td>
<td>9</td>
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</tr>
<tr>
<td>100024784-4</td>
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<td>(-)Quinpirole</td>
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<td>1.0E-07</td>
<td>67</td>
<td>66</td>
<td>67</td>
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<tr>
<td>(-)Quinpirole</td>
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<td>3.0E-07</td>
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<td>+(-)Sulpiride</td>
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<td>3.0E-06</td>
<td>29</td>
<td>5</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

The results are expressed as a percent of the control response to (-)Quinpirole (decrease in twitch contraction amplitude) (mean values; n=2)
Figure 11: Refined protocol; field stimulated rabbit ear artery assay results. D2 receptor (2015/1204930). Results are shown as a percentage of the response (decreased twitch contraction) of prototypical dopamine agonist (-)Quinpirole. (-)Sulpiride is a dopamine antagonist that was administered at a concentration of 3E-6 M.
IV. Overall Remarks

None

V. Summary and Conclusion

This study tested Afidopyropen (09/0676-1), and M440I002 (15/0197-1) field stimulated rabbit ear artery tissue bioassay with the D2 receptor.

The results of the first protocol confirmed earlier observed results: there was a concentration-dependent decrease in the twitch contraction amplitude, which was not blocked with further addition of (-)sulpiride at 3.0E-06 M. This is a repeat of the finding observed in the initial field-stimulated rabbit ear artery assay (2015/1117539) – i.e. dopamine agonism that was not reversed after addition of the dopamine antagonist.

In the second protocol: after a 20-minute pre-treatment with (-)sulpiride at 3.0E-06 M, Afidopyropen (09/0676-1) and M440I002 (15/0197-1) induced a concentration-dependent decrease in the twitch contraction amplitude that was right shifted when compared their effect after solvent pre-treatment (see. This demonstrated a dopamine agonist response that was reduced by pre-treatment with a dopamine antagonist.

Addition of (-)sulpiride 20 minutes before treatment with Afidopyropen and M440I002 decreases the dopamine agonist effect, while addition of (-)sulpiride after addition of the Afidopyropen and M440I002 has little effect.

In conclusion, Afidopyropen (09/0676-1) M440I002 (15/0197-1) behave as agonists at the D2 receptor in the tissue bioassay assay.
Study 10  [$^{14}$C] BAS 440 I: Study on absorption, distribution, metabolism and excretion in the F344 rat (Japanese clone) after combined dietary and oral administration.

Executive Summary

This report outlines the results of investigations on the plasma kinetics as well as absorption, distribution, and elimination of $^{14}$C-BAS 440 I in female F344 rats. This was a mechanistic study conducted with the primary intent of measuring the pharmokinetic properties of BAS 440 I in the strain and sex of rat as well as the doses that were relevant to the rat carcinogenicity studies conducted with BAS 440 I. The protocol of this study was discussed with the US EPA on February 3, 2015.

Another important aspect of this study was that this study measured the pharmacokinetic properties of a major metabolite M440I060 in the rat.

Plasma kinetics were investigated after 14 days of dietary intake followed with a single oral gavage administration of the radiolabeled test substance on Day 15. Target dose levels were 3, 15 and 50 mg/kg bw. At least 4 animals per dose group were included. Blood samples (approximately 300 µL) were taken on Day 1 [predose] and on Day 15 [predose, 1, 2, 4, 8, 24, 48 and 72 h after oral dosing]. The concentrations of the radioactive residues in plasma were analyzed by LSC.

In addition to measuring traditional PK parameters (related to total radioactivity measured in plasma – that does not allow to differentiation between parent compound and/or its metabolites), this study measured PK parameters in the plasma of four Afidopyropen-related residues. To obtain the maximum detection sensitivity as well as to accurately judge the impact of repeated dosing, analysis was conducted via LC/MS. The four residues analyzed were Afidopyropen, M440I017, M440I001 and M440I060.
Urine and feces were collected in the same animals in 8-24, 24-48 and 48-72 hour time intervals. The time points for the sampling of organs / tissues selected for the tissue distribution experiments were based on the results of the plasma kinetics and corresponded to the time points of the maximum plasma concentrations. Rats were sacrificed at 1 and 1.5 h for respectively 15 and 50 mg/kg bw BAS 440 I. After sacrifice, blood, liver and uterus were prepared and the radioactive residues of 14C-BAS 440 I were determined by LSC analyses after appropriate sample workups. These experiments allowed the evaluation of the distribution of the test substance into organs and tissues under the situation of defined plasma / blood concentrations.

In plasma kinetics, AUC values indicated an internal exposure that is clearly correlated to the dosing regimen of 14C-BAS 440 I. The AUC values of BAS 440 I increased with increasing dose level in an un-proportional manner. From 3 to 15 mg/kg the AUC was increased 13.5x, and for a 17x increase in dose (3 to 50 mg/kg) the AUC increased 53x.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Dose [mg/kg bw]</th>
<th>C_{max} [ng Eq/g]</th>
<th>t_{max} [h]</th>
<th>t_{last} [h]</th>
<th>terminal half-life [h]</th>
<th>AUC_{0-\text{last}} [ng Eq* h/g]</th>
<th>AUC_{0-\infty} [ng Eq* h/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>3, p.o.</td>
<td>262</td>
<td>1</td>
<td>24</td>
<td>8.17</td>
<td>728</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>2800</td>
<td>1</td>
<td>24</td>
<td>5.19^{*}</td>
<td>10400</td>
<td>10800^{*}</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>6980</td>
<td>1-2^{k}</td>
<td>24</td>
<td>4.21</td>
<td>41400</td>
<td>42300</td>
</tr>
<tr>
<td>Blood</td>
<td>3, p.o.</td>
<td>221</td>
<td>1</td>
<td>72</td>
<td>96.5^{*}</td>
<td>1600</td>
<td>3290^{*}</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>2110</td>
<td>1</td>
<td>72</td>
<td>55.2^{*}</td>
<td>11700</td>
<td>15500^{*}</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>5050</td>
<td>1-2^{k}</td>
<td>72</td>
<td>NR</td>
<td>43100</td>
<td>NR</td>
</tr>
</tbody>
</table>

*approximation; ^{k}range; NR not reported

In plasma kinetics of BAS 440 I and its metabolites (bioanalytical data), the AUC values of BAS 440 I increased with increasing dose level in an unproportional manner. From 3 to 15 mg/kg BAS 440 I the AUD was increased 44x. And for a 17x increase in dose (3 to 50 mg/kg) the AUC0-4 increased 199x. BAS 440 I and the metabolites M440I001 and M440I017 displayed comparable concentration time curves, while the metabolite M440I060 displayed a plateau level with a minor decrease after repeated administration.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Dose [mg/kg bw]</th>
<th>$c_{max}$ [ng/mL]</th>
<th>$t_{max}$ [h]</th>
<th>$t_{last}$ [h]</th>
<th>terminal half-life [h]</th>
<th>AUC$_{0-24}$ [ng*h/mL]</th>
<th>AUC$_{0-24}$ [ng*h/mL]</th>
<th>AUC [ng*h/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAS 4401</td>
<td>3, p.o.</td>
<td>24.7</td>
<td>1</td>
<td>4</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>1500</td>
<td>1</td>
<td>24</td>
<td>2.71</td>
<td>4470</td>
<td>4480</td>
<td>4530</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>4750</td>
<td>2</td>
<td>24</td>
<td>2.17</td>
<td>20600</td>
<td>20700</td>
<td>20700</td>
</tr>
<tr>
<td>M4401001</td>
<td>3, p.o.</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>72.5</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>153</td>
<td>1</td>
<td>24</td>
<td>4.41</td>
<td>576</td>
<td>590</td>
<td>635</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>457</td>
<td>2</td>
<td>24</td>
<td>3.59</td>
<td>2600</td>
<td>2630</td>
<td>2690</td>
</tr>
<tr>
<td>M4401017</td>
<td>3, p.o.</td>
<td>3.91</td>
<td>1</td>
<td>4</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>78.2</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>51.6</td>
<td>1</td>
<td>24</td>
<td>3.84</td>
<td>431</td>
<td>438</td>
<td>482</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>175</td>
<td>2</td>
<td>24</td>
<td>3.76</td>
<td>1280</td>
<td>1300</td>
<td>1350</td>
</tr>
<tr>
<td>CPCA-carnitine</td>
<td>3, p.o.</td>
<td>195</td>
<td>1</td>
<td>72</td>
<td>36.1*</td>
<td>2780</td>
<td>6300*</td>
<td>4930</td>
</tr>
<tr>
<td>M4401060</td>
<td>15, p.o.</td>
<td>1560</td>
<td>8</td>
<td>72</td>
<td>46.6*</td>
<td>22600</td>
<td>50200*</td>
<td>36200</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>4190</td>
<td>8</td>
<td>72</td>
<td>27.1*</td>
<td>71400</td>
<td>149000*</td>
<td>122000</td>
</tr>
</tbody>
</table>

*: approximation; n/c could not be calculated
Over an observation period of about 72 hours, respectively 0.9%, 1.3% and 1.6% of the dose at 3, 15, and 50 mg/kg was excreted via urine. Excretion via faeces was significantly higher and amounted for a period of about 72 hours to 85 %, 90 % and 65 % of the dose at respectively 3 mg/kg bw, 15 mg/kg bw and 50 mg/kg bw. The major part of faecal excretion occurred for all groups within 8-48 hours. Tissue sampling demonstrated distribution to the liver and, to a lesser extent, the uterus.

Cursory metabolic profiling (no metabolite ID) of each matrix was also conducted, and this data is detailed in the study report.

II. MATERIAL AND METHODS

A. MATERIALS

Test Material Equivalent to Submission Substance Identity:  Yes

Test Material  BAS 440 I (Addipropen)

Description:  $^{14}$C BAS 440 I, Solution in acetonitrile

Batch/purity  Batch 1055-0201. Chemical purity 95.6 %; radiochemical purity 99.0%. Specific activity 3.7 MBq/mg; concentration in acetonitrile 4.47 mg/g (16.7 MBq/g solution)
<table>
<thead>
<tr>
<th>Test Material</th>
<th>Non-radiolabeled BAS 440 l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Yellow solid</td>
</tr>
<tr>
<td>Batch/purity</td>
<td>Batch 080722. Chemical purity 93.23%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Material</th>
<th>M440I060 15/0440-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>White powder</td>
</tr>
<tr>
<td>Batch/purity</td>
<td>Batch L82-166. Chemical purity 97.8%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Reg.No. 6045738 (M440I017, 15/0199-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Yellowish solid</td>
</tr>
<tr>
<td>Batch/purity</td>
<td>Batch L82-178. Chemical purity 94.8%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Reg.No. 5741530 (M440I001, AS1561)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Yellowish solid</td>
</tr>
<tr>
<td>Batch/purity</td>
<td>L82-66</td>
</tr>
</tbody>
</table>

**Vehicle and/or positive control:** The non-radioactive dietary mixtures administered for the first 14 days were mixed once using powdered rodent diet (ground Kilba rat/mouse, Switzerland) and a premix. For the radioactive oral doses the acetonitrile was evaporated to dryness and then stock solutions were prepared in 0.5% carboxymethylcellulose in tap water.
Test animals

Species: Rat
Strain: F344 Rat (Japanese clone) (F344/DuCrIcrj)
Age: 13 – 16 weeks at dosing

B. STUDY DESIGN AND METHODS

1. Dates of work: 26-Jun-2015 to 11-Jan-2016 (end of analytical phase)

The study investigated the toxicokinetic parameters of adsorption, distribution, metabolism and excretion of BAS 440 I in the F344 rat after a 14-day dietary administration of BAS 440 I followed by an oral dose of ¹⁴C-BAS 4401 on Day 15.

After administration of the radio-active dose animals were place in macrolon cages and blood samples (300 µL) were taken from the tail vein into K₂-EDTA anticoagulant, as described in table 1. Blood, urine and faeces were collected form groups 1, 2 and 3. Groups 4 and 5 were sacrificed after dose administration and the liver and uterus were collected.

Plasma was separated from blood by centrifugation and radioactivity in plasma and urine was determined by solubilization/LSC. Faeces were homogenized and combusted prior to LSC. The liver and uterus were homogenized and solubilized using Hionoc Fluor as the scintillation fluid.

Quantification of BAS 440 I and three non-radiolabeled metabolites was conducted via LCMS to determine the PK properties of each of these molecules under the conditions of the study.

Metabolic profiling (not identification) was also conducted and is detailed in the study report.

Table 23: Experimental groups for each dose level

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Treatment</th>
<th>Sampling Times (h after dosing)</th>
<th>Sacrifice Time (h after last dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diet Days 1 – 14 (ppm)</td>
<td>Oral dose Day 15 (mg/kg bw)</td>
<td>Predose, Day 1 and Day 15</td>
</tr>
<tr>
<td>Excretion and kinetics</td>
<td></td>
<td></td>
<td></td>
<td>(A)</td>
</tr>
<tr>
<td>1</td>
<td>4 females</td>
<td>36</td>
<td>3</td>
<td>Predose, Day 1 and Day 15</td>
</tr>
<tr>
<td>2</td>
<td>4 females</td>
<td>600</td>
<td>50</td>
<td>Predose, Day 1 and Day 15</td>
</tr>
<tr>
<td>3</td>
<td>4 females</td>
<td>180</td>
<td>15</td>
<td>Predose, Day 1 and Day 15</td>
</tr>
<tr>
<td>Tissue distribution and metabolite profiling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4 females</td>
<td>600</td>
<td>50</td>
<td>Day 15 Predose</td>
</tr>
<tr>
<td>5</td>
<td>4 females</td>
<td>180</td>
<td>15</td>
<td>Day 15 Predose</td>
</tr>
</tbody>
</table>

n.a. = not applicable
(A) Urine and faeces was collected at 8- 24, 24-48 and 48-72 hours
(B) Liver and uterus removed for analysis
B. Kinetic calculations

Concentrations of radioactivity in blood and plasma were calculated as ng equivalents of BAS 440 I/g of sample, based on the specific activity of the radiolabelled BAS 440 I ([14C]-BAS 440 I) formulation. For each group, all toxicokinetic parameters were calculated from the curves constructed from individual animals using validated Phoenix WinNonlin 6.3. Non-compartmental analysis was applied. In addition the toxicokinetic parameters were calculated from the curves obtained from the bioanalytical results, BAS 440 I and 3 metabolites (constructed from the average values at each time point) using validated WinNonlin 6.3. Non-compartmental analysis was applied.

Parameters that were calculated include:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$</td>
<td>Maximum plasma concentration based on LSC-counts or bioanalytical results, i.e. not estimated based on curve-fitting</td>
</tr>
<tr>
<td>$t_{\text{max}}$</td>
<td>Time point at which maximum plasma concentration was reached, assessed directly from the data</td>
</tr>
<tr>
<td>$t_{\text{last}}$</td>
<td>Time point of last measurable plasma concentration.</td>
</tr>
<tr>
<td>$\text{AUC}_{\text{last}}$</td>
<td>Area under the plasma concentration-time curve from time of administration until the last measurable plasma concentration ($t_{\text{last}}$), calculated using the linear-logarithmic trapezoidal rule. Calculation of partial areas ($\text{AUC}_{0-24}$) was performed at the discretion of the study director.</td>
</tr>
<tr>
<td>$\text{AUC}_{\infty}$</td>
<td>Area under the curve after a single dose from time of administration until infinity, calculated as $\text{AUC}<em>{\text{last}} + C</em>{\text{last}}/\lambda_{z}$, where $C_{\text{last}}$ was the last measurable radioactive concentration. Extrapolations of more than 15% of the total AUC were reported as approximations.</td>
</tr>
<tr>
<td>$\text{AUD}$</td>
<td>Area under the data, calculated for the bioanalytical data (parent and 3 metabolites) using the linear-logarithmic trapezoidal rule by setting the reported LLOQ values after $C_{\text{max}}$ to the LLOQ value ($=0.75$ or $1.00$ ng/mL).</td>
</tr>
<tr>
<td>$\lambda_{z}$</td>
<td>Elimination rate constant, determined by linear regression of the terminal points of the log-linear concentration-time curve.</td>
</tr>
</tbody>
</table>
| $t_{1/2}$ | Elimination half-life, calculated as $\ln(2)/\lambda_{z}$. The following requirements had to be met for an acceptable calculation of $t_{1/2}$:  
  - at least three time points were used in the calculation  
  - correlation coefficient ($r_{2}$) was at least 0.9  
  - span of time points used in $t_{1/2}$ was at least twice the calculated value of $t_{1/2}$  
  Values that did not meet these criteria are reported as approximations. |

$C_{\text{max}}$ and $\text{AUC}$-values were also dose-normalized to a dose of 1 mg/kg bw. In addition the blood/plasma ratio was calculated.
C. Calculations

All calculations were performed with the complete numerical data. The numerical data were generally shown as rounded values (smaller degree of precision) to increase readability.

All results expressed in weight or concentration units are calculated with the assumption that the parent compound or metabolites have the same molecular mass. Since background radioactivity values of the particular matrices were not subtracted, radioactivity balances, mainly in the case of low concentrations, may exceed 100 %. The mean and standard deviation were used to characterize the data, where appropriate (i.e. radioactivity measurement, concentration, etc).

Cumulative amounts (expressed as percentage of the dose) excreted in urine and faeces were calculated by summation of the amounts excreted in the individual urine and faeces samples per collection period, respectively.

The following relationships were used for the calculation of results contained in the present report.

\[
\text{Total radioactivity concentration (TRR) in [mg/kg]} = \frac{\text{total counts per sample [Bq]}}{\text{sample weight [g] x spec. activity of dosed material [Bq/\mu g]}}
\]

\[
\text{Total radioactivity in [% of dose per matrix]} = \frac{\text{total counts per sample [Bq] x total matrix weight [g] x 100}}{\text{sample weight [g] x total dose [Bq]}}
\]

\[
\text{Relative peak area in HPLC elution profiles [% ROI]} = \frac{\text{area of particular peak x 100}}{\text{total peak area (sum of all ROIs)}}
\]

\[
\text{Concentration of metabolite [mg/kg]} = \frac{\text{relative amount of metabolite [% ROI] x concentration of radioactive residues [mg/kg]}}{100}
\]

\[
\text{Amount of metabolite in % of total radioactive residue [% TRR]} = \frac{\text{concentration of metabolite [mg/kg] x 100}}{\text{total radioactive residue [mg/kg] (TRR)}}
\]
III. RESULTS AND DISCUSSION

1. Concentration, homogeneity and stability of dosing suspensions
   The non-radiolabeled diets were 90-110% of the target concentration and confirmed to be homogeneous. The radiochemical purity of BAS 440 I in the dosing suspensions was 90.6 to 100%. Homogeneity of the dosing suspensions was confirmed by analysis of triplicate aliquots of each suspension (coefficient of variation ≤5%).

2. Mortality and clinical signs.
   There were no treatment-related clinical signs or mortality.

3. Body weight
   No abnormalities of the body weight were observed during the experiments.

4. Food Consumption
   No test substance related effect was noted on absolute food intake or on relative food consumption data (expressed as gram food/kg body weight/day).

5. Test substance intake

<table>
<thead>
<tr>
<th>Group</th>
<th>Nominal dietary dose [ppm]</th>
<th>Average intake [mg /kg bw/d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>600</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>51</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>14</td>
</tr>
</tbody>
</table>

6. Necropsy
   Necropsy was only conducted on groups 4 and 5. There were no treatment related changes observed.

7. Kinetics
   In plasma kinetics, AUC values indicated an internal exposure that is clearly correlated to the dosing regimen of \(^{14}\text{C}\)-BAS 440 I. The AUC values of increased with increasing dose level in an un-proportional manner. From 3 to 15 mg/kg the AUC was increased 13.5x and for a 17x increase in dose (3 to 50 mg/kg) the AUC increased 53x. In time, a distribution towards the red blood cells was noted. The kinetic data are presented in the following tables.
Table 24: Mean radioactivity concentrations in plasma of female rats having received an oral dose of 14C-BAS 440 I at 3, 15 or 50 mg/kg bw

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Plasma (Eq/g plasma)</th>
<th>Blood (Eq/g plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 mg/kg bw</td>
<td>15 mg/kg bw</td>
</tr>
<tr>
<td>1</td>
<td>262</td>
<td>2803</td>
</tr>
<tr>
<td>2</td>
<td>113</td>
<td>1942</td>
</tr>
<tr>
<td>4</td>
<td>38.0</td>
<td>1031</td>
</tr>
<tr>
<td>8</td>
<td>18.7</td>
<td>206</td>
</tr>
<tr>
<td>24</td>
<td>6.00</td>
<td>49.0</td>
</tr>
<tr>
<td>48</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td>72</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
</tbody>
</table>

Table 25: Pharmacokinetic parameters determined in female rats having received an oral dose of 14C-BAS 440 I at 3, 15 or 50 mg/kg bw

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Dose (mg/kg bw)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng Eq/g)</th>
<th>t&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>t&lt;sub&gt;last&lt;/sub&gt; (h)</th>
<th>terminal half-life (h)</th>
<th>AUC&lt;sub&gt;0-last&lt;/sub&gt; (ng Eq*h/g)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng Eq*h/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma</td>
<td>3</td>
<td>262</td>
<td>1</td>
<td>24</td>
<td>8.17</td>
<td>728</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2800</td>
<td>1</td>
<td>24</td>
<td>5.19*</td>
<td>10400</td>
<td>10800</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6980</td>
<td>1-2</td>
<td>24</td>
<td>4.21</td>
<td>41400</td>
<td>42300</td>
</tr>
<tr>
<td>blood</td>
<td>3</td>
<td>221</td>
<td>1</td>
<td>72</td>
<td>96.5*</td>
<td>1600</td>
<td>3290*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2110</td>
<td>1</td>
<td>72</td>
<td>55.2*</td>
<td>11700</td>
<td>15500*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5050</td>
<td>1-2</td>
<td>72</td>
<td>NR</td>
<td>43100</td>
<td>NR</td>
</tr>
</tbody>
</table>

* approximation

Table 26: Pharmacokinetics in Female F344 rats. Repeated dose (14D) at 3, 15 or 50 mg/kg bw. Study 2016/1019953

<table>
<thead>
<tr>
<th>External Dose</th>
<th>Plasma Afidopyropen</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg/bw/d</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ug-Eq/mL)</td>
</tr>
<tr>
<td>3</td>
<td>262</td>
</tr>
<tr>
<td>15</td>
<td>2800</td>
</tr>
<tr>
<td>50</td>
<td>6980</td>
</tr>
</tbody>
</table>
Table 27: Bioanalytical analysis: Pooled Plasma Concentration of BAS 440 I after Administration of 14C-BAS 440 I at Dose Levels of 3, 15 and 50 mg/kg bw (Oral Administration) to Female Rats

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>3 mg/kg bw</th>
<th>15 mg/kg bw</th>
<th>50 mg/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predose (Day 1)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Predose (Day 15)</td>
<td>&lt; 1</td>
<td>1.77</td>
<td>3.45</td>
</tr>
<tr>
<td>Plasma 1</td>
<td>24.7</td>
<td>1497</td>
<td>3250</td>
</tr>
<tr>
<td>Plasma 2</td>
<td>7.87</td>
<td>1085</td>
<td>4752</td>
</tr>
<tr>
<td>Plasma 4</td>
<td>1.70</td>
<td>427</td>
<td>2372</td>
</tr>
<tr>
<td>Plasma 8</td>
<td>&lt; 1</td>
<td>61.4</td>
<td>791</td>
</tr>
<tr>
<td>Plasma 24</td>
<td>&lt; 1</td>
<td>1.90</td>
<td>4.23</td>
</tr>
<tr>
<td>Plasma 48</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Plasma 72</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

<1: below lower limit of quantification (=1 ng/mL)

Table 28: Bioanalytical analysis: Pooled Plasma Concentration of metabolite M440I001 after Administration of 14C-BAS 440 I at Dose Levels of 3, 15 and 50 mg/kg bw (Oral Administration) to Female Rats

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>3 mg/kg bw</th>
<th>15 mg/kg bw</th>
<th>50 mg/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predose (Day 1)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Predose (Day 15)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>1.77</td>
</tr>
<tr>
<td>Plasma 1</td>
<td>2.10</td>
<td>153</td>
<td>277</td>
</tr>
<tr>
<td>Plasma 2</td>
<td>&lt; 1</td>
<td>111</td>
<td>457</td>
</tr>
<tr>
<td>Plasma 4</td>
<td>&lt; 1</td>
<td>57.5</td>
<td>344</td>
</tr>
<tr>
<td>Plasma 8</td>
<td>&lt; 1</td>
<td>12.6</td>
<td>95.8</td>
</tr>
<tr>
<td>Plasma 24</td>
<td>&lt; 1</td>
<td>1.84</td>
<td>6.10</td>
</tr>
<tr>
<td>Plasma 48</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Plasma 72</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

<1: below lower limit of quantification (=1 ng/mL)
Table 29: Bioanalytical analysis: Pooled Plasma Concentration of metabolite M440I017 after Administration of 14C-BAS 440 I at Dose Levels of 3, 15 and 50 mg/kg bw (Oral Administration) to Female Rats

Results expressed in ng/mL plasma

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>3 mg/kg bw</th>
<th>15 mg/kg bw</th>
<th>50 mg/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predose (Day 1)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Predose (Day 15)</td>
<td>&lt; 1</td>
<td>1.77</td>
<td>3.83</td>
</tr>
<tr>
<td>Plasma 1</td>
<td>3.91</td>
<td>51.6</td>
<td>107</td>
</tr>
<tr>
<td>Plasma 2</td>
<td>3.18</td>
<td>51.6</td>
<td>175</td>
</tr>
<tr>
<td>Plasma 4</td>
<td>1.27</td>
<td>47.9</td>
<td>130</td>
</tr>
<tr>
<td>Plasma 8</td>
<td>&lt; 1</td>
<td>22.6</td>
<td>75.6</td>
</tr>
<tr>
<td>Plasma 24</td>
<td>&lt; 1</td>
<td>1.26</td>
<td>3.53</td>
</tr>
<tr>
<td>Plasma 48</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Plasma 72</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

<1: below lower limit of quantification (=1 ng/mL)

Table 30: Bioanalytical analysis: Pooled Plasma Concentration of metabolite M440I060 (AS1574, CPCA-carnitine) after Administration of 14C-BAS 440 I at Dose Levels of 3, 15 and 50 mg/kg bw (Oral Administration) to Female Rats

Results expressed in ng/mL plasma

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>3 mg/kg bw</th>
<th>15 mg/kg bw</th>
<th>50 mg/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predose (Day 1)</td>
<td>1.48</td>
<td>1.12</td>
<td>1.05</td>
</tr>
<tr>
<td>Predose (Day 15)</td>
<td>39.3</td>
<td>253</td>
<td>1485</td>
</tr>
<tr>
<td>Plasma 1</td>
<td>195</td>
<td>729</td>
<td>1333</td>
</tr>
<tr>
<td>Plasma 2</td>
<td>182</td>
<td>1045</td>
<td>1653</td>
</tr>
<tr>
<td>Plasma 4</td>
<td>148</td>
<td>1022</td>
<td>2458</td>
</tr>
<tr>
<td>Plasma 8</td>
<td>139</td>
<td>1562</td>
<td>4187</td>
</tr>
<tr>
<td>Plasma 24</td>
<td>44.5</td>
<td>260</td>
<td>726</td>
</tr>
<tr>
<td>Plasma 48</td>
<td>26.4</td>
<td>208</td>
<td>695</td>
</tr>
</tbody>
</table>
Table 31: Bioanalytical analysis: Pharmacokinetic Parameters of BAS 440 I and its metabolites in Plasma after Oral Administration of 14C-BAS 440 I at Dose Levels of 3, 15 and 50 mg/kg bw to Female Rats

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Matrix</th>
<th>Dose</th>
<th>$C_{\text{max}}$</th>
<th>$t_{\text{max}}$</th>
<th>$t_{\text{half}}$</th>
<th>$AUC_{0-24}$</th>
<th>$AUC_{0-\infty}$</th>
<th>AUD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[mg/kg bw]</td>
<td>[ng/mL]</td>
<td>[h]</td>
<td>[h]</td>
<td>[ng*h/mL]</td>
<td>[ng*h/mL]</td>
<td>[ng*h/mL]</td>
</tr>
<tr>
<td>BAS 440 I</td>
<td>3, p.o.</td>
<td>24.7</td>
<td>1</td>
<td>4</td>
<td>n/c</td>
<td>4470</td>
<td>4480</td>
<td>4530</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>1500</td>
<td>1</td>
<td>24</td>
<td>2.71</td>
<td>20600</td>
<td>20700</td>
<td>20700</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>4750</td>
<td>2</td>
<td>24</td>
<td>2.17</td>
<td>20700</td>
<td>20700</td>
<td>20700</td>
</tr>
<tr>
<td>M440I001</td>
<td>3, p.o.</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>n/c</td>
<td>72.5</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>163</td>
<td>1</td>
<td>24</td>
<td>4.41</td>
<td>578</td>
<td>609</td>
<td>635</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>457</td>
<td>2</td>
<td>24</td>
<td>3.59</td>
<td>2600</td>
<td>2630</td>
<td>2690</td>
</tr>
<tr>
<td>M440I017</td>
<td>3, p.o.</td>
<td>3.91</td>
<td>1</td>
<td>4</td>
<td>n/c</td>
<td>72.5</td>
<td>78.2</td>
<td>78.2</td>
</tr>
<tr>
<td></td>
<td>15, p.o.</td>
<td>51.6</td>
<td>1</td>
<td>24</td>
<td>3.84</td>
<td>431</td>
<td>438</td>
<td>482</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>175</td>
<td>2</td>
<td>24</td>
<td>3.78</td>
<td>1280</td>
<td>1300</td>
<td>1350</td>
</tr>
<tr>
<td>AS1574</td>
<td>3, p.o.</td>
<td>195</td>
<td>1</td>
<td>72</td>
<td>36.1</td>
<td>2780</td>
<td>6300</td>
<td>4930</td>
</tr>
<tr>
<td>CPCAt-carnitine</td>
<td>15, p.o.</td>
<td>1560</td>
<td>8</td>
<td>72</td>
<td>46.6</td>
<td>22600</td>
<td>50200</td>
<td>36200</td>
</tr>
<tr>
<td></td>
<td>50, p.o.</td>
<td>4190</td>
<td>8</td>
<td>72</td>
<td>27.1</td>
<td>71400</td>
<td>149000</td>
<td>122000</td>
</tr>
</tbody>
</table>

*approximation; n/c could not be calculated

Table 32. Pharmacokinetics of individual residues in the F344 rat. Repeated dietary dose (14D) at 3, 15 or 50 mg/kg bw. Study 2016/1019953

<table>
<thead>
<tr>
<th>Analyte</th>
<th>External Dose</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg/bw/d</td>
<td>Difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afidopyropen</td>
<td>3</td>
<td>1X</td>
</tr>
<tr>
<td>(BAS 440 I)</td>
<td>15</td>
<td>5X</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16.7X</td>
</tr>
<tr>
<td>M440I001</td>
<td>3</td>
<td>1X</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5X</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16.7X</td>
</tr>
<tr>
<td>M440I017</td>
<td>3</td>
<td>1X</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5X</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16.7X</td>
</tr>
<tr>
<td>M440I060</td>
<td>3</td>
<td>1X</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5X</td>
</tr>
</tbody>
</table>
Excretion

The majority of radioactivity was excreted via the feces, with smaller amounts in the urine. The mean results are given in the below table.

Table 33: Mean excretion of retention of radioactivity in female rats having received an oral dose of 14C- BAS 440 I at 3, 15 or 50 mg/kg bw. Results are % of dose administered.

<table>
<thead>
<tr>
<th>Balance/excretion</th>
<th>3 mg/kg bw</th>
<th>15 mg/kg bw</th>
<th>50 mg/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine 8 – 24</td>
<td>0.293</td>
<td>0.322</td>
<td>0.720</td>
</tr>
<tr>
<td>Urine 24 – 48</td>
<td>0.387</td>
<td>0.639</td>
<td>0.560</td>
</tr>
<tr>
<td>Urine 48 – 72</td>
<td>0.237</td>
<td>0.342</td>
<td>0.360</td>
</tr>
<tr>
<td>Subtotal Urine</td>
<td>0.917</td>
<td>1.30</td>
<td>1.64</td>
</tr>
<tr>
<td>Faeces 8 – 24</td>
<td>34.3</td>
<td>42.5</td>
<td>43.9</td>
</tr>
<tr>
<td>Faeces 24 – 48</td>
<td>45.8</td>
<td>41.9</td>
<td>18.5</td>
</tr>
<tr>
<td>Faeces 48 – 72</td>
<td>4.70</td>
<td>5.14</td>
<td>2.65</td>
</tr>
<tr>
<td>Subtotal Faeces</td>
<td>84.7</td>
<td>89.5</td>
<td>65.0</td>
</tr>
<tr>
<td>Total</td>
<td>85.6</td>
<td>90.8</td>
<td>66.6</td>
</tr>
</tbody>
</table>

3. Tissue distribution and metabolite profiling

Analysis of the liver and uterus found the majority of radioactivity in the liver.

Table 34: Mean tissue concentrations of radioactivity in female rats having received an oral dose of 14C- BAS 440 I at 15 or 50 mg/kg bw.

<table>
<thead>
<tr>
<th>Dose level</th>
<th>Results expressed in µg Eq/g tissue</th>
<th>Results expressed in percentage of dose found in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 mg/kg bw</td>
<td>50 mg/kg bw</td>
</tr>
<tr>
<td>Time after administration (h)</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Blood cells</td>
<td>12.3</td>
<td>8.92</td>
</tr>
<tr>
<td>Plasma</td>
<td>3.45</td>
<td>8.62</td>
</tr>
<tr>
<td>Uterus</td>
<td>4.43</td>
<td>12.8</td>
</tr>
<tr>
<td>Liver</td>
<td>42.2</td>
<td>92.7</td>
</tr>
</tbody>
</table>

Individual results are given in the table below:

Table 35: Individual tissue concentrations of radioactivity in female rats having received an oral dose of 14C- BAS 440 I at 15 or 50 mg/kg bw. (% of dose found in tissue)

<table>
<thead>
<tr>
<th>animal number</th>
<th>15 mg/kg bw samples after 1 hour</th>
<th>50 mg/kg bw sampled after 1.5 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood cells</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>plasma</td>
<td>ns</td>
<td>0.603</td>
</tr>
<tr>
<td>uterus</td>
<td>0.060</td>
<td>0.055</td>
</tr>
<tr>
<td>liver</td>
<td>7.96</td>
<td>7.81</td>
</tr>
</tbody>
</table>

Results from the metabolic profile are presented in the study report.

OVERALL REMARKS, ATTACHMENTS
None

V. APPLICANT’S SUMMARY AND CONCLUSION

Over an observation period of about 72 hours, respectively 0.9%, 1.3% and 1.6% of the dose at 3, 15, and 50 mg/kg was excreted via urine. Excretion via faeces was significantly higher and amounted for a period of about 72 hours to 85%, 90% and 65% of the dose at respectively 3 mg/kg bw, 15 mg/kg bw and 50 mg/kg bw. The major portion of fecal excretion occurred for all groups within 8-48 hours. Tissue sampling demonstrated distribution to the liver and, to a lesser extent, the uterus.

Investigation of the adsorption, distribution, and elimination of $^{14}$C-BAS 440 I, given a single oral dose following 14 days dietary exposure, revealed AUC values that indicated an internal exposure clearly correlated to the dosing regimen. The AUC values of BAS 440 I increased with increasing dose level in an un-proportional manner. The repeated dose administration at 15 mg/kg bw showed the systemic dose was overproportional to the external dose (from 3 to 15 mg/kg) by about 3-fold. The repeated dose administration at 50 mg/kg bw showed the systemic dose was overproportional to the external dose (from 3 to 50 mg/kg) also by about 3-fold.

Table 36. Pharmacokinetics in Female F344 rats. Repeated dose (14D) at 3, 15 or 50 mg/kg bw. Study 2016/1019953

<table>
<thead>
<tr>
<th>External Dose</th>
<th>Plasma Afidopyropen</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg/bw/d</td>
<td>C$_{\text{max}}$ ug-Eq/mL</td>
</tr>
<tr>
<td>3</td>
<td>1X 262</td>
</tr>
<tr>
<td>15</td>
<td>5X 2800</td>
</tr>
<tr>
<td>50</td>
<td>16.7X 6980</td>
</tr>
</tbody>
</table>

In the bioanalytical phase of the report, the repeated dose administration at 15 mg/kg showed the Afidopyropen AUD was overproportional to the dose (from 3 to 15 mg/kg) by about 9-fold. At 50 mg/kg the AUD was overproportional to the dose (from 3 to 50 mg/kg) by about 12-fold.

For M440I017 the repeated dose administration at both 15 mg/kg and 50 mg/kg showed the AUD was approximately proportional to the dose, while the C$_{\text{max}}$ was overproportional by a little more than two fold. The repeated dose administration of M440I001 at 15 mg/kg showed the AUD was overproportional to the dose (from 3 to 15 mg/kg) by about 2-fold. At 50 mg/kg the AUD was also overproportional to the dose (from 3 to 50 mg/kg) by about 2-fold. With M440I060 (CPCA-Carnitine) pharmacokinetics were approximately overproportional to the dose (from 3 to 15 mg/kg and 3 to 50 mg/kg) by about 1.5-fold.

---

2 AUD is a conservative estimation of AUC, used when LCMS detection at a low dose was below LOQ for a normal AUC calculation. Area under the data, calculated for the bioanalytical data (parent and 3 metabolites) using the linear-logarithmic trapezoidal rule by setting the reported LLOQ values after Cmax to the LLOQ value (=0.75 or 1.00 ng/mL).
Table 37. Pharmacokinetics of individual residues in the F344 rat. Repeated dietary dose (14D) at 3, 15 or 50 mg/kg bw. Study 2016/1019953

<table>
<thead>
<tr>
<th>Analyte</th>
<th>External Dose</th>
<th>Plasma</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg/bw/d</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; ng-Eq/mL</td>
<td>Difference</td>
<td>AUD ng*h/mL</td>
<td>Difference</td>
<td></td>
</tr>
<tr>
<td>Afidopyropen (BAS 440 I)</td>
<td>3  1X</td>
<td>24.7</td>
<td>1X</td>
<td>104</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15  5X</td>
<td>1500</td>
<td>61X</td>
<td>4530</td>
<td>44X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50  16.7X</td>
<td>4750</td>
<td>192X</td>
<td>20700</td>
<td>199X</td>
<td></td>
</tr>
<tr>
<td>M440I001</td>
<td>3  1X</td>
<td>-</td>
<td>-</td>
<td>72.5</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15  5X</td>
<td>153</td>
<td>-</td>
<td>635</td>
<td>8.8X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50  16.7X</td>
<td>457</td>
<td>-</td>
<td>2690</td>
<td>37X</td>
<td></td>
</tr>
<tr>
<td>M440I017</td>
<td>3  1X</td>
<td>3.91</td>
<td>1X</td>
<td>78.2</td>
<td>1X</td>
<td></td>
</tr>
<tr>
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<td>175</td>
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<tr>
<td>M440I060</td>
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<td>1X</td>
<td>4930</td>
<td>1X</td>
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<tr>
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<td>4190</td>
<td>22X</td>
<td>122000</td>
<td>25X</td>
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Dosing with Afidopyropen resulted in high M440I060 levels in plasma. The plasma curve resulting from Afidopyropen dosed at 50 mg/kg bw (approximately 1000 ppm) indicates significant internal exposure to M440I060, and internal exposure to M440I060 is higher than exposure to either Afidopyropen or other Afidopyropen plasma metabolites.

Figure 12. Pooled Bioanalytical Plasma Concentrations of BAS 440 I and its metabolites after Oral Administration of 14C-BAS 440 I at a Dose Level of 50 mg/kg bw to Female Rats; Study 2016/1019953