

## Carcinogenicity study of 3-monochloropropane-1, 2-diol (3-MCPD) administered by drinking water to B6C3F1 mice showed no carcinogenic potential

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**Abstract** 3-Monochloropropane-1, 2-diol (or 3-chloro-1, 2-propanediol, 3-MCPD) is a well-known food processing contaminant found in a wide range of foods and ingredients. It has been classified as non-genotoxic carcinogen but its carcinogenic potential in the rodents has been controversial. The carcinogenicity to B6C3F1 mice by drinking water administration was assessed over a period of 104 weeks. Three groups, each comprising 50 male and 50

female mice received 3-MCPD at dosages of 30, 100 or 300 ppm up to Day 100 and 200 ppm onward (4.2, 14.3 and 33.0 mg/kg for males; 3.7, 12.2, and 31.0 mg/kg for females), were allocated. Survival was good, with at least 80% of males and 72% of females in each group surviving 104 weeks. Body weights and body weight gain were decreased in males and females receiving 200 ppm. Water and food consumptions of both sexes at 300/200 ppm were lowered. Emaciated or crouching position was observed for animals of both sexes exposed to 200 ppm. There were some differences in hematology and serum biochemistry compared with controls, although there was no histopathological evidence to support those changes. Histopathological examination did not reveal any neoplastic or non-neoplastic findings attributable to treatment with 3-MCPD. It is concluded that drinking water administration of 3-MCPD for 104 weeks revealed no evidence of carcinogenic potential.

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### Abbreviations

|        |                                                           |
|--------|-----------------------------------------------------------|
| CPN    | Chronic Progressive Nephropathy                           |
| KFDA   | Korea Food and Drug Administration                        |
| KIT    | Korea Toxicology Institute                                |
| NOAEL  | No-Observed-Adverse-Effect Level                          |
| 3-MCPD | 3-Monochloropropane-1, 2-diol                             |
| MTD    | Maximum Tolerated Dose                                    |
| OECD   | Organisation for Economic Co-operation and<br>Development |
| SCF    | Scientific Committee on Food                              |
| SD     | Spargue-Dawley                                            |
| TDI    | Tolerable Daily Intake                                    |

## Introduction

3-Monochloropropane-1, 2-diol (or 3-chloro-1,2-propanediol, 3-MCPD) is known as one of the most common chemical contaminants, called chloropropanols, which is found in a wide range of foods and ingredients (Olsen 1993). It is primarily formed in foods as a result of protein hydrolysis by adding hydrochloric acid to accelerate the reaction between a chlorine source in the food or a food contact material (Food Standard Agency 2001). Although the actual mechanisms of its formation are not completely understood (Scientific Committee on Food 2001), it is known that low levels of the contaminant are present in some heat-processed foods and sausages such as salami, roasted products (Pesselman and Feit 1988), and most notably in soy sauce (Macarthur et al. 2000).

3-MCPD is known to be mutagenic under *in vitro* (Henderson et al. 1987; Rossi et al. 1983) but not *in vivo* (El Ramy et al. 2007; Scientific Committee on Food 2001; Robjohns et al. 2003). In animal bioassay, it was considered to be carcinogenic to rats and highly suspected to be a non-genotoxic carcinogen (Scientific Committee on Food 2001). Until now, five long-term rodent carcinogenicity studies with 3-MCPD have been conducted (Cho et al. 2008b; Lynch et al. 1998; Olsen 1993; Sunahara et al. 1993; Van Duuren et al. 1974; Weisburger et al. 1981). Three rat studies were reported with controversial results; one dietary study with F344 rats induced Leydig cell tumors, mammary gland tumors, and kidney tumors (Sunahara et al. 1993); the other dietary study with SD rats did not induce any tumors (Weisburger et al. 1981); and another recent drinking water study using SD rats induced Leydig cell tumors and renal tubule tumors (Cho et al. 2008b). However, two Swiss mice carcinogenicity studies using the subcutaneous or intraperitoneal routes did not induce any tumors (Van Duuren et al. 1974). Unfortunately, those mice studies were conducted decades ago with inappropriate protocol (Cho et al. 2008b), and the route of administration did not mimic the most public exposure mode of human to 3-MCPD. Therefore, it is necessary to assay tumorigenic potential according to currently approved carcinogenicity bioassay guidelines (OECD 1981), and by drinking water as a route of administration to simulate the most common exposure route (i.e., soy sauce) to human. Thus, the carcinogenicity of 3-MCPD was investigated to B6C3F1 mice by drinking water, in order to clarify the possible involvement of the species- and strain- specific non-genotoxic carcinogenicity of 3-MCPD, according to the test guidelines from Korea Food and Drug Administration and the OECD Test Guideline 451 for 'Carcinogenicity studies' (OECD 1981).

## Materials and methods

### Test material and formulations analysis

3-MCPD (Cas No. 96-24-02), 98% pure, was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). The structure and purity were verified prior to use, with its stability confirmed at all concentrations. The dose formulations were prepared fresh every 2 weeks by dissolving 3-MCPD in deionized water and stored in amber glass vessels protected from the light at 4°C prior to use. The homogeneity and stability of the 3-MCPD were confirmed analytically by high-performance liquid chromatography (the expected concentration  $\pm 10\%$ ). Detailed records of compound usage were maintained. The amount of test substance necessary to prepare the formulations and the amount actually used were determined on each occasion. The difference between these amounts was checked before the formulations were dispensed. The solutions were given to the mice in water bottles with the solutions being replaced every 3 days. Periodic analyses of the dose formulations were conducted. Test formulations were prepared at concentrations of 30, 100, and 300  $\mu\text{g/ml}$  until Day 100 and then 200  $\mu\text{g/ml}$ . After 3 h, samples were taken from each of the two test formulations. The derivatized samples were analyzed by LC–MS (Hewlett-Packard 1100 series) with positive electrospray using Luna C18 column. The limits of analytical results for all dose formulations were within 10% of the theoretical concentrations.

### Animal husbandry and maintenance

Four-week-old male and female B6C3F1 mice strain were obtained from a specific pathogen-free colony at Orient Bio Inc. Korea, and quarantined for 14 days before the study. The animals were  $\sim 6$  weeks old on the first day of the study. The female mice were housed five per cage and the male mice were housed individually. Mice were fed UV-irradiated PMI Nutrition International diet (505 North 4th street Richmond, IN 47374, USA) *ad libitum*, with the exception of a one-night fast prior to their scheduled killing. Water was available *ad libitum*. The cages were changed twice weekly and the racks were changed every 2 weeks. The environmental conditions (temperature,  $23 \pm 1^\circ\text{C}$ ; relative humidity,  $55 \pm 5\%$ ; 12 h light/dark cycle) were monitored at 4 h cycles for 24 h/day and maintained within the acceptable ranges through the study. The mice were housed in an accredited Korea Institute of Toxicology (KIT) animal facility in accordance with the AAALAC International Animal Care Policies. Furthermore, all the study protocols were reviewed and approved by the Animal Care and Use Committee of the KIT.

### Experimental design and rationale for dose selection

The route of drinking water administration was chosen to simulate the most notable conditions of public exposure to 3-MCPD. Groups of 50 male and 50 female mice were exposed *ad libitum* to 0, 30, 100, and 300/200 ppm 3-MCPD in their drinking water over a 104-week period. Forty sentinel mice (20 males and 20 females) were allocated to monitor microbiology of animals. As a preliminary dose range-finding study for conducting the carcinogenicity study on 3-MCPD, a 13-week toxicity study was performed on B6C3F1 mice to survey target organs and select doses to be used for a 104-week study (Cho et al. 2008a). The results indicated that the MTD was 200 ppm; increased kidney weight was noted at 200 and 400 ppm. At 400 ppm, suppressed body weight gain, decreased sperm motility, testicular germinal epithelial degeneration, and delayed total estrus cycle were also reported. Therefore, initially, 300 ppm was selected as high dose level, where it was expected that a certain level of testicular seminiferous tubular degeneration would be induced. However, 300-ppm groups showed significant decreases in body weights (27 and 24% decrease compared to vehicle control for male and female, respectively), food consumption (19 and 21% decrease compared to vehicle control for male and female, respectively), and water consumption (17 and 25.5% decrease compared to vehicle control for male and female, respectively). Therefore, the high doses were reduced to 200 ppm from Day 101. The low dose was set at 30 ppm, with the estimation to provide information on the NOAEL. The mid-dose level of 100 ppm is approximately the geometric mean between the low and high dose levels.

### In-life phase examination

Animals were inspected visually at least once a day, and a weekly detailed physical examination was performed on each animal to monitor any superficial palpable swellings. Animals were killed for reasons of animal welfare where necessary, where possible, blood samples were taken. The location, size, consistency, and time of first observation of any swelling and subsequent history were recorded; special attention was paid to morbidity and mortality. The weight of water and food supplied to each cage and that remaining was recorded every week until 13 weeks and every month thereafter. From these records, the mean weekly consumption per animal (mg/mouse/week) was calculated. At the end of the study, all the animals were anesthetized with isoflurane, weighed, and blood samples were collected from the abdominal aorta for hematology and blood chemistry, and then killed by exsanguinations from the abdominal aorta.

### Urinalysis, hematology, and blood chemistry

Urine samples were collected on weeks 102 prior to terminal killing from 5 males and 5 females in each group. The individual samples were examined by urine chemistry analyser (ClinTek-500, Bayer) for volume, specific gravity, color, pH, glucose, protein, ketones, occult blood, bilirubin, urobilinogen, and nitrite. A microscopic examination of the urine sediment was also performed. Blood samples were taken one occasion only at the end of the treatment period.

Blood for the hematology determinations was placed in tubes containing potassium EDTA as an anticoagulant. The hematology determinations including the erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count and differential leukocytes count were performed on an Advia 120 hematology analyzer (Bayer, USA). The serum biochemistry parameters including alkaline phosphatase, total protein, albumin, blood urea nitrogen, and creatinine were evaluated using an autoanalyzer (Toshiba 200FR NEO, Toshiba, Japan).

### Pathology

All animals were subjected to a detailed necropsy. After a review of the history of each animal, a full macroscopic examination of the tissues was performed in accordance with the OECD Test Guideline 451 for 'Carcinogenicity studies' (OECD 1981) and SOPs of KIT. All external features and orifices were examined visually. Any abnormal position, morphology or interaction was recorded. Organs were weighed including adrenals, brain, epididymis, heart, kidneys, liver, lung, pituitary, ovaries, prostate, salivary glands, spleen, testis, thymus, thyroid, uterus, and cervix. Complete necropsies were performed on all mice, including those that died or became moribund. All organs/tissues were fixed in 10% neutral buffered formalin, with the exception of the testes, which were fixed in Bouin's solution, and the eyes and Harderian glands, which were fixed in Davidson's AFA fixative. Tissues with bone that required decalcification, such as the femur and spinal cord with bone, were treated with 7.5% nitric acid for approximately 4–5 h. All the organs and tissues were processed and trimmed, embedded in paraffin, sectioned to a thickness of 4–6  $\mu\text{m}$ , and stained with hematoxylin and eosin for the microscopic examination. Histopathological diagnosis and peer review were performed according to the Standardized System of Nomenclature and Diagnostic Criteria (SSNDC).

## Quality assurance and test guidelines

Quality assurance inspections and audits (undertaken at KIT) were conducted at each critical phases of the study; formulation analysis, dosing, necropsy, hematology, serum biochemistry, and report preparation properly according to their SOPs. The results of any inspection were reported promptly in writing to the appropriate management.

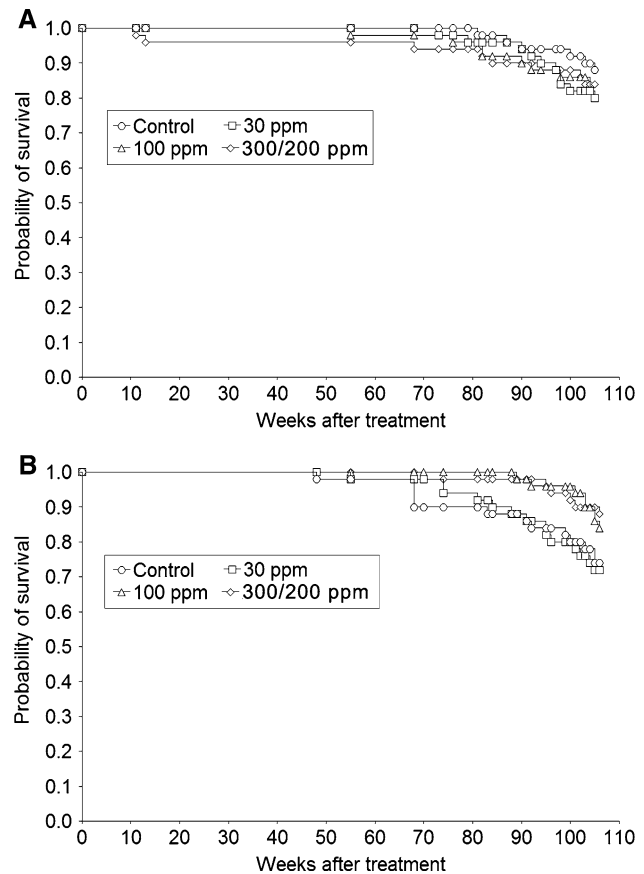
## Statistical analysis

Path/Tox System (version 4.2.2) was used for data management and statistical analysis of both in-life observation and pathology. All statistical analyses were carried out separately for males and females. For the bodyweight, blood chemistry, hematology, and urinalysis, analyzed separately at each time point. The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958). Statistical analyses for possible dose-related effects on survival were performed using Cox's (1972) method, for testing two groups for equality, and Tarone's (1975) life table test to identify dose-related trends. The body weight, food, and water consumption data were analyzed using an analysis of variance, followed by the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971). The Poly-*k*-test (Bailer and Portier 1988) was used to assess the incidences of neoplastic and non-neoplastic lesions. A  $P < 0.05$  was considered significant.

## Results

### In-life observations and formulation analysis

There was no significant treatment-related effects on the survival rate (Fig. 1, Table 1). Emaciated (39 in males; 35 in females) or crouching position (22 in males; 9 in females) were observed at infrequent intervals for males and females exposed to 300/200 ppm. All other signs observed in this study were of the types normally encountered in B6C3F1 at this laboratory and occurred at the expected frequencies. The body weights of both sexes in the 300/200 ppm groups were significantly decreased throughout the study compared to those of the control (Fig. 2). The water and food consumptions of both sexes administered 300/200 ppm were significantly lowered throughout the study than those of the control (Fig. 3). Concentrations of 30, 100 or 300/200 ppm 3-MCPD in the drinking water resulted in average daily consumptions of approximately 4.2, 14.3, and 33.0 mg/kg 3-MCPD for males and 3.7, 12.2 and 31.0 mg/kg 3-MCPD for females, respectively. The formulations investigated during the



**Fig. 1** Survival curves for male (a) and female (b) B6C3F1 mice in the 2-year drinking water study of 3-MCPD

study were found to comprise 3-MCPD within the range of 98.52–107.0% of nominal indicating satisfactory preparation of the dose formulations.

### Urinalysis, hematology, and serum chemistry

Urinalysis prior to terminal killing revealed no differences between controls and treated groups. Hematology results showed a decreased number of platelets at 100 or 300/200 ppm groups, decreased mean corpuscular hemoglobin (MCH), and increased red cell distribution width (RDW) at 300/200 ppm group in males, with a statistical significance. Statistically significant decreased monocyte counts were reported in females exposed to 300/200 ppm. Serum chemistry showed that BUN (in both sexes receiving 100 and 300/200 ppm), ALP (in both sexes receiving 300/200 ppm), and albumin (in male receiving 300/200 ppm) were significantly increased. In addition, triglyceride levels were significantly decreased for males in the 300/200 ppm group. A number of other differences in hematology and serum biochemistry from controls attained statistical significance, however, as degree of changes were slight, and lack of dose-relationship, all those were considered not to be treatment related.

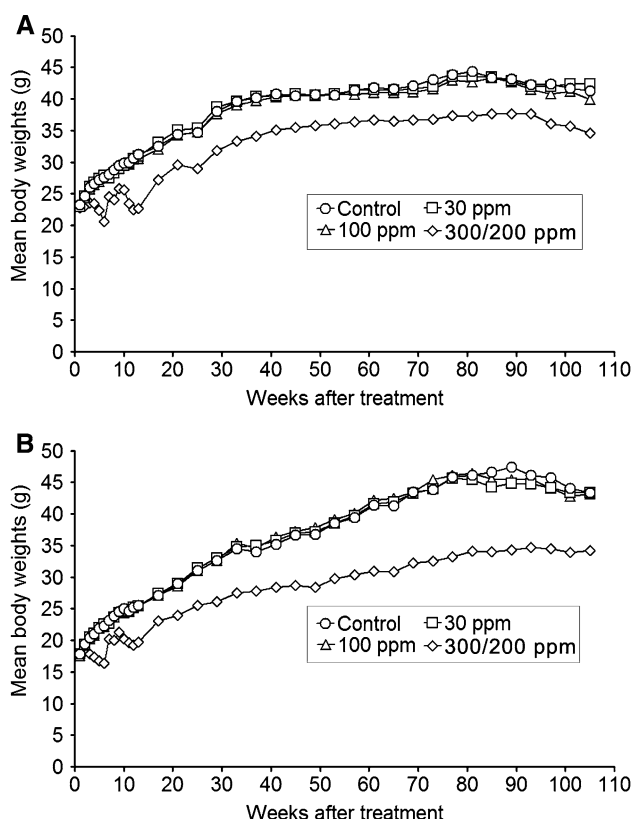
**Table 1** Survival of B6C3F1 mice exposed to 3-MCPD by drinking water for 104 weeks

| Sex                                          | Male          |             |             |             | Female        |             |               |               |     |
|----------------------------------------------|---------------|-------------|-------------|-------------|---------------|-------------|---------------|---------------|-----|
|                                              | Dosage (ppm)  | 0           | 30          | 100         | 200           | 0           | 30            | 100           | 200 |
| Animals initially on study                   | 50            | 50          | 50          | 50          | 50            | 50          | 50            | 50            | 50  |
| Humane kill                                  | 1             | 2           | 3           | 1           | 1             | 1           | 0             | 1             | 1   |
| Found dead                                   | 7             | 8           | 7           | 7           | 12            | 13          | 8             | 5             | 5   |
| Survival to terminal killing                 | 42            | 40          | 40          | 42          | 37            | 36          | 42            | 44            | 44  |
| Percent probability of survival <sup>a</sup> | 84            | 80          | 80          | 84          | 74            | 72          | 84            | 88            | 88  |
| Mean survival (days) <sup>b</sup>            | 719           | 714         | 708         | 692         | 698           | 704         | 731           | 728           | 728 |
| Survival analysis <sup>c</sup>               | $P = 0.979$ N | $P = 0.758$ | $P = 0.740$ | $P = 0.857$ | $P = 0.026$ N | $P = 0.999$ | $P = 0.224$ N | $P = 0.107$ N |     |

<sup>a</sup> Kaplan–Meier determinations

<sup>b</sup> Mean of all deaths (uncensored, censored, and terminal killing)

<sup>c</sup> The results of the life table trend test (Tarone 1975) is in the control column, and the results of the life table pairwise comparison (Cox 1972) with the controls are in the exposure group columns



**Fig. 2** Growth curves for male (a) and female (b) B6C3F1 mice in the 2-year drinking water study of 3-MCPD

### Pathology

There were no neoplastic findings considered to be related to treatment with 3-MCPD (Table 2). Statistical analysis of tumor incidence revealed that there were no statistically significant differences between the control and treated groups in either sex (Table 2). The summarized incidence of non-neoplastic lesions for some selected organs or

tissues were shown in Table 3, as 3-MCPD was expected to affect the reproductive system and the kidneys. The microscopic examination of tissues taken from animals revealed no lesions attributable to treatment with 3-MCPD. Given that incidence of those lesions was fall into background level, there were no significant difference in the incidence between the control and treatment groups, and lack of dose-relationship, all findings were considered to be incidental.

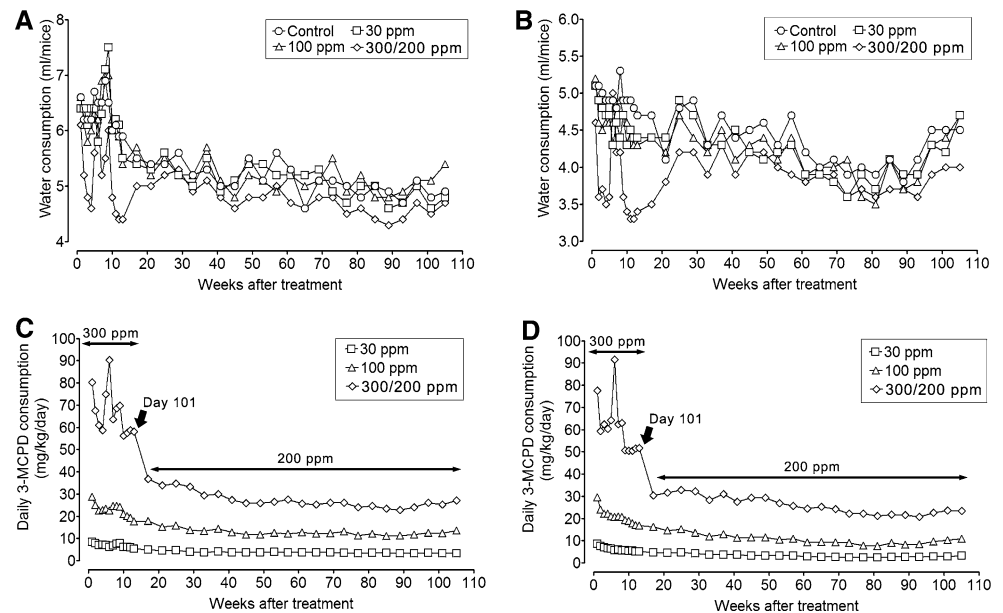
### Discussion

Administration of 3-MCPD was not associated with any neoplastic or non-neoplastic findings attributable to treatment with 3-MCPD. This investigation met all the OECD requirements of a satisfactory carcinogenicity study (OECD 1981; ICH S1C 2008).

The suppression of body weights at 300/200 ppm in both genders during the experiment is associated with sweetish taste of 3-MCPD and is consistent with previous 13-week B3C3F1 mice and 104-week SD rat study reported by us (Cho et al. 2008a; Cho et al. 2008b), and the results of the 2-year carcinogenicity study with F344 rats (Lynch et al. 1998) as well. Food and water consumption was decreased at 300/200 ppm groups for both sexes. A number of other differences in hematology (platelets, MCH, RDW and monocytes) and serum biochemistry (BUN, ALP and triglyceride) when compared to controls showed statistical significance. However, these variances were not consistent with the histopathological evidences and were not considered of great biological importance.

In view of the results seen in previous studies and those findings from our 13-week preliminary dose-range finding study, some changes in the reproductive organs and kidney were anticipated in this 104-week mice carcinogenicity

**Fig. 3** Water consumption curves and daily 3-MCPD consumption curves for male (a and c) and female (b and d) B6C3F1 mice in the 2-year drinking water study of 3-MCPD



study such as renal tubule tumors, germinal epithelial degeneration, or Leydig cell tumors, as Leydig cell tumor can be induced by testicular germinal cell degeneration by negative hormonal feedback mechanism.

At 13-week preliminary study conducted at the dose level of 5, 25, 100, 200 or 400 ppm 3-MCPD in the drinking water, delayed estrus cycle was observed at 400 ppm (Cho et al. 2008a).

Changes in reproductive organs were consistently reported in several reproductive toxicity studies with 3-MCPD (Gill and Guraya 1980; Kwack et al. 2004), and antioestrogenic effects were also reported in female rats (Lohika and Arya 1979). Furthermore, Leydig cell tumor was induced in the rat carcinogenicity studies (Cho et al. 2008b; Sunahara et al. 1993). The metabolites of 3-MCPD have an inhibitory activity on enzymes in the spermatozoa glycolysis, and the inhibition of sperm motility was partly due to the alkylation of spermatozoa cysteine by 3-MCPD (Jones 1983; Kalla and Bansal 1977). Significant delayed estrus cycle was an evidence of hormonal imbalance caused by 3-MCPD, which is suspected to have luteolytic and antioestrogenic effects in female rats (Lohika and Arya 1979).

In the kidney, tubule adenoma and carcinoma were induced by treatment with 3-MCPD in rat studies (Cho et al. 2008b; Sunahara et al. 1993) and also kidney weight increase without any histopathological changes (Cho et al. 2008a). The nephrotoxic mechanisms of 3-MCPD are thought to be due to the inhibition of glycolysis by metabolites associated with the  $\beta$ -chlorolactate pathway (Jones and Chantrill 1989). In addition, the accumulation of oxalic acid in the kidney also could contribute to chronic progressive nephropathy (CPN) (El Ramy et al. 2007;

Jones et al. 1981) and the subsequent stimulation of renal tubule hyperplasia and; finally, produce renal tubule adenomas and carcinomas (Cho et al. 2008b).

Interestingly, however, in this study, there was no evidence of 3-MCPD-related kidney change or any findings in reproductive organs.

There are five generally accepted approaches (ICH guidance for Industry S1C 2008) can be used for the selection of the high dosage: toxicity-based endpoints, pharmacokinetic endpoints, saturation of absorption, pharmacodynamic endpoints, and maximum feasible dosage. For 3-MCPD, the toxicity-based endpoint approach was considered not correctly suitable, although there were frank toxicities and the MTD was identified at the 13-week preliminary dose-range finding study using the same strain and species of animal. Based on our previous 13-week dose finding study, initially 300 ppm was chosen as high dose which showed some toxicity, although it was reduced to 200 ppm due to marked decreased body weight, food, and water consumption. In addition, 100 ppm was chosen as mid-dose which estimated to be tolerated without significant chronic physiological dysfunction and should permit data interpretation. The dosage of 100 ppm is also roughly the geometric mean between the proposed low dosage and high dosage levels. The low dosage was set at 30 ppm and it was estimated that this dosage was intended to provide information on the NOAEL. Given that rationale of dose level selection was justified, those changes seen at 13-week study was considered to be adaptive, and eventually no overt toxicological findings were observed at the 104 weeks of treatment. This is consistent with two previous mice studies by subcutaneous and intra-peritoneal route (Van Duuren et al. 1974) and one dietary rats study

**Table 2** Incidence of neoplastic changes in B6C3F1 mice exposed to 3-MCPD in drinking water for 104 weeks

| Sex                                              | Male    |         |        |         | Female |       |       |       |
|--------------------------------------------------|---------|---------|--------|---------|--------|-------|-------|-------|
|                                                  | 0       | 30      | 100    | 200     | 0      | 30    | 100   | 200   |
| <b>Alimentary system</b>                         |         |         |        |         |        |       |       |       |
| Esophagus                                        | (48)    | (47)    | (48)   | (49)    | (44)   | (46)  | (48)  | (49)  |
| Gall bladder                                     | (50)    | (50)    | (50)   | (50)    | (50)   | (50)  | (50)  | (50)  |
| Papilloma                                        | –       | –       | 1(1%)  | –       | –      | –     | –     | –     |
| Large intestine, cecum                           | (45)    | (47)    | (44)   | (44)    | (42)   | (41)  | (44)  | (45)  |
| Large intestine, colon                           | (45)    | (43)    | (47)   | (44)    | (42)   | (41)  | (47)  | (45)  |
| Large intestine, rectum                          | (46)    | (47)    | (48)   | (46)    | (44)   | (44)  | (47)  | (46)  |
| Liver                                            | (50)    | (49)    | (49)   | (49)    | (48)   | (50)  | (50)  | (50)  |
| Hemangioma                                       | –       | 2(4%)   | 1(2%)  | 1(2%)   | 2(4%)  | 1(2%) | –     | –     |
| Hepatocellular adenoma                           | 3(6%)   | 10(20%) | 6(12%) | 3(6%)   | 2(4%)  | 1(2%) | 2(4%) | 1(2%) |
| Hepatocellular carcinoma                         | 13(26%) | 7(14%)  | 4(8%)  | 13(27%) | –      | 1(2%) | 3(6%) | 1(2%) |
| Ito cell tumor                                   | –       | –       | 1(2%)  | –       | –      | 1(2%) | –     | –     |
| Squamous cell carcinoma, metastatic, forestomach | –       | –       | –      | 1(2%)   | –      | –     | –     | –     |
| Fibrosarcoma, metastatic, abdominal cavity       | –       | –       | –      | –       | –      | 1(2%) | –     | –     |
| Pancreas                                         | (49)    | (47)    | (47)   | (47)    | (46)   | (45)  | (48)  | (48)  |
| Hemangioma                                       | 2(4%)   | –       | –      | –       | –      | –     | –     | –     |
| Salivary gland                                   | (49)    | (48)    | (49)   | (48)    | (46)   | (47)  | (48)  | (49)  |
| Small intestine, duodenum                        | (45)    | (43)    | (46)   | (44)    | (42)   | (44)  | (44)  | (45)  |
| Adenocarcinoma                                   | –       | 1(2%)   | –      | –       | 1(2%)  | –     | –     | –     |
| Small intestine, ileum                           | (45)    | (43)    | (44)   | (45)    | (40)   | (41)  | (43)  | (45)  |
| Adenocarcinoma                                   | –       | –       | 1(2%)  | –       | –      | –     | –     | –     |
| Small intestine, jejunum                         | (44)    | (43)    | (45)   | (44)    | (41)   | (42)  | (44)  | (45)  |
| Adenoma                                          | –       | 1(2%)   | –      | –       | –      | –     | –     | –     |
| Stomach, forestomach                             | (47)    | (48)    | (47)   | (46)    | (43)   | (43)  | (45)  | (46)  |
| Squamous cell carcinoma                          | 1(2%)   | –       | –      | 1(2%)   | –      | –     | –     | –     |
| Stomach, glandular stomach                       | (47)    | (48)    | (47)   | (46)    | (43)   | (43)  | (45)  | (46)  |
| Tongue                                           | (50)    | (49)    | (49)   | (50)    | (47)   | (47)  | (49)  | (49)  |
| Bronchioloalveolar carcinoma, metastatic, lung   | –       | –       | –      | –       | 1(2%)  | –     | –     | –     |
| Tooth                                            | (50)    | (50)    | (50)   | (50)    | (50)   | (50)  | (50)  | (50)  |
| Body cavity                                      | (50)    | (50)    | (50)   | (50)    | (50)   | (50)  | (50)  | (49)  |
| Abdominal cavity Fibrosarcoma                    | –       | –       | –      | –       | –      | 1(2%) | –     | –     |
| Thoracic cavity                                  | (50)    | (50)    | (50)   | (50)    | (47)   | (50)  | (50)  | (48)  |
| Bronchiolo-alveolar carcinoma, metastatic, lung  | –       | –       | –      | –       | –      | 1(2%) | 1(2%) | –     |
| <b>Cardiovascular system</b>                     |         |         |        |         |        |       |       |       |
| Aorta                                            | (50)    | (50)    | (50)   | (50)    | (48)   | (49)  | (50)  | (49)  |
| Heart                                            | (47)    | (45)    | (45)   | (49)    | (46)   | (46)  | (47)  | (47)  |
| Hepatocellular carcinoma, metastatic, liver      | –       | –       | 1(2%)  | –       | –      | –     | –     | –     |
| Bronchioloalveolar carcinoma, metastatic, lung   | –       | –       | –      | –       | –      | 1(2%) | 1(2%) | –     |
| <b>Endocrine system</b>                          |         |         |        |         |        |       |       |       |
| Adrenal gland                                    | (49)    | (48)    | (49)   | (50)    | (48)   | (49)  | (50)  | (50)  |
| Cortical adenoma                                 | 1(2%)   | –       | –      | –       | –      | –     | –     | –     |
| Subcapsular cell adenoma                         | 4(8%)   | 5(10%)  | 3(6%)  | 2(4%)   | –      | –     | 2(4%) | –     |
| Benign pheochromocytoma                          | –       | –       | –      | –       | –      | –     | 2(4%) | –     |
| Pancreas, islet                                  | (50)    | (50)    | (50)   | (50)    | (50)   | (50)  | (50)  | (50)  |
| Islet cell adenoma                               | –       | 1(2%)   | –      | –       | –      | –     | –     | –     |
| Islet cell carcinoma                             | –       | –       | –      | –       | –      | 1(2%) | –     | –     |
| Parathyroid gland                                | (50)    | (50)    | (50)   | (50)    | (50)   | (50)  | (50)  | (50)  |

**Table 2** continued

| Sex                                                 | Male         |        |        |        | Female  |         |         |        |      |
|-----------------------------------------------------|--------------|--------|--------|--------|---------|---------|---------|--------|------|
|                                                     | Dosage (ppm) | 0      | 30     | 100    | 200     | 0       | 30      | 100    | 200  |
| Pituitary gland                                     | (49)         | (48)   | (48)   | (49)   | (48)    | (50)    | (50)    | (50)   | (50) |
| Adenoma, pars distalis                              | –            | –      | 1(2%)  | –      | 5(8%)   | 6(%)    | 8(16%)  | 4(8%)  |      |
| Thyroid gland                                       | (50)         | (49)   | (49)   | (49)   | (48)    | (49)    | (50)    | (50)   |      |
| Follicular cell adenoma                             | –            | –      | 1(2%)  | –      | –       | 1(2%)   | 1(2%)   | 1(2%)  |      |
| Follicular cell carcinoma                           | –            | –      | –      | –      | –       | 1(2%)   | –       | –      |      |
| Genital system                                      |              |        |        |        |         |         |         |        |      |
| Coagulating gland                                   | (50)         | (50)   | (50)   | (50)   |         |         |         |        |      |
| Epididymis                                          | (50)         | (50)   | (49)   | (50)   |         |         |         |        |      |
| Preputial gland                                     | (47)         | (49)   | (49)   | (50)   |         |         |         |        |      |
| Prostate                                            | (50)         | (50)   | (50)   | (48)   |         |         |         |        |      |
| Seminal vesicle                                     | (50)         | (50)   | (50)   | (49)   |         |         |         |        |      |
| Testes                                              | (50)         | (50)   | (50)   | (50)   |         |         |         |        |      |
| Interstitial cell adenoma                           | 1(2%)        | –      | 1(2%)  | 1(2%)  |         |         |         |        |      |
| Clitoral gland                                      |              |        |        |        | (48)    | (48)    | (50)    | (46)   |      |
| Squamous cell carcinoma                             |              |        |        |        | –       | 1(2%)   | –       | –      |      |
| Ovary                                               |              |        |        |        | (47)    | (47)    | (49)    | (46)   |      |
| Cystic adenoma                                      |              |        |        |        | 2(4%)   | –       | 1(2%)   | 1(2%)  |      |
| Benign sertoli cell tumor                           |              |        |        |        | 1(2%)   | –       | –       | –      |      |
| Uterus                                              |              |        |        |        | (48)    | (47)    | (49)    | (46)   |      |
| Leiomyoma                                           |              |        |        |        | 3(6%)   | –       | –       | 1(2%)  |      |
| Polypoid adenoma                                    |              |        |        |        | –       | 1(2%)   | –       | –      |      |
| Schwannoma                                          |              |        |        |        | 1(2%)   | –       | –       | –      |      |
| Hemangioma                                          |              |        |        |        | 1(2%)   | –       | 1(2%)   | –      |      |
| Bronchiolo-alveolar carcinoma, metastatic, lung     |              |        |        |        | –       | 1(2%)   | –       | –      |      |
| Vagina                                              |              |        |        |        | (48)    | (47)    | (49)    | (46)   |      |
| Basal cell carcinoma                                |              |        |        |        | 1(2%)   | –       | –       | –      |      |
| Squamous cell carcinoma, metastatic, clitoral gland |              |        |        |        | –       | 1(2%)   | –       | –      |      |
| Hematopoietic system#                               |              |        |        |        |         |         |         |        |      |
| Hematopoietic tissues                               | (50)         | (50)   | (50)   | (50)   | (50)    | (50)    | (50)    | (50)   |      |
| Lymphoma, all                                       | 8(16%)       | 6(12%) | 6(12%) | 7(14%) | 12(24%) | 10(20%) | 17(34%) | 8(16%) |      |
| Histiocytic sarcoma                                 | 3(6%)        | –      | 2(4%)  | 2(4%)  | 3(6%)   | 1(2%)   | 2(4%)   | 1(2%)  |      |
| Lymphocytic/lymphoblastic lymphoma                  | 1(2%)        | –      | 1(2%)  | 1(2%)  | 3(6%)   | 7(14%)  | 3(6%)   | 3(6%)  |      |
| Pleomorphic cell lymphoma                           | –            | –      | –      | –      | –       | –       | –       | –      |      |
| Lymphoma, NOS                                       | 4(8%)        | 5(10%) | 3(6%)  | 3(6%)  | 6(12%)  | 2(4%)   | 2(4%)   | 4(8%)  |      |
| Plasma cell tumor                                   | –            | –      | –      | 1(2%)  | –       | –       | –       | –      |      |
| Myeloid leukemia                                    | –            | 1(2%)  | –      | –      | 1(2%)   | 1(2%)   | –       | –      |      |
| Mandibular lymph node                               | (50)         | (48)   | (49)   | (48)   | (47)    | (45)    | (49)    | (47)   |      |
| Islet cell carcinoma, metastatic, pancreas          | –            | –      | –      | –      | –       | 1(2%)   | –       | –      |      |
| Mesenteric lymph node                               | (46)         | (44)   | (45)   | (46)   | (43)    | (40)    | (46)    | (46)   |      |
| Hemangioma                                          | 1(2%)        | –      | –      | –      | –       | –       | –       | 1(2%)  |      |
| Spleen                                              | (50)         | (49)   | (49)   | (49)   | (48)    | (49)    | (50)    | (50)   |      |
| Hemangioma                                          | 1(2%)        | –      | –      | –      | –       | –       | –       | –      |      |
| Hemangiosarcoma                                     | –            | –      | –      | –      | –       | –       | 1(2%)   | –      |      |
| Thymus                                              | (47)         | (49)   | (46)   | (45)   | (49)    | (50)    | (48)    | (49)   |      |
| Lymphocytic thymoma                                 | –            | –      | –      | –      | –       | –       | 1(2%)   | –      |      |
| Integumentary system                                |              |        |        |        |         |         |         |        |      |
| Mammary gland                                       | (50)         | (50)   | (50)   | (50)   | (49)    | (49)    | (49)    | (47)   |      |



**Table 2** continued

| Sex<br>Dosage (ppm)                            | Male   |        |        |       | Female |       |       |       |
|------------------------------------------------|--------|--------|--------|-------|--------|-------|-------|-------|
|                                                | 0      | 30     | 100    | 200   | 0      | 30    | 100   | 200   |
| Adenoma                                        | –      | –      | –      | –     | –      | –     | –     | 1(2%) |
| Adenocarcinoma                                 | –      | –      | –      | –     | 2(4%)  | 2(4%) | 2(4%) | –     |
| Bronchioloalveolar carcinoma, metastatic, lung | –      | –      | –      | –     | –      | 1(2%) | –     | –     |
| Skin                                           | (50)   | (50)   | (49)   | (50)  | (49)   | (50)  | (50)  | (50)  |
| Benign basal cell tumor                        | –      | –      | –      | 1(2%) | –      | –     | –     | –     |
| Fibrosarcoma                                   | –      | –      | –      | 1(2%) | 1(2%)  | –     | 1(2%) | –     |
| Lipoma                                         | –      | –      | –      | –     | –      | –     | –     | –     |
| Adenocarcinoma, metastatic, harderian gland    | –      | –      | –      | –     | 1(2%)  | –     | –     | –     |
| Musculoskeletal system                         |        |        |        |       |        |       |       |       |
| Bone                                           | (50)   | (50)   | (50)   | (50)  | (49)   | (50)  | (50)  | (50)  |
| Chondrosarcoma                                 | –      | –      | 1(2%)  | –     | –      | –     | –     | –     |
| Osteosarcoma                                   | –      | –      | –      | –     | –      | –     | –     | 1(2%) |
| Femur with marrow                              | (50)   | (50)   | (50)   | (50)  | (47)   | (50)  | (50)  | (50)  |
| Hemangioma                                     | 1(2%)  | –      | –      | 1(2%) | –      | –     | –     | –     |
| Muscle                                         | (50)   | (50)   | (50)   | (50)  | (48)   | (49)  | (50)  | (48)  |
| Bronchioloalveolar carcinoma, metastatic, lung | –      | –      | –      | –     | –      | 1(2%) | –     | –     |
| Nervous system                                 |        |        |        |       |        |       |       |       |
| Brain                                          | (50)   | (49)   | (49)   | (50)  | (46)   | (49)  | (50)  | (48)  |
| Spinal cord                                    | (50)   | (50)   | (50)   | (50)  | (48)   | (50)  | (50)  | (50)  |
| Sciatic nerve                                  | (50)   | (50)   | (49)   | (50)  | (48)   | (49)  | (50)  | (48)  |
| Respiratory system                             |        |        |        |       |        |       |       |       |
| Larynx                                         | (49)   | (49)   | (48)   | (47)  | (47)   | (47)  | (49)  | (49)  |
| Lung                                           | (50)   | (50)   | (50)   | (50)  | (48)   | (50)  | (50)  | (50)  |
| Bronchioloalveolar adenoma                     | 5(10%) | 5(10%) | 5(10%) | 2(4%) | 1(2%)  | 3(6%) | 3(6%) | 1(2%) |
| Bronchioloalveolar carcinoma                   | 8(16%) | 2(4%)  | 1(2%)  | 4(8%) | 1(2%)  | 3(6%) | 2(4%) | –     |
| Hepatocellular carcinoma, metastatic, liver    | –      | –      | –      | 1(1%) | –      | –     | –     | –     |
| Adenocarcinoma, metastatic, harderian gland    | –      | –      | –      | –     | 1(2%)  | –     | –     | –     |
| Nasal cavity                                   | (49)   | (48)   | (49)   | (50)  | (49)   | (50)  | (50)  | (49)  |
| Adenocarcinoma, metastatic, harderian gland    | –      | –      | –      | –     | 1(2%)  | –     | –     | –     |
| Trachea                                        | (48)   | (47)   | (49)   | (46)  | (45)   | (43)  | (46)  | (46)  |
| Special sense system                           |        |        |        |       |        |       |       |       |
| Eye                                            | (49)   | (50)   | (50)   | (50)  | (48)   | (50)  | (50)  | (50)  |
| Harderian gland                                | (50)   | (50)   | (49)   | (49)  | (49)   | (50)  | (50)  | (50)  |
| Adenoma                                        | 1(2%)  | 3(6%)  | 2(4%)  | 1(2%) | 1(2%)  | 3(6%) | 3(6%) | –     |
| Adenocarcinoma                                 | –      | –      | –      | –     | 2(4%)  | –     | 1(2%) | –     |
| Zymbal's gland                                 | (50)   | (49)   | (49)   | (49)  | (47)   | (45)  | (48)  | (48)  |
| Urinary system                                 |        |        |        |       |        |       |       |       |
| Kidney                                         | (50)   | (50)   | (48)   | (49)  | (45)   | (46)  | (47)  | (47)  |
| Tubule adenoma                                 | 1(2%)  | 1(2%)  | –      | –     | 1(2%)  | –     | –     | –     |
| Tubule carcinoma                               | –      | 1(2%)  | –      | –     | –      | –     | –     | –     |
| Bronchioloalveolar carcinoma, metastatic, lung | –      | –      | –      | –     | –      | –     | –     | –     |
| Fibrosarcoma, metastatic, skin                 | –      | –      | –      | –     | 1(2%)  | 1(2%) | –     | –     |
| Urethra                                        | (50)   | (49)   | (50)   | (49)  | (50)   | (50)  | (50)  | (50)  |
| Urinary bladder                                | (50)   | (49)   | (49)   | (49)  | (48)   | (49)  | (50)  | (50)  |
| Hemangioma                                     | 1(2%)  | –      | –      | –     | –      | –     | –     | –     |

**Table 2** continued

| Sex                                                 | Male |    |     |     | Female |    |       |     |
|-----------------------------------------------------|------|----|-----|-----|--------|----|-------|-----|
|                                                     | 0    | 30 | 100 | 200 | 0      | 30 | 100   | 200 |
| Dosage (ppm)                                        |      |    |     |     |        |    |       |     |
| Squamous cell carcinoma, metastatic, clitoral gland | –    | –  | –   | –   | –      | –  | 1(2%) | –   |

The data are reported as the number of animals with a neoplasm per number of animals examined microscopically, the % incidence (in parentheses)

# All hematopoietic tumors were recorded under the hematopoietic tissues rather than occurring individual organs/tissues

(Weisburger et al. 1981); all those studies showed no evidence of carcinogenic activity of 3-MCPD.

Based on those evidences from various genotoxic studies, 3-MCPD is considered as non-genotoxic carcinogen (El Ramy et al. 2007; SCF 2001). Due to the limited database and the lack of reproduction/development studies, Scientific Committee on Food (SCF) proposed to retain a lowest observed adverse effect level (LOAEL) of 1.1 mg/kg b.w./day with an uncertainty factor of 500 to establish

the tolerable daily intake (TDI) of 2 µg/kg b.w. (SCF 2001). In this study, we confirmed that B6C3F1 mice did not induce any tumors. Considering that highly species-specific tumor induction in long-term carcinogenicity studies, non-genotoxic nature of this compound and renal tumors maybe partially due to CPN, unless we provide clear human epidemiological evidence of 3-MCPD-caused carcinogenicity, it is still unclear whether 3-MCPD is genuine human carcinogen or not. Further mechanistic

**Table 3** Incidence of non-neoplastic changes for selected organs in B6C3F1 mice exposed to 3-MCPD in drinking water for 104 weeks

| Sex                               | Male    |         |         |         | Female  |         |         |         |
|-----------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|
|                                   | 0       | 30      | 100     | 200     | 0       | 30      | 100     | 200     |
| Dosage (ppm)                      |         |         |         |         |         |         |         |         |
| Testis                            | (50)    | (50)    | (50)    | (50)    |         |         |         |         |
| Arteritis/periarteritis           | 1(2%)   | –       | –       | –       |         |         |         |         |
| Interstitial cell hyperplasia     | 2(4%)   | –       | 5(10%)  | –       |         |         |         |         |
| Seminiferous tubular atrophy      | 5(12%)  | –       | 4(8%)   | 4(8%)   |         |         |         |         |
| Vascular degeneration             | 1(2%)   | 6(14%)  | –       | 3(6%)   |         |         |         |         |
| Spermatosis                       | 2(2%)   | –       | 2(4%)   | 1(2%)   |         |         |         |         |
| Seminiferous tubular dilatation   | 3(6%)   | –       | 1(2%)   | 4(8%)   |         |         |         |         |
| Rete testis dilatation            | –       | 3(6%)   | 1(2%)   | 1(2%)   |         |         |         |         |
| Rete testis hyperplasia           | –       | 1(2%)   | 1(2%)   | 2(4%)   |         |         |         |         |
| Ovary                             |         |         |         |         | (50)    | (50)    | (50)    | (50)    |
| Atrophy                           |         |         |         |         | 35(70%) | 38(76%) | 38(76%) | 37(74%) |
| Cyst                              |         |         |         |         | 14(28%) | 18(36%) | 16(32%) | 16(32%) |
| Mineralization                    |         |         |         |         | 1(2%)   | –       | 1(2%)   | –       |
| Tubular hyperplasia               |         |         |         |         | 2(4%)   | –       | 4(8%)   | 1(2%)   |
| Abscess                           |         |         |         |         | –       | 1(2%)   | –       | 1(2%)   |
| Kidney                            | (50)    | (50)    | (50)    | (50)    | (50)    | (50)    | (50)    | (50)    |
| Cortical tubular vacuolation      | 20(40%) | 18(36%) | 24(48%) | 15(30%) | 2(4%)   | –       | –       | 5(10%)  |
| Tubular degeneration/regeneration | 27(54%) | 16(32%) | 26(52%) | 33(66%) | 9(18%)  | 2(4%)   | 15(30%) | 15(30%) |
| Tubular hyaline droplets          | –       | –       | –       | 1(2%)   | 1(2%)   | –       | 1(2%)   | 1(2%)   |
| Cortical cysts                    | 10(20%) | 10(20%) | 11(22%) | 12(24%) | 1(2%)   | 2(4%)   | 3(6%)   | 1(2%)   |
| Cortical infarction               | –       | –       | –       | –       | –       | –       | 1(2%)   | –       |
| Extramedullar hematopoiesis       | –       | –       | –       | –       | 2(4%)   | –       | –       | 3(6%)   |
| Interstitial inflammation         | 18(36%) | 18(36%) | 20(40%) | 22(44%) | 16(32%) | 24(48%) | 28(36%) | 25(50%) |
| Nephropathy                       | 2(4%)   | 4(8%)   | 2(4%)   | 4(8%)   | –       | 4(8%)   | 4(8%)   | –       |
| Papillary necrosis                | 1(2%)   | –       | 1(2%)   | –       | –       | –       | 1(2%)   | –       |
| Mineralization                    | 37(74%) | 40(80%) | 35(70%) | 38(76%) | 3(6%)   | 1(2%)   | 5(10%)  | 3(6%)   |
| Pyelonephritis                    | –       | –       | –       | 1(2%)   | –       | 2(4%)   | –       | –       |

The data are reported as the number of animals with a neoplasm per number of animals examined microscopically, the % incidence (in parentheses)

studies will be elucidated those species and strain-specific tumor induction.

In summary, drinking water administration of 3-MCPD at dosages of 30, 100 or 300/200 ppm to B6C3F1 mice for 104 weeks revealed no evidence of overt toxicity or carcinogenic potential. The No-Observed-Adverse-Effect Level (NOAEL) in this study was, therefore, considered to be 300 ppm up to Day 100 and 200 ppm up to 104 weeks (33.0 mg/kg 3-MCPD for males and 31.0 mg/kg for females).

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