TWO-YEAR DIETARY CARCINOGENICITY STUDY OF ($\pm$)-4-DIETHYLAMINO-1,1-DIMETHYLBUT-2-YN-1-YL 2- CYCLOHEXYL-2-HYDROXY-2-PHENYLACETATE MONOHYDROCHLORIDE MONOHYDRATE (NS-21) , A NOVEL DRUG FOR URINARY FREQUENCY AND INCONTINENCE, IN RATS

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ABSTRACT— The oncogenic potential of ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate (NS-21), a new drug for the treatment of urinary frequency and incontinence, was assessed when it was administered in the diet of Charles River Fischer-344 rats for 2 years in dosages of 0, 10, 30 and 100 mg/kg/day.

No drug-related effects occurred on survival, appearance or behavior, or occurrence, location or number of palpable masses. Food efficiency and hematologic values also were apparently unaffected. Statistically significantly low mean weekly body weights and average food consumption values were observed in the all dose groups. The plasma concentrations of NS-21 and its active metabolite, RCC-36, in the treated groups were increased in a dose-dependent manner. Histopathological examinations disclosed test article-related increases in the incidence of periportal hypertrophy and midzonal hepatocellular vacuolization in the livers of the 100 mg/kg/day animals. There were no test article-related effects on the incidence or type of neoplastic lesions.

In conclusion, under the conditions of this study, no oncogenic effects were evident in Fischer-344 rats when NS-21 was administered in the diet in concentrations to produce an intake of up to 100 mg/kg/day for 2 years.

KEY WORDS : NS-21, Urinary frequency, Urinary incontinence, Carcinogenicity, Rats

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INTRODUCTION

NS-21, ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate, is a new drug from Nippon Shinyaku Co., Ltd. for the treatment of urinary frequency and incontinence. The drug ameliorates pollakiuria and urinary incontinence caused by hyperactivity of smooth muscle of the urinary bladder due to its anticholinergic and calcium antagonistic actions.

In a single-dose oral toxicity study of NS-21 (Nishiguchi *et al.*, 1997b), LD₅₀ values were 2839 and 1739 mg/kg in male and female rats, respectively. The no observed adverse effect level (NOAEL) obtained in repeated-dose toxicity studies in rats were 6 mg/kg for 13-week oral toxicity (Nishiguchi *et al.*, 1997a) and 5 mg/kg for 26-week oral toxicity (Okasaki *et al.*, 1997), respectively. Nine deaths occurred in the 750 mg/kg dose group of the 13-week toxicity study and 2 deaths occurred in the 500 mg/kg dose group of the 26-week oral toxicity study. The main toxicological findings observed in these studies were mydriasis, a decrease in body weight gain, increases in water consumption, urinary protein and BUN, stimulated thyroid follicles, hepatocellular hypertrophy and increases in incidence and severity of chronic progressive nephropathy.

The present paper describes the methods and findings obtained in a 2-year oncogenicity study of NS-21 in rats as a part of the safety evaluation of this drug.

Fig. 1. Chemical structure of ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate (NS-21).

MATERIALS AND METHODS

Test and Control Materials

The chemical structure of NS-21 is shown in Fig. 1. NS-21 occurs as white crystals or crystalline powder and its molecular weight is 440.02. It is very soluble in methanol and glacial acetic acid, freely soluble in dehydrated ethanol, soluble in acetone, slightly soluble in water and practically insoluble in ether. The 2% solution of NS-21 in dehydrated ethanol produces no optical rotation. The Lot No. of the test material used in this study was 20 (Purity; 99.9%).

Purina Certified Rodent Chow #5002 (Richmond, Virginia, U.S.A.) was the basal diet and served as the control material.

Animals and Husbandry

Two hundred fifty-seven male and 257 female Fischer-344 rats, 4 weeks of age, were received from Charles River Laboratories, Inc. (Portage, Michigan, U.S.A.). The rats were observed during a 17-day acclimation period for any clinical signs of disease. Five rats per sex were selected for a pretest viral health screen and were subsequently euthanized. The rats were individually housed in stainless steel wire mesh-bottom cages in an environmentally controlled room with a 12-hr photoperiod, a temperature range of 18-25°C and a humidity range of 40-66 percent. Purina Certified Rodent Chow #5002 and water were available *ad libitum*.

Group Assignment and Dose Levels

Following an examination for general physical conditions, 200 rats per sex were randomly divided into 4 groups each containing 50 males and 50 females.

A potential route of administration to humans is by the oral route. Therefore, this study in rats employed the same route.

Dose levels were based upon the results of a preliminary range-finding toxicity study. In the preliminary study, the administration of NS-21 in the diet to rats for 13 weeks at doses of 0, 4, 20, 100 and 500 mg/kg/day produced no apparent effects on appearance, behavior or survival. Animals receiving 500 mg/kg/day had substantially reduced body weight gains and slightly

reduced food consumption. There were no clear effects on food efficiency although this tended to be below in the 500 mg/kg/day dose group. There were no treatment-related lesions noted at necropsy. Histopathological examination revealed hepatocellular hypertrophy in the 20, 100 and 500 mg/kg/day groups, stimulated thyroid follicles and increased regenerative renal tubules in the 100 and 500 mg/kg/day groups and increased hyaline casts in the tubular lumen in the 500 mg/kg/day group. Increased regenerative tubules and hyaline casts of kidney in the 100 or 500 mg/kg/day group suggest that the long-term administration of more than 100 mg/kg/day of NS-21 increases the incidence of chronic progressive nephropathy and mortality.

Based on this preliminary study, dosage levels of 0, 10, 30 and 100 mg/kg/day were selected for the present 2-year oncogenicity study in rats.

Compound Formulation and Administration

Prior to weighing, the test article was ground using a Waring blender (New Hartford, Connecticut, U.S.A.) and sieved through a #40 sieve. The test article:diet mixtures were prepared at concentrations to yield dosage levels of 10, 30 and 100 mg/kg/day. The test article was ground with a small amount of Certified Rodent Chow #5002 using a mortar and pestle. This premix was added to additional diet and mixed using a Hobart mixer (Troy, Michigan, U.S.A.) for 5 min. The resulting premix was then blended with an appropriate additional amount of diet in a twin shell blender for 10 min with an intensifier bar operated for the entire blending period. Fresh test article:diet mixtures were prepared weekly. Untreated diet was dispensed for the control group. Control and prepared test diets were stored at room temperature. The test article concentrations were adjusted weekly according to group body weights and food intake values.

Beginning on November 13, 1992, the test diets were administered. The control group received untreated diet. Following 2 years of treatment, all surviving rats were euthanized and necropsied on November 11-12 and 14-17, 1994.

Sampling and Analysis of Test Diets

The identity and purity of test article samples were verified upon delivery by Nippon Shinyaku

Co., Ltd. (the first delivery on November 4, 1992, the second one on March 8, 1993 and the third one on January 24, 1994). Bulk analyses for the assessment of test article stability were also conducted.

Homogeneity analyses using high performance liquid chromatography (HPLC) (Model. 730, Waters Corporation, Milford, Massachusetts, U.S.A.) of representative test diet formulations were conducted before initiation of treatment. Stability analyses of test article-containing diet preparations were also conducted by HPLC prior to initiation of treatment. Samples of the test diets from all dose levels prepared for study weeks 1-4 and every 4 weeks thereafter were analyzed for test article concentrations by HPLC.

Analyses indicated that the prepared diets were homogeneous and accurately prepared. Stability data indicated that the formulations were stable under study conditions for up to 10 days. Periodic analyses for dose verification for study weeks 1-4 and every 4 weeks thereafter showed dietary concentrations of NS-21 ranging from 84 to 112 % of the desired concentrations of test article.

Mortality and Clinical Signs

The rats were observed for mortality and clinical signs twice daily throughout the study.

The rats were observed for signs of overt toxicity at the times of the morbidity/mortality checks. Detailed observations of appearance and condition, behavior and activity, excretory functions, respiration, orifices, eyes and palpable masses were conducted once weekly.

Individual body weights were recorded pretest, weekly for the first 14 weeks of study and every 2 weeks thereafter.

Individual food consumption was measured weekly for the first 14 weeks of study and every 2 weeks thereafter. From these values and the body weights, compound consumption was calculated for the same intervals. Food efficiency was calculated weekly for the first 14 weeks of study.

Clinical Hematology

Clinical hematological studies were conducted on all surviving animals at termination of study. Blood samples were collected from animals

euthanized *in extremis* during the study when possible from 8 A.M. to 5 P.M. (normal working hours). Animals euthanized *in extremis* during other than normal working hr had a blood smear made at necropsy and differential leukocyte counts were determined. Blood samples were obtained from the orbital sinus. The animals used for plasma collection were also used for hematology, but blood from these 6/sex/group were collected by cardiac puncture. The animals had free access to food and water prior to the blood collection. The following hematological variables were evaluated: leukocyte count (total and differential), erythrocyte count, hemoglobin concentration, hematocrit, MCV, MCH, MCHC and platelet count.

Analysis for Plasma Concentrations of Test Article and its Active Metabolite

Blood samples were collected from three CO₂-anesthetized animals/sex/group at 10 P.M. and at 1 A.M. (different 3 animals/sex/group) immediately before necropsy at the termination of study. Blood samples were obtained by cardiac puncture. Samples were processed for plasma. The concentrations of NS-21 and its active metabolite, ($\pm$)-4-ethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride (RCC-36), in plasma samples were determined using HPLC (LC-10A, Shimadzu corporation, Kyoto, JAPAN) by Nippon Shinyaku Co., Ltd..

Pathology

All animals received a complete postmortem examination. All survivors and all animals found *in extremis* were euthanized by carbon dioxide inhalation. The animals were examined carefully for external abnormalities including palpable masses. The skin was reflected from a ventral midline incision and any subcutaneous abnormalities were identified. The abdominal, thoracic and cranial cavities were examined for abnormalities *in situ* and the organs were removed and examined. All abnormalities were recorded.

The following organs and tissues were collected from all animals and placed in phosphate-buffered neutral formalin: adrenals, aorta, bone and bone marrow (femur, sternum), brain (fore, mid and hind), esophagus, stomach

(glandular and nonglandular), duodenum, jejunum, ileum, cecum, colon, rectum, ovaries, testes with epididymides, heart, kidneys, liver (3 sections collected, 2 sections examined microscopically), lungs with mainstem bronchi (collected whole, 2 sections examined microscopically), lymph nodes (mandibular, mesenteric and regional when applicable), mammary gland, pancreas, pituitary, prostate and seminal vesicles, salivary glands (mandibular with sublingual), sciatic nerve, skeletal muscle (thigh), skin, spinal cord (cervical, thoracic and lumbar), spleen, thymus, thyroid/parathyroids, tongue, trachea, urinary bladder, uterus (horns and cervix), vagina, tissue masses and gross lesions. Eyes including optic nerve and Harderian gland were fixed in Davidson's fixative.

All preserved organs and tissues from the control and 100 mg/kg/day rats and from all rats that were euthanized or died on study were embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined microscopically. Additionally, all tissue masses, gross lesions and potential target organs from all dose groups were processed, stained and examined. The potential target organs were liver and thyroid gland, as determined by treatment-related stimulated thyroid follicles observed in the repeated-dose toxicity studies in rats (Nishiguchi *et al.*, 1997a; Okasaki *et al.*, 1997) and treatment-related hepatocellular hypertrophy and vacuolization observed in the 100 mg/kg/day group of the present study.

Statistical Analysis

Body weights, food consumption, food efficiency and hematological values were analyzed using analysis of variance (one-way classification) (Gad and Weil, 1988a) and Bartlett's test for homogeneity of variance (Gad and Weil, 1988b). Treatment groups were compared to the control group, by sex, using the appropriate t-statistic (for equal or unequal variance) (Steel and Torrie, 1980). Dunnett's multiple comparison tables (Dunnett, 1964) or pairwise comparisons with a Bonferroni correction (Neter and Wasserman, 1974) were used to determine the significance of differences. Where non-parametric statistical procedures were appropriate, rank

transformation methods (Conover and Iman, 1981) were employed. Analysis of tumor incidence data was performed as described by Huff *et al.* (1988). These procedures included life table tests, the Hoel-Walburg "incidental tumor" tests, Fisher's exact tests and Cochran-Armitage

trend tests.

RESULTS

Mortality and Clinical Signs

The test article had no effect on survival. The

Fig. 2. Group mean body weights of male rats given NS-21 for 2 years.

Fig. 3. Group mean body weights of female rats given NS-21 for 2 years.

numbers of animals that died or were euthanized *in extremis* through 2 years of the study for the control, 10, 30 and 100 mg/kg/day groups were 23, 15, 16, 17 for males and 12, 12, 11, 12 for females, respectively.

The test article had no apparent effect on clinical appearance or behavior of the animals or on the occurrence, location or number of palpable masses.

Fig. 4. Group mean food consumptions of male rats given NS-21 for 2 years.

Fig. 5. Group mean food consumptions of female rats given NS-21 for 2 years.

Body Weights

Mean group body weights are illustrated in Figs. 2 and 3.

As the study progressed, statistically significantly low mean body weights were observed in the male and female rats of all treated groups in comparison to means of the control rats. Mean weights of the 100 mg/kg/day males and females were statistically significantly less than control means for nearly the entire study. In the 30 mg/kg/day males and females, significantly low mean weights were observed from week 2 or 3 for nearly the entire study. In the 10 mg/kg/day males, low mean weights in comparison to means of the control animals were observed during weeks 2-80. In the 10 mg/kg/day females, low mean weights in comparison to means of the control animals were observed during weeks 26-50.

Food and Compound Consumptions and Food Efficiency

Mean food consumption data are illustrated in Figs. 4 and 5.

As the study progressed, food consumption

values decreased statistically significantly in all treated male and female groups. Mean food consumption values of the 100 mg/kg/day males and females were statistically significantly less than control means for nearly the entire study. In the 30 mg/kg/day males and females, low mean food consumption values in comparison to means of the control group were observed for nearly the entire study. Fewer low food consumption values were observed in the 30 mg/kg/day group than in the 100 mg/kg/day group. In the 10 mg/kg/day males, low mean food consumption values in comparison to means of the control animals were observed during weeks 1-13, 20-42, and 50-70. In the 10 mg/kg/day females, low mean food consumption values in comparison to means of the control animals were observed during weeks 20-26 and 90-98.

Calculated overall mean compound consumption values through 2 years for the control, 10, 30 and 100 mg/kg/day groups were 0, 8.8, 26.4 and 89.0 mg/kg/day for males and 0, 8.8, 25.9 and 88.8 mg/kg/day for females, respectively.

Mean food efficiency values during weeks 1-14 varied considerably within and among groups. Statistically significant differences were either

Table 1. Summary of drug concentrations in plasma.

		Male				Female			
Group No.	Dose (mg/kg/day)	NS-21		RCC-36		NS-21		RCC-36	
		22:00	1:00	22:00	1:00	22:00	1:00	22:00	1:00
2	10 ^{*)}	N.D.	N.D.	14.13	12.04	32.41	N.D.	39.87	10.74
		26.07	N.D.	5.14	16.85	N.D.	N.D.	6.46	24.81
		N.D.	N.D.	34.07-	12.79	N.D.	N.D.	12.08	13.48
		Mean	8.69	0	17.78	13.89	10.80	0	19.47
3	30 ^{*)}	70.42	55.87	23.33	76.00	N.D.	N.D.	54.31	68.43
		N.D.	N.D.	36.85	35.64	55.61	N.D.	14.74	11.62
		N.D.	N.D.	39.64	63.46	N.D.	N.D.	49.34	54.04
		Mean	23.48	18.62	33.28	58.37	18.54	0	39.46
4	100 ^{*)}	100.91	N.D.	89.01	111.11	83.67	154.49	51.28	193.96
		N.D.	N.D.	175.51	97.61	N.D.	N.D.	61.30	70.04
		62.10	147.55	123.53	207.70	156.03	N.D.	78.28	138.02
		Mean	54.34	49.18	129.35	138.81	79.90	51.50	63.62

N.D. : Not determined. (NS-21 < 25ng/ml).

*) : N = 3 rats per test materials and interval.

Unit : ng/ml.

Table 2-1. Location and incidence of types of primary neoplasms in rats given NS-21 for 2 years.

Sex	Male				Female			
Dose (mg/kg/day)	0	10	30	100	0	10	30	100
No. of animals	50	50	50	50	50	50	50	50
No. of tumor-bearing animals	50	49	50	50	39	40	40	43
Hemolymphoreticular system								
Mononuclear cell leukemia	22	9	10	13	11	9	8	8
Malignant lymphoma, lymphoblastic	0	0	0	1	0	0	0	0
Histiocytic sarcoma	0	0	2	0	0	0	1	2
Hemangiosarcoma	1	0	2	0	0	0	0	0
Thyroid gland								
C-cell adenoma	10	9	15	8	2	8	4	8
Follicular adenoma	1	0	0	2	0	0	1	0
C-cell carcinoma	3	1	1	1	2	2	1	1
Follicular carcinoma	0	1	1	1	0	0	0	0
Parathyroid gland								
Adenoma	2	0	0	0	0	1	0	0
Mandibular salivary gland								
Malignant neurilemmoma	0	0	0	0	0	1	0	0
Lung								
Alveolar/bronchiolar carcinoma	0	0	0	0	0	0	1	0
Liver								
Hepatocellular adenoma	0	4	1	1	0	1	0	0
Hemangioma	1	0	0	0	0	0	0	0
Hepatocellular carcinoma	0	0	0	0	0	1	0	0
Kidney								
Renal cell adenoma	0	0	1	0	0	0	0	0
Pancreas								
Islet cell adenoma	5	1	1	2	1	0	1	0
Islet cell carcinoma	1	0	0	0	0	0	0	0
Stomach, nonglandular								
Squamous cell papilloma	1	0	0	1	0	0	0	0
Jejunum								
Adenocarcinoma	0	0	0	1	0	0	0	0
Leiomyosarcoma	0	0	0	0	0	0	0	1
Ileum								
Undifferentiated sarcoma	0	0	0	0	0	0	1	0
Rectum								
Leiomyosarcoma	1	0	0	0	0	0	0	0
Urinary bladder								
Papilloma	0	0	0	0	0	0	1	0

All animals in the 0 and 100 mg/kg/day dose groups and unscheduled deaths from all groups were examined. In addition, liver, thyroid gland, tissue masses and gross lesions of all animals from the 10 and 30 mg/kg/day groups were also examined.

Table 2-2. Location and incidence of types of primary neoplasms in rats given NS-21 for 2 years.

Sex	Male				Female			
Dose (mg/kg/day)	0	10	30	100	0	10	30	100
No. of animals	50	50	50	50	50	50	50	50
Adrenal gland, medulla								
Benign pheochromocytoma	7	1	5	2	0	0	0	2
Complex pheochromocytoma	1	1	0	1	0	0	0	0
Testis								
Benign interstitial cell tumor	42	43	47	40	-	-	-	-
Malignant mesothelioma	0	1	1	0	-	-	-	-
Epididymis								
Malignant mesothelioma	1	1	0	0	-	-	-	-
Ovary								
Benign Sertoli cell tumor	-	-	-	-	0	0	0	2
Uterus								
Endometrial polyp	-	-	-	-	9	8	7	13
Adenocarcinoma	-	-	-	-	1	0	1	0
Endometrial sarcoma	-	-	-	-	1	0	0	1
Cervix								
Adenocarcinoma	-	-	-	-	1	0	0	0
Brain								
Astrocytoma	0	0	0	0	0	0	0	1
Oligodendroglioma	0	0	0	1	0	0	0	0
Pituitary gland								
Adenoma	20	22	8	18	20	16	16	20
Craniopharyngioma	0	0	0	0	1	0	0	1
Bone (Pelvis)								
Osteosarcoma	0	0	0	0	0	1	0	0
Bone (Skull)								
Osteosarcoma	1	1	0	0	0	0	0	0
Skin								
Sebaceous adenoma	0	0	0	0	1	0	0	0
Benign basal cell tumor	0	0	0	0	1	0	0	0
Keratoacanthoma	1	1	1	2	0	0	1	0
Squamous cell papilloma	0	0	0	1	0	1	0	0
Squamous cell carcinoma	0	0	1	0	0	1	0	0
Neurofibroma	0	0	0	0	0	0	0	1
Skin, subcutis								
Fibroma	2	2	1	0	0	0	0	0
Hemangioma	1	0	0	0	0	0	0	0
Neurofibroma	0	0	1	0	0	0	0	0

All animals in the 0 and 100 mg/kg/day dose groups and unscheduled deaths from all groups were examined. In addition, liver, thyroid gland, tissue masses and gross lesions of all animals from the 10 and 30 mg/kg/day groups were also examined.

greater or less than control group values.

Clinical Hematology

At the terminal interval, males in the 30 and 100 mg/kg/day groups had statistically significantly higher mean erythrocyte counts and lower MCH (30 mg/kg/day group only) and MCHC when compared to control group males.

Plasma Concentrations of Test Article and its Active Metabolite

Plasma concentrations of NS-21 and its active metabolite, RCC-36, are summarized in Table. 1. There were many animals in which NS-21 and RCC-36 were not detected in the plasma concentrations which were under the limit of quantification. In addition, there was individual variation in plasma NS-21 and RCC-36 concentrations. However, a dose-response relationship

was shown for both NS-21 and RCC-36 plasma concentrations in each dose group.

Gross Pathology

No apparent treatment-related lesions were seen at necropsy.

Histopathology

Tables 2-1 to 2-3 present the incidences of types of primary neoplasms in control and treatment groups. Photographs of representative lesions are presented (Photos 1-7).

There was an increased incidence of thyroid C-cell adenomas in all treated females (4 %, 16 %, 8 %, and 16 %, respectively, for the 0, 10, 30 and 100 mg/kg/day groups). Although the increased incidences of C-cell adenomas in the 10 and 100 mg/kg/day groups were statistically significant by Fisher's exact test (p-values are both

Table 2-3. Location and incidence of types of primary neoplasms in rats given NS-21 for 2 years.

Sex	Male				Female			
Dose (mg/kg/day)	0	10	30	100	0	10	30	100
No. of animals	50	50	50	50	50	50	50	50
Skin, subcutis								
Fibrosarcoma	0	0	1	0	0	0	0	1
Neurofibrosarcoma	1	0	0	1	0	0	0	0
Osteosarcoma	0	0	1	0	0	0	0	0
Skin, ear								
Neurofibroma	0	0	2	0	0	0	0	0
Neurofibrosarcoma	0	0	0	0	0	0	1	0
Zymbal's gland								
Zymbal's gland adenoma	1	1	0	0	0	0	0	1
Zymbal's gland carcinoma	0	1	0	1	0	0	0	0
Preputial gland								
Adenoma	3	0	0	2	-	-	-	-
Clitoral gland								
Adenoma	-	-	-	-	0	2	3	0
Squamous cell papilloma	-	-	-	-	1	0	0	0
Mammary gland								
Fibroadenoma	1	1	0	2	5	7	7	8
Adenoma	0	0	0	0	2	3	0	0
Adenocarcinoma	0	0	0	0	1	1	0	0

All animals in the 0 and 100 mg/kg/day dose groups and unscheduled deaths from all groups were examined. In addition, liver, thyroid gland, tissue masses and gross lesions of all animals from the 10 and 30 mg/kg/day groups were also examined.

Photo 1. Liver from a female rat showing midzonal hepatocellular vacuolation. The rat received 100 mg/kg/day of NS-21. H.E. staining. $\times 400$.

Photo 2. Liver from a female rat showing periportal hypertrophy. The rat received 100 mg/kg/day of NS-21. H.E. staining. $\times 400$.

Photo 3. Thyroid gland from a female rat showing C-cell hyperplasia. The rat received 10 mg/kg/day of NS-21. H.E. staining. $\times 200$.

Photo 4. Thyroid gland from a male rat showing follicular hyperplasia. The rat received 100 mg/kg/day of NS-21. H.E. staining. $\times 400$.

Photo 5. Thyroid gland from a male rat showing C-cell adenoma. The rat served as a control.
H.E. staining. $\times 400$.

Photo 6. Thyroid gland from a male rat showing follicular adenoma. The rat served as a control.
H.E. staining. $\times 400$.

Photo 7. Uterus from a female rat showing endometrial polyp. The rat served as a control.
H.E. staining. $\times 40$.

Table 3. Non-neoplastic histopathological findings of thyroid gland in rats given NS-21 for 2 years.

Sex		Male				Female			
Dose (mg/kg/day)		0	10	30	100	0	10	30	100
No. of animals		50	50	50	50	50	50	50	50
	Grade								
Follicular hyperplasia	+	2	0	0	2	0	0	0	1
	++	1	0	1	0	0	0	0	0
Cystic follicular hyperplasia	++	0	0	0	0	0	0	1	0
C-cell hyperplasia	+	16	18	21	19	16	19	25	17
	++	20	16	11	11	28	22	16	17
	+++	1	1	2	1	2	4	4	0
	++++	0	1	0	0	0	0	0	0
Ultimobranchial cyst	+	1	1	0	1	0	0	3	0
	++	0	0	1	2	1	1	0	0
	+++	1	0	0	0	0	0	1	0
Follicular cyst	+	1	0	1	1	0	3	0	0
	++	1	1	0	1	0	0	0	0
Mononuclear cell infiltration	+	1	0	0	0	1	0	1	0
	++	0	1	0	0	0	0	0	1
Inflammation	+	0	0	0	0	1	0	0	0
	++	0	0	0	0	0	0	0	0
Brown pigment deposition	+	9	3	2	11	2	0	0	2
	++	0	1	1	0	0	0	0	0

Grade of histopathological findings : +, trace; ++, mild; +++, moderate; +++, severe.

0.049), these increased incidences were not statistically significant when evaluated by the Cochran-Armitage trend test (p-value is 0.134). Table 3 presents the incidences of non-neoplastic lesions of the thyroid gland. There were no treatment-related increases in incidence of any non-neoplastic lesions including hyperplastic changes such as C-cell and follicular hyperplasia. In addition, there was an increased incidence of uterine endometrial polyps in the 100 mg/kg/day females (18 % and 26 % in the 0 and 100 mg/kg/day groups, respectively). This increased incidence was not statistically significant by Fisher's exact test or Cochran-Armitage trend test (p-values are 0.235 and 0.457, respectively).

The other commonly observed primary neoplasms observed in this study were mononuclear cell leukemia in the hemolymphoreticular system, benign pheochromocytoma in the adrenal gland, benign interstitial cell tumor in the testis, adenoma in the pituitary gland and fibroadenoma in the mammary gland. The occurrences of these types of neoplasms in the control and treated animals were within the ranges expected for this strain of rats.

In the liver, trace periportal hypertrophy and trace to mild midzonal hepatocellular vacuolization were observed with increased incidence in the 100 mg/kg/day males and females. Both the periportal hepatocellular hypertrophy and the midzonal hepatocellular vacuolization were more prominent in females than in males of the 100 mg/kg/day group. The hepatocellular vacuoles were discrete, single, often very large and nonstaining on hematoxylin and eosin sections, and were consistent with lipid vacuoles.

Other non-neoplastic microscopic observations seen were considered to be spontaneous and incidental, and usual for this strain and age of rats.

DISCUSSION

The only effects of the test article on in-life observations were decreased body weights and decreased food consumptions in all three treated groups of both sexes. However, there was no clear and uniform effect of the test article on mean food efficiency values in weeks 1-14. The plasma concentrations of NS-21 and its active metabolite,

RCC-36, in the treatment groups were increased in a dose-dependent manner.

Clinical hematology showed that males in the 30 and 100 mg/kg/day groups had statistically significantly higher mean erythrocyte counts and lower MCH (30 mg/kg/day group only) and MCHC. These differences were small in magnitude and were not considered to be associated with test article administration. These differences were considered to be due to a higher incidence of mononuclear cell leukemia (as identified on histopathologic evaluation) and resultant lower erythrocyte counts in the control group males at the terminal interval.

Histopathological examination revealed an increased incidence of thyroid C-cell adenomas in all treated females (4%, 16%, 8% and 16%, respectively, for the 0, 10, 30 and 100 mg/kg/day groups) and uterine endometrial polyps in the 100 mg/kg/day females (18% and 26% in the 0 and 100 mg/kg/day groups, respectively). Both of these neoplasms are common lesions in aging Fischer-344 rats. Although the incidences of these neoplasms in the treated groups are outside of the historical control ranges (C-cell adenoma; 0-14%, uterine endometrial polyp; 10-24%) for two year studies in Fischer-344 rats conducted at MPI, historical control data for Fischer-344 rats in National Toxicology Program (NTP) studies show incidences up to 34 % for C-cell adenomas and 37 % for uterine endometrial polyps (Haseman *et al.*, 1990). Additionally, the incidence of the thyroid tumor was not dose-related, there were no increased incidences of hyperplastic changes or no evidence of a trend toward malignancy for either tumor in any group, and the increased incidence of thyroid C-cell adenomas was not observed in males. Therefore, the increased numbers of these neoplasms were considered to be incidental, and a result of normal biologic variation, and not a direct effect of the administration of the test article.

The repeated-dose toxicity studies in rats (Nishiguchi *et al.*, 1997a; Okasaki *et al.*, 1997) revealed an increased incidence of stimulated thyroid follicles and hepatocellular hypertrophy caused by proliferation of smooth endoplasmic reticulum. The mechanism of these changes was considered to be induction of hepatic T₄UDP-GT

which resulted in a compensatory stimulation of thyroidal function by an increased secretion of pituitary TSH in response to increased blood thyroid hormone metabolism (Ishibashi *et al.*, 1997). However, there were no differences between the control and the treated animals receiving 500 mg/kg/day of NS-21 after 13 weeks in the labelling index of thyroid follicular cells using proliferation cell nuclear antigen (PCNA) immunocytochemistry (Ishibashi *et al.*, 1997). There was no increased incidence of thyroid follicular hyperplasia or tumors induced by NS-21 treatment in the present study. Thus, we have concluded that the degree of thyroid follicular cell proliferation induced by NS-21 is minimal.

Apart from thyroid C-cell adenomas and uterine endometrial polyps described above, various neoplasms were observed, among which the most common were those in the testis, pituitary gland, hemolymphoreticular system, mammary gland, skin including subcutis, and adrenal gland. The type and number of these neoplasms seen in this study are common in aged Fischer-344 rats (Coleman G. L. *et al.*, 1977; Goodman D. G. *et al.*, 1979; Haseman J. K., 1983; Haseman J. K. *et al.*, 1984; Jacobs B. B. and Huseby R. A., 1967; Maekawa A. *et al.*, 1983; Maita K. *et al.*, 1987; Sacksteder M. R., 1976; Sass B., *et al.*, 1975; Sher S. P., *et al.*, 1982; Tarone R. E. *et al.*, 1981). They were distributed throughout the dosage groups without an apparent relationship to the dose of NS-21 administered.

Histopathological examination of liver sections revealed test article-related trace periportal hyper-trophy and trace to mild midzonal hepatocellular vacuolization (morphologically compatible with lipid) in the 100 mg/kg/day males and females. Hepatocellular vacuoles (lipid vacuoles) in the 100 mg/kg/day group indicate that the 100 mg /kg/day of NS-21 was mildly hepatotoxic, because fatty change is commonly observed as a hepato-cellular response to toxicants (Gopinath C. *et al.*, 1987). Morphologically similar hepatocellular vacuoles, which stained positive for lipid, were observed in the companion 18-month mouse carcinogenicity study of this compound in B6C3F₁ mice (Hatch, R.C *et al.*, 1997).

In conclusion, under the conditions of this

study, no oncogenic effects were evident with dietary intake of NS-21 up to 100 mg/kg/day of NS-21 in male and female Fischer rats for 2 years.

REFERENCES

- Conover, W.J. and Iman, R. L. (1981) : Rank transformations as a bridge between parametric and nonparametric statistics. *The American Statistician*, 35, 124-133.
- Coleman, G.L., Barthold, S.W., Osbaldiston, G.W., Foster, S.J. and Jonas, A.M. (1977) : Pathological changes during aging in barrier-reared Fischer 344 male rats. *J. Gerontol.*, 32, 258-278.
- Dunnett, C.W. (1964) : New tables for multiple comparisons with a control. *Biometrics*, 20, 482-491.
- Gad, C.S. and Weil, S.C. (1988a) : Hypothesis testing : univariate parametric tests. *In Statistics and Experimental Design for Toxicologists* (Gad, C.S. and Weil, S.C. eds.), pp. 70-92, Telford Press, Caldwell, New Jersey.
- Gad, C.S. and Weil, S.C. (1988b) : Methods for data preparation and exploration *In Statistics and Experimental Design for Toxicologists* (Gad, C.S. and Weil, S.C. eds.), pp. 40-52, Telford Press, Caldwell, New Jersey.
- Goodman, D.G., Ward, J.M., Squire, R.A., Chu, K.C. and Linhart, M.S. (1979) : Neoplastic and non-neoplastic lesions in aging F344 rats. *Toxicol. Appl. Pharmacol.*, 48, 237-248.
- Gopinath, C., Prentice, D.E. and Lewis, D.J. (1987) : The liver. *In Atlas of Experimental Toxicological Pathology* (Gopinath, C., Prentice, D.E. and Lewis, D.J. eds.), pp. 43-60, MTP Press limited, Norwell.
- Haseman, J.K. (1983) : Patterns of tumor incidence in two-year cancer bioassay feeding studies in Fischer 344 rats. *Fundam. Appl. Toxicol.*, 3, 1-9.
- Haseman, J.K., Eustis, S.C. and Arnold, J. (1990) : Tumor incidences in control F344 rats from NTP carcinogenesis studies. *In Pathology of the Fischer Rat* (Boorman, G.A., Eustis, S.L., Elwell, M.R., Montgomery, C.A. and MacKenzie, W.F. eds.) pp.555-564, Academic Press Inc., San Diego, CA.

- Haseman, J.K., Huff, J. and Boorman, G.A. (1984) : Use of historical control data in carcinogenicity studies in rodents, *Toxicol. Pathol.*, 12, 126-135.
- Hatch, R.C., Regan, K.S., Ishibashi, S., Nakazawa, M., Iwakura, K. and Sumi N. (1997) : Seventy-eight week dietary carcinogenicity study of ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate (NS-21), a novel drug for urinary frequency and incontinence, in mice. *J. Toxicol. Sci.*, 22, Suppl. I, 275-287.
- Huff, J.E., McConnell, E.E., Haseman, J.K., Boorman, G.A., Eustis, S.L., Schwetz, B.A., Rao, G.N., Jameson, C.W., Hart, L.G. and Rall, D.P. (1988) : Carcinogenesis studies: results of 398 experiments on 104 chemicals from the U.S. National Toxicology Program. *In Annals of the New York Academy of Sciences* (Cesare Maltoni and Irving J. Selikoff, eds.), vol.534, pp.1-30, the New York Academy of Sciences, New York.
- Ishibashi, S., Nakazawa, M., Tawaratani, T., Yoshida, M., Tamura, H., Adachi, T., Kawaguchi, K., Iwakura, K., Sumi, N. and Wakabayashi, K. (1997) : The mechanism of increased thyroidal function caused by ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate (NS-21), a novel drug for urinary frequency and incontinence, administration in rats *J. Toxicol. Sci.*, 22, Suppl. I, 187-199.
- Jacobs, B.B. and Huseby, R.A. (1967) : Neoplasms occurring in aged Fischer rats, with special reference to testicular, uterine, and thyroid tumors. *J. Natl. Cancer Inst.*, 39, 303-309.
- Maekawa, A., Kurokawa, Y., Takahashi, M., Kokubo, T., Ogiu, T., Onodera, H., Tanigawa, H., Ohno, Y., Furukawa, F. and Hayashi, Y. (1983) : Spontaneous tumors in F-344/DuCrj rats. *Gann*, 74, 365-372.
- Maita, K., Hirano, M., Harada, T., Mitsumori, K., Yoshida, A., Takahashi, K., Nakashima, N., Kitazawa, T., Enomoto, A., Inui, K. and Shirasu Y. (1987) : Spontaneous tumors in F344/DuCrj rats from 12 control groups of chronic and oncogenicity studies. *J. Toxicol. Sci.*, 12, 111-126.
- Neter, J. and Wasserman, W. (1974) : Analysis of factory effects. *In Applied Linear Statistical Models*, pp.582-584, Richard C. Irwin, Inc., Homewood, Illinois.
- Nishiguchi, Y., Adachi, T., Nakazawa, M., Tawaratani, T., Uchimoto, H., Yoshida, M., Ishibashi, S., Kitayama, E., Iwakura, K. and Sumi, N. (1997a) : A 13-week oral toxicity study of ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate (NS-21), a novel drug for urinary frequency and incontinence, in rats followed by a 5-week recovery test. *J. Toxicol. Sci.*, 22, Suppl. I, 27-57.
- Nishiguchi, Y., Yoshida, M., Tarui, M., Nakazawa, M., Oka, T., Iwakura, K. and Sumi, N. (1997b) : Single-dose toxicity studies of ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate (NS-21), a novel drug for urinary frequency and incontinence, in mice, rats and dogs. *J. Toxicol. Sci.*, 22, Suppl. I, 1-13.
- Okasaki, K., Baba, S., Ikeda, H., Chihaya, Y., Satake, S., Nagata, R., Ishibashi, S., Iwakura, K. and Sumi N. (1997) : A 26-week oral toxicity study of ($\pm$)-4-diethylamino-1,1-dimethylbut-2-yn-1-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate monohydrochloride monohydrate (NS-21), a novel drug for urinary frequency and incontinence, in rats followed by a 9-week recovery test. *J. Toxicol. Sci.*, 22, Suppl. I, 59-92.
- Sacksteder, M.R. (1976) : Occurrence of spontaneous tumors in the germfree F344 rat. *J. Natl. Cancer Inst.*, 57, 1371-1373.
- Sass, B., Rabstein, L.S., Madison, R., Nims, R.M., Peters, R.L. and Kelloff, G.J. (1975) : Incidence of spontaneous neoplasms in F344 rats throughout the natural life-span. *J. Natl. Cancer Inst.*, 54, 1449-1456.
- Sher, S.P., Jensen, R.D. and Bokelman, D.L. (1982) : Spontaneous tumors in control F344 and Charles River-CD rats and Charles River CD-1 and B6C3HF₁ mice. *Toxicol. Lett.*, 11, 103-110.

Steel, R.G.D. and Torrie, J.H. (1980) : Multiple comparisons. *In* Principles and Procedures of Statistics (Steel, R.G.D. and Torrie, J.H., eds) 2nd ed, pp.172-194, McGraw-Hill book company, Inc., New York.

Tarone, R.E., Chu, K.C. and Ward, J.M. (1981) : Variability in the rates of some common naturally occurring tumors in Fischer 344 rats and (C57BL/6N $\times$ C3H/HeN)F₁(B6C3F₁) mice. *J. Natl. Cancer Inst.*, 66, 1175-1181.