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Original Article

A 28-day repeated oral-dose toxicity study of insecticide synergist N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2] oct-5-ene-2,3-dicarboximide in rats

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ABSTRACT — N-(2-Ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide (Synepirin 500; CAS: 13358-11-7) is used as a synergist, a chemical that makes pesticide ingredients more effective. People can be exposed to Synepirin 500 by using insecticides containing this chemical or from residues in food. The Japanese government chose this chemical as a target substance in its existing chemical testing program. Crl:CD(SD) rats were administered 0, 40, 200, and 1000 mg/kg/day Synepirin 500 by gavage for 28 days, followed by a 14 day recovery period. Diarrhea or soft feces were observed in both sexes at 1000 mg/kg/day. Absolute and/or relative liver weights significantly increased at ≥ 40 mg/kg/day in females and at ≥ 200 mg/kg/day in males. Absolute and/or relative thyroid weights significantly increased in both sexes at 1000 mg/kg/day. These changes were still significant at the end of the recovery period in females. Significantly prolonged prothrombin time and activated partial thromboplastin time were observed in males receiving ≥ 40 mg/kg/day. Histopathological changes in the liver and thyroid were observed in both sexes at 1000 mg/kg/day. On the basis of the effects on the liver, the level of the lowest observed adverse effect from repeated doses of Synepirin 500 was judged to be 40 mg/kg/day for rats.

Key words: CAS No. 13358-11-7, Synepirin 500, Synergist, OECD TG 407, Repeated dose toxicity

INTRODUCTION

For the proper use and management of chemical substances or the products containing them, safety information on chemicals is essential. Under the Chemical Substance Control Law, the Japanese government continues its chemical testing program to compile safety information related to health risks on existing chemicals to which humans could be exposed.

N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2] oct-5-ene-2,3-dicarboximide (Synepirin 500; CAS: 13358-11-7) is used as a synergist, a chemical that lacks its own pesticidal properties but makes the other ingredients in pesticides more effective. Synepirin 500 is a dicarboximide, which has two carboxamide groups and is structurally similar to octyl-bicycloheptene-dicarboximide (MGK264; CAS: 113-48-4, also known as Synepirin 222). MGK264 is toxicologically well evaluated by the US Environmental Protection Agency (USEPA) in its Reregistration Eligibility Decision (USEPA, 2004, 2006) and under the Endocrine Disruptor Screening Program Tier 1 assessment and is selected on the basis of expected exposures (USEPA, 2015). The chemical structures of Synepirin 500 and MGK264 are provided in Fig. 1.

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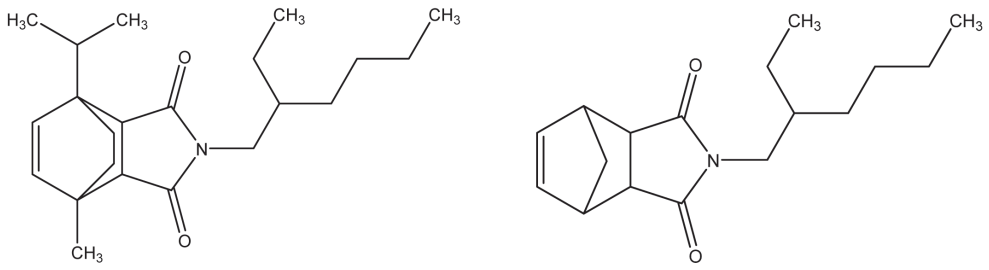
Both Synepirin 500 and MGK264 are considered to inhibit microsomal enzymes in pests by binding to these enzymes and inhibiting the breakdown of pesticide ingredients, such as pyrethrins and pyrethroids (Bernard and Philogene, 1993; Ono *et al*., 1995; Raffa and Priester, 1985; Yamamoto, 1973), but the exact mode of action for synergists remains under investigation (Sarwar, 2016).

The oral-dose LD_{50} MGK264 was reported to be 2800 mg/kg in rats and 1000 mg/kg in mice (Lewis, 2004). Although acute toxicity of MGK264 is low in laboratory animals, the adverse effects on the liver were observed in repeated-dose studies in mice, rats, and dogs (Blair, 1989, 1991a, 1991b; Goldenthal, 1993; Schardein, 1991). The lowermost Lowest Observed Adverse Effect Level (LOAEL) of 61 mg/kg/day was obtained in a two-generation rat study in which decreased body weight was observed in the parental animals and pups, and hepatocellular hypertrophy was observed in the parental animals (Schardein, 1991). From this result, the point of departure of 6.1 mg/kg/day has been used for the USEPA human health risk assessment of MGK264. In a chronic mouse study of MGK264, statistically significant increases in liver adenomas were observed in males and an increased tendency was observed in females at 400 and 800 mg/kg/day (Blair, 1991a). The thyroid is also considered the target organ of MGK264 toxicity in rats. Treatment of MGK264 significantly increased thyroid follicular hypertrophy in both sexes (Schardein, 1991) and thyroid follicular tumors in males (Goldenthal, 1993). Male and female pubertal rats treated with MGK264 also showed a decrease in the mean colloid area in the thyroid (Zorrilla, 2012b).

Many products containing Synepirin 500 are commercially available and used both inside and outside of the home, and some are directly applied on pets to control fleas and ticks. People can be directly exposed to Synepirin 500 by using these insecticides. Synepirin 500 was judged to be non-biodegradable and with moderate bioconcentration by the Ministry of Economy, Trade and Industry, Japan (NITE, 2016). The chemical residues in food and runoff from outdoor pest control in the drinking water can expose humans to ingestion of these substances; however, the only available toxicological information on Synepirin 500 is from the oral LD_{50} of 20,000 mg/kg in mice (Yakkyoku, 1981). On the basis of the structural similarity and the same uses of Synepirin 500 and MGK264, repeated-dose toxicity of Synepirin 500 was anticipated. Thus, this chemical was selected by the Japanese government as a target substance in its existing chemical testing program, and a 28.0 day repeated oral-dose toxicity study, a bacterial reverse mutation test, and an *in vitro* mammalian chromosome aberration test were conducted. In this paper, we report the results of the 28-day repeated oral-dose toxicity study of Synepirin 500.

MATERIALS AND METHODS

The present study was conducted at the Safety Research Institute for Chemical Compounds Co., Ltd., Sapporo, Japan. The study was designed to meet Japan's guidelines in its "Methods of Testing New Chemical Substances" (Yakushokuhatsu No. 1121002, Seikyoku No. 2, Kanpokihatsu No. 031121002, revision November 20, 2006) and the "Organisation for Economic Co-operation and Development Guidelines for the Testing of Chemicals" (TG 407, adopted on July 27, 1995) and was conducted in compliance with good laboratory practice standards criteria at test facilities for carrying out tests on new chemical substances in Japan (Yakushokuhatsu No. 1121003, Seikyoku No. 3, and Kanpokihatsu No. 031121004, revised July 4, 2008). The use and care of animals complied with the "Act on Welfare and Management of Animals" (Japanese Animal Welfare Law, Act No. 105, revised June 22, 2005), "Standards Relating to



Synepirin 500 (CAS No. 13358-11-7) MGK264 (CAS No. 113-48-4)

Fig. 1. Chemical structures of N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide (Synepirin 500, CAS No. 13358-11-7) and octyl-bicycloheptene-dicarboximide (MGK264; CAS No. 113-48-4).

the Care and Management of Laboratory Animals and Relief of Pain" (Announcement No. 88, Ministry of the Environment, Japan, April 28, 2006), and "Guidelines for Proper Conduct of Animal Experiments" (Kahatsu No. 061005, June 1, 2006).

Test substances and reagent

Synepirin 500 (CAS No. 13358-11-7), lot no. Y225, purity 99.7%, is a clear yellow liquid with a slight odor. It was obtained from API Corporation (Tokyo, Japan) and was stored in the light-shielding test substance room at 1-8°C. The stability of the prepared sample was confirmed before use. Corn oil (lot no. V5R8265), used as a vehicle, was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Animals

Eighty-four specific-pathogen-free 4-week old male and female Sprague-Dawley rats [Crl: CD (SD)] were purchased from Atsugi Breeding Center of Charles River Japan, Inc. (Kanagawa, Japan). All animals were acclimated to the testing environment for 6-7 days before examination. Administration of the test substance was initiated at 5 weeks of age. The ranges in body weights for males and females on initiation of treatment were 134-156 and 113-131 g, respectively. Animals were housed individually in wire-mesh steel cages (240 × 380 × 180 mm) and kept in an environmentally controlled room as follows: temperature, 21-24°C; humidity, 39-54%; ventilation, 10-15 times/hr; and lighting, 12 hr/d (light on/off, 08:00/20:00). The animals were fed a pellet diet after γ-ray irradiation (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and given tap water *ad libitum*.

Selection of dose levels

Dose levels were selected on the basis of the results obtained from a 14-day dose range-finding study using the same strain of rats (three animals/sex/dose) at dose levels of 0 (corn oil), 100, 300, and 1000 mg/kg/day. In the dose range-finding study, diarrhea and soft feces were observed at 300 mg/kg/day and were the same in both sexes. Significant decreases in absolute and relative spleen weights were observed at ≥ 300 mg/kg/day in males. Significant increases in absolute and relative liver weights were observed in both sexes, and significant increases in alanine aminotransferase (ALT) and total cholesterol levels were observed in females at 1000 mg/kg/day. In the present study, the high dose was set at 1000 mg/kg/day, and the middle and low doses were set 200 and 40 mg/kg/day, respectively, using a common ratio of 5.

Experimental design

Rats (6 or 12 animals/sex/dose) were administrated Synepirin 500 by gavage once daily at 0 (vehicle control by corn oil), 40, 200, and 1000 mg/kg/day for 28 days. The main group comprised of six male and six female rats, and the recovery group comprised of six male and six female rats in the highest and control groups. The dosing volume was set at 10 mL/kg body weight. On the day after the last dosing, six males and six females from each group were euthanized for a hematology assessment, blood biochemistry, organ weights, and macroscopic and microscopic findings as a main group. After the administration period, the remaining six rats/sex at 0 and 1000 mg/kg/day were kept without treatment for 14 days as a recovery group and then fully examined.

Observations

All animals were observed daily in their cages for clinical signs of toxicity. Detailed clinical observations in the cage, on the hand, and in the open field were carried out before the administration and on days 7, 14, 21, and 28 of the administration period and days 7 and 14 of the recovery period. Functional observation battery (FOB) was recorded at week 4 of the administration period and at week 2 of the recovery period as follows: reactivity to visual, touch, auditory, pain, or proprioceptive stimulus and righting reflex. Body weight and food consumption were recorded on days 1, 7, 14, 21, and 28 of the administration period and on days 7 and 14 of the recovery period.

A urinalysis was performed at week 4 of the administration period and at week 2 of the recovery period. Urine was collected for 3 hr under non-fasting conditions using a KN-646, B-1 type rat metabolism cage (Natsume Seisakusho Co., Ltd., Tokyo, Japan) and analyzed for dipstick parameters, such as pH, protein, glucose, ketone bodies, urobilinogen, bilirubin, occult blood, and color using Multistix (Bayer Medical Ltd., Osaka, Japan). Urine volume was measured using a 21 hr urine, and specific gravity was analyzed with the 21 hr urine sample using an ATAGO urine specific gravity refractometer URC-S (ATAGO Co., Ltd., Tokyo, Japan).

The day after the end of the administration and recovery periods, blood was collected from the abdominal aorta under deep anesthesia after overnight fasting. One portion of the blood was treated with EDTA-2K using a venoject II vacuum blood collection tube (Terumo Co., Tokyo, Japan) and examined for hematologic parameters, such as red blood cells (RBCs), hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticu-

locytes, platelets, white blood cells, and differential leukocytes using the F-820 automated multi-item blood cell analyzer (Sysmex Co., Hyogo, Japan). Another blood sample was treated with 3.8% sodium citrate and blood-clotting parameters, such as prothrombin time (PT) and activated partial thromboplastin time (APTT), were analyzed using the KC4-Delta automated blood coagulation analyzer (Trinity Biotech Plc., Wicklow, Ireland).

Serum from the remaining portion of blood was analyzed for blood biochemistry, such as aspartate aminotransferase, ALT, alkaline phosphatase, gamma-glutamyl transpeptidase (γ-GTP), glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen, creatinine, sodium, potassium, chlorine, calcium (Ca), inorganic phosphorus, total protein, protein fraction, albumin/globulin (A/G) ratio, and albumin, using the 7080 type automatic biochemical analyzer (Hitachi High-Tech Solutions Corporation, Tokyo, Japan) and the AES320 automatic electrophoresis system (Mishima Olympus Corporation, Tokyo, Japan).

After blood collection, all animals were sacrificed by exsanguination, and all the organs and tissues, including the head, breast, and abdomen, were observed macroscopically. The brain (cerebrum, cerebellum, and medulla oblongata), pituitary gland, spinal cord, thymus, thyroid glands, parathyroid, adrenals, spleen, heart, tongue, esophagus, stomach, liver, pancreas, duodenum, jejunum, ileum (including Peyer's patch), cecum, colon, rectum, mesenteric lymph nodes, submandibular lymph nodes, trachea, lung, kidneys, bladder, testes, epididymides, prostate, seminal vesicles (including coagulating glands), ovaries, vagina, eyes, Harderian gland, femur (including bone marrow, right), and sciatic nerve were then removed and fixed in 10.0% neutral-buffered formalin. The eye and Harderian gland were fixed in Davidson solution, and the testes and epididymis were fixed in Bouin solution.

The brain, pituitary gland, thyroid, adrenals, spleen, heart, liver, kidneys, thymus, testes, epididymides, prostate, seminal vesicles (including coagulating glands), ovaries, and uterus were weighed using the ER-180A electronic balance (A&D Company Limited, Tokyo, Japan) before fixation. In addition, relative organ weights were calculated from the ratios of organ weights to body weight.

All fixed organs and tissues were routinely prepared and stained with hematoxylin and eosin, and histopathological examination was conducted on both the control and high-dose groups. Treatment-related histopathological changes were found in the liver and thyroid in the high-dose group; these organs were then examined for the low- and middle-dose groups. Tissue sections of the liver of three males and four to six females who received 1000.0 mg/kg/day were stained with Oil Red O, which confirmed the presence of neutral fat.

Data analyses

Parametric data, such as grip strength, motor activity, body weight gain and rate, food consumption, urine volume, hematology, blood biochemistry, and absolute and relative organ weights, were analyzed by Bartlett's test for homogeneity of distribution. When homogeneity was recognized ($P > .05$), a one-way analysis of variance was performed. When a significant difference ($P \leqq .10$) was observed, Dunnett's multiple comparison test was conducted for comparing the control and administration groups. No-homogeneous data, values of detailed clinical observation and FOB, and qualitative data and specific gravity in the urinalysis were analyzed using the Kruskal-Wallis ranking test. When a significant difference ($P \leqq .10$) was detected, the Mann-Whitney *U*-test was conducted for comparison among the control and administration groups.

RESULTS

Repeated doses of Synepirin 500 caused no deaths of either sexes. During the administration period, diarrhea or soft feces were sporadically observed in both sexes at 40 and 200 mg/kg/day and in all males except one and in all females at 1000 mg/kg/day. During the recovery period, these changes were soon reversed. In the FOB test at the end of the recovery period, significant increases in motor activity counts were observed in males who received 40 mg/kg/day, but these were considered incidental because no effects were found during the administration period. There were no changes related to the test substance in detailed clinical observation, body weight, and food consumption during the administration and recovery periods (data not shown).

No significant changes were observed in the urinalyses at the end of the administration period. At the end of the recovery period, specific gravity was significantly increased, but this change was considered incidental because no related toxicological effects on the kidneys were observed at the end of the administration and recovery periods (data not shown). Hematological results are summarized in Table 1. Dose dependent significantly prolonged PT and APTT were observed in males who received ≥ 40 mg/kg/day. At the end of the recovery period, a significantly prolonged PT was still observed in males at 1000 mg/kg/day. Other changes observed

Table 1. Hematological findings of male and female rats after 28 days repeated oral doses of N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide.

Dose (mg/kg/d)	At the end of the administration period				At the end of the recovery period	
	0	40	200	1000	0	1000
MALE						
No. of animals examined	6	6	6	6	6	6
RBC, $\times10^4/\mu L$	795.0 ± 20.6	790.0 ± 21.8	758.5 ± 26.2*	786.3 ± 26.9	867.7 ± 24.7	863.8 ± 23.9
Hematocrit, %	45.57 ± 1.67	46.40 ± 0.57	45.82 ± 1.21	44.90 ± 1.05	46.62 ± 1.74	47.10 ± 2.05
Hemoglobin, g/dL	15.97 ± 0.40	16.37 ± 0.20	16.17 ± 0.20	16.02 ± 0.55	17.08 ± 0.63	17.03 ± 0.71
MCV, fL	57.35 ± 2.47	58.78 ± 2.07	60.45 ± 2.09	57.13 ± 2.12	53.73 ± 2.16	54.52 ± 1.86
MCH, pg	20.10 ± 0.69	20.72 ± 0.54	21.30 ± 0.60*	20.40 ± 0.88	19.70 ± 0.78	19.72 ± 0.40
MCHC, g/dL	35.07 ± 0.49	35.28 ± 0.41	35.30 ± 0.63	35.67 ± 0.42	36.65 ± 0.37	36.15 ± 0.80
WBC, $\times10^2/\mu L$	128.7 ± 43.3	134.0 ± 51.9	139.8 ± 20.9	125.8 ± 15.8	144.2 ± 19.1	151.5 ± 25.5
Platelet, $\times10^4/\mu L$	119.40 ± 13.98	131.68 ± 13.25	124.22 ± 10.66	135.72 ± 14.87	123.10 ± 17.63	116.10 ± 8.41
Reticulocyte, ‰	27.8 ± 5.0	25.7 ± 4.3	28.3 ± 7.3	26.3 ± 7.1	26.7 ± 5.1	26.5 ± 3.1
PT, sec.	16.60 ± 0.97	19.22 ± 1.56†	21.88 ± 3.57††	26.42 ± 6.80††	19.10 ± 1.31	23.37 ± 2.60**
APTT, sec.	25.28 ± 2.17	28.68 ± 1.24†	34.35 ± 3.93††	43.53 ± 6.62††	25.98 ± 1.71	27.65 ± 3.43
Differential leukocyte count (%)						
Neutrophil stab form	0.93 ± 0.48	1.00 ± 0.33	1.00 ± 0.42	0.80 ± 0.57	1.07 ± 0.70	1.73 ± 1.12
Neutrophil segmented	10.47 ± 3.42	10.80 ± 3.40	11.73 ± 2.95	12.27 ± 3.20	7.33 ± 1.89	11.27 ± 3.36*
Eosinophil	0.40 ± 0.51	0.53 ± 0.65	0.07 ± 0.16	0.40 ± 0.25	0.93 ± 0.48	0.60 ± 0.49
Basophil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.16	0.00 ± 0.00
Monocyte	2.33 ± 1.30	2.47 ± 1.14	3.00 ± 0.87	2.27 ± 1.00	1.07 ± 0.41	1.20 ± 0.62
Lymphocyte	85.87 ± 2.71	85.20 ± 4.06	84.20 ± 3.17	84.27 ± 4.53	89.53 ± 2.46	85.20 ± 2.76*
Others	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
FEMALE						
No. of animals examined	6	6	6	6	6	6
RBC, $\times10^4/\mu L$	770.8 ± 47.9	791.5 ± 25.5	753.0 ± 29.2	753.2 ± 14.2	774.2 ± 17.2	725.0 ± 48.9
Hematocrit, %	43.22 ± 1.99	43.65 ± 1.16	42.32 ± 1.46	41.93 ± 0.70	41.88 ± 1.24	39.27 ± 2.58*
Hemoglobin, g/dL	15.82 ± 0.80	15.83 ± 0.39	15.42 ± 0.58	15.38 ± 0.29	16.00 ± 0.54	15.10 ± 0.70*
MCV, fL	56.12 ± 1.39	55.15 ± 0.75	56.22 ± 1.11	55.68 ± 0.50	54.10 ± 0.85	54.20 ± 1.55
MCH, pg	20.53 ± 0.47	20.03 ± 0.52	20.47 ± 0.42	20.45 ± 0.29	20.67 ± 0.44	20.87 ± 0.69
MCHC, g/dL	36.60 ± 0.48	36.27 ± 0.44	26.42 ± 0.73	36.68 ± 0.38	38.20 ± 0.48	38.50 ± 0.94
WBC, $\times10^2/\mu L$	88.2 ± 18.3	78.8 ± 34.6	89.8 ± 23.7	102.8 ± 26.5	100.8 ± 17.9	96.2 ± 32.8
Platelet, $\times10^4/\mu L$	129.42 ± 15.00	134.00 ± 16.72	133.75 ± 9.20	147.02 ± 22.39	122.08 ± 10.93	134.17 ± 22.41
Reticulocyte, ‰	24.0 ± 7.0	25.5 ± 5.5	23.0 ± 4.0	27.3 ± 4.3	28.7 ± 5.2	28.5 ± 3.9
PT, sec.	17.40 ± 0.38	16.28 ± 0.63††	15.87 ± 0.65††	17.60 ± 1.61	17.37 ± 0.92	16.63 ± 1.06
APTT, sec.	19.28 ± 1.88	20.33 ± 2.86	22.23 ± 1.61	29.07 ± 5.08**	19.15 ± 1.39	20.23 ± 1.95
Differential leukocyte count (%)						
Neutrophil stab form	1.13 ± 1.37	0.87 ± 0.47	1.33 ± 0.65	0.87 ± 0.47	0.80 ± 0.76	1.00 ± 0.55
Neutrophil segmented	14.73 ± 13.75	11.93 ± 7.148	12.80 ± 4.48	11.67 ± 2.31	8.00 ± 3.65	8.93 ± 6.35
Eosinophil	1.13 ± 0.99	0.60 ± 0.66	1.20 ± 0.67	0.40 ± 0.51	1.13 ± 0.59	1.00 ± 0.61
Basophil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Monocyte	2.93 ± 1.57	2.53 ± 1.78	2.67 ± 0.79	2.07 ± 0.99	1.27 ± 0.82	1.13 ± 0.47
Lymphocyte	80.13 ± 14.74	84.07 ± 8.21	82.00 ± 5.23	85.00 ± 3.41	88.80 ± 4.06	87.93 ± 5.96
Others	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values are expressed as the mean ± standard deviation.
*Significantly different from the control group at $P \leqq .05$ by the Dunnett multiple comparison test.
**Significantly different from the control group at $P \leqq .01$ by the Dunnett multiple comparison test.
†Significantly different from the control group at $P \leqq .05$ by the Mann-Whitney *U*-test.
††Significantly different from the control group at $P \leqq .01$ by the Mann-Whitney *U*-test.

at the end of the recovery period, such as the percentage of segmented neutrophils and lymphocytes in males and the hemoglobin level in females, were within the range of background data and not considered as adverse effects. In females, APTT was significantly prolonged at 1000 mg/kg/day; however, unlike males, prolonged PT was not observed. Significantly shorter PT in females at 40 and 200 mg/kg/day were not dose dependent and not considered to be adverse effects of Synepirin 500. Statistically significant differences in RBC and MCH were observed in males at 200 mg/kg/day, but these changes were not dose dependent and not considered toxicological effects of Synepirin 500.

Clinical biochemistry results are summarized in Table 2. At the end of the administration period, the following changes were observed. Significant decreases in albumin fraction was observed in males at 1000 mg/kg/day and in females at ≥ 200 mg/kg/day, whereas significant increases in α_2- and β-globulin fractions were observed at 200 and/or 1000 mg/kg/day in both sexes; A/G ratios were significantly decreased in males at 1000 mg/kg/day and in females at ≥ 200 mg/kg/day. Significant increases in total protein levels and γ-GTP were observed in males at 1000 mg/kg/day and in females at ≥ 200 mg/kg/day. Total bilirubin levels significantly decreased in males at 1000 mg/kg/day and in females at ≥ 200 mg/kg/day but were considered a non-toxicological effect. Triglyceride levels significantly decreased in males but increased in females at 1000 mg/kg/day. Total cholesterol significantly increased in females, and Ca levels significantly increased in both sexes at 1000 mg/kg/day. These changes were observed after the administration period but were reversed by the end of the recovery period.

The results of organ weight analyses are summarized in Tables 3 and 4. Significant increases in absolute and relative liver weights were observed in both sexes who received ≥ 200 mg/kg/day, and relative liver weight significantly increased at 40 mg/kg/day in females. In females, absolute and relative weights of the liver significantly increased and remained increased at the end of the recovery period. Significant increases in absolute and/or relative thyroid weights were observed at the end of the administration period in both sexes who received 1000 mg/kg/day. A significant decrease in absolute testis weight was observed in the group who received 200 mg/kg/day, but this effect was not dose dependent and not considered a toxicological effect. Absolute heart weight significantly increased at 1000 mg/kg/day in males; however, this effect was not considered toxicologically important because the relative weight of the heart was not affected. Increases in absolute and relative uterus weights were observed at the end of the recovery period in females receiving 1000 mg/kg/day, but these values were within background data ranges (absolute mean: 0.510 g [0.118-0.902 g]; relative mean: 0.220% [0.044-0.397%]).

The results of histopathological findings are summarized in Table 5. At the end of the administration period, slight hypertrophy in centrilobular hepatocytes was observed in five males and six females, and slight periportal fatty change was observed in two males at 1000 mg/kg/day. Slight hypertrophy in the thyroid follicular cells was observed in three males who received 200 mg/kg/day and in six males and four females who received 1000 mg/kg/day. There were no changes in other organs and tissues in any of the groups. No effects were observed at the end of the recovery period at 1000 mg/kg/day.

DISCUSSION

To obtain information on the possible repeated-dose oral toxicity of Synepirin 500, rats were administered the test substance by gavage once daily at 0, 40, 200, and 1000 mg/kg/day for 28 days, followed by a 14-day recovery period. No adverse effects were observed on detailed clinical observations, FOB, body weight, food consumption, and urinalysis. During the administration period, diarrhea or soft feces was sporadically observed in both sexes at 40 and 200 mg/kg/day and in all males except one and in all females at 1000 mg/kg/day; however, these changes were soon reversed during the recovery period, indicating that these effects were a result of direct action on the gastrointestinal organs induced by the test substance. No other clinical effects were observed.

The liver is the primary target organ of Synepirin 500, which is the same as that of MGK264, a structurally similar synergist. Increases in absolute and relative liver weights were observed in both sexes who received ≥ 200 mg/kg/day, and relative liver weight increased at 40 mg/kg/day in females. Increased absolute and relative weights of the liver were still observed at the end of the recovery period in females but recovered in males at 1000 mg/kg/day. Slight hypertrophy in centrilobular hepatocytes was also observed in both sexes at 1000 mg/kg/day. Given the mode of action of Synepirin 500 as a synergist, it was presumed that the microsomal enzyme was altered by Synepirin 500, but it is difficult to discuss how it is related to the liver toxicity observed in rats because no further information on the mode of action is available.

Microsomal enzyme inducers are known to cause hepatomegaly as an adaptive change without any evidence of liver injury in rats (Amacher *et al*., 1998). Syn-

Table 2. Biochemical findings of male and female rats after 28 days repeated oral doses of N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide.

Dose (mg/kg/day)	At the end of the administration period				At the end of the recovery period	
	0	40	200	1000	0	1000
MALE						
No. of animals examined	6	6	6	6	6	6
Total protein, g/dL	5.53 ± 0.05	5.58 ± 0.15	5.56 ± 0.22	6.03 ± 0.29††	5.77 ± 0.22	5.77 ± 0.18
Albumin, g/dL	2.970 ± 0.055	3.035 ± 0.082	2.958 ± 0.121	2.992 ± 0.260	3.003 ± 0.146	2.962 ± 0.160
A/G ratio	1.165 ± 0.060	1.193 ± 0.050	1.102 ± 0.102	0.985 ± 0.092**	1.088 ± 0.050	1.062 ± 0.096
Protein fraction %						
Albumin	53.80 ± 1.29	54.38 ± 1.03	52.35 ± 2.24	49.53 ± 2.33**	52.08 ± 1.09	51.40 ± 2.26
α_1-globulin	20.62 ± 1.67	19.05 ± 1.14	19.63 ± 3.41	20.12 ± 2.05	20.97 ± 2.29	21.85 ± 1.76
α_2-globulin	7.38 ± 0.21	7.63 ± 0.61	8.18 ± 0.83	9.05 ± 0.60**	7.42 ± 0.47	7.43 ± 0.50
β-globulin	15.63 ± 1.00	16.55 ± 0.50	17.40 ± 0.98*	19.12 ± 1.24**	16.65 ± 0.90	16.57 ± 0.61
γ-globulin	2.57 ± 0.37	2.38 ± 0.35	2.43 ± 0.34	2.18 ± 0.61	2.88 ± 0.51	2.75 ± 0.52
AST, IU/L	61.2 ± 3.1	58.0 ± 5.0	57.5 ± 6.6	58.8 ± 6.6	75.0 ± 16.0	72.7 ± 5.3
ALT, IU/L	23.0 ± 2.5	23.8 ± 3.1	24.2 ± 2.9	25.7 ± 6.1	28.7 ± 3.9	26.0 ± 1.5
ALP, IU/L	715.0 ± 142.9	652.3 ± 167.3	611.5 ± 110.3	543.7 ± 99.0	515.5 ± 90.4	492.0 ± 70.7
γ-GTP, IU/L	1.02 ± 0.21	0.82 ± 0.26	1.00 ± 0.25	2.48 ± 0.82††	0.43 ± 0.16	0.43 ± 0.22
Total bilirubin, mg/dL	0.040 ± 0.011	0.032 ± 0.010	0.032 ± 0.008	0.025 ± 0.005*	0.062 ± 0.004	0.060 ± 0.011
Glucose, g/dL	163.3 ± 10.3	173.7 ± 14.2	165.7 ± 15.4	156.5 ± 22.2	182.3 ± 23.0	177.5 ± 16.2
Total cholesterol, mg/dL	50.3 ± 6.9	64.2 ± 16.4	60.8 ± 5.0	60.2 ± 17.3	68.2 ± 18.7	60.2 ± 17.3
Triglyceride, mg/dL	54.5 ± 23.3	47.5 ± 9.8	50.0 ± 14.6	22.8 ± 7.0**	52.0 ± 21.1	45.8 ± 21.6
Urea nitrogen, mg/dL	8.88 ± 1.55	9.58 ± 1.64	8.43 ± 0.88	10.32 ± 1.01	13.70 ± 2.78	13.88 ± 2.38
Creatinine, mg/dL	0.525 ± 0.016	0.507 ± 0.040	0.513 ± 0.039	0.545 ± 0.044	0.547 ± 0.043	0.522 ± 0.034
Na, mEq/L	143.7 ± 1.4	143.5 ± 1.2	143.7 ± 1.8	143.5 ± 1.5	144.7 ± 1.8	1044.5 ± 0.8
K, mEq/L	4.925 ± 0.224	4.948 ± 0.324	4.768 ± 0.195	4.712 ± 0.270	4.728 ± 0.106	4.657 ± 0.268
Cl, mEq/L	105.8 ± 1.7	106.2 ± 1.6	105.7 ± 1.2	105.3 ± 1.6	105.3 ± 1.9	105.2 ± 1.0
Ca, mg/dL	9.67 ± 0.33	9.83 ± 0.26	9.92 ± 0.38	10.13 ± 0.18*	9.88 ± 0.13	0.97 ± 0.26
IP, mg/dL	8.92 ± 0.70	8.43 ± 0.26	8.37 ± 0.43	8.35 ± 0.36	8.08 ± 0.27	8.00 ± 0.40
FEMALE						
No. of animals examined	6	6	6	6	6	6
Total protein, g/dL	5.68 ± 0.04	5.90 ± 0.28	6.08 ± 0.08††	6.02 ± 0.18††	5.90 ± 0.26	6.25 ± 0.33
Albumin, g/dL	3.267 ± 0.111	3.268 ± 0.184	3.090 ± 0.207	2.952 ± 0.215*	3.173 ± 0.208	3.237 ± 0.199
A/G ratio	1.358 ± 0.108	1.242 ± 0.071	1.038 ± 0.118**	0.970 ± 0.099**	1.175 ± 0.116	1.075 ± 0.061
Protein fraction %						
Albumin	57.50 ± 2.01	55.37 ± 1.45	50.82 ± 3.04**	49.08 ± 2.54**	53.82 ± 2.44	51.82 ± 1.39
α_1-globulin	15.82 ± 2.49	16.98 ± 0.86	19.87 ± 3.20*	19.12 ± 1.80	20.05 ± 2.44	22.10 ± 1.34
α_2-globulin	7.40 ± 0.63	7.52 ± 0.50	7.92 ± 0.72	9.30 ± 1.08**	6.37 ± 0.71	5.82 ± 0.48
β-globulin	15.42 ± 0.87	16.30 ± 0.59	17.67 ± 0.50**	19.33 ± 1.09**	15.58 ± 1.06	15.58 ± 0.90
γ-globulin	3.87 ± 1.11	3.83 ± 0.71	3.73 ± 0.76	3.17 ± 0.37	4.18 ± 0.79	4.68 ± 1.01
AST, IU/L	57.8 ± 6.1	62.0 ± 8.2	55.2 ± 2.2	52.0 ± 5.9	60.2 ± 6.8	63.2 ± 18.8
ALT, IU/L	21.0 ± 3.3	23.8 ± 3.4	22.0 ± 1.8	21.2 ± 3.2	21.8 ± 2.1	29.0 ± 18.9
ALP, IU/L	392.5 ± 66.4	466.0 ± 81.4	367.2 ± 98.1	341.5 ± 116.4	253.5 ± 79.6	224.3 ± 47.1
γ-GTP, IU/L	0.90 ± 0.23	1.15 ± 0.14	2.03 ± 0.53††	5.47 ± 1.35††	0.82 ± 0.19	0.85 ± 0.16
Total bilirubin, mg/dL	0.047 ± 0.014	0.037 ± 0.010	0.025 ± 0.008**	0.028 ± 0.008*	0.087 ± 0.016	0.067 ± 0.016
Glucose, g/dL	130.8 ± 9.7	146.8 ± 10.9	147.0 ± 23.4	142.0 ± 11.5	151.0 ± 15.9	164.7 ± 23.8
Total cholesterol, mg/dL	59.3 ± 8.5	66.7 ± 13.0	66.5 ± 15.6	117.0 ± 17.0**	78.2 ± 18.1	87.5 ± 15.8
Triglyceride, mg/dL	11.8 ± 2.8	10.2 ± 6.8	10.0 ± 4.2	29.7 ± 18.3†	12.5 ± 5.4	19.2 ± 7.8
Urea nitrogen, mg/dL	11.90 ± 2.17	12.03 ± 1.93	11.92 ± 2.46	11.38 ± 2.29	13.92 ± 2.29	16.37 ± 1.83
Creatinine, mg/dL	0.543 ± 0.033	0.555 ± 0.040	0.572 ± 0.048	0.577 ± 0.037	0.538 ± 0.023	0.538 ± 0.019
Na, mEq/L	143.3 ± 1.6	143.8 ± 1.2	143.3 ± 1.4	142.8 ± 1.7	143.2 ± 0.8	142.8 ± 1.2
K, mEq/L	4.805 ± 0.270	4.663 ± 0.370	4.493 ± 0.094	4.610 ± 0.208	4.600 ± 0.246	4.670 ± 0.271
Cl, mEq/L	108.2 ± 1.9	108.2 ± 1.7	107.0 ± 1.3	106.8 ± 1.0	106.0 ± 1.7	104.8 ± 0.8
Ca, mg/dL	9.50 ± 0.33	9.68 ± 0.13	9.82 ± 0.16	10.13 ± 0.28**	9.83 ± 0.27	10.02 ± 0.44
IP, mg/dL	7.28 ± 0.70	7.33 ± 0.42	6.90 ± 0.62	7.52 ± 0.52	6.88 ± 0.58	7.13 ± 0.34

Values are expressed as the mean ± standard deviation.
*Significantly different from the control group at $P \leqq .05$ by the Dunnett multiple comparison test.
**Significantly different from the control group at $P \leqq .01$ by the Dunnett multiple comparison test.
†Significantly different from the control group at $P \leqq .05$ by the Mann-Whitney *U*-test.
††Significantly different from the control group at $P \leqq .01$ by the Mann-Whitney *U*-test.

Table 3. Absolute and relative organ weights of male rats after 28 days repeated oral doses of N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide.

Dose (mg/kg/day)		At the end of the administration period				At the end of the recovery period	
		0	40	200	1000	0	1000
No. of animals examined		6	6	6	6	6	6
Body weight	(g)	360.8 ± 25.8	358.8 ± 31.1	362.7 ± 27.1	338.0 ± 23.9	430.5 ± 31.6	415.2 ± 32.0
Liver	(g)	12.155 ± 1.653	13.567 ± 2.319	14.898 ± 0.826*	17.408 ± 1.477**	13.577 ± 1.562	13.572 ± 1.955
	(%)	3.358 ± 0.234	3.767 ± 0.390	4.117 ± 0.224**	5.152 ± 0.318**	3.148 ± 0.159	3.257 ± 0.220
Kidney	(g)	2.742 ± 0.273	2.795 ± 0.364	2.870 ± 0.202	2.770 ± 0.261	3.187 ± 0.342	3.302 ± 0.296
	(%)	0.760 ± 0.052	0.778 ± 0.059	0.793 ± 0.052	0.818 ± 0.036	0.738 ± 0.053	0.798 ± 0.052
Spleen	(g)	0.645 ± 0.109	0.620 ± 0.107	0.617 ± 0.083	0.617 ± 0.089	0.732 ± 0.163	0.730 ± 0.103
	(%)	0.178 ± 0.023	0.170 ± 0.019	0.170 ± 0.023	0.183 ± 0.023	0.168 ± 0.025	0.173 ± 0.015
Heart	(g)	1.253 ± 0.063	1.262 ± 0.156	1.090 ± 0.308	1.145 ± 0.021†	1.388 ± 0.414	1.453 ± 0.106
	(%)	0.348 ± 0.026	0.352 ± 0.022	0.305 ± 0.093	0.340 ± 0.018	0.322 ± 0.023	0.350 ± 0.023
Brain	(g)	2.063 ± 0.096	2.057 ± 0.037	2.158 ± 0.079	2.048 ± 0.042	2.200 ± 0.054	2.148 ± 0.106
	(%)	0.573 ± 0.047	0.577 ± 0.044	0.598 ± 0.032	0.610 ± 0.046	0.512 ± 0.037	0.518 ± 0.043
Pituitary gland	(mg)	10.43 ± 1.78	11.53 ± 1.68	10.55 ± 1.33	11.42 ± 1.57	13.57 ± 2.25	12.95 ± 2.48
	(10^{-3}%)	2.907 ± 0.542	3.202 ± 0.241	2.913 ± 0.354	3.373 ± 0.345	3.147 ± 0.458	3.112 ± 0.460
Thymus	(mg)	632.2 ± 159.2	661.0 ± 263.1	599.8 ± 98.2	552.0 ± 167.3	593.3 ± 122.7	534.7 ± 126.0
	(10^{-3}%)	117.507 ± 54.405	180.677 ± 56.742	165.173 ± 22.195	161.982 ± 38.879	136.737 ± 19.541	129.760 ± 34.712
Thyroid	(mg)	19.32 ± 4.00	17.40 ± 3.45	20.85 ± 2.50	26.65 ± 8.74	21.25 ± 3.99	23.78 ± 3.95
	(10^{-3}%)	5.380 ± 1.200	4.898 ± 1.117	5.792 ± 0.994	7.853 ± 2.488*	4.922 ± 0.714	5.753 ± 0.972
Adrenal	(mg)	52.8 ± 7.8	53.5 ± 6.7	51.0 ± 9.5	52.0 ± 6.3	62.3 ± 9.4	61.7 ± 4.6
	(10^{-3}%)	14.613 ± 1.668	14.913 ± 1.410	14.057 ± 2.300	15.452 ± 2.303	14.500 ± 2.012	14.970 ± 2.081
Testis	(g)	3.255 ± 0.151	3.123 ± 0.111	2.910 ± 0.107**	3.102 ± 0.161	3.223 ± 0.274	3.288 ± 0.157
	(%)	0.905 ± 0.061	0.875 ± 0.071	0.807 ± 0.072	0.922 ± 0.081	0.753 ± 0.092	0.797 ± 0.088
Epididymis	(g)	0.747 ± 0.078	0.827 ± 0.086	0.712 ± 0.072	0.762 ± 0.092	1.052 ± 0.104	1.060 ± 0.054
	(%)	0.205 ± 0.014	0.232 ± 0.035	0.198 ± 0.024	0.227 ± 0.023	0.243 ± 0.027	0.257 ± 0.025
Prostate	(mg)	411.7 ± 96.0	439.0 ± 111.6	499.3 ± 56.5	352.8 ± 70.8	512.3 ± 110.8	547.8 ± 55.3
	(10^{-3}%)	113.180 ± 19.106	122.142 ± 28.164	138.795 ± 22.291	104.715 ± 21.694	118.872 ± 23.322	132.772 ± 18.111
Seminal vesicle	(g)	1.282 ± 0.249	1.218 ± 0.158	1.365 ± 0.164	1.327 ± 0.188	1.645 ± 0.197	1.413 ± 0.259
	(%)	0.353 ± 0.054	0.338 ± 0.028	0.378 ± 0.051	0.393 ± 0.056	0.382 ± 0.047	0.343 ± 0.076

Values are expressed as the mean ± standard deviation.
*Significantly different from the control group at $P \leqq .05$ by the Dunnett multiple comparison test.
**Significantly different from the control group at $P \leqq .01$ by the Dunnett multiple comparison test.
†Significantly different from the control group at $P \leqq .05$ by the Mann-Whitney *U*-test.

epirin 500 decreased A/G ratios, increased total protein levels, and increased γ-GTP in both sexes, and Synepirin 500 appeared to have a specific mechanism to induce liver injury at high doses. The only observed effects at 40 mg/kg/day were increased relative liver weight in females and prolonged PT and APTT in males. These effects were considered an adaptive response because there was no indication of these on the histopathology of the liver; however, as a screening assessment, we evaluated these changes as adverse effects to be on the safe side by considering the toxicity information on the analog chemical of MGK264 and the results obtained from only a limited period of exposure to Synepirin 500.

On the basis of the findings of liver weight and the above-mentioned biochemical parameters, females appeared to be more susceptible to liver toxicity from Synepirin 500; however, it was not that simple because a significant increase in PT was observed only in males. It is interesting that triglyceride levels showed a clear sex difference. More specifically, triglyceride levels significantly decreased in males but increased in females at 1000 mg/kg/day. Total cholesterol levels also showed different properties by sex. Sex hormones and their receptors regulate lipid, glucose, and cholesterol homeostasis in the liver (Shen and Shi, 2015); therefore, sex hormones might play some roles in the hepatotoxicity of Synepirin 500.

Increases in absolute and/or relative thyroid weight were observed in males and in females who received 1000 mg/kg/day at the end of the administration period.

Table 4. Absolute and relative organ weights of female rats after 28 days repeated oral doses of N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide.

Dose (mg/kg/day)		At the end of the administration period				At the end of the recovery period	
		0	40	200	1000	0	1000
No. of animals examined		6	6	6	6	6	6
Body weight	(g)	204.5 ± 18.7	231.5 ± 14.7	211.8 ± 27.6	214.0 ± 18.3	235.3 ± 15.5	239.7 ± 14.5
Liver	(g)	6.472 ± 0.841	7.652 ± 0.872	9.560 ± 1.598**	12.803 ± 1.116**	6.978 ± 0.647	8.768 ± 0.855**
	(%)	3.158 ± 0.176	3.578 ± 0.224*	4.507 ± 0.342**	5.987 ± 0.243**	2.960 ± 0.125	3.660 ± 0.326**
Kidney	(g)	1.643 ± 0.143	1.678 ± 0.106	1.685 ± 0.211	1.687 ± 0.151	1.798 ± 0.136	1.878 ± 0.125
	(%)	0.803 ± 0.035	0.788 ± 0.048	0.797 ± 0.025	0.788 ± 0.052	0.763 ± 0.036	0.783 ± 0.033
Spleen	(g)	0.423 ± 0.074	0.397 ± 0.048	0.420 ± 0.082	0.395 ± 0.071	0.475 ± 0.049	0.462 ± 0.073
	(%)	0.205 ± 0.026	0.187 ± 0.022	0.197 ± 0.020	0.183 ± 0.020	0.202 ± 0.013	0.192 ± 0.028
Heart	(g)	0.740 ± 0.066	0.758 ± 0.044	0.798 ± 0.166	0.763 ± 0.062	0.863 ± 0.064	0.888 ± 0.076
	(%)	0.362 ± 0.012	0.355 ± 0.023	0.377 ± 0.043	0.360 ± 0.030	0.368 ± 0.004	0.370 ± 0.014
Brain	(g)	1.895 ± 0.102	1.912 ± 0.096	1.902 ± 0.198	1.907 ± 0.060	1.988 ± 0.088	1.963 ± 0.048
	(%)	0.930 ± 0.066	0.902 ± 0.088	0.903 ± 0.060	0.895 ± 0.064	0.848 ± 0.066	0.822 ± 0.035
Pituitary gland	(mg)	12.42 ± 3.48	13.98 ± 2.78	11.55 ± 1.34	12.95 ± 1.71	14.15 ± 1.56	15.65 ± 1.81
	(10^{-3}%)	12.42 ± 3.48	13.98 ± 2.78	11.55 ± 1.34	12.95 ± 1.71	6.032 ± 0.699	6.522 ± 0.568
Thymus	(mg)	481.5 ± 141.8	481.3 ± 47.3	504.5 ± 116.0	481.7 ± 72.9	435.2 ± 65.3	430.0 ± 106.2
	(10^{-3}%)	233.180 ± 49.457	226.179 ± 26.104	236.592 ± 34.264	227.478 ± 46.966	185.308 ± 27.746	180.157 ± 46.696
Thyroid	(mg)	15.047 ± 4.62	13.42 ± 2.16	15.60 ± 1.58	21.78 ± 4.46**	14.47 ± 2.56	17.32 ± 1.74*
	(10^{-3}%)	7.322 ± 1.974	6.362 ± 1.412	7.533 ± 1.615	10.182 ± 1.852*	6.140 ± 0.915	7.228 ± 0.642*
Adrenal	(mg)	63.2 ± 5.7	63.8 ± 5.9	61.2 ± 5.9	63.8 ± 8.9	68.2 ± 8.2	71.8 ± 9.6
	(10^{-3}%)	31.078 ± 3.813	29.948 ± 2.741	29.523 ± 6.231	29.915 ± 3.971	29.042 ± 3.663	29.928 ± 3.173
Ovary	(g)	85.5 ± 14.3	80.8 ± 13.9	95.5 ± 20.7	85.5 ± 16.5	99.8 ± 16.0	96.3 ± 19.6
	(10^{-3}%)	41.845 ± 6.306	38.012 ± 7.011	44.997 ± 7.403	39.817 ± 5.813	42.310 ± 5.181	39.943 ± 5.585
Uterus	(g)	0.433 ± 0.123	0.607 ± 0.297	0.495 ± 0.135	0.472 ± 0.123	0.398 ± 0.080	0.563 ± 0.122*
	(%)	0.208 ± 0.043	0.288 ± 0.155	0.238 ± 0.067	0.220 ± 0.058	0.172 ± 0.029	0.237 ± 0.061*

Values are expressed as the mean ± standard deviation.
*Significantly different from the control group at $P \leqq .05$ by the Dunnett multiple comparison test.
**Significantly different from the control group at $P \leqq .01$ by the Dunnett multiple comparison test.

At the end of the recovery period, increased absolute and relative thyroid weights were still observed in females at 1000 mg/kg/day. Slight hypertrophy in thyroid follicular cells was observed in three males who received 200 mg/kg/day and in six males and four females who received 1000 mg/kg/day. Calcium levels, mediated by a thyroid hormone, increased in both sexes at 1000 mg/kg/day. Microsomal enzyme inducers increase thyroid follicular cell proliferation in rodents through the induction of thyroxine (T_4) glucuronidation, the reduction of serum T_4, and an increase in serum thyroid-stimulating hormone (TSH) (de Sandro *et al*., 1991; Hood *et al*., 2003). By calculating the thyroid gland-liver response ratio, defined by Yamada *et al*. (2013), the effect on the thyroid caused by Synepirin 500 was suggested to be indirect. According to USEPA studies, treatment with MGK264 significantly increased thyroid follicular hypertrophy and follicular tumors in rats (Goldenthal, 1993; Schardein, 1991). USEPA further evaluated for toxic effects of MGK264 on the thyroid pathway, but there were no effects seen on serum T_4 or TSH levels in male and female pubertal rats (Zorrilla, 2012b). Further studies will be necessary to discuss the extrapolation of thyroid toxicity caused by Synepirin 500.

USEPA (2015) also evaluated the potential that MGK264 interacts with estrogen and androgen pathways. There was no evidence of potential interaction with the androgen pathway (Wagner, 2012; Willoughby, 2012a; Zorrilla, 2012a), and *in vitro* estrogen receptor binding, estrogen receptor translational activation and steroidogenesis, and *in vivo* uterotrophic assays were negative (Willoughby, 2012b, 2012c; Zorrilla, 2012c); however, MGK264 was positive for aromatase inhibition in an *in vitro* assay (Wilga, 2012). In a female pubertal rat assay at 800 mg/kg/day, the vaginal opening and mean age of the first estrus were significantly delayed without a growth effect (Zorrilla, 2012b). In a two-generation rat study, decreased absolute and relative ovary weights were observed in the P generation, and altered luteinization of the corpora lutea in the P and F1 females were observed

Table 5. Histopathological findings of male rats after 28 days repeated oral doses of N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide.

Organ	Findings	Grade	At the end of the administration period				At the end of the recovery period	
	Dose (mg/kg/day)		0	40	200	1000	0	1000
MALE								
No. of animals examined			6	6	6	6	6	6
Lung	Foam cell accumulation	+	0	-	-	1	0	0
	Mineralization in artery	+	0	-	-	1	0	1
Liver	Centrilobular hypertrophy of hepatocyte	+	0	0	0	5	0	0
	Periportal fatty change	+	0	0	0	2	0	1
	Microgranuloma	+	5	3	2	2	4	1
Heart	Focal mycocardial degeneration	+	0	-	-	1	0	0
Kidney	Hyaline droplet in proximal tubular epithelium	+	0	-	-	0	1	2
	Eosinophilic body in proximal tubular epithelium	+	0	-	-	0	1	2
	Tubular epithelium regeneration	+	0	-	-	0	3	0
	Mineralization in papilla	+	1	-	-	0	0	0
Prostate	Inflammatory cell infiltration	+	2	-	-	2	2	3
Thyroid	Hypertrophy of follicular cell	+	0	0	3	6	0	0
FEMALE								
No. of animals examined			6	6	6	6	6	6
Lung	Foam cell accumulation	+	1	-	-	1	0	0
	Inflammatory cell infiltration	+	1	-	-	0	0	0
Liver	Centrilobular hypertrophy of hepatocyte	+	0	0	0	6	0	0
	Periportal fatty change	+	3	5	5	4	1	2
	Microgranuloma	+	6	4	4	2	2	3
Heart	Focal mycocardial degeneration	+	1	-	-	0	0	0
Kidney	Tubular epithelium regeneration	+	0	-	-	1	0	1
Thyroid	Hypertrophy of follicular cell	+	0	0	0	4	0	0

Values are number of animals with findings.
-not examined
Grade; +: slight change.

at 952 mg/kg/day (Schardein, 1991). These results suggested that MGK264 inhibited estrogen synthesis. USEPA concluded that MGK264 at relatively high doses has the potential to interact with the estrogen pathways.

Repeated doses of Synepirin 500 caused adverse effects on the liver and thyroid in rats as expected, but sex differences were also recognized with their toxic responses. In this study, the no-observed adverse effect was not obtained. As a conservative assessment, the lowest observed adverse effect level of Synepirin 500 was judged to be 40 mg/kg/day for both sexes of rats on the basis of the effects on the liver, such as increased relative weight of the liver in females and changes in the blood parameters related to liver toxicity in males at ≥ 40 mg/kg/day. Taken into account the findings of the estrogen pathway and two-generation study with MGK264, there are some concerns about Synepirin 500's interaction with endocrine-related processes and its effects on reproduction. To clarify the effects of Synepirin 500 on human health, a reproductive study, including endocrine disruptor relevant endpoints, is recommended.

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

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