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Thirteen-week oral toxicity study of difluoromethylornithine in combination with tamoxifen citrate in female dogs

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Abstract *Purpose*: Cancer chemoprevention is the use of pharmacologic or natural agents to inhibit the development of cancer. Difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase, the rate-limiting enzyme in the biosynthesis of polyamines. DFMO has demonstrated chemopreventive efficacy in animal models of tumorigenesis. Tamoxifen (TAM), a nonsteroidal antiestrogen, is approved for use in the treatment of estrogen receptor-positive breast carcinoma and has demonstrated efficacy in chemoprevention of breast cancer in women at high risk for the disease. The administration of TAM with DFMO is being considered for development by the National Cancer Institute as a potential drug regimen for the chemoprevention of breast carcinoma. *Methods*: The toxicity of DFMO in combination with TAM was evaluated in female Beagle dogs following 13 weeks of daily oral administration by capsule. Dose levels in milligrams per kilogram body weight per day were: 0 (vehicle control), 100 DFMO, 0.1 TAM, 1.0 TAM, 0.1 TAM + 100 DFMO and 1.0 TAM + 100 DFMO. *Results*: No mortalities occurred. Diarrhea was produced by TAM and vaginal discharge, due to reproductive tract lesions, was produced by both DFMO and TAM, either alone or in combination. DFMO decreased reticulocyte counts and TAM increased counts of mature neutrophils. DFMO alone resulted in lesions to the intestines and ovaries, and cornified epithelium of vagina and cervix. TAM produced cornified epithelium of vagina and cervix, and numerous lesions in the ovaries, fallopian tube, uterus, cervix and vagina which were likely due to an estrogen agonist effect. Coadministration of DFMO increased the incidence and/or severity of these reproductive tract lesions. Each compound alone produced ovarian atrophy, and antral follicles and corpora lutea were completely absent in the 1.0 TAM + 100 DFMO group. *Conclusions*: Coadministration of DFMO and TAM resulted in additive toxicity involving the female reproductive system.

Key words Tamoxifen · Difluoromethylornithine · Dogs · Reproductive system toxicity · GI toxicity

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Introduction

Cancer chemoprevention is the use of pharmacologic or natural agents to inhibit the development of cancer by either inhibiting the initial phases of carcinogenesis or arresting the process of neoplastic growth and development [12, 14]. The Chemoprevention Program of the National Cancer Institute, NIH, is organized as a drug development program focusing on chemoprevention of cancer. To date, this program has identified various naturally occurring and synthetic compounds as well as micronutrients that may prevent or delay neoplastic progression in humans, particularly those populations at high risk [14]. Currently, chemopreventive drugs are under development with mechanisms of action that include, for example, antioxidant activity, induction of drug metabolizing enzymes, the reversal of abnormal differentiation of neoplastic cells, the suppression of

cellular replication, and the induction of apoptosis in neoplastic cells [12, 15].

2-Difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase [8]. Ornithine decarboxylase is the rate-limiting enzyme in the biosynthesis of polyamines (putrescine, spermidine and spermine), which are small cationic molecules that are required for cell proliferation and differentiation [2]. Polyamine synthesis increases during the G1 phase of the cell cycle [23]. Intracellular levels of polyamines and ornithine decarboxylase activity have been observed to be elevated in neoplastic cells or cells undergoing neoplastic transformation [22]. DFMO is decarboxylated by ornithine decarboxylase, resulting in a reactive intermediate that alkylates and inactivates the enzyme [21]. DFMO can induce a depletion of intracellular pools of polyamines, inhibit cellular proliferation and elicit apoptosis [9, 17, 23]. DFMO is currently approved for use in the treatment of African sleeping sickness caused by *Trypanosoma brucei gambiense* [17], and has demonstrated chemopreventive efficacy in various animal models of tumorigenesis which include mouse skin, colon and bladder, and rat colon, liver, stomach, mammary gland and bladder [8, 19, 22]. Recent studies have indicated that DFMO may also be a potential antitumor agent. DFMO has been shown to induce significant regression of epidermal squamous papillomas in a murine model, presumably due to inhibition of polyamine synthesis [20]. DFMO is currently under investigation for safety and chemopreventive efficacy in clinical trials [4, 18]. In a phase I dose de-escalation trial in patients with grade 3 cervical intraepithelial neoplasia, DFMO was well tolerated at doses up to 1 g/m^2 per day for 31 days (adverse effects included nausea, diarrhea, stomatitis and dizziness), and regression of neoplastic lesions was seen at doses of $\leq$250 mg/m^2 per day [18].

Tamoxifen is a nonsteroidal antiestrogen that is widely prescribed for the treatment of breast cancer. Tamoxifen is the initial hormonal treatment of choice in both pre- and postmenopausal women with estrogen receptor-positive breast carcinoma [13] and is believed to produce its antitumor effect by inhibiting estrogen-induced cellular proliferation of neoplastic cells [13]. Tamoxifen exhibits complex pharmacological actions, acting as an estrogen antagonist, or partial or full agonist, depending on the target tissue or species studied [6]. Tamoxifen has recently demonstrated efficacy in chemoprevention of breast cancer. In the Breast Cancer Prevention Trial (conducted during the period 1992–1998), daily administration of 20 mg tamoxifen in women with a high risk of breast cancer resulted in a 45% reduction of invasive breast carcinoma [10, 24].

The administration of tamoxifen with DFMO is being considered by the National Cancer Institute for development as a potential drug regimen for the chemoprevention of breast carcinoma. In a rat model of mammary carcinogenesis, DFMO in combination with tamoxifen reduced tumor incidence and increased the latency period for tumor development [25]. The current study was conducted to determine whether the toxicity of tamoxifen citrate in combination with DFMO was additive, synergistic, or abrogated in female dogs following 13 weeks of daily oral administration by capsule, and was conducted in accordance with the Food and Drug Administration Good Laboratory Practices Regulations for Nonclinical Laboratory Studies. Oral administration of DFMO with tamoxifen citrate is the intended clinical route for these drugs.

Materials and methods

Drugs

DFMO was received from McKesson Bioservices, Rockville, Md., and was stored in an amber bottle at ambient temperature and humidity. Tamoxifen citrate (mole fraction 0.66), was also received from McKesson Bioservices, and was stored at ambient temperature and humidity. Tamoxifen was initially identified by gas chromatography-mass spectrometry and DFMO was initially identified by solid probe-mass spectrometry. The purities of both drugs were determined by high performance liquid chromatography prior to the initiation of dosing and after the completion of dosing. The initial purity of DFMO was 97.5 $\pm$ 0.63% and the final purity was 97.0 $\pm$ 1.31%. The initial purity of tamoxifen citrate was 99.8 $\pm$ 0.01% and the final purity was 99.8 $\pm$ 0.03%. Thus, both compounds were stable under the storage conditions used in this study.

Animals

Twenty eight female Beagle dogs were obtained from Marshall Farms, North Rose, NY. The animals were approximately 6–7 months old upon arrival at the University of Illinois at Chicago AAALAC Intl.-accredited animal facility. Each animal was given a facility-unique animal number upon arrival. This number was coded on a subcutaneously implanted microchip. All dogs were housed in a temperature-controlled (65–84°F) and humidity-controlled (50 $\pm$ 20%) room with a 12-h light/12-h dark cycle. They were housed one or two per run during the quarantine period, and were singly housed following randomization into treatment groups. The run size, at least 15 $feet^2$, (3 feet $\times$ 5 feet) was adequate to house dogs at the upper weight range as described in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. All runs were cleaned and fresh bedding was provided daily. The runs were sanitized once every 2 weeks. General procedures for animal care and housing were in accordance with the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996, and the study was approved by the University of Illinois at Chicago Animal Care Committee.

Certified Canine Diet No. 5007 (PMI Feeds, St. Louis, M), approximately 400 g, was provided daily from arrival until termination. Exactly 400 g were given when food consumption was measured. The food was removed for an overnight fast (about 16–20 h) prior to blood collection for clinical pathology measurements and/or scheduled sacrifice. Tap-water from an automatic watering system in which the room distribution lines were flushed daily was provided *ad libitum* from arrival until termination. There were no known contaminants in the feed or water which could have been expected to influence the study.

The animals were quarantined for 3 weeks. During that time, the animals were observed daily for signs of illness. Body weights and physical examinations were done upon arrival at the animal facility. Each dog was lightly treated upon arrival for fleas, lice, and ticks. All dogs had been previously vaccinated by the animal supplier against canine distemper, infectious canine hepatitis, oral

papilloma, leptospirosis, parainfluenza, parvo and rabies. Blood samples were collected within a few days of arrival for quarantine clinical chemistry and hematology tests, and fecal samples were collected for internal parasite examinations. Animals were examined during quarantine and approved for use by a clinical veterinarian prior to being utilized for the study.

Experimental design

Near the end of the quarantine period, 24 animals were selected for the study on the basis of quarantine data which included body weights, food consumption, clinical pathology measurements, electrocardiograph data, and ophthalmology examinations. These animals were randomized into six groups of four dogs per dose group using a restricted randomization procedure stratified on the basis of body weight. No litter mates were included in the same dose group. Dose levels in the study were 0 mg/kg body weight per day (vehicle control), 100 mg DFMO per kg body weight per day, 0.1 mg tamoxifen per kg body weight per day, 1.0 mg tamoxifen per kg body weight per day, 0.1 mg tamoxifen plus 100 mg DFMO per kg body weight per day and 1.0 mg tamoxifen plus 100 mg DFMO per kg body weight per day. Tamoxifen dose levels are expressed as the free base, although tamoxifen citrate was given. Tamoxifen is typically given clinically in 20-mg doses. This is equivalent to 0.3 mg/kg body weight for a 60-kg woman or approximately 12 mg/m^2 [7]. The doses of tamoxifen used in the current study of 0.1 and 1.0 mg/kg body weight/day are approximately equivalent to 2 and 20 mg/m^2 per day, respectively, and bracket the clinical dose. DFMO is currently being investigated in clinical trials at doses up to 500 mg/m^2 per day or approximately 14 mg/kg body weight per day [18]. The DFMO dose of 100 mg/kg body weight per day used in this study is approximately equivalent to 2000 mg/m^2 per day, which is greater than the dose level being examined in the clinic. Therefore, the study was designed to characterize how a high dose of DFMO could effect the toxicity of clinical doses of tamoxifen.

The neat drugs were administered once daily in separate gelatin capsules for 13 weeks. Tamoxifen was administered in a size 000 gelatin capsule (Torpac, Fairfield, NJ) and DFMO in a size 13 gelatin capsule (Torpac). The specific quantity in each capsule was adjusted for the purity of the drugs, mole fraction of tamoxifen, and each animal's most recent weekly body weight. Day 1 was the first day of dosing. Control animals received the vehicle (two empty gelatin capsules). All animals received empty gelatin capsules for 3 days prior to the first day of dosing in order to acclimate the animals to the dosing procedure. The animals were dosed up to and including the day prior to scheduled necropsy (at least 91 days). The animals weighed 5.5–8.1 kg on day −3 (the most recent body weight prior to dosing), and were approximately 7–8 months old at initiation of treatment.

Measurements

Non-fasted body weights were recorded at randomization in week −1, weekly thereafter, and at termination. All animals were observed once daily for clinical signs of toxicity about 1–2 h after dosing. Additionally, the animals were observed immediately prior to dosing in the morning and in the afternoon, at least 6 h apart, for moribundity/mortality. Physical examinations which included examination of eyes and all orifices were conducted during the quarantine period and once weekly commencing in week −1. Food consumption was measured over a 24-h period twice in the quarantine period and once weekly after initiation of treatment.

Ophthalmology examinations were performed during the quarantine period and in week 13, and included examination of the cornea, iris, lens, fundus, and anterior and posterior chambers. Electrocardiographic examinations were conducted during the quarantine period and approximately 2 h after dosing in week 13 using a Vetronics Computerized ECG Analyzer (Vetronics, Lafayette, Md.). Recordings were taken from leads I, II, III, aV_F, aV_L and aV_R over an approximate 10-min period. Analysis included heart rate and duration of the P wave, and PR, QRS and QT intervals. The animals were neither sedated nor trained for the electrocardiographic measurements. Hematology (complete blood count with differential), coagulation, clinical chemistry and urinalysis parameters were measured in weeks −3 (except urinalysis), −1, 4 and 13. Sufficient blood was collected from the jugular vein of unanesthetized, overnight-fasted animals (water was available *ad libitum* during all fasting periods) for clinical pathology measurements. The following clinical chemistry parameters were measured: alanine aminotransferase, albumin, albumin/globulin ratio, alkaline phosphatase, aspartate aminotransferase, urea nitrogen (BUN), creatinine, BUN/creatinine ratio, calcium, chloride, cholesterol, glucose, inorganic phosphate, potassium, sodium, total bilirubin, total protein, and triglycerides. The following coagulation parameters were measured: activated partial thromboplastin time, fibrinogen and prothrombin time. Urinalysis parameters were measured from samples collected overnight (about 16–20 h) in a metabolism cage. The animals did not receive food during the overnight urine collection, but water was available.

Pathology

All animals were killed and necropsied in random order on study days 92 and 93. Euthanasia was accomplished by sodium pentobarbital anesthesia (approximately 20–30 mg/kg, i.v.) and exsanguination. Terminal body weights were collected prior to routine sacrifice. The necropsy procedure was a thorough and systematic examination and dissection of the animal viscera and carcass which included the external surface, all orifices, the cranial cavity, external surface of the brain, cross section of the spinal cord, the nasal cavity and nasal turbinates, thoracic, abdominal and pelvic cavities and their viscera, and cervical tissues and organs, and was performed under the direction and supervision of a veterinary pathologist. More than 40 tissues/organs were collected for histopathology evaluations. Tissues, organs and gross lesions were collected and fixed in 10% neutral buffered formalin, with the exception of the eyes, which were fixed in 3% glutaraldehyde. Organ weights were determined (paired organs were weighed as a unit) and expressed as percentage of brain weight. A bone marrow smear was prepared from the rib. Histopathologic observations were made on all collected tissues in the control and in the 1.0 mg tamoxifen plus 100 mg DFMO per kg per day dose groups. Tissues from organs found to be normal in the 1.0 mg tamoxifen plus 100 mg DFMO per kg per day dose group were not examined histologically in the other dose groups. However, the following tissues were found to be abnormal and were subsequently examined in all remaining dose groups: vagina, cervix, ovaries, fallopian tube, uterus, duodenum, jejunum and cecum. All gross lesions were examined histologically.

Statistical analyses

Analysis of variance tests were conducted on body weight, electrocardiographic measurements, hematology, clinical chemistry, coagulation, urinary pH and specific gravity, and organ weight data. Organ weight analysis considered weights relative to brain weight (% brain weight). If a significant F ratio was obtained ($P \leq 0.05$), Dunnett's t-test was used for pair–wise comparisons with the concurrent control group. Food consumption data were analyzed by the Kruskal-Wallis test ($P \leq 0.05$). If a significant effect was seen, the Mann-Whitney U-test was used for pair-wise comparisons with the concurrent control group.

Results

Mortality and clinical signs

No mortalities occurred in the study. Diarrhea was observed in all dose groups receiving tamoxifen and was most frequent in the combination low-dose group (0.1 mg tamoxifen plus 100 mg DFMO per kg per day).

Vaginal discharge/secretion, first observed on day 53, was seen in all drug-treated dose groups but was most prevalent in the tamoxifen plus DFMO dose groups. Excess eye secretions were seen periodically in dose groups receiving DFMO, and excess salivation was seen periodically in the DFMO-alone, tamoxifen-alone, and combination low-dose groups. Emesis was observed infrequently in the control, DFMO-alone and combination low-dose groups.

Body weights and food consumption

Failure to gain weight occurred in the DFMO-alone dose group and minimal weight gain occurred in the combination high-dose group (1.0 mg tamoxifen plus 100 mg DFMO per kg per day), although the changes were not statistically significant (Table 1). Body weight changes in the other dose groups were not seen. Food consumption in the drug-treated dose groups was generally decreased in comparison with the control group, although statistically significant differences were not seen (data not shown).

Clinical pathology, cardiology and ophthalmology

In week 4, total protein was slightly increased in the tamoxifen high-dose group (1.0 mg tamoxifen per kg per day; Table 2). No other changes in clinical chemistry parameters were considered to be treatment-related. Several hematology parameters were affected by treatment (Table 2). In week 4, reticulocyte counts were decreased in all dose groups and the decrease was significant in the dose groups receiving DFMO. In week 13, reticulocyte counts were decreased in all but the tamoxifen high-dose group and the decrease was significant in the combination low-dose group. Increased leukocyte counts were seen in week 4 in the tamoxifen low- and high-dose, and combination high-dose groups. In week 13, the effect persisted in all tamoxifen-treated groups and was significant in the combination high-dose group. The increased leukocyte counts were due to increases in mature neutrophil counts. One animal each in the tamoxifen low- and high-dose groups, and two animals in the combination high-dose group had increased numbers of leukocytes in urine specimens collected in week 13 (data not shown). No other changes in clinical pathology parameters were considered to be treatment-related. Sporadic increases and decreases were seen but were not considered to be biologically significant. No treatment-related cardiology or ophthalmology changes were seen.

Organ weights

The weights of the ovaries were decreased in all drug-treated dose groups in comparison with the control

Table 1 Effect of drug administration on body weights (kg). Values are the means ± standard deviation of the mean. The differences in body weights were not statistically significant. Body weight measurements from days 5–82 are not presented for brevity

	DFMO: tamoxifen (mg/kg/day)					
	0:0	100:0	0:0.1	0:1.0	100:0.1	100:1.0
Day −3	6.9 ± 0.9	6.8 ± 0.7	6.8 ± 0.5	6.7 ± 0.7	6.6 ± 0.6	6.5 ± 0.7
Day 89	7.6 ± 1.0	6.4 ± 0.9	7.9 ± 0.8	7.6 ± 0.9	7.1 ± 0.7	6.6 ± 1.0
Total weight gain	0.7 ± 0.7	−0.4 ± 0.5	1.1 ± 0.4	0.9 ± 0.7	0.5 ± 0.2	0.1 ± 0.6

Table 2 Select clinical pathology parameters. Values are the means ± standard deviation of the mean

	DFMO: tamoxifen (mg/kg/day)					
	0:0	100:0	0:0.1	0:1.0	100:0.1	100:1.0
Total protein (g/dl)						
Week −1	6.1 ± 0.3	6.1 ± 0.3	6.1 ± 0.2	6.5 ± 0.3	6.4 ± 0.2	6.1 ± 0.2
Week 4	5.6 ± 0.4	5.8 ± 0.2	6.0 ± 0.1	6.4 ± 0.1*	6.0 ± 0.2	5.8 ± 0.5
Week 13	6.2 ± 0.4	6.1 ± 0.3	6.1 ± 0.6	6.7 ± 0.2	6.3 ± 0.2	6.3 ± 0.3
Reticulocytes (% red blood cells)						
Week −1	0.58 ± 0.2	0.63 ± 0.3	0.63 ± 0.2	0.65 ± 0.2	0.40 ± 0.2	0.48 ± 0.3
Week 4	0.78 ± 0.5	0.25 ± 0.2*	0.30 ± 0.2	0.30 ± 0.1	0.23 ± 0.1*	0.13 ± 0.2*
Week 13	0.93 ± 0.4	0.40 ± 0.2	0.50 ± 0.3	0.83 ± 0.4	0.28 ± 0.2*	0.40 ± 0.1
Leukocytes (10^3/μl)						
Week −1	9.3 ± 1.2	8.1 ± 1.3	9.0 ± 0.3	7.5 ± 0.6	8.3 ± 1.9	7.5 ± 0.7
Week 4	8.9 ± 1.4	8.8 ± 2.0	12.0 ± 1.7*	12.3 ± 0.4*	10.9 ± 1.8	12.6 ± 1.0*
Week 13	10.3 ± 1.9	7.7 ± 1.6	12.4 ± 1.1	12.8 ± 1.7	12.0 ± 0.7	14.5 ± 2.8*
Neutrophils (10^3/μl)						
Week −1	6.3 ± 1.2	5.4 ± 0.9	6.0 ± 0.8	4.7 ± 1.0	5.8 ± 2.1	4.2 ± 0.6
Week 4	5.7 ± 1.0	6.2 ± 1.5	9.5 ± 2.5*	9.3 ± 0.5*	7.6 ± 1.4	8.6 ± 1.4
Week 13	6.1 ± 1.5	4.7 ± 1.5	8.3 ± 1.0	8.6 ± 2.7	8.7 ± 1.2	9.4 ± 2.6

* $P < 0.05$ vs dose group (0:0)

Table 3 Treatment-related changes in organ weights (% brain weight). Values are the means ± standard deviation of the mean

	DFMO: tamoxifen (mg/kg/day)					
	0:0	100:0	0:0.1	0:1.0	100:0.1	100:1.0
Ovaries	1.60 ± 0.46	0.94 ± 0.22*	0.67 ± 0.28*	0.76 ± 0.25*	0.83 ± 0.28*	0.62 ± 0.09*
Uterus	12.9 ± 9.1	4.5 ± 2.8	31.4 ± 19.7	26.0 ± 17.3	20.7 ± 4.3	26.2 ± 14.9
Vagina	25.6 ± 11.1	21.2 ± 5.9	37.6 ± 7.7	38.9 ± 6.3	38.6 ± 5.3	32.9 ± 15.1

* $P < 0.05$ vs 0:0 dose group (ANOVA followed by Dunnett's *t*-test)

group (Table 3). Ovarian weight was decreased to the greatest extent in the combination high-dose group. Both the uterus and vagina had increased weights in dose groups receiving tamoxifen in comparison with the controls, although the differences were not statistically significant (Table 3). The increases in uterine and vaginal weights did not occur in a dose-dependent fashion.

Pathology

During necropsy, a clear uterine wall was seen in one animal in the tamoxifen high-dose group and in one animal in the combination high-dose group. Clear uterine wall correlated with a microscopic diagnosis of edema. Green fluid present in the lumen of the uterus and vagina of one animal in the tamoxifen low-dose group was consistent with acute inflammation and epithelial necrosis observed in the uterus.

Histologic evaluation showed that the duodenum, jejunum, cecum, ovary, vagina, cervix, fallopian tube and uterus were target organs of toxicity (Tables 4 and 5). Microabscesses were observed in the crypts of the duodenum, jejunum and cecum in animals from all DFMO dose groups (Table 4). These microabscesses were characterized by the presence of focally dilated crypts that were filled with cell debris and neutrophils (Fig. 1).

Ovarian atrophy was present in all drug-treated animals and was associated with the reduced presence of corpora lutea and/or antral follicles (Table 5). Ovaries from animals in the combination high-dose group were completely devoid of corpora lutea and antral follicles (Fig. 2B). Both primordial and growing follicles were present in all animals. Serosal hyperplasia was present in ovaries from animals receiving tamoxifen (either alone or with DFMO), with increased incidence and severity in the combination low-dose group compared with the tamoxifen low-dose group. Serosal hyperplasia was characterized by the presence of papillae of thickened serosal epithelium on the surface of the ovary (Fig. 2B). Oocyte mineralization was produced by both DFMO and tamoxifen alone, with increased incidence in the combination low- and high-dose groups. Mineralization was characterized by the presence of a focus of deeply basophilic granular material in the central region of an ovarian follicle where an oocyte is normally located. A luteal cyst observed in one animal in the combination low-dose group was characterized by the presence of an empty space in the ovary surrounded by a rim of luteal cells.

Cornified epithelium of the vagina was seen in one animal receiving DFMO alone and in all animals receiving tamoxifen, either alone or with DFMO (Table 5). Cornified epithelium of the cervix was seen in two animals receiving DFMO, and in all animals receiving tamoxifen, either alone or with DFMO (Table 5). Edema of the vagina and cervix was seen in animals receiving tamoxifen (either alone or with DFMO), with increased incidence in the combination low-dose group compared with the tamoxifen low-dose group. Edema was characterized by thickened vaginal or cervical walls with increased amounts of clear space in fascial planes and around muscle bundles. Subacute inflammation of the vagina, due to focal inflammation of subepithelial stroma with lymphocytes, plasma cells and a few neutrophils, was seen in all dose groups receiving tamoxifen (either alone or with DFMO), with increased incidence in the combination dose groups.

Both edema and dilatation of the fallopian tube were present in animals receiving tamoxifen (either alone or with DFMO), with the incidence and severity of the lesions dependent upon the dose level of tamoxifen (Table 5). The tamoxifen-induced changes in the fallopian tubes were not modified by DFMO coadministration.

Numerous lesions were observed in the uterus of animals receiving tamoxifen, either alone or in combination with DFMO (Table 5, Fig. 3B). Lumen dilata-

Table 4 Summary of treatment-related intestinal lesions. Values are incidence and values in parentheses are mean group severity scores (1 = minimal, 2 = mild, 3 = moderate, 4 = marked)

Organ	Observation	DFMO:tamoxifen (mg/kg/day)					
		0:0	100:0	0:0.1	0:1.0	100:0.1	100:1.0
Small intestine, duodenum	Microabscess, crypt	1/4 (0.25)	4/4 (2.00)	1/4 (0.25)	0/4	4/4 (1.50)	4/4 (1.75)
Small intestine, jejunum	Microabscess, crypt	0/4	4/4 (1.25)	0/4	0/4	2/4 (1.00)	3/4 (1.00)
Large intestine, cecum	Microabscess, crypt	1/4 (0.25)	2/4 (0.75)	0/4	0/4	2/4 (0.50)	4/4 (1.25)

Table 5 Summary of treatment-related reproductive system lesions. Values are incidence and values in parentheses are mean group severity score (1 = minimal, 2 = mild, 3 = moderate, 4 = marked)

Organ	Observation	DFMO: tamoxifen (mg/kg/day)					
		0:0	100:0	0:0.1	0:1.0	100:0.1	100:1.0
Vagina							
	Cornified epithelium	0/4	1/4	4/4	4/4	4/4	4/4
	Edema	0/4	0/4	2/4 (1.25)	4/4 (1.50)	4/4 (1.50)	4/4 (1.50)
	Inflammation, subacute	0/4	0/4	1/4 (0.25)	1/4 (0.50)	2/4 (0.50)	2/4 (0.75)
Cervix							
	Cornified epithelium	0/4	2/4	4/4	4/4	4/4	4/4
	Edema	0/4	0/4	3/4 (1.00)	4/4 (2.50)	4/4 (2.75)	4/4 (2.25)
Ovary							
	Corpora lutea present	4/4	2/4	1/4	0/4	1/4	0/4
	Growing follicles present	4/4	4/4	4/4	4/4	4/4	4/4
	Primordial follicles present	4/4	4/4	4/4	4/4	4/4	4/4
	Antral follicles present	4/4	4/4	3/4	2/4	3/4	0/4
	Atrophy	0/4	4/4 (1.75)	4/4 (3.75)	4.4 (4.00)	4/4 (3.75)	4/4 (4.00)
	Hyperplasia, serosa	0/4	0/4	2/4 (1.00)	4/4 (1.75)	3/4 (2.00)	4/4 (2.25)
	Mineralization, oocyte	0/4	2/4 (0.50)	1/4 (0.25)	0/4	3/4 (1.00)	2/4 (0.50)
	Cyst, luteal	0/4	0/4	0/4	0/4	1/4 (0.75)	0/4
Fallopian tube							
	Dilatation	0/4	0/4	3/4 (1.50)	4/4 (1.75)	2/4 (1.25)	4/4 (2.00)
	Edema	0/4	0/4	3/4 (1.75)	4/4 (3.25)	3/4 (2.25)	4/4 (3.00)
Uterus							
	Dilatation, lumen	1/4 (0.50)	0/4	2/4 (1.75)	1/4 (0.75)	1/4 (0.25)	4/4 (1.75)
	Edema	0/4	0/4	4/4 (2.25)	4/4 (3.50)	4/4 (2.75)	4/4 (4.00)
	Metaplasia, squamous	0/4	0/4	1/4 (0.50)	3/4 (2.25)	0/4	4/4 (2.75)
	Inflammation, acute	0/4	0/4	2/4 (1.50)	2/4 (1.25)	1/4 (0.25)	4/4 (2.25)
	Necrosis, epithelium	0/4	0/4	2/4 (1.25)	1/4 (0.25)	2/4 (0.75)	3/4 (1.50)
	Ulceration	0/4	0/4	2/4 (1.50)	1/4 (0.75)	0/4	1/4 (0.75)
	Dilatation, gland	0/4	0/4	3/4 (0.75)	4/4 (2.00)	4/4 (1.75)	4/4 (2.50)

tion, due to increased lumenal diameter, was considered to be treatment-related in the combination high-dose group. Edema was seen in dose groups receiving tamoxifen (either alone or with DFMO), with increased severity in the combination dose groups compared with tamoxifen alone. Squamous metaplasia was seen in the

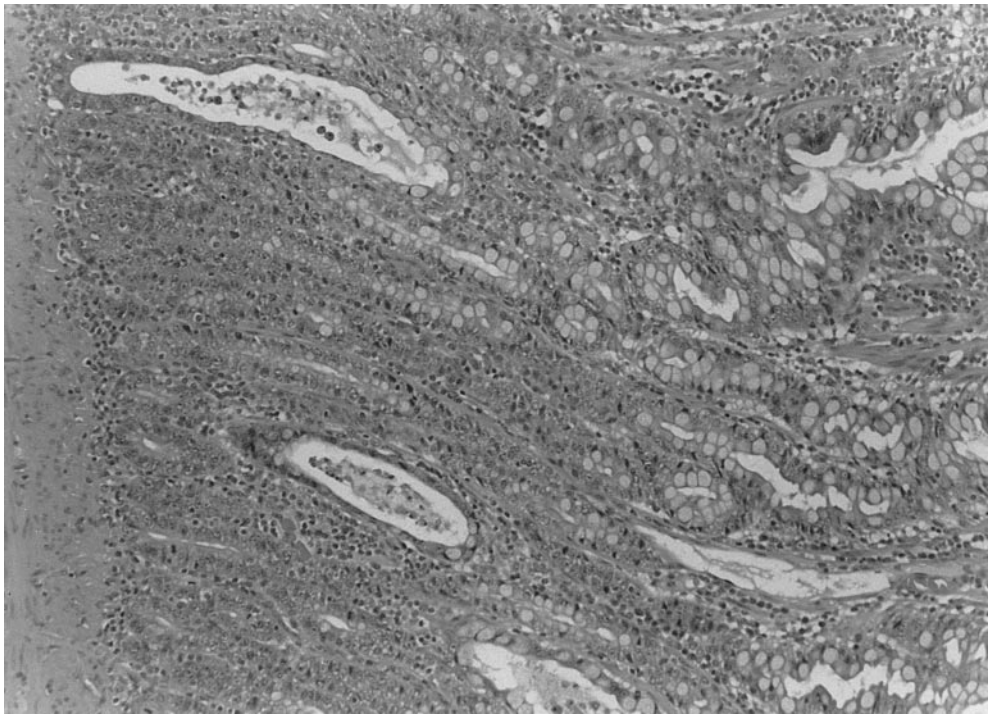

Fig. 1 Jejunum from an animal in the combination high-dose group with focal crypt micro-abscesses containing cell debris and neutrophils. (× = 165)

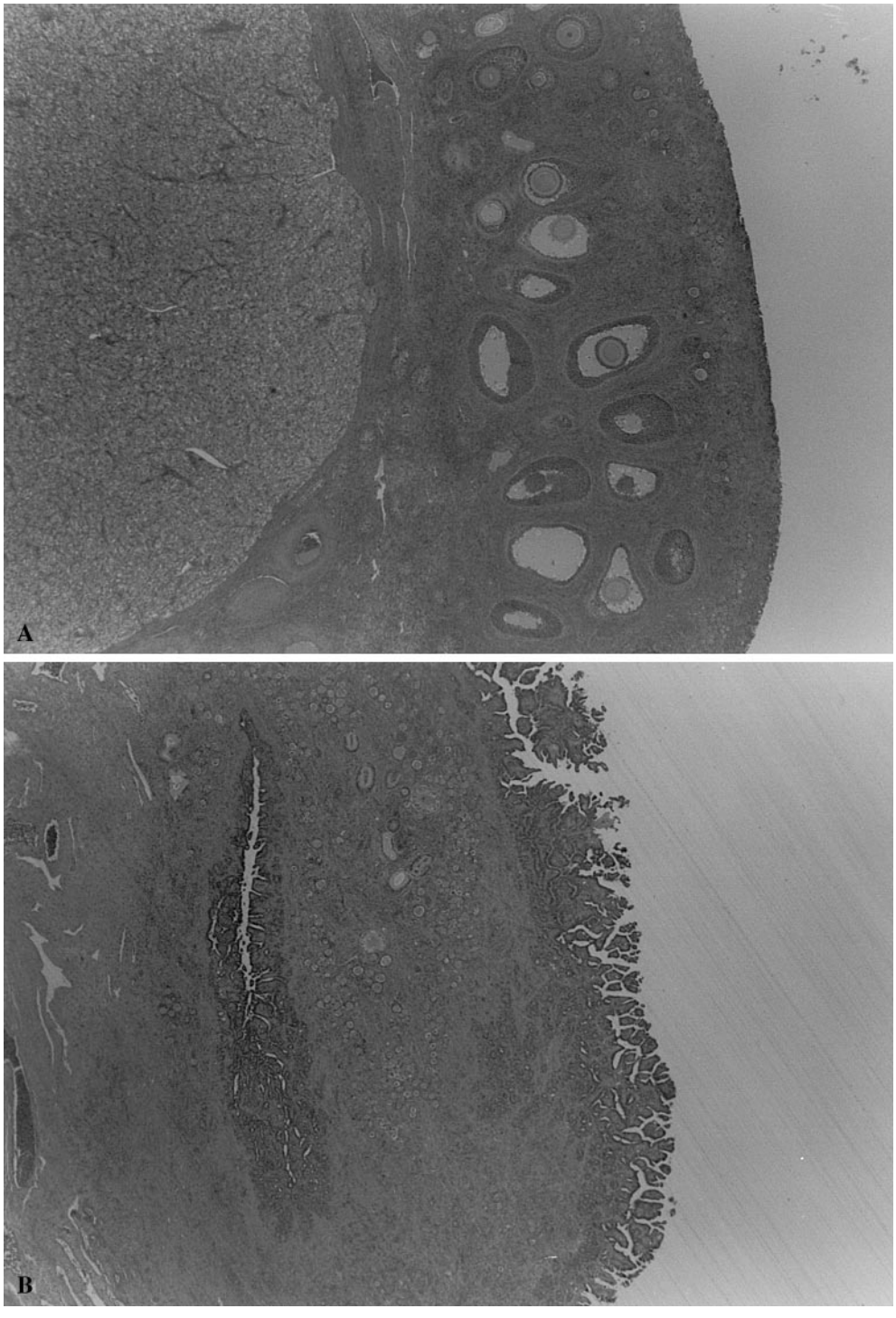

Fig. 2 A Ovary from a control animal. Follicles in various stages of development along with corpora lutea are present. **B** Ovary from an animal in the combination high-dose group. Ovarian atrophy and serosal hyperplasia are present ($\times = 33$)

tamoxifen-alone and combination high-dose groups, with increased incidence in the combination high-dose group. Squamous metaplasia was characterized by the presence of stratified squamous epithelium on the lumenal surface of the uterus and lining of the lumenal surface of uterine glands. Acute inflammation was seen in dose groups receiving tamoxifen (either alone or with DFMO), with increased incidence in the combination high-dose group compared with the tamoxifen high-dose group. Acute inflammation was characterized by the presence of neutrophils in the uterine lumen and uterine mucosal surface epithelium. Epithelial necrosis of the uterus was seen in dose groups receiving tamoxifen (either alone or with DFMO), with increased incidence and severity in the combination high-dose group compared with the tamoxifen high-dose group. Epithelial necrosis was characterized by the presence of cell debris in and around the mucosal surface epithelium. Ulceration of the uterus was seen in the tamoxifen-alone and combination high-dose groups, and was diagnosed when the

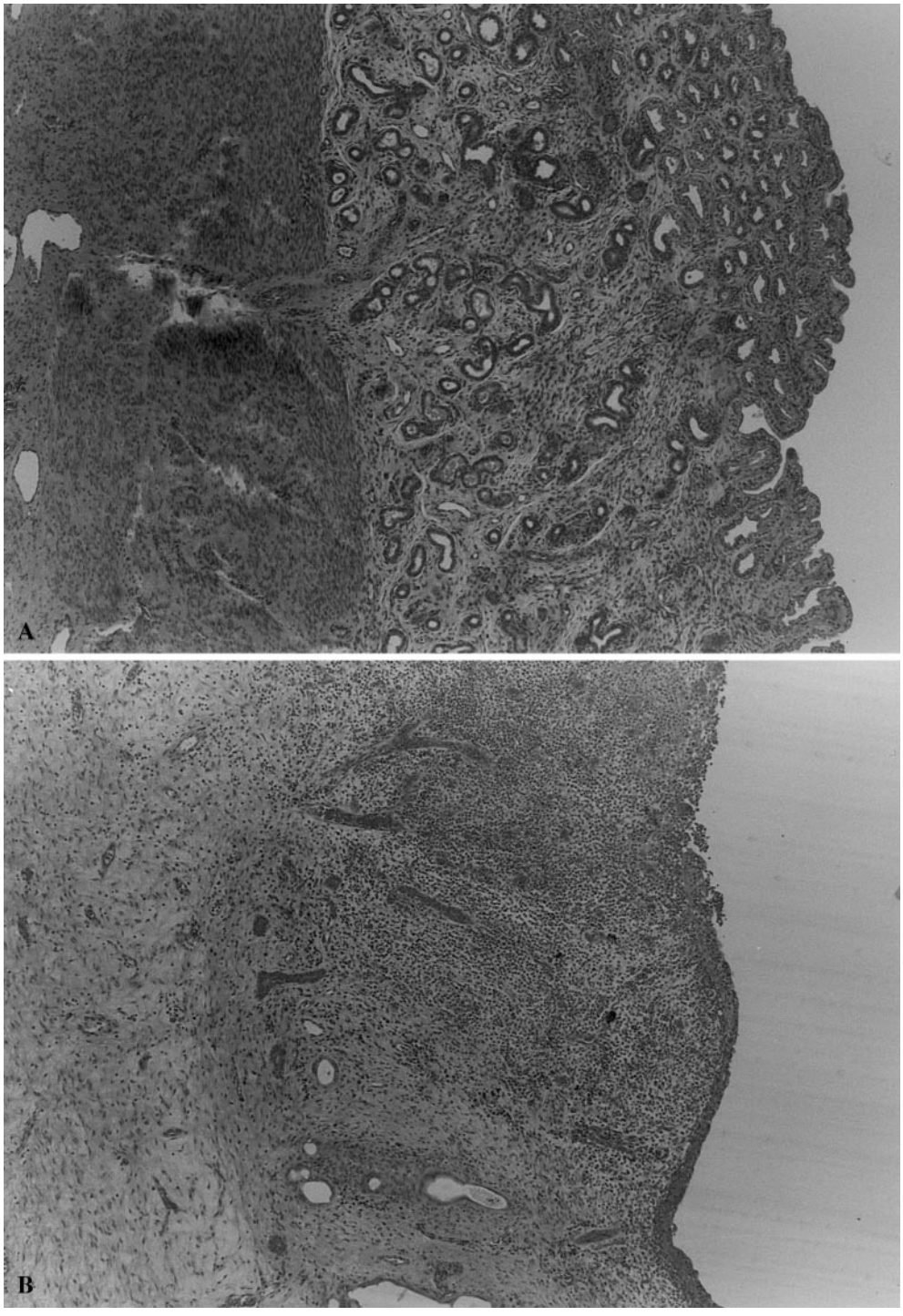

Fig. 3 A Uterine wall from a control animal. **B** Uterine wall from an animal in the combination high-dose group. Lumen dilatation, edema, squamous metaplasia, acute inflammation, epithelial necrosis and ulceration are present (× = 66)

epithelial tissue was absent in the inflamed region. Ulceration was not modified by DFMO coadministration. Uterine gland dilatation, characterized by the presence of dilated empty uterine glands in the uterine mucosa, was seen in dose groups receiving tamoxifen (either alone or with DFMO), with increased incidence and severity in the combination low-dose group in comparison with the tamoxifen low-dose group.

Discussion

DFMO is currently under investigation as a cancer chemopreventive agent. Tamoxifen is the chemotherapeutic agent of choice for the treatment of estrogen-dependent breast carcinoma and has recently been demonstrated to be efficacious in the prevention of breast carcinoma in women at high risk. These agents are under consideration for use, possibly at lower dose

levels than when used singly, as a combination regimen for the primary prevention or secondary treatment of breast cancer. This study was conducted to determine the toxic potential of coadministration of DFMO with tamoxifen and was performed in female Beagle dogs following 13 weeks of daily oral dosing.

Vaginal discharge/secretion was seen in all drug-treated dose groups but was most prevalent following administration of DFMO plus tamoxifen. Vaginal discharge was first observed on day 53 and was consistent with the pathologic changes that were present in the reproductive tract at week 14. Diarrhea was observed in the dose groups that received tamoxifen. Both nausea and vaginal bleeding/discharge have occurred as side effects to tamoxifen therapy in women [13]. Vaginal discharge has also been observed in a previous 13-week toxicity study of tamoxifen in adult Beagle dogs [11]. Excess eye secretions were seen in dose groups receiving DFMO, although no ophthalmologic changes were seen. In a previous chronic toxicity study in dogs, DFMO at a dose level of 100 mg/kg per day produced ocular discharge [8].

Minimal hematologic changes occurred in the study and included decreased reticulocyte counts in groups receiving DFMO. In a previous phase I clinical trial, DFMO administered at doses of 3000 mg/m^2 per day (approximately 81 mg/kg body weight per day) or higher produced dose-limiting thrombocytopenia that appeared to be due to decreased numbers of bone marrow megakaryocytes [1]. However, platelet counts were unaffected in the current study and biologically significant changes in hematologic parameters were not seen. Administration of tamoxifen produced increased leukocyte counts, due to increases in mature neutrophils, that were likely related to the inflammatory events that occurred in the uterus and vagina. Also, increased numbers of leukocytes were seen in urine specimens in week 13 from animals receiving tamoxifen alone and in combination with DFMO which may have been derived from the vaginal discharge observed in the study.

Administration of 100 mg/kg per day DFMO, either alone or coadministered with tamoxifen, resulted in intestinal tract lesions consisting of crypt microabscesses. Lesion severity and incidence were not affected by coadministration of tamoxifen. In a previous chronic toxicity study in dogs, DFMO at a dose level of 100 mg/kg per day has also been shown to produce cystic crypts in the intestinal tract [8]. The intestinal tract has a significant demand for polyamines due to the high rate of epithelial cell division, and therefore may be susceptible to changes induced by polyamine depletion [23]. In a rat model of gastric mucosal injury, early mucosal repair was dependent upon induction of ornithine decarboxylase with subsequent actin polymerization. In that study, DFMO pretreatment inhibited actin polymerization and mucosal repair [3]. The crypt microabscesses observed in the current study may be related to the inhibition of ornithine decarboxylase in the intestinal tract tissue by DFMO administration. In a previous phase I clinical trial, administration of DFMO at doses of 500 mg/m^2 per day (14 mg/kg body weight per day) or greater resulted in gastrointestinal toxicity manifested as nausea, diarrhea, and flatus [16].

Numerous lesions were produced in reproductive organs by both DFMO and tamoxifen. Ovarian atrophy was present in all drug-treated animals and was associated with reduced numbers of corpora lutea and antral follicles, and decreased ovarian weights. Decreased numbers of corpora lutea can result in decreased production of progesterone, which can have an affect on reproductive organs. Both corpora lutea and antral follicles were completely absent in ovaries from animals in the DFMO plus 1.0 mg tamoxifen per kg per day dose group. Although corpora lutea and antral follicles were decreased in numbers or absent completely, both primordial and growing follicles were present in all animals, suggesting that DFMO and tamoxifen were inhibiting follicular development. Tamoxifen induced serosal hyperplasia of the ovaries, and this response was potentiated by DFMO coadministration. Oocyte mineralization was produced by both DFMO and tamoxifen alone, and the incidence was increased by coadministration. The presence of mineralized oocytes indicates that destruction of oocytes had occurred. Therefore, upon cessation of drug administration, the ovaries would have a reduced number of oocytes. However, the number of mineralized oocytes was quite small compared with the number of primary and secondary oocytes. Therefore, prolonged treatment would be required to attain clinical significance. Tamoxifen has been shown to act as an estrogen agonist on the ovaries, resulting in interruption of follicular development [26]. Tamoxifen may also act as an estrogen agonist on the hypothalamus, inducing downregulation of FSH and LH production and release from the anterior pituitary [27]. This would result in interruption of follicular development and inhibition of ovulation. However, hormone levels were not measured in this study and the ovarian toxicity produced by tamoxifen may have resulted from other mechanisms of action. DFMO appears to interact with tamoxifen in a negative fashion on the ovaries. The lesions observed in the other reproductive tissues may have arisen, in part, as responses secondary to the ovarian lesions.

Administration of either DFMO or tamoxifen alone resulted in cornified epithelium of the vagina and cervix, with the greatest effect produced by tamoxifen. Cornified, stratified squamous epithelium is normally only present during the estrus portion of the reproductive cycle [5]. Cornified epithelium of the vagina and cervix were not observed in any dogs in the control group. Assuming that none of the dogs in the study were in normal estrus, these findings suggest that cornification of the epithelium was a treatment-related response. Tamoxifen appears to have an estrogenic effect on the vagina and cervix, resulting in histologic changes (edema, subacute inflammation) which are increased by DFMO coadministration.

Numerous lesions were observed in the uterus from animals receiving tamoxifen which included edema, squamous metaplasia, uterine gland dilatation, acute inflammation, epithelial necrosis and ulceration. During proestrus in the dog, which is estrogen-dependent, edema, congestion and hemorrhage occur and are signals for the start of estrus [5]. The uterine changes observed in the current study appear to result from tamoxifen's estrogenic actions either due to an agonist effect on the uterus or as a response secondary to the ovarian lesions [26, 27]. DFMO alone did not produce any uterine changes, but increased the incidence and/or severity of the uterine lesions elicited by tamoxifen.

In summary, administration of DFMO alone resulted in lesions to the intestines and ovaries, and cornified epithelium of vagina and cervix. Tamoxifen administration produced cornified epithelium of vagina and cervix, and numerous lesions in the reproductive system. The lesions produced by tamoxifen were likely due to an estrogen agonist effect. Coadministration of DFMO increased the incidence and/or severity of the reproductive system lesions in an additive fashion. These results suggest that administration of a high dose of DFMO with clinically relevant doses of tamoxifen results in additive toxicity. Therefore, clinical trials of the coadministration of DFMO and tamoxifen for cancer chemoprevention would be warranted and could include lower dose levels of the drugs.

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