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Difluoromethylornithine in combination with tamoxifen in female rats: 13-week oral toxicity study

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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) is currently used for 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 tamoxifen 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 rats following 13 weeks of daily administration by gavage. Dose groups were vehicle control, DFMO (1000 mg/kg per day), low TAM (0.25 mg/kg per day), high TAM (2.5 mg/kg per day), low combination (1000 + 0.25) and high combination (1000 + 2.5). *Results*: No mortalities occurred in the study. Clinical signs of toxicity were limited to dermal lesions consisting of scab formation and abrasions produced by DFMO. Administration of either DFMO or TAM resulted in decreased body weight gains, with coadministration having an additive effect. Serum albumin, total protein, cholesterol and triglyceride levels were decreased in all drug-treated dose groups, although histologic evidence of liver lesions were not seen. TAM resulted in increased numbers of red blood cells, whereas DFMO produced a slightly anemic response. DFMO produced lesions in the small intestine consisting of necrosis of crypt epithelium and crypt microabscess, which were enhanced by TAM coadministration. Administration of TAM resulted in histologic changes in the ovaries, fallopian tube, vagina, cervix and uterus, indicating that inhibition of ovulation and reproductive cycle arrest in the proestrus stage had occurred. Coadministration with DFMO did not affect the changes to the reproductive system induced by TAM. *Conclusions*: Coadministration of DFMO with tamoxifen did not result in toxicity unique to the combination drug regimen, but rather toxicity resulted from administration of each drug. Under the conditions of the study, the overall toxicity produced by dual administration of DFMO with tamoxifen was additive with respect to the toxicity associated with each agent alone.

Key words Tamoxifen · Difluoromethylornithine · Rats · Reproductive system toxicity · GI toxicity · Dermal toxicity · Ornithine decarboxylase · Estrogen

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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 [15, 17]. 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 [17]. 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 [15, 18].

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the biosynthesis of polyamines (putrescine, spermidine and spermine), which are small cationic molecules required for cell proliferation and differentiation [2]. Polyamine synthesis increases during the G1 phase of the cell cycle [28]. Intracellular levels of polyamines and ODC activity have been observed to be elevated in neoplastic cells or cells undergoing neoplastic transformation [27]. Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC through a mechanism whereby DFMO is decarboxylated by ODC to a reactive intermediate that alkylates and inactivates the enzyme [10, 26]. DFMO can induce a depletion of intracellular pools of polyamines and elicit apoptosis [11, 21, 28].

DFMO is currently approved for use in the treatment of African sleeping sickness caused by *Trypanosoma brucei gambiense* [16], 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 [10, 23, 27]. In a recent study, DFMO administration in mice elicited regression of epidermal squamous papillomas, suggesting that DFMO may have antitumor activity [24]. DFMO is currently under investigation for safety and cancer chemopreventive efficacy in clinical trials [4, 22]. 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 (27 mg/kg per day) for 31 days (adverse effects included nausea, diarrhea, stomatitis and dizziness), and regression of neoplastic lesions was seen at doses of 60–250 mg/m^2 per day (1.62–6.76 mg/kg per day) [22].

Tamoxifen, which is a nonsteroid, is the initial treatment of choice in both pre- and post-menopausal women with estrogen receptor-positive breast carcinoma and is believed to exhibit its antitumor effect by inhibiting estrogen-induced cellular proliferation of neoplastic cells [16]. Tamoxifen exhibits complex pharmacologic effects, acting as an estrogen antagonist, or partial or full estrogen agonist, depending on the target tissue or species examined [16, 25]. Tamoxifen has recently been shown to demonstrate efficacy in chemoprevention of breast cancer. In the Breast Cancer Prevention Trial (conducted from 1992 to 1998), daily administration of tamoxifen in women with a high risk of breast cancer resulted in a 45% reduction of invasive breast carcinoma [13, 29].

The oral administration of tamoxifen with DFMO is being considered for development by the National Cancer Institute as a potential drug regimen for the primary prevention and secondary treatment 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 [30]. In a previously conducted 13-week oral toxicity study in female dogs, coadministration of DFMO (100 mg/kg per day) with tamoxifen (1 mg/kg per day) was well tolerated [5]. The current study was conducted to determine whether DFMO alters the toxicity of tamoxifen in female rats following 13 weeks of daily oral administration by gavage.

Materials and methods

Drugs

2-Difluoromethylornithine (DFMO) and tamoxifen citrate (mole fraction 0.66) were received from McKesson Bioservices (Rockville, Md.) and stored in amber bottles at ambient temperature and humidity. Tamoxifen dose levels are expressed as the free base, although tamoxifen citrate was given. The initial purity of DFMO was 97.03 ± 1.31% and the final purity was 93.19 ± 0.50%. The initial purity of tamoxifen citrate was 99.84 ± 0.03% and the final purity was 99.48 ± 0.07%. Both drugs were prepared as suspensions in 1% methylcellulose/0.2% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) based upon purity and base mole fraction of tamoxifen. The suspensions were prepared every 2 weeks, stored at 2–8 °C, and allowed to warm to room temperature and stirred continuously before and during administration.

Animals

Female CD Virus Antibody Free (VAF) rats were obtained from Charles River Breeding Laboratories (Kingston, N.Y.) at approximately 5 weeks of age. All rats were singly housed in polycarbonate cages with Anderson Bed-o-cob bedding (Heinold, Kankakee, Ill.) in a temperature- and humidity-controlled room (65–78 °F, 30–70%) with a 14-h light/10-h dark cycle. 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. Certified Rodent Chow No. 5002 (PMI Feeds, St. Louis, Mo.) and tap water were provided *ad libitum*.

Experimental design

Near the end of the 2-week quarantine period the animals were randomized into six dose groups (20 rats/group), stratified on the basis of body weight. Dose levels in milligrams per kilogram body weight per day were: vehicle control (0), DFMO (1000), low tamoxifen (0.25), high tamoxifen (2.5), low combination (1000 + 0.25) and high combination (1000 + 2.5). The drugs were administered daily by gavage for 13 weeks in a dose volume of 5 ml/kg body weight per day. Control animals received the vehicle alone (1.0% methylcellulose/0.2% Tween 80). The dose volume was adjusted weekly, based on each animal's most recent body weight.

Measurements

All animals were also observed twice daily for morbidity/mortality, and once daily for clinical signs of toxicity approximately 1–2 h after dosing. Body weight and food consumption measurements, and physical examinations were conducted weekly. All rats were examined by indirect ophthalmoscopy prior to study initiation. However, only the last ten animals in each group were examined during week 13. Ophthalmologic examinations were not performed

on animals previously bled from the orbital sinus for clinical pathology (first ten animals in each group).

Clinical pathology

Hematology (complete blood count with differential) and standard clinical chemistry parameters were measured in weeks 4 and 12 in the first ten animals in each group. The animals were anesthetized by carbon dioxide inhalation and sufficient blood was collected from each animal from the orbital sinus. The following clinical chemistry parameters were measured: alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALKP), aspartate aminotransferase (AST), calcium, chloride, cholesterol, creatinine, glucose, inorganic phosphate, potassium, sodium, total bilirubin, total protein, triglycerides and urea nitrogen (BUN). At termination in week 14, blood was collected from the inferior vena cava from the first ten animals in each group, and prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were determined as standard coagulation measurements.

Pathology

All animals were sacrificed by carbon dioxide asphyxiation and necropsied in random order over two consecutive days (days 92 and 93) under the direction of a veterinary pathologist. More than 40 tissues/organs were collected for histopathologic evaluations, and organ weights were determined (paired organs were weighed as a unit). Histopathologic examinations were conducted on all collected tissues in the control group and in the high combination dose groups. Tissues from organs found to be normal in the high combination 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 the remaining dose groups: vagina, cervix, fallopian tube, ovary, uterus, duodenum, jejunum, ileum, and bone marrow from femur and sternum. In addition, all skin gross lesions collected were examined microscopically.

Statistical analyses

Statistical data are presented as the mean ± standard deviation of the mean. For each sex, Analysis of variance (ANOVA) tests were conducted on body weight, food consumption, clinical pathology and organ weight data. Organ weight analysis considered weights relative to brain weights. If a significant F ratio was obtained ($P \leq 0.05$), Dunnett's t-test was used for pair-wise comparisons to the control group. Interactions of DFMO coadministered with tamoxifen were examined by pair-wise comparisons of the combination dose groups with the respective DFMO-alone and tamoxifen-alone groups using Duncan's multiple range test (significance at $P < 0.05$).

Results

Mortality/clinical observations

No mortalities occurred in the study and daily cage-side clinical signs of toxicity were not seen 1–2 h after dosing. However, when weekly physical examinations were performed, dermal lesions, consisting of scab formation and abrasions, were seen throughout the study in 19/20 animals in each of the DFMO-alone and combination dose groups. Scab formation occurred primarily around the mouth, the chin area, and on the front legs and feet. Abrasions were seen primarily on the front feet. These dermal lesions were seen on rare occasions in a few animals (one or two animals) in the control and tamoxifen-alone dose groups. Treatment-related ophthalmologic changes were not apparent.

Body weights/food consumption

Administration of either DFMO or tamoxifen resulted in decreased body weight gain in all drug-treated dose groups with body weight changes occurring to the greatest extent in the combination dose groups (Fig. 1). Total weight gains at the end of the study (day 92) were reduced 13%, 41%, 36%, 55% and 48% below

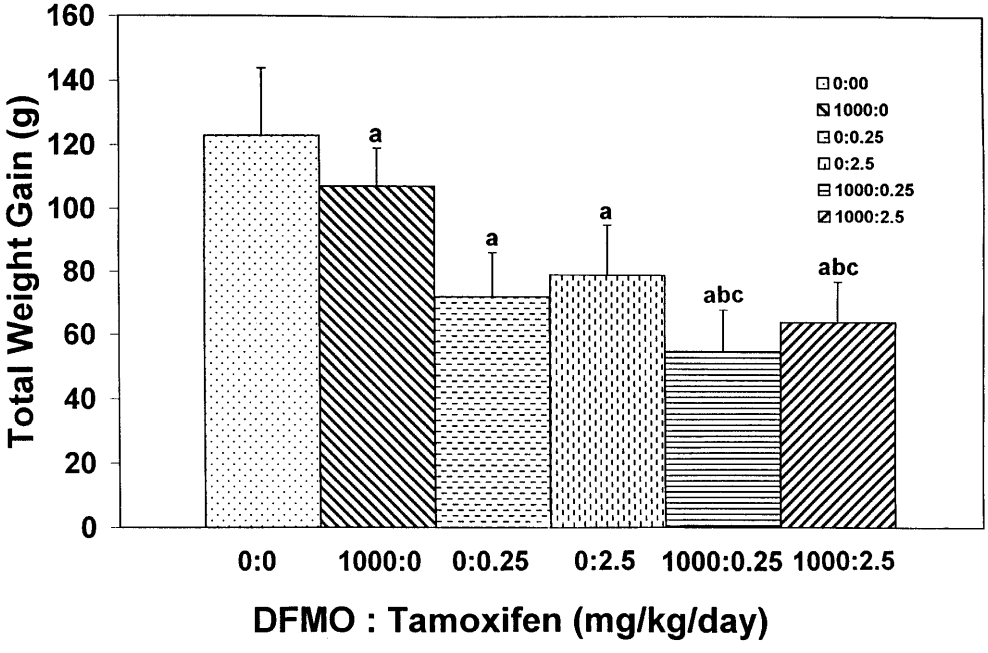

Fig. 1 Treatment-related changes in total weight gains. Total weight gains were determined from day 1 (initiation of treatment) to day 92 (end of study). [a]$P < 0.05$ vs control (0:0) dose group, [b]$P < 0.05$ vs DFMO alone (1000:0) dose group, [c]$P < 0.05$ vs respective group without DFMO

control in the DFMO-alone, low tamoxifen (0.25 mg/kg per day), high tamoxifen (2.5 mg/kg per day), low combination (0.25 mg tamoxifen plus 1000 mg DFMO/kg per day), and high combination (2.5 mg tamoxifen plus 1000 mg DFMO/kg per day) dose groups, respectively.

Decreased food consumption was seen throughout the study in all dose groups receiving tamoxifen and on several occasions in the DFMO-alone dose group (data not shown). Decreased food consumption was independent of the dose of tamoxifen and was not affected by coadministration with DFMO.

Clinical pathology

Administration of DFMO and tamoxifen resulted in several changes in clinical chemistry parameters which were most apparent in week 12 compared with week 4 (Table 1). In week 4, serum albumin was significantly decreased 9–19% in all drug-treated dose groups. This was reflected by decreased serum total protein values, which were significantly decreased in the combination dose groups. In week 12, serum albumin was significantly decreased 20–27% and serum total protein levels were significantly decreased 13–17% in all drug-treated dose groups. Serum cholesterol was decreased 26–47% and serum triglycerides were decreased 44–54% in all drug-treated dose groups in week 12, but were not affected in week 4. Serum ALT values were decreased in week 12 in the low-tamoxifen and combination dose groups, possibly due to an effect on enzyme synthesis or low protein levels.

Administration of DFMO and tamoxifen resulted in numerous changes in hematology parameters which were most pronounced in week 12 (Table 2). Administration of tamoxifen alone or in combination with DFMO resulted in slight, but statistically significant, nondose-related increases in red blood cell (RBC) counts in weeks 4 and 12 with the effect being greater at the later timepoint. While this may have been a hemoconcentration effect, as significant reductions in body weight gains occurred, RBCs in tamoxifen-treated rats were also hypochromic (decreased mean corpuscular hemoglobin). Because these hypochromic RBCs were increased in numbers, total hemoglobin concentration was not altered. Hematocrit was increased, however, in week 12 in the tamoxifen alone groups. This did not occur in the combination dose groups, because DFMO produced a counteractive anemic response by week 12 (but not earlier in week 4). In week 12, microcytic (decreased mean corpuscular volume), hypochromic RBCs were seen in the DFMO alone dose group. This resulted in corresponding decreases in hematocrit and total hemoglobin concentration. RBCs were hypochromic and microcytic to the greatest extent in the combination dose groups in week 12. DFMO administration resulted in increased white blood cell (WBC) counts. WBC counts were increased 27% in the high combination dose group in week 4, and were increased 43–58% in week 12 in all dose groups receiving DFMO (data not shown). The increased WBC counts in week 12 were due to significantly increased lymphocyte and mature neutrophil counts (data not shown).

Administration of tamoxifen resulted in changes in coagulation parameters, irrespective of dose level (Table 2). PT was increased 1.7–2.6 s and plasma fibrinogen levels were increased 26–37% in all dose groups receiving tamoxifen at necropsy in week 14. These changes did not appear to be affected by DFMO coadministration. APTT was not affected by drug administration (data not shown).

Table 1 Select clinical chemistry parameters. Values are means ± standard deviation (*ALT* alanine aminotransferase)

	DFMO:Tamoxifen (mg/kg/day)					
	0:0	1000:0	0:0.25	0:2.5	1000:0.25	1000:2.5
Albumin (g/dl)						
Week 4	4.3 ± 0.3	3.9 ± 0.3[*1]	3.7 ± 0.2[*1]	3.7 ± 0.3[*1]	3.7 ± 0.1[*1]	3.5 ± 0.2[*1,*2]
Week 12	4.9 ± 0.5	3.9 ± 0.5[*1]	3.9 ± 0.2[*1]	3.8 ± 0.2[*1]	3.6 ± 0.3[*1]	3.6 ± 0.2[*1]
Total protein (g/dl)						
Week 4	7.6 ± 0.6	7.1 ± 0.5	7.2 ± 0.4	7.1 ± 0.5	7.0 ± 0.4[*1]	6.8 ± 0.3[*1]
Week 12	8.6 ± 0.7	7.5 ± 0.9[*1]	7.5 ± 0.2[*1]	7.3 ± 0.4[*1]	7.1 ± 0.6[*1]	7.3 ± 0.4[*1]
Cholesterol (mg/dl)						
Week 4	61 ± 16	48 ± 16	54 ± 21	47 ± 20	47 ± 16	43 ± 9
Week 12	76 ± 15	56 ± 18[*1]	51 ± 10[*1]	46 ± 16[*1]	48 ± 9[*1]	40 ± 10[*1,*2]
Triglyceride (mg/dl)						
Week 4	156 ± 110	126 ± 41	129 ± 34	125 ± 41	109 ± 42	137 ± 39
Week 12	302 ± 126	153 ± 70[*1]	154 ± 48[*1]	169 ± 44[*1]	138 ± 55[*1]	142 ± 43[*1]
ALT (IU/l)						
Week 4	64 ± 12	54 ± 19	59 ± 9	58 ± 7	44 ± 7[*1,*3]	65 ± 12
Week 12	79 ± 36	63 ± 38	54 ± 9[*1]	57 ± 7	38 ± 6[*1,*3]	44 ± 6[*1]

[*1] $P < 0.05$ vs control (0:0) dose group, [*2] $P < 0.05$ vs DFMO alone (1000:0) dose group, [*3] $P < 0.05$ vs respective group without DFMO

Table 2 Select hematology and coagulation parameters. Values are means ± standard deviation (*MCH* mean corpuscular hemoglobin, *MCV* mean corpuscular volume, *RBC* red blood cell)

	DFMO:Tamoxifen (mg/kg/day)					
	0:0	1000:0	0:0.25	0:2.5	1000:0.25	1000:2.5
RBC count (10^6/μl)						
Week 4	7.66 ± 0.27	7.59 ± 0.29	8.10 ± 0.37	8.18 ± 0.59[*1]	8.12 ± 0.32[*1]	7.96 ± 0.43[*2,*3]
Week 12	7.77 ± 0.17	7.71 ± 0.40	8.57 ± 0.49[*1]	8.30 ± 0.47[*1]	8.37 ± 0.51[*1,*2,*3]	8.34 ± 0.55[*1,*2]
Hematocrit (%)						
Week 4	43.6 ± 1.8	42.7 ± 1.4	45.1 ± 1.2	45.7 ± 2.8	45.3 ± 2.2[*2]	44.0 ± 2.4[*2,*3]
Week 12	42.9 ± 1.2	40.2 ± 2.3[*1]	45.9 ± 1.8[*1]	45.4 ± 2.2[*1]	43.1 ± 2.3[*2,*3]	43.2 ± 2.9[*2,*3]
Hemoglobin (g/dl)						
Week 4	16.4 ± 0.7	16.2 ± 0.6	16.7 ± 0.4	16.7 ± 1.1	16.5 ± 0.6	16.1 ± 0.8
Week 12	16.1 ± 0.5	14.9 ± 0.8[*1]	16.6 ± 0.4	16.3 ± 0.9	15.6 ± 0.8[*2,*3]	15.4 ± 1.0[*2,*3]
MCV (fl)						
Week 4	57.0 ± 1.3	56.2 ± 1.1	55.7 ± 1.6	56.0 ± 1.4	55.8 ± 1.0	55.3 ± 0.9
Week 12	55.2 ± 1.4	52.2 ± 1.7[*1]	53.6 ± 1.7	54.7 ± 1.5	51.5 ± 1.3[*1,*2,*3]	51.8 ± 1.3[*1,*3]
MCH (pg)						
Week 4	21.4 ± 0.6	21.3 ± 0.6	20.6 ± 0.8[*1]	20.4 ± 0.5[*1]	20.3 ± 0.4[*1,*2,*3]	20.3 ± 0.4[*1,*2,*3]
Week 12	20.8 ± 0.7	19.4 ± 0.5[*1]	19.5 ± 0.8[*1]	19.6 ± 0.4[*1]	18.6 ± 0.6[*1,*2,*3]	18.4 ± 0.5[*1,*2,*3]
Prothrombin time (s)	16.5 ± 0.7	17.4 ± 1.3	18.8 ± 0.6[*1]	18.7 ± 0.6[*1]	18.2 ± 1.4[*1,*2,*3]	19.1 ± 1.3[*1,*2,*3]
Fibrinogen (mg/dl)	198 ± 11	207 ± 14	272 ± 48[*1]	252 ± 21[*1]	249 ± 23[*1,*2,*3]	254 ± 55[*1,*2]

[*1] $P < 0.05$ vs control (0:0) dose group, [*2] $P < 0.05$ vs DFMO alone (1000:0) dose group, [*3] $P < 0.05$ vs respective group without DFMO

Organ weights

Administration of both DFMO and tamoxifen produced organ weight changes (Table 3). The weights (as percent of brain weights) of the ovaries plus fallopian tubes were decreased 33–43% and the uterus plus vagina were decreased 39–47% in all dose groups receiving tamoxifen. These organ weight changes were not affected by DFMO coadministration. The weight of the spleen was increased 16% in the DFMO alone dose group.

Pathology

Administration of tamoxifen resulted in histologic changes in the reproductive tract that were unaffected by DFMO coadministration (Table 4). Vacuolated, uncornified epithelium of the vagina and cervix was present in most rats dosed with tamoxifen and in only 1 or 2 out of 20 rats in the DFMO alone dose group. These changes were diagnosed when the cornified surface of the stratified epithelium was lacking and the surface layer of epithelial cells contained large vacuoles in its cytoplasm. Decreased corpora lutea were observed in most rats given tamoxifen and in 1/20 rats in the DFMO alone dose group. Ovarian cyst, characterized by the presence of a distended capsule around the ovary, and oocyte mineralization, 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 present, were seen in some animals that had received tamoxifen. The presence of mineralized oocytes indicates that destruction of oocytes had occurred. Vacuolation of fallopian tube epithelium was seen in most animals that had received tamoxifen. Uterine atrophy was seen in all animals that had received tamoxifen. Squamous metaplasia, characterized by the presence of stratified squamous epithelium on the lumenal surface of the uterus, and the lining of the lumenal surface of uterine glands, was seen in some animals that had received tamoxifen. Uterine dilatation, which is typically present in low incidence in a population of normally cycling rats, was seen in the control (6/20 rats) and DFMO (3/20 rats) dose groups, but not in dose groups that had received tamoxifen.

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

	DFMO:Tamoxifen (mg/kg/day)					
	0:0	1000:0	0:0.25	0:2.5	1000:0.25	1000:2.5
Uterus plus vagina	45.4 ± 11.2	46.1 ± 8.2	27.5 ± 7.5[*1]	23.9 ± 4.7[*1]	24.9 ± 4.9[*1,*2]	25.3 ± 5.7[*1,*2]
Ovaries plus fallopian tubes	8.35 ± 1.97	9.02 ± 2.35	5.42 ± 1.18[*1]	5.28 ± 1.71[*1]	4.73 ± 0.87[*1,*2]	5.61 ± 1.43[*1,*2]
Spleen	30.4 ± 4.3	35.3 ± 8.3[*1]	28.2 ± 3.4	28.7 ± 3.5	31.6 ± 4.4[*2]	33.6 ± 5.7[*3]

[*1] $P < 0.05$ vs control (0:0) dose group, [*2] $P < 0.05$ vs DFMO alone (1000:0) dose group, [*3] $P < 0.05$ vs respective group without DFMO

Table 4 Treatment-related histologic changes

Organ – observation	DFMO:Tamoxifen (mg/kg/day)					
	0:0	1000:0	0:0.25	0:2.5	1000:0.25	1000:2.5
Vagina						
– Vacuolated uncornified epithelium	0/20	2/20 (0.10)[a]	19/20 (1.80)	18/19 (1.95)	17/20 (1.65)	18/20 (2.00)
Cervix						
– Vacuolated uncornified epithelium	0/20	1/20 (0.05)	12/20 (0.75)	15/20 (1.05)	16/20 (0.95)	17/20 (1.30)
Ovary						
– Decreased corpora lutea	0/20	1/20 (0.15)	20/20 (3.55)	19/20 (3.70)	20/20 (3.90)	20/20 (3.30)
– Cyst	0/20	0/20	1/20 (0.10)	4/20 (0.40)	0/20	3/20 (0.50)
– Mineralization, oocyte	0/20	0/20	2/20 (0.10)	1/20 (0.05)	0/20	1/20 (0.05)
Fallopian tube						
– Vacuolation epithelium	0/20	0/20	18/20 (1.15)	18/20 (1.05)	17/20 (0.85)	17/20 (1.35)
Uterus						
– Atrophy	0/20	0/20	20/20 (2.40)	20/20 (3.45)	20/20 (3.20)	20/20 (3.15)
– Squamous metaplasia	0/20	0/20	0/20	5/20 (0.25)	1/20 (0.05)	2/20 (0.10)
Duodenum						
– Necrosis, crypt epithelium	0/20	13/20 (0.80)	1/20 (0.05)	0/20	17/20 (1.20)	18/20 (0.95)
– Microabscess, crypt	0/20	1/20 (0.05)	0/20	1/20 (0.05)	4/20 (0.20)	4/20 (0.20)
Jejunum						
– Necrosis, crypt epithelium	0/20	13/20 (0.80)	0/20	0/20	15/20 (1.20)	19/20 (1.25)
– Microabscess, crypt	0/20	0/20	0/20	0/20	2/20 (0.10)	5/20 (0.25)
Ileum						
– Necrosis, crypt epithelium	0/20	11/20 (0.60)	0/20	0/20	18/20 (1.55)	17/20 (1.05)
– Microabscess, crypt	0/20	0/20	0/20	0/20	3/20 (0.15)	5/20 (0.25)
Skin, lip						
– Ulceration	–	0/2	–	–	2/4 (1.00)	2/2 (2.00)
– Inflammation, chronic, active	–	2/2 (3.00)	–	–	4/4 (2.75)	2/2 (3.00)
– Abscess	–	1/2 (1.00)	–	–	2/4 (0.75)	1/2 (1.00)
Bone marrow						
– Hyperplasia, femoral marrow	0/20	10/20 (0.50)	0/20	0/20	12/20 (0.70)	11/20 (0.55)
– Hyperplasia, sternal marrow	0/20	9/20 (0.45)	0/19	0/20	13/20 (0.65)	9/20 (0.45)

[a] Incidence (mean group severity score)
Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

Administration of DFMO, either alone or in combination with tamoxifen, resulted in lesions in the small intestine (Table 4; Fig. 2). Crypt microabscess, seen as dilated crypts filled with cell debris and neutrophils, was present in the duodenum, jejunum and ileum (Fig. 2B). Necrosis of crypt epithelium, characterized by the presence of small pockets of cell debris replacing epithelial cells in crypt regions of the intestinal mucosa, was seen in the duodenum, jejunum and ileum (Fig. 2C). The incidence and severity scores of the intestinal tract lesions were greatest in the combination dose groups.

At necropsy, gross skin lesions located on the lip and axillary regions were observed in some animals that had received DFMO, irrespective of tamoxifen coadministration (Table 4; Fig. 3). The skin lesions (minimal to moderate in severity) consisted of ulceration, chronic active inflammation and abscess formation. Ulceration was characterized by the absence of the epithelial layer with associated inflammation of the dermis and thickened epithelium at the margins of the ulcer. Chronic active inflammation was characterized by infiltration of the dermis with macrophages, lymphocytes and neutrophils. Abscess formation was due to the presence of discrete foci of neutrophils and cell debris in the dermis of the skin.

Minimal bone marrow hyperplasia in the femur and sternum, due to a relative lack of fat cells and increased volume of hematopoietic tissue, was present in several animals dosed with DFMO and was not affected by tamoxifen co-administration (Table 4).

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 high-risk women [13, 16, 29]. 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. Tamoxifen is typically given clinically at doses of 20 mg/day which is equivalent to 0.3 mg/kg per day for a 60-kg woman or approximately

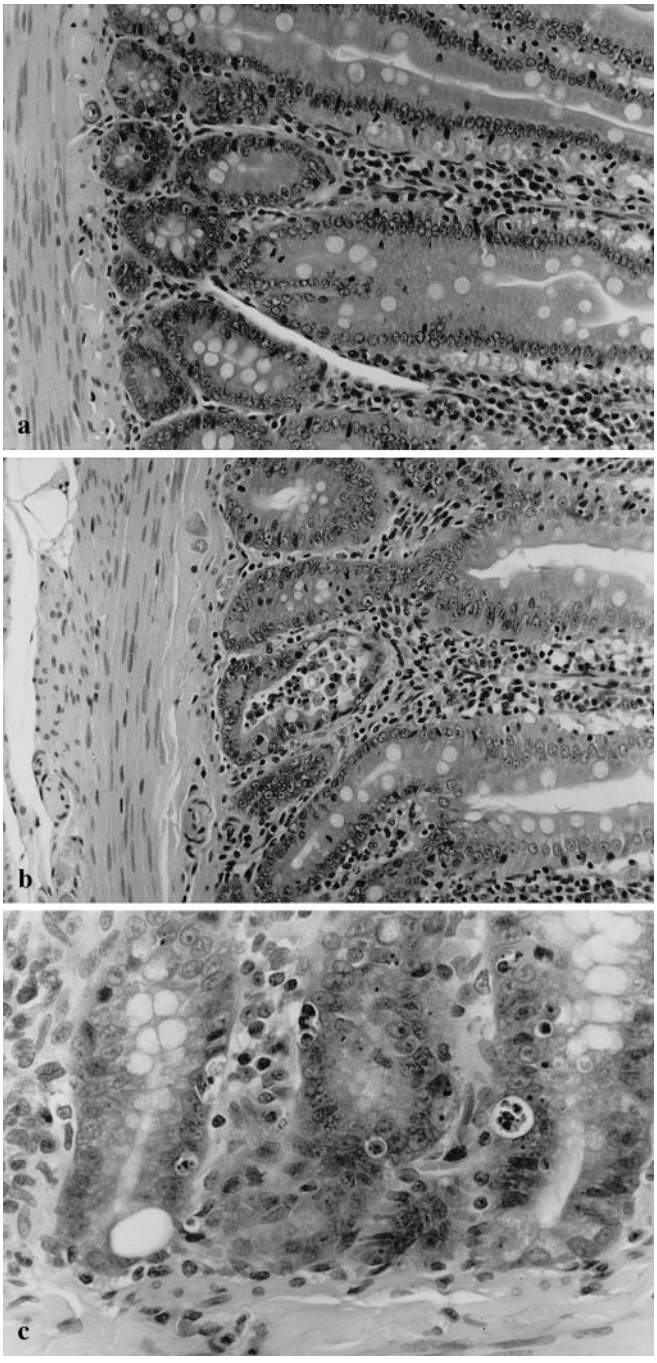

Fig. 2 **a** small intestine (duodenum) from a rat in the control group. **b** Small intestine (duodenum) from a rat in the 2.5 mg tamoxifen plus 1000 mg DFMO/kg per day dose group with a crypt microabscess (×240). **c** Small intestine (jejunum) from a rat in the 2.5 mg tamoxifen plus 1000 mg DFMO/kg per day dose group with necrosis of crypt epithelium (×480)

12 mg/m^2 per day [7]. The doses of tamoxifen used in the current study of 0.25 and 2.5 mg/kg per day are approximately equivalent to 1.5 and 15 mg/m^2 per day, respectively. DFMO is currently being investigated in clinical trials at doses up to 500 mg/m^2 per day or approximately 14 mg/kg per day [22]. The DFMO dose of 1000 mg/kg per day in the current study is approximately equivalent to 5900 mg/m^2 per day. Therefore, the study was designed to characterize how a high dose of DFMO alters the toxicity of clinically relevant doses of tamoxifen.

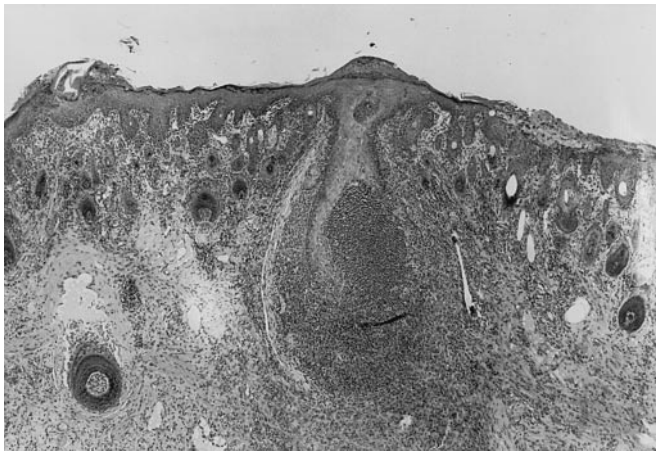

Fig. 3 Skin from the lip of a rat in the 2.5 mg tamoxifen plus 1000 mg DFMO/kg per day dose group with abscess, ulceration and chronic active inflammation (×48)

Dermal lesions were produced by administration of DFMO, irrespective of tamoxifen coadministration. Histologic evaluation of these lesions indicated the presence of dermal ulceration, chronic active inflammation and abscess formation. Dermal lesions, similar to those seen in the current study, were observed in a 1-year oral toxicity study of DFMO in male and female rats at 800 mg/kg per day [10]. In response to the dermal and subsequently discussed intestinal tract lesions, WBC counts were increased in the high combination dose group in week 4 and in all dose groups receiving DFMO in week 12. DFMO administration also resulted in minimal bone marrow hyperplasia, which was likely a response to the inflammatory changes seen in the dermis and intestinal tract.

Administration of either DFMO or tamoxifen resulted in reduced body weight gains, with tamoxifen having a greater effect at the dose levels tested. Coadministration of DFMO with tamoxifen had an additive effect on the body weight changes, as body weights were decreased to the greatest extent in the combination dose groups. The body weight changes observed may have been due to decreased food consumption and/or absorption of nutrients from the GI tract. Decreases in serum albumin, total protein, cholesterol and triglycerides were seen in all drug-treated dose groups, and may have also resulted from decreased food consumption and/or absorption of nutrients from the GI tract. Estrogens typically decrease serum cholesterol and triglyceride levels, and tamoxifen has been previously shown to decrease serum cholesterol and phospholipid [16, 20]. Serum ALT levels were decreased in week 12 in the low-tamoxifen and combination dose groups, likely due to decreased enzyme synthesis as a result of decreased food consumption and/or altered liver function.

Changes in coagulation parameters were produced by tamoxifen. Both PT and plasma fibrinogen levels were increased in all dose groups receiving tamoxifen, which may reflect altered liver function. However, histologic changes in the liver were not seen in the study. Tamoxifen has been shown to produce significant changes in rat liver including induction of hepatocellular carcinoma in a time- and dose-dependent fashion. Daily administration of tamoxifen to female Sprague-Dawley rats for 52 weeks at 45 mg/kg per day produced hepatocellular carcinoma [14]. In another study, daily administration of tamoxifen to female Sprague-Dawley rats for 12 months at 11.3 mg/kg per day produced hepatocellular carcinoma whereas a dose level of 2.8 mg/kg per day did not cause neoplastic changes [31]. Therefore, based upon these results, administration of tamoxifen for 13 weeks at 2.5 mg/kg per day (high dose level in current study) would not be expected to produce neoplasia in the liver.

Administration of DFMO, either alone or in combination with tamoxifen, resulted in lesions in the small intestine consisting of necrosis of the crypt epithelium and crypt microabscess. Although these lesions were minimal in severity, the incidence and severity scores were greatest in the combination dose groups, indicating that tamoxifen can enhance the development of DFMO-induced intestinal tract lesions. Intestinal tract lesions, similar to those observed in the current study, have also been seen in female dogs following administration of DFMO for 13 weeks at 100 mg/kg per day [5]. 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 [28]. In a rat model of gastric mucosal injury, early mucosal repair was dependent upon induction of ODC with subsequent actin polymerization [3]. In that model, DFMO pretreatment inhibited actin polymerization and mucosal repair. The intestinal tract lesions observed in the current study may be related to the inhibition of ODC by DFMO. In a previous phase I clinical trial, administration of DFMO at doses of 500 mg/m^2 per day (14 mg/kg per day) or higher resulted in gastrointestinal toxicity manifest as nausea, diarrhea and flatus [19].

Administration of tamoxifen produced histologic changes in the reproductive tract that were unaffected by DFMO coadministration. Decreased corpora lutea in the presence of primordial, growing and antral follicles was observed, suggesting that inhibition of ovulation had occurred. Numbers of corpora lutea were also decreased in a previous 14-day toxicity study in female rats at dose levels of 0.5 and 5.0 mg/kg per day [20]. Although tamoxifen exhibits antitumor activity by acting as an estrogen antagonist on mammary carcinoma cells, estrogenic effects can be elicited on other tissues [25]. Tamoxifen has been shown to act as an estrogen agonist on the ovaries, resulting in interruption of follicular development [32]. Tamoxifen may also act as an estrogen agonist on the hypothalamus, inducing downregulation of FSH and LH production and release from the anterior pituitary due to the presence of nonphysiologic levels of a synthetic estrogen [33]. This would result in interruption of follicular development and inhibition of ovulation. Vacuolated, uncornified epithelium of the vagina and cervix were seen in the tamoxifen dose groups. Cornified epithelium is typically present in rodent vagina except during the brief proestrus portion of the reproductive cycle. These results suggest that tamoxifen administration in the rat results in reproductive cycle arrest in the proestrus stage.

The reproductive system changes seen in the current study are important considering what is observed with the clinical use of tamoxifen. Tamoxifen has been shown to produce endocrine effects in women depending upon their menopausal status. In premenopausal women treated with tamoxifen, serum estrogen and progesterone levels have been observed to be elevated with little or no effect on serum gonadotrophin levels, and menstrual disturbances have been noted [6]. Tamoxifen administration in postmenopausal women has been associated with decreased serum levels of FSH and LH, likely due to an estrogenic effect on the hypothalamus [6, 16]. In addition, tamoxifen therapy has been associated with increased incidences of endometrial cancer, likely due to its estrogenic effects [12, 13]. In the current study, ovarian cysts were observed in some rats given tamoxifen. Similarly, tamoxifen treatment has been shown to increase the incidence of ovarian cysts in premenopausal breast cancer patients [8, 9].

Administration of both DFMO and tamoxifen resulted in changes in erythrocyte parameters. Tamoxifen produced increased numbers of RBCs and decreases in MCH. Erythrocyte counts were also increased in a 14-day toxicity study of tamoxifen in female rats at 5 mg/kg per day [20]. DFMO produced an anemic condition by week 12 and RBCs were hypochromic and microcytic to the greatest extent in the combination dose groups in week 12. In a previous phase I clinical trial of DFMO, anemia and dose-limiting thrombocytopenia were produced at dose levels of 6000 mg/m^2 per day (162 mg/kg per day) and higher [1]. Because rapidly dividing cells require polyamines, inhibition of ODC in bone marrow by DFMO may result in hematologic changes.

In summary, administration of either DFMO or tamoxifen resulted in decreased body weight gains with coadministration having an additive effect. Administration of both drugs resulted in RBCs that were hypochromic. DFMO produced lesions in the small intestine, which were enhanced by tamoxifen coadministration, and dermal lesions. Although administration of tamoxifen produced reproductive system changes indicating that inhibition of ovulation and reproductive cycle arrest in the proestrus stage had occurred, coadministration with DFMO did not affect these changes. Thus, coadministration of DFMO with tamoxifen did not result in toxicity unique to the combination drug regimen, but rather toxicity resulting from administration of each drug. Under the conditions of the study, the overall

toxicity produced by dual administration of DFMO with tamoxifen was additive with respect to the toxicity associated with each agent alone. In conclusion, based upon the results observed in this study and the clinical experience with each drug alone, clinical trials of the coadministration of DFMO and tamoxifen for cancer chemoprevention would be warranted.

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