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Comprehensive toxicity evaluation of cyclopentyl methyl ether (CPME) for establishing a permitted daily exposure level

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ABSTRACT — Cyclopentyl methyl ether (CPME, CAS No. 5614-37-9) is used as an alternative to other ethereal solvents in pharmaceutical chemical process development. Although brief summaries on the repeated dose toxicity or genotoxicity of CPME are available, no detailed toxicity information has been reported. This study performed a detailed hazard characterization of CPME using new reproductive/developmental toxicity data and existing data for a 28-day repeated dose toxicity and genotoxicity study of CPME. Sprague-Dawley (SD) rats were treated with CPME by gavage at doses of 50, 150, or 450 mg/kg/day in the reproductive/developmental toxicity study or at doses of 15, 150, and 700 mg/kg/day in the 28-day study. In the reproductive/developmental toxicity study, lower body weight in males and longer gestational lengths were observed in the F0 animals receiving the 450 mg/kg/day dose. In the F1 animals, lower body weight gain during days 1-7 of life was detected in the 450 mg/kg/day groups in both sexes. In the 28-day study, 6 of 10 males showed poor clinical conditions, resulting in unscheduled deaths in the 700 mg/kg/day group. Based on these findings, the NOAELs for both the reproductive/developmental and 28-day repeated dose toxicity studies were estimated at 150 mg/kg/day. The results of all genotoxicity studies including the bacterial reverse mutation test, *in vitro* mammalian cell chromosome aberration test, and *in vivo* mouse micronucleus test were negative. A permitted daily exposure (PDE) of 15 mg/day was suggested based on the present findings to help determine the PDE for the ICH guidelines on impurities in pharmaceuticals.

Key words: Cyclopentyl methyl ether (CPME), Reproductive/developmental toxicity, Repeated dose toxicity, Genotoxicity, Permitted daily exposure (PDE) level

INTRODUCTION

Cyclopentyl methyl ether (CPME: CAS 5614-37-9) is an ethereal solvent that is widely used in chemical synthesis as an alternative to hazardous solvents (Watanabe, 2007). CPME has been available in commercial quantities since 2005 from Zeon Corporation (Tokyo, Japan). The chemical property of CPME was introduced by Watanabe *et al*. (2007). According to the report, a high boiling point and preferable characteristics such as its low formation of peroxides and relative stability under acidic and basic conditions allows CPME to serve as an alternative to other ethereal solvents such as tetrahydrofuran (THF), 2-methyl tetrahydrofuran (2-MeTHF), dioxane (carcinogenic), and 1,2-dimethoxyethane (DME). Since CPME has been used in the pharmaceutical chemical process development, the residual level and toxicity of CPME should be measured and evaluated according to the ICH guidance of Q3A(R2) and Q3C(R4).

Some summary reports on CPME toxicity have already been published (Antonucci *et al*., 2011; Watanabe *et al*., 2013). In these reports, several types of acute toxicity and skin sensitization studies for CPME have been reported, and CPME was judged to have a low acute toxicity with moderate to severe dermal and eye irritation and no potential to induce skin sensitization. These reports also

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contained summary results of repeated dose toxicity and genotoxicity studies for CPME. For example, a 28-day oral toxicity study (according to the Organization for Economic Co-operation and Development (OECD) test guideline 407) and 90-day subchronic inhalation (according to the OECD Test Guideline 413) or oral toxicity studies had previously been performed to assess repeated dose toxicity (Antonucci *et al*., 2011; Watanabe *et al*., 2013). According to the summary reports, the no observed adverse effect level (NOAEL) for the 28-day oral toxicity study was 150 mg/kg/day based on the high mortality and clinical signs observed at a dose of 700 mg/kg/day. In the 90-day subchronic inhalation toxicity study, the no observed effect level (NOEL) was 0.87 mg/L for male rats and 0.84 mg/L for female rats based on clinical signs, lower body weight, and histopathological findings in the kidneys and urinary bladder observed at a dose of 4.0 mg/L (Watanabe *et al*., 2013). In a 3-month repeated dose oral toxicity study, male and female Crl:CD (SD) rats did not display any adverse toxicity effects of CPME at a dose up to 31 mg/kg/day by gavage (Antonucci *et al*., 2011). Thus, toxicity data for CPME have accumulated over the past several years. However, detailed data on each of the test items in these toxicity studies were not fully published and are insufficient for a full toxicity evaluation of CPME because of the lack of detailed reproductive/developmental toxicity data. Recently, we have obtained new data from a reproductive/developmental toxicity study on CPME. Therefore, in the present study, we published all reliable data of a repeated dose oral toxicity, reproductive/developmental toxicity, and genotoxicity study and performed a detailed toxicity evaluation to better understand the toxicity profiles of CPME and to help establish a permitted daily exposure (PDE) level for the ICH guidelines on impurities in pharmaceuticals.

MATERIALS AND METHODS

In the following toxicity studies, CPME was supplied by ZEON Corporation (Tokyo, Japan) and all the animal studies were conducted in accordance with the applicable sections of the United Kingdom Animals (Scientific Procedures) Act 1986.

Reproductive screening toxicity and 28-day repeated dose toxicity studies

Chemicals

CPME (Batch number: 1820146, purity: 99.97% for the reproductive toxicity study; Batch number: 020618, purity: 99.8% for the 28-day repeated dose toxicity study) was the solvent and appeared as a colorless transparent liquid. The CPME was prepared for administration as a series of graded concentrations using corn oil as a vehicle.

The test substance was used as supplied. All formulations were prepared freshly each week and were prepared up to one day in advance of the first day of dosing. The formulations were stored in a refrigerator (approximately 2-8°C). The formulations were warmed to room temperature, mixed by inversion, and magnetically stirred for at least 5 min before the dosing commenced and throughout the dosing procedure each day. The control animals received the vehicle (corn oil) alone at the same dosage volume.

In both studies, the homogeneity and stability of the formulations during storage were confirmed by Huntingdon Life Sciences (Cambridgeshire, UK). The formulation was determined to be stable for up to 2 days when stored at room temperature and for up to 8 days when refrigerated at nominal concentrations of 2 mg/mL or 200 mg/mL. During weeks 1, 2, 3, and 4 of treatment and the last week of dosing, samples from each concentration were analyzed to determine the achieved concentration of the test substance. Four samples were taken (nominally 1 mL accurately weighed) from each group. During week 1, following a low result for the concentration achieved in group 2, the dose was reformulated, and an additional 4 samples were taken for analysis. Two samples were assayed from each test group and one sample was assayed from the control group. The mean concentrations of CPME in the test formulations analyzed for the study were within +10%/−15% of nominal concentrations, confirming accurate formulation, with the exception of the formulation prepared for dose week 1, group 2, days 1-4, which was approximately 39% below nominal. To maintain the concentration, the formulation procedure was revised and applied to all formulations dosed from week 3 onwards.

Animal treatment

Each toxicity study was designed to meet the requirements of the following testing guidelines: OECD: Testing of Chemicals, Guideline 421 “Reproductive/Developmental Toxicity Screening Test” or Guideline 407 “Repeated Dose 28-day Oral Toxicity Study in Rodents (revised 1995)”, respectively. Both studies were conducted in accordance with the requirements of current internationally recognized Good Laboratory Practice Standards such as the OECD Principles of Good Laboratory Practice (as revised in 1997).

Reproductive screening toxicity study

The reproductive/developmental screening toxicity study was performed by Huntingdon Life Sciences (Suffolk, UK). Crl:CD (SD) rats (45 males and 45 females) were obtained from Charles River Ltd. (UK). The rats were approximately 65 days old, and after a 6-day acclimation period, 40 animals of each sex were selected for this study. The body weights of the selected animals ranged from 341 to 405 g for males and 234 to 268 g for females. They received a standard rodent diet SDS VRF1 Certified diet (Special Diet Service, Essex, UK) and tap water *ad libitum* during the present study. Five rats were housed per polycarbonate cage (pre-pairing period) with sterilized softwood chips as bedding in a barrier-sustained animal room conditioned at 19-23°C and 40%-70% humidity, on a 12 hr light/dark cycle.

Selected F0 animals were randomly divided into 4 groups (10 animals/group/sex) and given 0 (control), 50, 150, or 450 mg/kg/day of CPME by gavage. In the preliminary study to determine the dose setting, a 4-week treatment of 700 mg/kg/day of CPME by gavage was not tolerated by the males and resulted in a significant decrease in weight gain during the final 2 weeks of treatment in females. Therefore, the NOAEL was identified as 150 mg/kg/day; a middle dose from the preliminary study, 450 mg/kg/day, was selected as the high dose level; 150 mg/kg/day was selected as the intermediate dose level; and 50 mg/kg/day was selected as the low dose level for the present study. The animals received the test material or vehicle orally at a volume of 5 mL/kg body weight, using a suitably graduated syringe and a rubber catheter. The volume administered to each animal was calculated from the most recently recorded scheduled body weight. CPME was administered to the males for a minimum of 4 weeks and to the females daily for 15 days before pairing and throughout pairing until day 6 after the birth of the F1 generation. F0 females did not receive any doses if parturition was in progress at the scheduled time of administration. Animals in the F1 generation did not receive any doses.

Mating, parturition, and gestation

For 15 days before pairing, daily vaginal smears were obtained from all females to assess the estrous cycle. After pairing with the male, smearing was continued using pipette lavage until evidence of mating was observed.

After 15 days of treatment, males and females from the same treatment groups were paired on a one-to-one basis until mating occurred or for a period of up to 2 weeks. The day on which evidence of mating was determined was designated day 0 of gestation. Once mating occurred, the males and females were separated and smearing was discontinued. The precoital interval was calculated for each female as the time elapsing between initial pairing and the detection of mating. From day 20 after mating, the females were inspected thrice daily for evidence of parturition. The progress and completion of parturition was monitored, and the numbers of live and dead offspring were recorded. All litters were examined at approximately 24 hr after birth (day 1 of age) and then daily thereafter.

Body weight and food consumption

The F0 males and females were weighed weekly throughout the study or until mating was detected, respectively. The F0 females were also weighed on days 0, 3, 7, 10, 14, 17, and 20 after mating and on days 1, 4, and 7 of lactation.

Food consumption was measured by weighing the food supplied to each cage, the food remaining, and an estimate of any spilled food on a weekly basis from the beginning of treatment for F0 males and females until the animals were paired for mating. From these records, the mean weekly consumption per animal (g/animal/week) was calculated for each cage.

Clinical observation

The animals were observed visually at least twice daily for evidence of ill health or reaction to treatment. The cages were inspected daily for evidence of animal ill health among the occupant(s). In addition, a more detailed physical examination was performed weekly for F0 adults and on days 0, 7, 14, and 20 after mating and days 1 and 7 of lactation for F0 females to monitor general health.

Detailed observations were also recorded regarding dose administration at the following times: immediately before and after dosing, completion of dosing in each group, between 1 and 2 hr after the completion of dosing of all groups, and as late as possible in the working day. F0 males and females were observed daily during the first week of treatment and once a week beginning in week 2. For F0 females, observations were also recorded on days 0, 7, 14, and 20 after mating and on days 1 and 6 of lactation.

All litters of the F1 animals were examined at approximately 24 hr after birth (day 1) and then daily thereafter. The records maintained were as follows: clinical signs, litter size from days 1 to 7, mortality, sex ratio on days 1, 4, and 7, and body weight on days 1, 4, and 7.

Necropsy and pathological examination

To perform euthanasia, the F0 animals inhaled car-

bon dioxide gas and were then sacrificed at the following times: during week 5 of treatment for the F0 males and on day 7 of lactation for the F0 females. Females whose litter died before day 7 of lactation were sacrificed on the day the last offspring died.

All F0 animals were subjected to a detailed necropsy. A full macroscopic examination of the tissues was performed in each animal. For F0 females, the number of implantation sites in each uterine horn was determined. The following organs were taken from the F0 animals: the testes, epididymides, pituitary, prostates, and seminal vesicles for males and the ovaries, vagina, and pituitary for females. In females whose litter all died or who had suspect fertility, the caudal mammary area or uterus with cervix and oviducts were taken, respectively. The testes, epididymides, and pituitary were weighed and calculated to determine the relative weight compared to the body weight. For the histological examination, the testes were fixed in modified Davidson's fluid, and the other tissues were preserved in 10% neutral buffered formalin. The epididymides, ovaries, and testes were the primary organs subject to histological processing. The tissue samples of these organs were dehydrated, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin, except for the testes, which were stained using a standard periodic acid-Schiff (PAS) method. Microscopic examination in the epididymides, ovaries, and testes was performed for all F0 animals from the control and 450 mg/kg/day groups. The findings were either reported as "present" or assigned a severity grade. The following five grades were used to indicate the severity of findings: minimal, slight, moderate, marked, or severe. A reviewing pathologist performed a peer review of the microscopic findings. Macroscopic abnormalities in the tissues were also examined.

Sodium pentobarbitone was intraperitoneally injected into the offspring, which were sacrificed on the seventh day of life. For the offspring who survived to scheduled termination, a careful external examination was performed for gross abnormalities. When an external abnormality was observed in an offspring, the offspring was examined macroscopically, and the abnormal tissues were retained in an appropriate fixative and examined. Additionally, the offspring that died before day 7 and were applicable for a morphological examination were assessed with milk present in the stomach, if possible.

Statistics

Variances in the data for body weight, food consumption, litter size, survival indices, and organ weight were checked for homogeneity by Bartlett's procedure (1937). When Bartlett's test was not significant at the 1% level, a parametric analysis was performed. If the F1 approximate test for monotonicity of the dose-response was not significant at the 1% level, Williams' test (Williams, 1971, 1972) for a monotonic trend was applied. If the F1 approximate test was significant, suggesting that the dose-response was not monotonic, Dunnett's test (Dunnett, 1955, 1964) was performed instead. When Bartlett's test was significant at the 1% level, a nonparametric analysis was performed. If the H1 approximate test for monotonicity of the dose-response was not significant at the 1% level, Shirley's test (Shirley, 1977) for a monotonic trend was applied. If the H1 approximate test was significant, suggesting that the dose-response was not monotonic, Steel's test (Steel, 1959) was performed instead.

If 75% of the survival indices data (across all groups) were the same value, Fisher's exact test (Fisher, 1973a and 1973b) was performed. Treatment groups were compared using pairwise comparisons of each dose group against the control. For the organ weight data, an analysis of covariance was performed using terminal body weight as the covariate (Angervall and Carlström, 1963). The treatment comparisons were made based on adjusted group means to allow for differences in body weight that might influence the organ weights. Sex ratios were analyzed by generalized mixed linear models with binomial errors, a logit link function, and litter as a random effect (Lipsitz *et al.,* 1991). Each treatment group was compared to the control using a Wald chi-square test. The numerator was the number of males, and the denominator was the number of live fetuses.

To assess gestation length, an exact two-tailed linear-by-linear test (Cytel, 1995), with equally spaced scores, was applied to all groups. If the test was statistically significant ($p < 0.05$), the highest dose group was excluded and the test was reapplied. This "step-down" process was repeated until the test was no longer statistically significant ($p \geq 0.05$). If the exact version of the linear-by-linear test could not be calculated (because of the size of the table containing the data), then the asymptotic version was used instead.

The 28-day repeated dose toxicity study

The 28-day repeated dose toxicity study was performed by Huntingdon Life Sciences (Cambridgeshire, UK). Crl:CD (SD) rats (35 males and 35 females) were obtained from Charles River Ltd (UK). They were approximately 6 weeks old, and their body weight ranged from 208.4 to 242.7 g for males and from 164.2 to 203.1 g for females, respectively, at the start of treatment. After a 6-day acclimation period, 60 animals were selected and randomly allocated to the 4 treatment groups and 2 recov-

ery groups, so that each group contained 5 males and 5 females. At the start of treatment, individual body weights were within ± 20% of the group mean for each sex. These animals were kept in stainless steel cages with stainless steel grid floors in a barrier-sustained animal room conditioned at 19-23°C and 40%-70% humidity, on a 12 hr light/dark cycle. They received pelleted SDS rat and Mouse No. 1 modified maintenance diet (LBS Biotechnology, UK) and tap water ad libitum during the present study. The animals were given 0 (control), 15, 150, and 700 mg/kg/day of CPME by gavage once daily, 7 days a week, for a total of 4 weeks, with the exception of the highest dose group of males, which was treated for 15 days. In a 7-day preliminary study to determine the dose setting, 1000 mg/kg/day was not tolerated and resulted in deaths; because 700 mg/kg/day was tolerated in male rats, the highest dose for the present study was decided to be 700 mg/kg/day. The low and middle doses were selected based on the key dosages relative to the EU labelling requirements. A constant dosage volume of 5 mL/kg body weight was used. Clinical signs and mortality in all animals were observed at least twice each day from arrival to termination. During the pretreatment period, weeks 1-4 of treatment, and weeks 1 and 2 of the recovery periods, a detailed physical examination and arena-based behavioral observations were performed on all surviving animals. During week 4 of treatment, sensory reactivity, grip strength, and motor activity assessments were performed prior to any laboratory investigations and before dosing. In addition, in week 2 of the recovery period, motor activity was assessed in females due to the higher activity observed at week 4 of the treatment period. All animals were weighed when allocated to treatment groups on arrival, prior to dosing on day 1 and on days 8, 15, and 28 (prior to overnight starvation for clinical pathology) during the treatment period. The final body weights of all animals were also measured before necropsy. The consumed volume of food in each cage was measured weekly, and the food intake per rat (g/rat/week) and food conversion efficiency (%) were calculated using the data for body weight gain (g) and food consumed (g). Water consumption was measured on days 23-25 (at week 4) due to the apparent hemoconcentration noted in the blood of the male 700 mg/kg/day group that was terminated early on day 16 of treatment. Blood samples were obtained from all animals under anesthesia with isoflurane on day 16 for the 2 surviving males in the 700 mg/kg/day group, on day 19 for the other males and all females, or on day 15 of the recovery period. The following items were assessed in the hematological analysis: hematocrit (Hct), hemoglobin concentration (Hb), erythrocyte count (RBC), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), mean cell volume (MCV), total leucocyte count (WBC), differential leucocyte counts including neutrophils (N), lymphocytes (L), eosinophils (E), basophils (B), monocytes (M), and large unstained cells (LUC), and platelet count (Plt). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were also analyzed from the blood samples. For serum biochemistry, serum separated from each blood sample was used to analyze the following items: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (Bili), urea (Urea), creatinine (Creat), glucose (Gluc), total cholesterol (Chol), triglycerides (Trig), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), phosphorus (Phos), total protein (Total Prot), albumin (Alb), and the albumin/globulin (A/G) ratio.

A urinalysis was performed by collecting overnight urine samples from all surviving animals of the main group on day 29, all male recovery groups on day 30, and all female recovery group on day 15 of the recovery period. Food and water were removed during urine sampling, and all animals were allowed access to water for one hour prior to blood sampling. The following parameters were analyzed in the urinalysis: appearance such as color and clouds, volume, specific gravity, protein, sodium, potassium, and chloride.

To perform the necropsy, the 2 surviving males in the 700 mg/kg/day group were sacrificed early on day 16 because of their deteriorating condition. The treatment for the other 2 males in the same dose group for recovery was stopped on day 16, and all males in the recovery groups including the controls were sacrificed on day 30 after the 2-week recovery period. The males in the control, 15, and 150 mg/kg/day groups and the females in the main treatment groups were sacrificed on day 29 after a 4-week treatment period. The females in the recovery group were also sacrificed on day 43 after a 2-week recovery period. To sacrifice each animal, carbon dioxide was used under anesthesia with isoflurane.

During the necropsy, the brain, heart, thymus, spleen, liver, kidneys, adrenal glands, testes, epididymides, seminal vesicles, and ovaries were removed and weighed. In addition, the head, pituitary, eyes with optic nerves, Harderian glands, spinal cord (cervical, thoracic, and lumbar portions), salivary glands, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), pancreas, urinary bladder, skin, mammary glands, mandibular and mesenteric lymph nodes, trachea, esophagus, thyroid glands with parathyroids, tongue, aorta, thigh muscle, sternum, femur, sciatic nerves, epidi-

dymides, seminal vesicles, prostate (ventral and dorsolateral lobes), uterus with cervix, ovaries, vagina, and macroscopically abnormal tissues were also removed. All organs/tissues were fixed in 10% neutral buffered formalin except the testes and epididymides, which were fixed in Bouin's solution and then transferred to 70% Industrial Methylated Spirits. The eyes were fixed in Davidson's fluid. Tissue slices of all organs/tissues were routinely processed for paraffin embedding, and the sections were prepared and stained with hematoxylin and eosin. The testes tissue sections were stained by the PAS method. Histopathological assessment was first performed on all tissues of the control and highest dose group animals. If a chemical treatment-related change appeared at the highest dose, the relevant tissue(s) from the lower dose groups were then also examined. In addition, the adrenals, epididymides, heart, kidneys, liver, spleen, stomach and testes were examined for all animals in the main treatment groups receiving 15 and 150 mg/kg/day and all recovery animals.

The same sequential tests used in the reproductive screening toxicity study were performed for the statistical analyses of body weight, neurobehavioral screening, hematology, blood chemistry, urinalysis, organ weight, and pathology data. However, some tests differed from those used in the reproductive screening toxicity study. Dunn's test (Dunn, 1964) was used instead of Steel's test as the nonparametric test. The results of the recovery control and high dose groups were compared by the Student's t-test. Bartlett's test was first applied for the grip strength data and Coulbourne activity data, and then the treatment groups were compared to the control group, incorporating adjustments for multiple comparisons where necessary.

Genotoxicity

Bacterial reverse mutation test (Ames test)

To assess the mutagenic potential of CPME, a bacterial reverse mutation test was performed by Huntingdon Life Sciences Ltd. (Cambridgeshire, England), according to OECD Test Guideline 471 (OECD, 1997). Briefly, amino acid requiring strains of *Salmonella typhimurium* TA98, TA100, TA1537, and TA1535 (obtained from the National Collection of Type Cultures, London, England) and *Escherichia coli*, strain WP2*uvrA*/pKM101 (obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland) were exposed to CPME (purity 99.6%) at doses of 0 (untreated control), 5, 15, 50, 150, 500, 1500, and 5000 μg/plate with or without S9 mix (a range-finding test). The doses in the main test were determined based on the results of this test performed using the standard incorporation method. Since the results of all 7 doses including those in the 5000 μg/plate, the highest dose tested, were negative in the preliminary study, the highest dose for the main study was determined to be 5000 μg/plate, and the other doses were determined to be the same doses as the 4 lower doses of the preliminary study. The main test was performed using the preincubation method (Maron and Ames, 1983). Dimethyl sulfoxide (DMSO, Aldrich, ACS spectrophotometric grade) was used as a solvent (i.e., untreated control) for this study. The S9 fraction of male Sprague-Dawley rats was prepared according to the methods described by Ames, McCann, and Yamasaki (1975), and the S9 mix containing S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADPH (4 mM), and NADH (4 mM) was used in the present study. For the positive controls, the following chemicals were chosen: sodium azide (purity 99.5%, Sigma Chemical; 0.5 μg/plate was applied for the strains TA1535 and TA100), 9-aminoacridine (purity > 97%, Sigma Chemical; 30 μg/plate was applied for the strain TA1537), 2-nitrofluorene (purity 98%, Aldrich Chemical Company; 1 μg/plate was applied for the strain TA98), and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) (purity 98%-102%, Wako Pure Chemical Industries Ltd.; 0.05 μg/plate was applied for the strain WP2*uvrA*/pKM101) in the absence of S9 mix. In the presence of S9 mix, benzo[a]pyrene (purity 98%, Aldrich Chemical Company; 5 μg/plate was applied for the strains TA1537, TA98, and TA100) and 2-aminoanthracene (purity 96%, Aldrich Chemical Company; 2 and 10 μg/plate were applied for the strain TA1535 and WP2*uvrA*/pKM101, respectively) were used for the positive control. The mixtures of bacteria, buffer, or S9 mix and test dilution were preincubated at 37°C for 30 min with shaking before the addition of the agar overlay. After the plates were incubated at 37°C for 72 hr, the number of revertant colonies for all treatment groups was counted using an automated colony counter. Simultaneously, growth inhibition and the precipitation on the plates were examined. The results were judged positive when there were at least twice as many colonies as the untreated control plates and in a dose-dependent manner. Each dose was tested in triplicate both for the range-findings and the main test.

In vitro chromosome aberration test

To evaluate the ability of CPME to induce chromosomal aberrations *in vitro*, a mammalian chromosome aberration test using Chinese hamster lung (CHL) cells was performed in Huntington Life Sciences Ltd. (Suffolk, England), according to the OECD Test Guideline 473

(OECD, 1997). CHL cells obtained from Safepharm Laboratories Ltd. (Derby, United Kingdom) were cultured in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and some amino acids. The cells were incubated at 37°C in a humid atmosphere containing 5% carbon dioxide. The highest concentration used in this test was 10 mM (1001.6 μg/mL) in accordance with the OECD guidelines (OECD, 1997). In the first test, 5 mL of the cell suspension (5×10^4 cells/mL) was added to 25-cm^2 tissue culture flasks. After 24 hr, the culture medium was replaced with 5 mL of fresh medium, and 50-μL aliquots of CPME (purity 99.8%) were added to one set of duplicate cultures to reach final concentrations of 7.83, 15.65, 31.3, 62.6, 125.2, 250.4, 500.8, and 1001.6 μg/mL. DMSO in 50-μL aliquots was added to 2 cultures as a negative control. Mitomycin C (Sigma Chemical Co. Ltd.) at a final concentration of 0.1 μg/mL was added to duplicate cultures as a positive control in the absence of S9 mix. Immediately before treatment of the second set of cultures, the culture medium was replaced with 5 mL of fresh medium containing S9 mix followed by 50-μL aliquots of the same series of final concentrations of CPME as described above. S9 mix containing S9 fraction (5% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), and NADP (4 mM) was used in the present study (Natarajan *et al*., 1976). Cyclophosphamide (Asta Medica Ltd., UK) was added to duplicate cultures at a final concentration of 5 μg/mL as a positive control, and DMSO (50 μL) was added to 2 cultures again as a negative control in the presence of S9 mix. Three hours after dosing, the treated medium was replaced with fresh medium, which was incubated for a further 12 hr. To arrest mitotic activity, Colcemid® (Sigma) was added to each culture at a final concentration of 0.1 μg/mL and the cells were incubated for 2 hr. After 2 mL of 0.25% trypsin treatment for 1 min, the cells were resuspended in the medium and trypsin solution. An aliquot of the cell suspension (1 mL) was removed from all samples and added to 9 mL of Isoton®, an azide-free electrolyte balanced solution, and counted using an electronic particle counter (Coulter counter) to measure cell toxicity. The remaining cell suspensions were then centrifuged for 5 min at 200 *g*. The supernatant was discarded, and the cells were resuspended in 5 mL of pre-warmed 0.075 M KCl, a hypotonic solution. After a 10 min incubation at 37°C, 0.5 mL of fixative (3 parts methanol: 1 part glacial acetic acid) was added, centrifuged for 5 min at 200 *g*, and resuspended in 4 mL of fixative, which was replaced a further 3 times. After the fixation, two or three drops of the cell suspensions were placed onto microscope slides and then air-dried. The slides were stained in 10% Giemsa prepared in buffered water (pH 6.8) and then air-dried after rinsing and mounted in DPX (Merck). In the light microscopy examination, the dose level causing a reduction of approximately 50% of the negative control value, or, if there was no increase, the maximum achievable concentration, was used as the highest dose level for the metaphase analysis. The slides that were subjected to observation were then coded. Metaphase cells were identified using a low-power objective and examined at a magnification of × 1000 using an oil immersion objective. One hundred metaphase figures were examined from each culture. Chromosome aberrations were scored according to the classification of the ISCN (1985). Only cells with 23-27 chromosomes were analyzed. Polyploid and endoreduplicated cells were noted when observed. The incidence of polyploid metaphase cells among the total 500 metaphase cells was quantitatively evaluated in all cultures for chromosomal aberrations. The number of aberrant metaphase cells (excluding gaps) in each treatment group was compared with the negative control values using Fisher's test (Fisher, 1973). Moreover, a second test was performed to evaluate a continuous treatment under the following experimental conditions: 15 hr of continuous treatment without S9 mix and 3 hr of treatment with S9 mix. The following concentrations of CPME were used in the second test: 62.6, 125.2, 250.4, 500.8, and 1001.6 μg/mL. Three hours after dosing, the treated medium in the presence of S9 mix was replaced with fresh medium, and the cells were incubated for a further 12 hr. The following process in the second test was same as that in the first test.

In vivo mouse micronucleus test

To assess the potential induction of micronuclei by CPME, the bone marrow micronucleus test was performed using mice, according to the OECD Test Guideline 474 (OECD, 1997). Male CD-1 mice weighing between 28 and 32 g were obtained from Charles River UK Limited (Kent, England). Since there were no differences in the toxicological effects between males and females in a preliminary study (data not shown), males were used for the present study in line with current guidelines. The animals received a rat basal pellet diet and Mouse No. 1 maintenance diet (SQC grade, obtained from Special Diets Services Ltd. Essex, UK) and tap water *ad libitum* during the acclimation period for at least 5 days. They were kept in an animal room conditioned at 21 ± 2°C and 55 ± 15% humidity, on a 12 hr light/dark cycle. After acclimation, the animals were divided into five groups: three treatment groups, a vehicle group, and a positive control group. Each group contained 5 (the positive control group), 7

(the 2 lower dose treatment groups) or 14 (the vehicle control and highest dose treatment group) animals, respectively. The animals in the treatment groups were administered CPME (purity 99.96%) by single gavage with a volume of 20 ml/kg at doses of 500, 1000, and 2000 mg/kg. The highest dose was determined to be 2000 mg/kg since it was clarified that this dose level was tolerated in mice and was an appropriate maximum dose level for the main study (OECD, 1997). As the vehicle or positive control, 12 mg/kg of Mitomycin C or corn oil was used and administered to the animals in the corresponding groups, respectively. Following dosing, the animals were checked regularly, and mortalities and clinical signs were recorded. At 24 or 48 hr after dosing, the animals were anesthetized with carbon dioxide by inhalation and sacrificed by cervical dislocation. The bone marrow of both femurs was flushed out and pooled in a total volume of 2 mL of pre-filtered fetal calf serum. The cells were sedimented by centrifugation, and after the supernatant was discarded, the cells were resuspended in a small volume of fresh serum. Smears were prepared on glass microscopic slides, then fixed in methanol and stained with 10% Giemsa. After staining, the smears were rinsed, air-dried, and mounted with cover slips using DPX. The incidence of micronucleated cells per 2000 polychromatic erythrocytes (immature erythrocytes) per animal was examined by light microscopy (under code). The proportion of immature erythrocytes for each animal was assessed by examining at least 1000 erythrocytes. For the statistical analysis, nonparametric statistical methods were chosen when the results of the treatment groups were compared with those of the concurrent control group. To assess the incidences of micronucleated immature erythrocytes, exact one-sided p-values were calculated by permutation (StatXact, CYTEL Software Corporation, MA, US). To assess the effects on the proportion of immature erythrocytes, equivalent permutation tests such as the exact version of the Wilcoxon rank sum test and the Jonckheere trend test were used.

RESULTS

The reproductive screening toxicity study

F0 animals

Mortality and clinical observation

One male rat in the 450 mg/kg/day group displayed a poor clinical condition on day 18 and was sacrificed that same day. The animal showed underactive behavior, hunched posture, fast breathing, poor reflexes, and partially closed eyelids. A macroscopic examination revealed dark liver without histopathological changes and dark adrenals caused by minimal congestion, reduced stomach and cecal content, and abnormal orange viscous fluid in the jejunum with the colon and rectum devoid of content. One female became suddenly thin and displayed a hunched posture, resulting in sacrifice for welfare reasons on day 4 of lactation. Other males and females in the control and treatment groups survived throughout the study.

Some clinical symptoms were observed after the daily administration of CPME. On day 1, 3 males in the 450 mg/kg/day group had an unsteady gait, and one of those animals was also underactive. On day 2, 30 min after receiving 450 mg/kg/day of CPME, 2 males in that group displayed unsteady gait, and 1 was the same animal that had displayed that symptom on day 1. On day 3, a different animal was underactive. These signs were no longer observed after day 4 of treatment. Males and females in the 150 or 450 mg/kg/day groups showed an increased incidence of chin rubbing and/or increased salivation after administration compared to the controls. The incidence of these symptoms was not continuous but was sporadic in both sexes (data not shown).

There were no other signs observed in association with dose administration or during the routine physical examinations that could be related to the CPME treatment.

Body weight and food consumption

The overall body weight gain for males receiving 450 mg/kg/day was significantly lower than that of the controls ($p < 0.01$) (Table 1). A decreased body weight gain was observed from week 1 throughout the study period, which resulted in a significant decrease of the mean body weight from week 3 in males in the 450 mg/kg/day group. In the male low- and middle-dose groups, there were no significant changes in the mean body weight and body weight gain throughout the study period. In females, the body weight gain for the 2-week period before pairing, during gestation, and up to day 7 of lactation showed no statistically significant decrease in all treated groups (data not shown).

During the 2-week period before pairing, both males and females receiving 450 mg/kg/day had low food consumption (g/animal/week) compared to the controls, corresponding to approximately 93% and 88% of that consumed by controls during week 1 and 85% and 88% of that consumed by controls during week 2 for males and females, respectively (Table 2). Food consumption for males and females receiving 50 or 150 mg/kg/day was similar to the controls during the 2-week period before pairing. During gestation and up to day 3 of lactation, food consumption (g/animal/day) for the treated females

Table 1. Mean body weight and body weight change from Weeks 0 to 4 in F0 males.

Dose (mg/kg/day)	Number of animals examined	Mean body weight (g) Week 4	Body weight change (g) Weeks 0-4
0	10	472.0 ± 26.8[a]	104.0 ± 19.5
50	10	461.0 ± 32.4	89.0 ± 25.4
150	10	470.0 ± 38.1	99.0 ± 23.8
450	9	433.0 ± 23.6**	60.0 ± 22.5**

[a]: Mean ± SD.
**: Significantly different from the controls at $p < 0.01$.

Table 2. Mean food consumption of male and female F0 rats treated with CPME before pairing (for two weeks).

Week	Dose (mg/kg/day)			
	0	50	150	450
Males				
Week 1	183.5 ± 4.9[a]	186.5 ± 0.7	190.0 ± 15.6	171.5 ± 3.5
Week 2	177.0 ± 2.8	167.0 ± 4.2	172.5 ± 16.3	150.5 ± 0.7
Females				
Week 1	121.0 ± 14.1	122.5 ± 3.5	128.5 ± 3.5	106.0 ± 5.7
Week 2	123.5 ± 12.0	125.0 ± 5.7	131.5 ± 2.1	109.0 ± 5.7

[a]: Mean ± SD.
Number of cages examined was two in each group of both sexes.

was similar to that of the controls. However, during days 4-6 of lactation, food consumption in the treated females was slightly but significantly higher than that of the controls ($p < 0.05$) without dose-dependency.

Estrous cycle, mating performance, fertility, gestation length, and gestation index

All females showed regular (4- or 5-day) estrus cycles, except for one female in the 150 mg/kg/day group, whose cycle was at least 10 days without estrus. The precoital interval was 1-4 days for all females except for one female in the 450 mg/kg/day group whose precoital interval was 13-14 days. Mating performance and fertility were not affected by the treatment. The percentages of mating, conception rate (%), and fertility index (%) were 100% in all groups including the controls. The gestation length of the treated females was within the normal range of 22-23.5 days. However, females receiving 450 mg/kg/day showed a slight but significant shift in the duration of gestation ($p < 0.05$), with a higher proportion showing longer gestational lengths than the controls (Table 3). The length of gestation was not affected by the CPME treatment at 50 or 150 mg/kg/day.

Final body and organ weights and histopathology

Final body, testes, epididymides, and pituitary weights were measured in the F0 males. The final body weight in the 450 mg/kg/day group was statistically significantly lower (432 ± 24 g) than that of the control group (474 ± 26 g). The absolute weights of the testes, epididymides, and pituitary in the treated groups were not affected by the treatment. Histopathologically, spermatocele granulomas were observed in the epididymides in one male receiving 50 mg/kg/day. No other histopathological findings were detected in the testes or epididymides in all groups including the controls. In addition to the endocrine organs, one male in the 450 mg/kg/day group had a scab on the dorsal skin. In the skin lesion, minimal epidermal hyperplasia, folliculitis, and inflammation were observed histopathologically. The skin lesion was first observed as a cut on day 30, and the cause could not be identified because there was no evidence of fighting or other problems.

F1 animals

Clinical signs, litter size, survival rate, and sex ratio

There were no statistically significant findings in the F1 animals related to the treatment regarding clinical signs, litter size, offspring survival, and sex ratio.

The viability index on day 7 of lactation at 450 mg/kg/day was slightly but not significantly lower (88.9%) than that of the controls (97.5%). This occurred because one litter was found dead or sacrificed for welfare reasons on day 4.

Table 3. Gestation length (numbers and percentage of animals showing each gestation length) and gestation index- F0 animals.

Dose (mg/kg/day)	Number of pregnant animals	Gestation length (days)				Number of live litters born	Gestation index (%)
		22	22.5	23	23.5		
0	10	5 (50)[a]	3 (30)	2 (20)	0	10	100
50	10	4 (40)	5 (50)	1 (10)	0	10	100
150	10	4 (40)	4 (40)	2 (20)	0	10	100
450	10	1 (10)	4 (40)	4 (40)	1 (10)	10	100

[a:] The number in parenthesis indicates percentage of animals showing each gestation length in the corresponding group.

Body weight

In the F1 animals, the body weight on day 1 did not differ statistically between the controls and the treated groups, suggesting no adverse effects from maternal treatment. The body weight gain during days 1-7 for offspring in the 450 mg/kg/day group was approximately 92% (6.0 ± 1.0 g) and 90% (5.8 ± 1.3 g) that of the control male (6.5 ± 1.0 g) and female (6.4 ± 1.0 g) offspring, respectively. In the 50 and 150 mg/kg/day groups, the offspring weight gain was marginally superior to that of the controls, indicating no adverse effects of maternal treatment.

Macropathology

The stomachs of the litter that was found dead or sacrificed for welfare reasons on day 4 contained no milk. In the offspring sacrificed at scheduled termination, 1 and 2 litters had a small build in the 50 and 450 mg/kg/day groups, respectively. However, the findings did not show dose-dependency, and there were no macroscopic alterations in the other treated offspring, suggesting no effects from parental treatment.

28-day Repeated Dose Toxicity Study

Mortality and clinical signs

In the male 700 mg/kg/day group, 6 unscheduled deaths were observed between days 12 and 15. Three of the six dead animals were from the main group and the others were from the recovery group. These animals showed poor or deteriorating clinical conditions such as underactive behavior, piloerection, abnormal gait, body tremors, convulsion, hunched posture, fast respiration, and thin appearance. When poor conditions were observed and animals were considered unlikely to survive, the males in the 700 mg/kg/day group were rapidly sacrificed for ethical reasons. The remaining 2 males in the 700 mg/kg/day treatment main group also showed same clinical condition as the sacrificed males, but the serious clinical signs appeared later than in the others, and those males were sacrificed when the clinical signs were observed (day 16). In the 2 males in the 700 mg/kg/day recovery group, no serious clinical conditions were found during the study. In the other male groups and all female groups, no unscheduled deaths occurred during the experimental period. In the assessment of clinical signs, salivation was observed in the treated groups. In the 700 mg/kg/day group, both pre- and post- or pre-dosing salivation was noted between days 6 and 14 and between days 14 and 25 in males and females, respectively. Interestingly, the 2 males sacrificed on day 16 and the 1 male sacrificed on day 12 in the 700 mg/kg/day group did not show salivation during the treatment period. In the male 150 mg/kg/day group, post-dose salivation was observed in one animal on days 10 and 17, respectively. A wet coat was also observed between days 20 and 28 in the female 700 mg/kg/day group. The wet coat was detected between 1 and 2 hr after dosing and was present in 3-8 animals on each day from days 20 to 28.

In the neurobehavioral screening assessment, there were no treatment-related adverse effects in all treated groups of both sexes.

Body weight, food consumption, and water consumption

The body weight gain in the male 700 mg/kg/day group was statistically significantly lower than that of the controls during days 1 to 8 (Table 4, Fig. 1). The body weight during days 8 to 15 did not increase, and the mean body weight gain was −5.9 g in the male 700 mg/kg/day group. Simultaneously, the mean food consumption and food conversion efficiency had clearly decreased. These changes in body weight and food consumption were related to the deteriorating clinical condition and unscheduled death.

Table 4. Body weight gain of male rats treated with CPME for 28 days.

Sex/dose (mg/kg/day)	Days				
	1-8	8-15	15-22	22-28	1-28
Males					
0	56.0 ± 7.1[a]	46.2 ± 10.4	35.2 ± 8.2	26.9 ± 9.2	164.3 ± 26.7
15	57.3 ± 7.8	49.9 ± 8.2	37.2 ± 7.2	30.2 ± 4.6	174.5 ± 21.2
150	53.5 ± 6.7	45.8 ± 5.4	28.8 ± 10.0	27.1 ± 3.4	155.2 ± 23.0
700	41.1 ± 5.4**	-5.9 ± 21.9**	NA	NA	NA
Females					
0	21.0 ± 6.5	19.8 ± 8.4	17.9 ± 5.0	11.8 ± 6.4	70.5 ± 14.4
15	25.3 ± 6.4	24.6 ± 8.2	22.8 ± 3.1	7.8 ± 7.0	80.5 ± 10.6
150	20.8 ± 7.6	22.7 ± 3.4	19.6 ± 2.2	7.9 ± 3.3	71.0 ± 10.0
700	25.2 ± 5.7	18.5 ± 3.8	11.0 ± 4.3**	5.2 ± 6.6**	59.8 ± 6.3*

[a]: Mean ± SD.
*, **: Significantly different from the controls at $p < 0.05$ and $p < 0.01$, respectively.
NA: not analyzed.

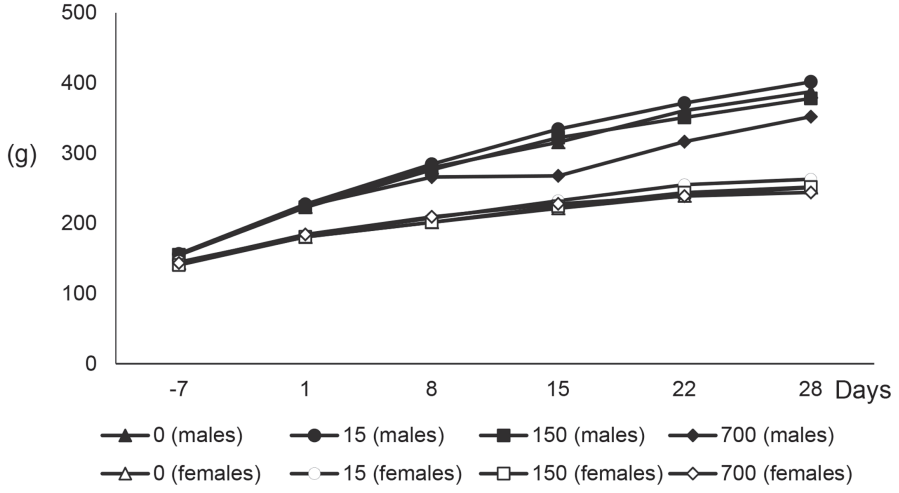

Fig. 1. Mean body weight of rats treated with CPME for 28 days.

The body weight of the 2 surviving males treated with 700 mg/kg/day CPME for 15 days recovered comparably to the control level after the 14-day recovery period from days 16 to 30. The food consumption for the two surviving males was comparable to that of the controls in the recovery period. In the female 700 mg/kg/day group, the body weight gain progressively decreased compared to that of the controls from days 15 to 28 with statistical significance (Table 4). After the 14-day recovery period, the body weight in the female 700 mg/kg/day group became comparable to that of the corresponding control group. The changes of the food conversion efficiency in the female 700 mg/kg/day group were very similar to that of body weight (Table 5): the food conversion efficiency was gradually decreased from days 15 to 28 but was comparable with that of the controls in the recovery period; nevertheless, the food consumption level from weeks 1 to 4 was higher than or comparable to the controls. In the 15 and 150 mg/kg/day group, there were no treatment-related changes in body weight and food consumption in both sexes.

Water consumption was measured on days 23-25 (week 4), and there were no treatment-related changes in all treatment groups of both sexes compared to the controls.

Hematology (Table 6)

The 2 males in the 700 mg/kg/day group that were sac-

Table 5. Food conversion efficiency in the 28-day repeated dose toxicity study.

Dose (mg/kg/day)	0	15	150	700
Males				
Week				
1	29.9[a]	29	27.9	22.1
2	23.5	23.9	23.6	-19.3
3	19.1	19.1	16.4	(17.4)[b]
4	19.9	23.5	19.3	(22.6)[b]
Females				
Week				
1	18.6	21.1	17.6	18.9
2	16.2	18.9	17.6	13.3
3	14.2	17.5	14.9	7.9
4	11.6	8.2	8.2	5.3

[a]: Group mean values (%) calculated by the following formula; Body weight gain (g) devided by food consumed (g) x 100.
[b]: Data in parentheses are from survived two animals after Day 16 of treatment.

rificed on day 16 had higher levels of RBC, Hct, Hb, and MCV and lower MCHC levels than the male controls terminated on day 29 (data not shown). In addition, obviously lower total white blood cell counts, mainly due to lower lymphocytes and eosinophil counts, were noted in the same group. These changes, which were observed in the 2 surviving males of the 700 mg/kg/day group, became comparable to the controls after the recovery period. Similar changes were also observed in the female 700 mg/kg/day group, including a statistically significantly higher mean RBC, Hct, and Hb values and a slightly lower mean WBC count, which resulted from lower lymphocyte and eosinophil counts, compared to the controls on day 29. These changes in females showed reversibility after the recovery period. There were some statistically significant differences in the RBC values in the female 15 and 150 mg/kg/day groups and the WBC count in the female 150 mg/kg/day group. However, these changes were not accompanied with other related changes or dose-dependency. Therefore, these changes observed in the female 15 and 150 mg/kg/day groups were judged to be incidental. In conclusion, there were no treatment-related hematological changes in the 15 and 150 mg/kg/day groups of both sexes.

Serum biochemistry (Table 7)

The 2 surviving males in the 700 mg/kg/day group that were sacrificed on day 16 displayed higher mean values of ALP, ALT, AST, glucose, potassium, calcium, phosphorous, total protein, and albumin compared to the controls sacrificed on day 29. Since the data for the males receiving 700 mg/kg/day were derived from two animals, the statistical significance was not evaluated. In the female 700 mg/kg group, total cholesterol and albumin levels were statistically significantly higher than those of the controls. However, the values for total cholesterol and albumin became comparable after the recovery period. At the end of the recovery period, the ALT and urea levels in the female 700 mg/kg/day group were statistically significantly lower than those of the controls, although the values of these parameters were not affected during the treatment period. No statistically significant or dose-related changes were observed in the 15 and 150 mg/kg/day groups after the treatment period in both sexes.

Urinalysis

A urinalysis assessment was not performed in the male 700 mg/kg/day group because of the poor clinical condition of those animals. After the recovery period, data from the male 700 mg/kg/day group were obtained but could not be statistically analyzed because of the lack of animals examined. In the female 700 mg/kg/day group, an increased mean protein value (0.50 g/L) and a small amount of ketones in all animals were detected compared to the controls (0.32 g/L protein level and 0 ketones). However, the values of these items became comparable to those of the controls after the recovery period. There were no statistically significant changes in both sexes in the 50 and 150 mg/kg/day groups (data not shown).

Organ weights (Table 8)

Although a statistical analysis was not performed for the 2 males in the 700 mg/kg/day group because of the insufficient number of animals examined, the absolute weights of measured organs, except for the adrenal glands, were lower than the control values. Both the

Table 6. Hematological data for rats treated with CPME for 28 days.

Dose (mg/kg/day)		0	15	150	700
Males (No. of animals examined)		5	5	5	2[b]
Hct	L/L	0.43 ± 0.01[a]	0.43 ± 0.01	0.43 ± 0.02	0.57[c]
Hb	g/dL	15.1 ± 0.5	14.9 ± 0.5	15.2 ± 0.6	17.7
RBC	$x10^{12}$/L	7.44 ± 0.22	7.24 ± 0.30	7.37 ± 0.31	8.62
MCH	Pg	20.3 ± 0.3	20.5 ± 0.4	20.6 ± 0.5	20.6
MCHC	g/dL	35.5 ± 0.3	34.8 ± 0.5	35.5 ± 0.5	30.9
MCV	fL	57.1 ± 0.7	59.1 ± 1.8	58.2 ± 1.2	66.6
WBC	$x10^9$/L	12.7 ± 3.6	15.1 ± 3.8	12.8 ± 3.1	4.13
N	$x10^9$/L	1.39 ± 0.61	1.65 ± 0.25	1.74 ± 0.67	1.07
L	$x10^9$/L	10.6 ± 2.9	12.7 ± 3.8	10.4 ± 2.6	2.73
E	$x10^9$/L	0.11 ± 0.03	0.10 ± 0.02	0.11 ± 0.06	0.03
B	$x10^9$/L	0.04 ± 0.02	0.06 ± 0.03	0.03 ± 0.02	0.02
M	$x10^9$/L	0.21 ± 0.06	0.27 ± 0.04	0.23 ± 0.06	0.17
LUC	$x10^9$/L	0.28 ± 0.11	0.30 ± 0.12	0.27 ± 0.07	0.13
Plt	$x10^9$/L	997 ± 232	926 ± 62	1062 ± 136	1192
PT	sec	14.6 ± 0.6	14.7 ± 0.4	14.7 ± 0.7	NA
APTT	sec	19.6 ± 1.9	17.9 ± 2.0	21.3 ± 2.8	NA
Dose (mg/kg/day)		0	15	150	700
Females (No. of animals examined)		5	5	5	5
Hct	L/L	0.40 ± 0.011	0.40 ± 0.016	0.41 ± 0.0062	0.43 ± 0.016**
Hb	g/dL	14.2 ± 0.58	14.6 ± 0.53	14.8 ± 0.19	15.5 ± 0.57**
RBC	$x10^{12}$/L	6.9 ± 0.22	7.3 ± 0.18*	7.2 ± 0.21*	7.7 ± 0.24**
MCH	Pg	20.6 ± 0.60	20.1 ± 0.25	20.5 ± 0.54	20.3 ± 0.26
MCHC	g/dL	36.4 ± 0.56	36.3 ± 0.19	36.6 ± 0.42	36.1 ± 0.39
MCV	fL	56.4 ± 0.86	55.4 ± 0.87	56.1 ± 1.82	56.1 ± 0.38
WBC	$x10^9$/L	9.22 ± 1.24	12.0 ± 1.76	12.7 ± 2.31*	7.60 ± 2.24
N	$x10^9$/L	1.1 ± 0.10	1.3 ± 0.38	1.3 ± 0.38	0.95 ± 0.22
L	$x10^9$/L	7.65 ± 1.38	10.2 ± 1.63	10.7 ± 1.88	6.26 ± 2.39
E	$x10^9$/L	0.16 ± 0.084	0.14 ± 0.025	0.20 ± 0.068	0.08 ± 0.026
B	$x10^9$/L	0.02 ± 0.011	0.03 ± 0.011	0.05 ± 0.015	0.02 ± 0.010
M	$x10^9$/L	0.17 ± 0.042	0.16 ± 0.046	0.20 ± 0.031	0.16 ± 0.040
LUC	$x10^9$/L	0.15 ± 0.044	0.18 ± 0.044	0.21 ± 0.059	0.12 ± 0.026
Plt	$x10^9$/L	1005 ± 94.5	1045 ± 160.6	1102 ± 65.9	998 ± 80.3
PT	sec	16.2 ± 0.39	16.0 ± 0.20	15.9 ± 0.78	15.7 ± 0.48
APTT	sec	14.7 ± 1.86	17.7 ± 1.10	17.3 ± 1.23	14.7 ± 3.08

[a]: Mean ± SD.
[b]: The data from two males were obtained on Day 16.
[c]: Mean values only due to insufficient number of animals examined.
*, **: Significantly different from the controls at $p < 0.05$ and $p < 0.01$, respectively.
NA: not analyzed.

absolute and relative weights of the adrenal glands were higher than the controls. After the recovery period, the absolute weights of the measured organs in the 2 males, except for the thymus and seminal vesicles, were also lower than those of the controls in the male 700 mg/kg/day group (data not shown). These lower absolute organ weights might be caused by the lower terminal body weight in the male 700 mg/kg/day group in both the treatment and recovery periods. The terminal body weights were 394.8 ± 14.3 g and 328.3 ± 10.8 g in the control and 700 mg/kg/day groups, respectively. In the female 700 mg/kg/day group, the absolute brain weight was statistically significantly lower, but the relative weight of the brain was comparable with the controls. Lower absolute or relative weights were not observed in other organs in the female 700 mg/kg/day group although the terminal body weight in that group was obviously lower than that of the controls. Additionally, all organ weights were compara-

Table 7. Serum biochemistry data for rats treated with CPME for 28 days.

Dose (mg/kg/day)			0	15	150	700
Number of animals examined			5	5	5	2[b]
Males	ALP	U/L	190 ± 23.6[a]	201 ± 43.7	189 ± 54.6	268[c]
	ALT	U/L	36 ± 4.9	37 ± 4.3	37 ± 10.4	52
	AST	U/L	75 ± 7.9	64 ± 8.3	77 ± 13	95
	γ-GT	U/L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0
	Bili	µmol/L	1.0 ± 0.5	1.0 ± 0.5	1.0 ± 0.5	1.0
	Urea	mmol/L	3.8 ± 0.63	3.9 ± 0.41	3.8 ± 0.54	5.1
	Creat	µmol/L	47 ± 2.3	45 ± 3.8	45 ± 1.8	49
	Gluc	mmol/L	5.5 ± 0.51	6.9 ± 0.58**	5.5 ± 0.63	22
	Chol	mmol/L	1.6 ± 0.30	1.7 ± 0.31	1.8 ± 0.44	2.8
	Trig	mmol/L	0.68 ± 0.19	0.98 ± 0.19	0.90 ± 0.36	0.50
	Na	mmol/L	140 ± 1.80	139 ± 2.80	141 ± 2.50	146
	K	mmol/L	3.8 ± 0.27	4.0 ± 0.08	3.8 ± 0.34	9.7
	CL	mmol/L	105 ± 1.1	105 ± 2.2	105 ± 2.4	104
	Ca	mmol/L	2.8 ± 0.055	2.8 ± 0.057	2.8 ± 0.061	3.3
	Phos	mmol/L	2.8 ± 0.17	2.9 ± 0.18	2.8 ± 0.22	5.4
	TP	g/L	61 ± 3.6	61 ± 2.9	63 ± 3.0	71
	Alb	g/L	35 ± 1.1	35 ± 1.7	35 ± 1.3	40
	A/G	Ratio	1.4 ± 0.11	1.3 ± 0.14	1.3 ± 0.13	1.3
Females	ALP	U/L	116 ± 26.5	141 ± 21.4	109 ± 29.2	108 ± 8.50
	ALT	U/L	32 ± 8.1	33 ± 3.6	27 ± 6.8	30 ± 4.3
	AST	U/L	67 ± 4.7	76 ± 6.3	68 ± 8.1	65 ± 4.6
	γ−GT	U/L	2.0 ± 4.0	0.0 ± 0.4	0.0 ± 0.4	0.0 ± 0.0
	Bili	µmol/L	1 ± 0.5	1 ± 0.4	2 ± 0.5	2 ± 0.4
	Urea	mmol/L	5.0 ± 0.38	5.7 ± 0.45	5.2 ± 0.43	5.6 ± 1.1
	Creat	µmol/L	51 ± 2.6	54 ± 2.9	53 ± 2.6	54 ± 5.1
	Gluc	mmol/L	5.8 ± 0.65	5.9 ± 0.59	5.3 ± 0.32	5.9 ± 0.31
	Chol	mmol/L	1.6 ± 0.35	1.9 ± 0.25	2.1 ± 0.30	2.7 ± 0.52**
	Trig	mmol/L	0.64 ± 0.31	0.53 ± 0.15	0.57 ± 0.14	0.42 ± 0.047
	Na	mmol/L	140 ± 2.1	139 ± 1.1	140 ± 0.8	139 ± 0.9
	K	mmol/L	3.5 ± 0.25	3.5 ± 0.31	3.4 ± 0.35	3.5 ± 0.32
	CL	mmol/L	106 ± 3.4	104 ± 1.3	104 ± 1.1	104 ± 1.1
	Ca	mmol/L	2.8 ± 0.029	2.8 ± 0.073	2.8 ± 0.10	2.8 ± 0.071
	Phos	mmol/L	2.3 ± 0.18	2.0 ± 0.19	2.2 ± 0.21	2.2 ± 0.25
	TP	g/L	68 ± 7.5	67 ± 4.7	69 ± 2.2	72 ± 3.6
	Alb	g/L	37 ± 1.0	37 ± 2.7	39 ± 2.0	40 ± 1.9*
	A/G	Ratio	1.2 ± 0.26	1.3 ± 0.12	1.3 ± 0.12	1.3 ± 0.017

[a]: Mean ± SD.
[b]: Number of survival animals in 700 mg/kg/day group.
[c]: Mean values only due to insufficient number of animals examined.
*, **: Significantly different from the controls at $p < 0.05$ and $p < 0.01$, respectively.

ble to the control values after the recovery period (data not shown). Therefore, the lower absolute brain weight in the female 700 mg/kg/day group might not be treatment-related. There were no statistically significant changes in any measured organs in the 15 and 150 mg/kg/day groups of both sexes.

Macroscopic and microscopic findings (Table 9)

The following findings were observed in the macroscopic examination of the male 700 mg/kg/day group that was sacrificed in extremis or on day 16: dark adrenal glands in 3/8 animals, pale spleen in 4/8 animals, and depression in the mucosa of the antrum or corpus ventriculi in the stomach in 2/8 or 1/8 animals, respectively. There were no treatment-related findings in any body

Table 8. Terminal body and organ weight of rats treated with CPME for 28 days.

Dose (mg/kg/day)			0	15	150	700
No. of animals examined			5	5	5	2 (males) or 5 (females)
Males	BW	g	342.8 ± 18.5[a]	385.6 ± 27.5	352.5 ± 25.3	244.1
	Brain	g	1.91 ± 0.05	1.92 ± 0.08	1.89 ± 0.03	1.65
		g/100 g BW	0.56 ± 0.04	0.50 ± 0.02	0.54 ± 0.03	0.68
	Adrenals	g	0.052 ± 0.002	0.052 ± 0.006	0.053 ± 0.004	0.054
		g/100 g BW	0.015 ± 0.001	0.014 ± 0.002	0.015 ± 0.002	0.022
	Heart	g	1.42 ± 0.15	1.35 ± 0.05	1.35 ± 0.14	1.09
		g/100 g BW	0.42 ± 0.05	0.35 ± 0.02	0.38 ± 0.01	0.44
	Kidneys	g	3.09 ± 0.25	3.11 ± 0.23	2.94 ± 0.18	2.31
		g/100 g BW	0.90 ± 0.07	0.81 ± 0.03	0.83 ± 0.04	0.95
	Liver	g	15.4 ± 0.9	16.6 ± 1.6	16.2 ± 2.4	12.5
		g/100 g BW	4.5 ± 0.2	4.3 ± 0.2	4.6 ± 0.4	5.1
	Spleen	g	0.58 ± 0.05	0.62 ± 0.09	0.56 ± 0.09	0.28
		g/100 g BW	0.17 ± 0.01	0.16 ± 0.02	0.16 ± 0.01	0.11
	Thymus	g	0.44 ± 0.05	0.60 ± 0.16	0.46 ± 0.11	0.24
		g/100 g BW	0.13 ± 0.01	0.15 ± 0.04	0.13 ± 0.02	0.10
	Testes	g	3.16 ± 0.16	3.35 ± 0.22	3.18 ± 0.18	2.82
		g/100 g BW	0.92 ± 0.06	0.87 ± 0.06	0.91 ± 0.10	1.16
	Epididymides	g	0.91 ± 0.10	0.93 ± 0.08	0.85 ± 0.03	0.57
		g/100 g BW	0.27 ± 0.04	0.24 ± 0.02	0.24 ± 0.01	0.23
	Seminal vesicles	g	0.93 ± 0.10	1.03 ± 0.16	0.97 ± 0.19	0.57
		g/100 g BW	0.27 ± 0.03	0.27 ± 0.04	0.28 ± 0.08	0.24
Females	BW	g	241.8 ± 23.8	252.3 ± 9.2	241.8 ± 15.7	229.2 ± 9.0
	Brain	g	1.84 ± 0.08	1.85 ± 0.05	1.81 ± 0.08	1.71 ± 0.03**
		g/100 g bw	0.77 ± 0.06	0.73 ± 0.03	0.75 ± 0.06	0.75 ± 0.02
	Adrenals	g	0.072 ± 0.014	0.062 ± 0.004	0.068 ± 0.007	0.070 ± 0.003
		g/100 g bw	0.030 ± 0.003	0.025 ± 0.002	0.028 ± 0.004	0.031 ± 0.002
	Heart	g	0.99 ± 0.11	0.97 ± 0.07	0.93 ± 0.08	0.93 ± 0.03
		g/100 g bw	0.41 ± 0.03	0.38 ± 0.03	0.38 ± 0.01	0.41 ± 0.02
	Kidneys	g	2.05 ± 0.21	2.09 ± 0.11	2.14 ± 0.08	2.05 ± 0.07
		g/100 g bw	0.85 ± 0.05	0.83 ± 0.06	0.89 ± 0.03	0.90 ± 0.05
	Liver	g	10.3 ± 1.7	10.1 ± 0.4	10.5 ± 0.8	10.7 ± 0.7
		g/100 g bw	4.3 ± 0.3	4.0 ± 0.2	4.3 ± 0.2	4.7 ± 0.4
	Spleen	g	0.48 ± 0.09	0.51 ± 0.07	0.52 ± 0.08	0.41 ± 0.07
		g/100 g bw	0.20 ± 0.02	0.20 ± 0.03	0.21 ± 0.03	0.18 ± 0.03
	Thymus	g	0.46 ± 0.08	0.49 ± 0.08	0.55 ± 0.07	0.40 ± 0.07
		g/100 g bw	0.19 ± 0.02	0.19 ± 0.03	0.23 ± 0.04	0.18 ± 0.03
	Ovaries	g	0.091 ± 0.015	0.107 ± 0.010	0.096 ± 0.009	0.095 ± 0.012
		g/100 g bw	0.038 ± 0.006	0.042 ± 0.004	0.040 ± 0.004	0.041 ± 0.004

[a]: Mean ± SD.

**: Significantly different from the controls at $p < 0.01$ (Williams's test).

parts in the male 15 and 150 mg/kg/day groups and all female treated groups.

Microscopically, necrosis in the mucosa and lamina propria (erosion) in the glandular stomach was observed in 4 of the decedent animals (3 and 1 were sacrificed on days 12 and 14, respectively) in the male 700 mg/kg/day group. In the same group, a higher incidence of adrenal cortical and medullary congestion was noted among the decedent and terminal males compared to the controls. Additionally, a reduction of severity (slight to minimal) for extramedullary hematopoiesis in the spleen was observed in all the decedent and terminal males in the 700 mg/kg/day group. In the lungs, some microscopical findings including peribronchiolar and perivascular

Table 9. Histopathological findings in rats treated with CPME for 28 days.

Finding	Males					Females			
Dose (mg/kg/day)	0	15	150	700[a]	700[b]	0	15	150	700
Number of animals examined	5	5	5	6	2	5	5	5	5
Stomach									
Ectopic nonglandular epithelium in grandular mucosa, minimal, focal	1	1	2	3	0	0	1	1	0
Necrosis of mucosa and lamina propria (Erosion), slight to moderate, focal or multifocal	0	0	0	4	0	0	0	0	0
Inflammatory cells in submucosa	0	0	0	2	0	0	0	0	0
Mineralization	0	1	2	1	0	0	0	0	0
Dilatated gastric gland	1	0	1	0	0	0	0	0	0
Adrenals									
Cortical and medullary congestion, minimal, multifocal	0	2	3	5	2	3	4	4	3
Cortical vacuolation	0	0	2	1	1	0	0	0	0
Cortical inflammatory cell aggregation	1	0	0	0	0	1	0	1	0
Mineralization of medulla	0	0	0	0	0	1	0	0	0
Lungs and Bronchi									
Peribronchiolar and perivascular inflammatory cell infiltration	5	NA	NA	3	2	5	NA	NA	5
Alveolar hemorrage, minimal, multifocal	4	NA	NA	2	0	1	NA	NA	3
Prominent alveolar macrophages, minimal, multifocal	5	NA	NA	2	2	4	NA	NA	4
Hemoglobin crystals, minimal, focal	0	NA	NA	0	1	0	NA	NA	0
Foamy alveolar macrophages, minimal, focal	0	NA	NA	0	1	0	NA	NA	0
Arterial mural mineralization	2	NA	NA	0	0	1	NA	NA	0
Alveolar septal thickening	1	NA	NA	0	0	1	NA	NA	0
Pleural thickening and subpleural inflammatory cell foci	2	NA	NA	0	0	3	NA	NA	4
Spleen									
Reduction of severity for extramedullary hemopoiesis, minimal, multifocal	0	0	0	5[c]	2	0	0	0	0
Decreased cellularity of white pulp, generalised	0	0	0	1	1	0	0	0	0
Hemosiderosis	0	0	0	0	0	3	1	3	5

[a]: Males sacrificed or died during the treatment period.
[b]: Males sacrificed after 15 days of treatment.
[c]: The spleen in one animal sacrificed in extremis was not examined due to tissue missing at the necropsy.
NA: not analyzed.

inflammatory cell infiltration, alveolar hemorrhage, and prominent alveolar macrophages were found in the male 700 mg/kg/day group, but the same findings were also observed in the controls without a significant difference. The adrenal glands displayed a dark color in the macroscopic examination, and cortical and medullary congestion was observed in these glands in the decedent and terminal males receiving 700 mg/kg/day. No treatment-related microscopic findings were observed in the male 15 and 150 mg/kg/day groups and all female treated groups after the treatment and recovery periods.

Genotoxicity

Bacterial reverse mutation test (Ames test)

Since the reproducibility between the preliminary and main studies was confirmed, the results of the main study are shown in Table 10. In the present bacterial reverse mutation test, precipitation of the test chemical or growth inhibition was not observed. In the treatment groups, no substantial increase (more than twice) in revertant colony numbers compared to the untreated controls was observed in any test strains at any concentration with or without S9 mix. The mean revertant colony counts for the untreated controls (DMSO) were within the 99% confidence limits of the current historical control laboratory range. However, the revertant colony numbers of the positive controls showed remarkable increases. The sensitivity of cultures and activity of the S9 mix was confirmed to be relevant in this study. These results suggested that CPME does not have mutagenicity in the bacterial system under the

Table 10. The bacterial reverse mutation test with CPME.

Dose	S9	Mean revertant colonies per plate (mean ± SD, n = 3)				
(μg/plate)	mix	TA100	TA98	TA1535	TA1537	WP2*uvrA*/pkM101
0[a]	-	129 ± 5	36 ± 4	23 ± 4	12 ± 2	132 ± 6
50	-	103 ± 12	36 ± 10	14 ± 2	11 ± 2	124 ± 27
150	-	105 ± 18	34 ± 3	21 ± 8	12 ± 3	119 ± 10
500	-	126 ± 10	32 ± 9	17 ± 5	9 ± 1	116 ± 19
1500	-	116 ± 5	34 ± 5	18 ± 4	11 ± 4	113 ± 23
5000	-	117 ± 10	32 ± 4	17 ± 4	10 ± 2	128 ± 18
2 NF 1.0	-		444 ± 49			
AF-2 0.05	-					1094 ± 291
SAZ 0.5	-	1327 ± 245		989 ± 125		
9-AA 30.0	-				1113 ± 412	
0[a]	+	132 ± 9	43 ± 6	27 ± 1	16 ± 2	163 ± 11
50	+	135 ± 5	43 ± 5	23 ± 7	17 ± 4	160 ± 13
150	+	135 ± 1	43 ± 5	21 ± 8	12 ± 4	156 ± 14
500	+	135 ± 5	42 ± 4	21 ± 6	14 ± 7	131 ± 14
1500	+	125 ± 11	41 ± 3	23 ± 6	13 ± 3	130 ± 13
5000	+	126 ± 4	41 ± 2	15 ± 2	14 ± 4	136 ± 23
BaP 5.0	+	753 ± 93	524 ± 78		126 ± 2	
2AA 2.0	+			215 ± 56		
2AA 10.0	+					822 ± 60

[a]: Negative control, Dimethyl sulfoxide.
2NF, 2-Nitrofluorene; AF-2, 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide; SAZ, Sodium azide; 9-AA, 9-Aminoacridine; BaP, Benzo[a] pyrene; 2AA, 2-Aminoanthracene.

Table 11. *In vitro* chromosomal aberration test in CHL cells with CPME (first test).

Dose	S9	No.of cells	Survival	The number of structural aberrations (n)					TA	POLY
(μg/mL)	mix[b]	examined	(%)[c]	g	ctb	cte	csb	cse	(%)	(%)
0[a]	-	200	100	6	3	0	0	0	1.5	0.1
250.4	-	200	98	14	9	0	1	0	4.5	0.3
500.8	-	200	92	12	1	0	1	0	1.0	0.2
1001.6	-	200	96	12	7	0	2	0	4.5	0.2
MMC	-	200	76	25	56	18	1	0	26.0***	0.0
0[a]	+	200	100	5	1	0	1	0	1.0	0.0
250.4	+	200	71	4	1	0	3	0	1.5	0.2
500.8	+	200	67	10	5	0	4	0	4.0	0.1
1001.6	+	200	61	8	9	0	2	0	4.0	0.2
CP	+	200	63	27	20	5	3	0	11.5***	0.2

[a]: Negative control, Dimethyl sulfoxide. [b]: Sampling time (hr), 3 hr treatment and 12 hr recovery. [c]: Survival (%) shows the ratio of the number of cells treated or positive control cultures for that of the negative control cultures.
Abbreviations: g, chromatid and chromosome gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; MMC, Mitomycin C; CP, Cyclophosphamide.
TA, total aberrant cells excluding gap; POLY, polyploid cells.
***: Significantly different from the control group at $P < 0.001$ (Fisher's test).

present test conditions.

In vitro chromosomal aberration test

The results of the first and second tests for *in vitro* chromosomal aberration are shown in Tables 11 and 12. In the first and second tests, less than 50% decrease of cell count, an indicator of cytotoxicity, was not observed under the 3-hr treatment of CPME with or without S9 mix and the continuous 15-hr treatment compared to the negative control. Therefore, the doses of CPME for the met-

Table 12. *In vitro* chromosomal aberration test in CHL cells with CPME (second test).

Dose (μg/mL)	S9 mix	No.of cells examined	Survival (%)[d]	The number of structural aberrations (n)					TA (%)	POLY (%)
				g	ctb	cte	csb	cse		
0[a]	-[b]	200	100	2	5	0	0	0	2.5	0.1
250.4	-	200	80	9	4	0	1	0	2.0	0.2
500.8	-	200	86	5	3	0	0	0	1.5	0.0
1001.6	-	200	80	3	9	0	0	0	4.0	0.0
MMC	-	200	77	15	53	10	7	0	19.5***	0.0
0[a]	+[c]	200	100	3	3	0	2	0	2.0	0.0
250.4	+	200	88	8	4	0	5	0	3.5	0.1
500.8	+	200	104	10	8	0	5	0	6.0	0.3
1001.6	+	200	96	5	11	1	2	0	6.0	0.1
CP	+	200	100	12	27	1	9	0	15.0***	0.1

[a]: Negative control, Dimethyl sulfoxide. [b]: Sampling time (hr), 15 hr continuous treatment, [c]: 3 hr treatment and 12 hr recovery. [d]: Survival (%) shows the ratio of the number of cells treated or positive control cultures for that of the negative control cultures.
Abbreviations: g, chromatid and chromosome gap; ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; MMC, Mitomycin C; CP, Cyclophosphamide.
TA, total aberrant cells excluding gap; POLY, polyploid cells.
***: Significantly different from the control group at $P < 0.001$ (Fisher's test).

Table 13. *In vivo* mouse micronucleus test with CPME.

Dose (mg/kg)	No. of Dosing	No. of animals	Treatment Time (hr)	Mean ± SD (%)	
				%MNIE[c]	%IE[d]
0[a]	1	7	24	0.06 ± 0.05	35.1 ± 2.7
500	1	7	24	0.04 ± 0.06	37.9 ± 3.9
1000	1	7	24	0.04 ± 0.03	34.3 ± 2.1
2000	1	7	24	0.04 ± 0.04	35.4 ± 4.3
0[a]	1	7	48	0.03 ± 0.03	37.3 ± 3.9
2000	1	7	48	0.01 ± 0.02	38.6 ± 2.6
MMC[b]	1	5	24	1.36 ± 0.34**	40.6 ± 1.1

[a]: Negative control, corn oil. [b]: Positive control, Mitomycin C (MMC). [c]: % of micronucleated cells observed per 2000 immature erythrocytes. [d]: Proportion of immature erythrocytes.
**: $P < 0.01$(nonparametric method).

aphase analysis in both tests were determined to be 250.4, 500.8, and 1001.6 μg/mL (the highest dose corresponded to 10 mM). In the metaphase analysis, the percentages of structural chromosomal aberrations and of polyploid cells were less than 4.5% and 0.3% in the first test or 6.0% and 0.3% in the second test, respectively, with or without S9 mix, and there were no dose-dependent alterations in the induction of chromosomal aberrations and polyploid cells, suggesting that these are negative results under the experimental conditions of both tests. The percentage of structural chromosomal aberrations for the negative controls was within the current historical control laboratory range. In the positive controls, a statistically significant increase in the percentage of chromosomal aberrations was observed. Based on these results, it was confirmed that CPME does not induce chromosomal aberrations in CHL cells under the present experimental conditions.

In vivo mouse micronucleus test

The results of the *in vivo* mouse micronucleus test are shown in Table 13. The animals in the 1000 and 2000 mg/kg groups showed some clinical signs such as piloerection, under- or overactivity, and fast respiration, but there was no mortality or decrease of mean body weights in the present test. In the assessment of the effects of CPME on the micronucleus, the mean percentages of micronucleated cells observed per 2000 immature erythrocytes were less than 0.04%, and there were no statistically significant increases or dose-dependent alterations in any test conditions. In addition, a significant decrease in the proportion of immature erythrocytes was not induced under the present experimental conditions. In the positive control, a mean of 1.36% of cells were micronucleated, indicating a statistically significant increase compared to the negative control. These results suggested that

there was no possibility for CPME to induce chromosomal aberrations in mouse bone marrow cells when the mice were treated with CPME by gavage under the present experimental conditions.

DISCUSSION

In this study, we demonstrated the new test results of reproductive and developmental toxicity study of CPME, an ethereal solvent in pharmaceutical chemical process development. We also presented the dose-response data of repeated dose toxicity and genotoxicity studies for which summary data only has been already published. Then detailed toxicity evaluation of CPME was performed using these reliable test data for the setting a PDE level.

In both the reproductive/developmental toxicity and repeated dose toxicity studies, serious poor clinical conditions accompanied by decreased body weight gain resulting in unscheduled sacrifice was observed only in males. One male in the 450 mg/kg/day group in the reproductive/developmental toxicity study showed underactive behavior, hunched posture, fast breathing, poor reflexes, and partially closed eyelids only on day 18 without a change in body weight. In the same study, several males in the 450 mg/kg/day group showed some clinical signs such as an unsteady gait during the first 3 days. In the 28-day repeated dose toxicity study, 6 out of 10 animals in the 700 mg/kg/day group showed underactive behavior, piloerection, abnormal gait, body tremors, convulsion, hunched posture, fast respiration, and thin appearance, resulting in unscheduled deaths during days 12 to 15. The other 2 males in the main group receiving 700 mg/kg/day also showed similar clinical conditions on day 16 and were sacrificed on the same day, which was earlier than scheduled. Conversely, none of the females in the same dose group showed such poor clinical conditions although their body weight and food consumption decreased. These findings indicate that CPME might be a cause of these clinical conditions and that males are more susceptible than females. Although the doses in the reproductive/developmental toxicity study were decided based on the 28-day repeated dose study, 450 mg/kg/day of CPME, which was lower than the highest dose in the 28-day study, affected the clinical condition of males. Therefore, the effect of CPME on poor clinical conditions has a degree of uncertainty. In the present study, a primary cause of the poor clinical condition and a higher susceptibility in males could not be identified. In the 28-day repeated dose toxicity study, the slight to moderate erosion in the stomach observed in the male 700 mg/kg/day group could affect clinical conditions. However, the incidence of erosion in the stomach was not observed in all males; it was found in four of the six males sacrificed because of poor clinical conditions. Additionally, 2 males in the recovery group receiving 700 mg/kg/day did not show such poor clinical conditions, and their body weight was not affected by the treatment. Therefore, there is a slight possibility that the technique of administration might affect the condition of males in the highest dose group, in addition to the irritation caused by CPME.

The significantly lower viability index of the F1 animals receiving 450 mg/kg/day on day 7 of lactation in the reproductive/developmental toxicity study was caused by one litter that was found dead or sacrificed for welfare reasons on day 4. A dam of the litter was acyclic (at least ten days without estrus) during pairing and had a longer precoital interval (14 days), and on day 4 of the lactation period, the dam suddenly became thin and displayed a hunched posture, resulted in sacrifice for welfare reasons. Moreover, a predominant decrease of body weight and food consumption was observed on day 4 or on days 1-3 of the lactation period, respectively. Since the findings in the dam were suddenly observed during the lactation period only, CPME treatment is not seemed to be the cause of the poor clinical condition. In addition, since there was no milk in the stomach of the litter, the cause of the lower viability index might not be the direct effects of CPME but rather malnutrition caused by the poor systemic condition of their dam. The findings of the acyclic estrus cycle and longer precoital interval in the dam were observed in only one among 10 animals in the 450 mg/kg/day group, and the dam did not show any systemic abnormalities including changes in body weight. Therefore, the acyclic estrus cycle and longer precoital interval might be derived from an endocrine unbalance in the dam rather than CPME treatment, although the sexual hormone levels in the serum were not examined.

Pre- and/or post-dosing salivation was observed in the 700 mg/kg/day groups of both sexes in the 28-day study. The same finding was also detected in the 150 and 450 mg/kg/day groups in the reproductive/developmental toxicity study. However, the frequency was not continuous but was sporadic, and the incidence (number of animals showing salivation) was not constant on each observed day. Therefore, the cause of salivation was considered to be an irritation from CPME rather than a direct toxic effect of CPME. The only difference between males and females was the timing of salivation. In the 700 mg/kg/day group, males displayed pre-and post-dosing salivation whereas females displayed only pre-dosing salivation. The cause of this difference could not be

clarified in the present study, but this would also provide evidence that salivation is not an adverse effect of CPME treatment.

In the 28-day study, some hematological alterations were observed in both sexes receiving 700 mg/kg/day. The RBC, Hb, and Htc levels were higher and the WBC and some of its fractions were lower than the controls in the two surviving males and the female mean values. In the assessment of alterations in the hematopoietic organs, the reduction in the severity of extramedullary hematopoiesis, which might be one of the causes of decreased weight and pale appearance in the spleen, was only observed in the decedent and terminal males of the 700 mg/kg/day group. However, these alterations are opposite reactions to the findings in the RBC and its related items. Additionally, since it was unclear which fraction of blood was decreased in the extramedullary hematopoiesis in the spleen, the present study could not conclude that the alteration in the WBC was caused by the findings in the spleen. Therefore, the cause of the hematological alteration observed in the 700 mg/kg/day group might be the deterioration of systemic conditions including decreased body weight gain.

In the serum biochemistry assessment and urinalysis in the 28-day study, some alterations were induced in the 700 mg/kg/day groups of either both the sexes or males, respectively. However, there were no histopathological findings in the liver and kidneys, the related organs of serum biochemistry items such as ALP, ALT, AST, total cholesterol, and albumin, and of urinalysis items such as protein and ketones. Based on these findings, the alterations in the serum biochemistry and urinalysis induced by 700 mg/kg/day CPME are not considered biologically significant.

The summary of a 3-month repeated dose oral toxicity study was described in previously published reports (Antonucci *et al.*, 2011). However, it was no clear whether those studies were performed according to the OECD test guidelines for repeated dose oral toxicity studies. And the summary of a 90-day subchronic inhalation study (OECD TG413) was described in the report (Watanabe, 2013). No detailed data to judge the reliability of the study for both study were publicity available. Hence, we decided that these studies were not suitable for derivation of the PDE in the present study, although the NOAELs of these studies were clarified in the summary reports.

Based on the results of the Ames test, *in vitro* chromosomal aberration test and *in vivo* micronucleus test described in the present study, it can be concluded that CPME is not mutagenic or genotoxic. According to the results of the reproductive/developmental toxicity study and the 28-day study described in the present article, the NOAELs for both studies for CPME were 150 mg/kg/day. These NOAELs were based on lower body weight in F0 males, lower body weight gain in F1 animals of both sexes and poor clinical condition in males observed at the highest doses in the reproductive/developmental toxicity and the 28-day studies, respectively. These NOAEL values are higher than those of other reported CPME toxicity studies. In the 90-day subchronic inhalation toxicity study (OECD 413), the NOEL of CPME was 0.87 mg/L for male rats and 0.84 mg/L for female rats (Watanabe, 2013), which corresponds to 115.4 mg/kg/day for males and 111.4 mg/kg/day for females when the NOAELs of the inhalation study are converted to those used for oral values. In the 90-day subchronic oral toxicity study, the NOEL of CPME was 31 mg/kg/day (Antonucci *et al.*, 2011). However, since the reliability of both 3-month repeated dose oral toxicity study and a 90-day subchronic inhalation study reported by Watanabe or Antonucci could not be confirmed as described previously. Therefore, the rat oral 28-day study and reproductive/developmental toxicity study can be used as key studies for the present study. We suggest that the NOAELs of 150 mg/kg/day for the rat oral 28-day study and reproductive/developmental toxicity study are suitable to serve as the PDE for CPME. Although the poor clinical signs were observed at the 700 mg/kg/day in the 28 days study, no severer toxicological effects were caused at the 450 mg/kg/day of F0 animals in the reproductive screening toxicity study. Therefore, we propose that the factor of F4 for PDE calculation according to the ICH Q3C guideline is 1. A candidate PDE would be calculated as follows:

$$\text{PDE} = \frac{150 \times 50}{5 \times 10 \times 10 \times 1 \times 1} = 15 \text{ mg/day}$$

F1 = 5 to account for the extrapolation from rats to humans
F2 = 10 to account for differences between individual humans
F3 = 10 because duration of treatment (28 days)
F4 = 1 because no severe effects were observed
F5 = 1 because a NOAEL was established

The present report supplies new reproductive/developmental toxicity data and detailed results from a rat oral 28-day repeated dose study and genotoxicity studies for CPME. These data might be able to contribute to the full toxicity evaluation of CPME and to support the establishment of an official PDE level for ICH guidelines on impurities in pharmaceuticals.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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