Tamoxifen-induced Antitumorigenic/Antiestrogenic Action Synergized by a Selective Aryl Hydrocarbon Receptor Modulator

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ABSTRACT

Tamoxifen (TAM) is a highly effective selective estrogen receptor (ER) modulator used extensively for the treatment and prevention of breast cancer. However, prolonged treatment of women with TAM may be a risk factor for endometrial cancer, and research in our laboratory is focused on the development of selective aryl hydrocarbon receptor modulators that can be used in combination with TAM to improve its efficacy in the breast and inhibit TAM-induced endometrial effects. This study investigated the effects of the selective aryl hydrocarbon receptor modulators 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) alone and in combination with TAM in the carcinogen-induced mammary tumor model and in the ovariectomized uterotropic assay using female Sprague Dawley rats. The lowest effective dose of 6-MCDF that inhibited tumor growth was 50 μg/kg/day, and TAM was antitumorigenic at a dose of 100 μg/kg/day. In animals cotreated with TAM + 6-MCDF at doses of 50, 100, or 25 μg/kg/day of each compound, complete inhibition of mammary tumor growth was observed at all doses, and the results are consistent with a more than additive antitumorigenic response for the low dose group (25 + 25 μg/kg) and additive interactions at the 50 and 100 μg/kg doses. In a separate experiment, 6-MCDF (800 μg/kg) inhibited TAM-induced peroxidase activity and progesterone receptor binding in the ovariectomized rat uterus but did not affect TAM-induced bone growth in ovariectomized rats. This study also investigated the effects of TAM and 6-MCDF alone and in combination on ERα protein levels in MCF-7 human breast cancer cells as a model for studying interactions between these compounds. The results show that 6-MCDF decreased TAM-induced ERα levels in the absence or presence of 17β-estradiol through proteasome activation, and these interactions may contribute to the observed combined antitumorigenic effects of these compounds.

INTRODUCTION

Breast cancer is among the leading causes of premature death in women, and a high proportion of early stage mammary tumors are ERα-positive and amenable to endocrine therapy (1). SERMs, such as the antiestrogen TAM, are used extensively for successful treatment of breast cancer and as chemopreventive agents for women at high risk for this disease (2–4). However, there is concern regarding prolonged treatment of women with TAM due to its ER agonist activity in the uterus and the potential increased risk for endometrial cancer (5). New SERMs such as raloxifene that may pose lower risks for endometrial cancer are being developed as alternative treatments. Additionally, ligands for other receptors including the retinoic acid receptor, peroxisome proliferator-activated receptor, and the vitamin D receptor are also being developed as indirect antiestrogens (6, 7) because these ligand-activated receptors inhibit estrogen action through complex receptor cross-talk pathways. Because TAM is highly effective for treatment and prevention of breast cancer, it is also important to develop new drugs that can be used in combination with TAM, not only to improve its efficacy in the breast but also to diminish possible adverse side effects, such as endometrial cancer.

Research in this laboratory has focused on AhR-mediated inhibition of E2-induced gene expression and development of SAhRMs for treatment of breast cancer (8). The AhR was characterized initially by its high binding affinity for the environmental toxicant TCDD (9), and inhibitory AhR-ERα cross-talk has been investigated extensively both in vitro and in vivo. In Sprague Dawley rats, TCDD inhibits spontaneous and carcinogen-induced mammary cancers, which are known to be E2 dependent (10, 11), and TCDD prevents E2-dependent tumor growth in immuno-suppressed B6C3F1 mice bearing MCF-7 breast cancer cell xenographs (12). In the rodent uterus, TCDD blocks E2-induced hypertrophy. PR and ER binding, peroxidase activity, and expression of the epidermal growth factor receptor and fos genes (11, 13). TCDD inhibits E2-induced proliferation, DNA synthesis, and expression of PR and other E2-responsive genes in MCF-7 human breast cancer cells and Ishikawa human endometrial adenocarcinoma cells (13, 14). The mechanisms underlying the inhibitory cross-talk between the AhR and ER appear to be complex. TCDD activation of the AhR induces proteasome-mediated degradation of ERα in breast cancer cells (15) and induces cytochrome P450 enzymes that metabolize E2 and lower cellular levels of this hormone (16). Another mechanism of inhibitory AhR-ER cross-talk involves direct interactions of the AhR complex with inhibitory dioxin-responsive elements in the 5′-regulatory regions of E2-responsive genes, resulting in inhibition of E2-induced gene expression through multiple gene promotor-specific pathways (17–19).

There is evidence that AhR agonists such as TCDD and polynuclear aromatic hydrocarbons inhibit mammary and endometrial tumor formation in humans (8, 20, 21). Our laboratory has developed two classes of relatively nontoxic AhR agonists, namely, alternate substituted alkyl polychlorinated dibenzo-furans and diindolylmethane analogues, that inhibit growth of DMBA-induced mammary tumor in female Sprague Dawley rats (22–25); moreover, these compounds also exhibit antitumor activity in the rodent uterus (8, 23, 25, 26). Both classes of SAhRMs have minimal effects on classical AhR-mediated toxic responses observed in most rodent and cell culture models. Moreover, 6-MCDF, the prototypical SAhRM used in this study, inhibits TCDD-induced CYP1A1 gene expression, immunotoxicity, porphyria, and teratogenicity in mice through partial AhR antagonism (8).

This study describes inhibition of carcinogen-induced mammary tumor growth in female Sprague Dawley rats by TAM, 6-MCDF, and their combination and antagonism of TAM-induced uterine effects by 6-MCDF in the ovariectomized rat model, suggesting that both drugs in combination may be highly effective for treating breast cancer.

MATERIALS AND METHODS

Cell Maintenance and Western Blot Analysis. MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Manassas,
Fig. 1. ERα protein levels are modulated through the ER and AhR pathways. Western blot analysis was performed on 30 μg of protein from each treatment group separated by SDS PAGE, and the resulting autoradiograph was quantitated using a densitometer. C, control treatment; E, treatment with 1 nM E2; M, treatment with 1 μM 6-MCDF; and 4T, treatment with 4-OHTAM. STD, the loading of pure Sp1 protein and in vitro translated ERα as standards. E2 and 6-MCDF treatment significantly (P < 0.05) decreased ERα levels compared with control treatment, and treatment with E2 + 6-MCDF resulted in a further decrease in ERα levels. 4-OHTAM treatment significantly (P < 0.05) increased ERα levels compared with controls. ERα down-regulation by E2 was blocked after cotreatment with 4-OHTAM. 6-MCDF significantly (P < 0.05) decreased induction of ERα protein by 4-OHTAM in the presence or absence of E2. Results are the means ± SEs for at least three separate experiments for each treatment group, and representative Western blots are shown. Means with P < 0.05 less than controls are indicated with an a, means with P < 0.05 less than E2 treatment are indicated with an b, and means with P < 0.05 less than 4-OHTAM treatment are indicated with a c.

VA). Cells were grown on monolayer cultures in DMEM:Ham’s F-12 media with phenol red supplemented with 2.2 grams/liter sodium bicarbonate, 0.2 gram/liter BSA, 10 mg/liter apo-transferrin, 5% fetal bovine serum (Intergen, Purchase, NY), and antibiotic-antimycotic solution (pH 7.4). Cells were seeded into 35-mm 6-well plates in phenol-free media containing 2.5% charcoal-stripped fetal bovine serum. The next day, cells were treated in fresh media for 1 h with intermittent vortexing followed by centrifugation (40,000 × g, 10 min, 4°C). Equal amounts of protein from each treatment group, as well as pure Sp1 protein (Promega Corp., Madison, WI) and in vitro translated ERα (15) standards, were separated by SDS-PAGE in a 7.5% acrylamide/bisacrylamide gel and electrophoresed to polyvinylidene difluoride membrane in transfer buffer (48 mM Tris, 39 mM glycine, and 0.025% SDS). Membranes were blocked for 15 min in Blotto (5% milk + TBS) and then probed with the polyclonal antibodies for ERα (sc-544; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:1,000 dilution in Blotto or with antibodies for Sp1 at a 1:10,000 dilution (sc-59; Santa Cruz Biotechnology, Inc.). After 5 h, the membrane was washed twice (5 min each time) in TBS + 0.05% Tween 20, probed with secondary peroxidase-conjugated antibody (diluted 1:5,000 in Blotto) for 2 h, washed three times (5 min each time) in TBS + 0.05% Tween 20, washed once for 5 min in TBS, and then visualized using the enhanced chemiluminescence detection system (New England Nuclear, Boston, MA). ERα protein levels in each treatment group were determined relative to the equal amounts of protein (Promega Corp., Madison, WI) and in vitro translated ERα (15) standards, as determined through the vehicle control (DMSO) group, which were set at 100%. Results are expressed as the means ± SE for at least three separate determinations for each treatment group. The Western blot analysis of Sp1 protein was derived from a single experiment.

Mammary Tumor Studies. Virgin female Sprague Dawley rats were obtained from Harlan (Houston, TX) and treated p.o. at 50 days of age with a single dose of DMBA (20 mg/rat). Within 45–75 days, tumors could be detected by palpation; when the tumors reached a small predetermined size (100–200 mm3), rats were treated by gavage with corn oil (vehicle) or test compounds dissolved in corn oil daily for 20 days at a final volume of 2 ml/kg, with 8–10 rats/treatment group (22–24). The assay was normally limited to 1 tumor/animal; in a few cases where two tumors of equal size were observed in the same animal, the daily tumor volumes were averaged to give a single data point per animal. Tumors were measured with calipers, and volume was calculated by the formula: (length/2) × (width/2) × (length/2) × π.

Fig. 2. Inhibition of DMBA-induced rat mammary tumor growth by TAM and 6-MCDF. Rats were treated by gavage with corn oil (vehicle) or test compounds in corn oil daily for 20 days; four rats received control vehicle; five rats were treated with 400 μg/kg TAM, five rats were treated with 800 μg/kg 6-MCDF; and six rats were treated with 200 μg/kg 6-MCDF. Rats were treated by gavage or gavage with corn oil (vehicle) or test compounds dissolved in corn oil daily for 20 days and euthanized on day 21. Tumors and selected organs were excised, weighed, and processed for histopathological examination. Hepatic microsomal extracts were prepared and analyzed for EROD activity as described previously (22–24).

Uterine and Bone Study in Ovariectomized Rats. Cycling female virgin Sprague Dawley rats were ovariectomized at approximately 90 days of age. Animals were allowed to acclimate for 3 weeks before treatments to allow serum hormone levels to normalize and so that the age of the ovariectomized rats would correspond to the age of rats in the tumor studies. Rats were treated by gavage with corn oil (vehicle) or test compounds in corn oil daily for 20 days; four rats received control vehicle; five rats were treated with 400 μg/kg TAM, five rats were treated with 800 μg/kg 6-MCDF; and six rats were treated with 200 μg/kg 6-MCDF.
The effects of TAM, 6-MCDF, or TAM on tumor growth were not affected by treatment in MCF-7 human breast cancer cells. E2 and 6-MCDF alone each and 25 \( \mu \)g/kg cotreated with 400 \( \mu \)g/kg TAM + 800 \( \mu \)g/kg 6-MCDF. On day 21, animals were euthanized, and uterus were immediately excised, weighed, bisected, and processed; livers were weighed and frozen in liquid nitrogen so that hepatic microsomes could be prepared and tested for EROD activity, and other organs (heart, spleen, and kidneys) were weighed and checked for gross pathology. The uterine cytosolic progesterone receptor assay, calculated as fmol receptor/uterus, and the uterine nuclear peroxidase activity assay, calculated as absorbance units/min/uterus, were performed as described previously (23). Uterine wet weight was measured after blotting and before bisecting each uterus. Left and right femurs were excised, and their length was measured with calipers; the femurs were then washed for 4 h with PBS (pH 2.0) to soften muscle and connective tissue, allowing bones to be completely cleaned before blot drying and measurement of wet weight. Femurs were dried for 12 h at 130°C and allowed to cool, and dry weight was measured. Data were normalized to body weight and are expressed as a percentage of control.

Data Analysis. Statistical differences among groups were determined using ANOVA and Duncan’s new multiple range. Results are expressed as the means ± SE. The fractional method of Webb (27, 28) and the simple isobologram method of Lowe (27, 29) were used to determine synergistic interactions of 6-MCDF and TAM. The Q test for removal of outliers was performed for the interaction studies, and one rat from the control group, one rat from the TAM (50 \( \mu \)g/kg)-treated group, and one rat from the 6-MCDF (25 \( \mu \)g/kg)–treated group, were removed.

RESULTS

Activation of Proteasome Pathways by 6-MCDF. Fig. 1 shows the separate and combined effects of a 24-h treatment with E2, 6-MCDF, and 4-OHTAM on intracellular ERα and Sp1 protein levels in MCF-7 human breast cancer cells. E2 and 6-MCDF alone each decreased ERα levels relative to control, and treatment with E2 + 6-MCDF caused a further decrease in ERα levels. Treatment with proteasome inhibitors blocked ER down-regulation by E2, 6-MCDF, and AhR agonists (data not shown; Ref. 15). 4-OHTAM, the active metabolite of TAM, significantly increased ERα levels and prevented the E2-induced degradation of ER and cotreatment with 6-MCDF partially reversed this effect of 4-OHTAM, in both the presence and absence of E2. Levels of Sp1, a transcription factor important in basal expression of many different genes, were not affected by treatment and serve as a control for this study as described previously (15).

Interaction of TAM and 6-MCDF on Inhibition of Mammary Tumor Growth. The effects of TAM, 6-MCDF, or TAM + 6-MCDF on the growth of DMBA-induced mammary tumors (adenocarcinomas) in Sprague Dawley rats are summarized in Fig. 2 and Table 1. The lowest effective doses of TAM and 6-MCDF that inhibited mammary tumor growth were 100 and 50 \( \mu \)g/kg/day, respectively, whereas doses of 50 and 25 \( \mu \)g/kg/day (TAM) or 25 \( \mu \)g/kg/day (6-MCDF) were inactive. In contrast, combined treatment with TAM + 6-MCDF using doses of 100, 50, and 25 \( \mu \)g/kg/day was effective at inhibiting mammary tumor growth.

<p>| Table 1: Effects of TAM, 6-MCDF, and their combinations on final body weight, tumor volumes, and weights in rats bearing mammary tumors |</p>
<table>
<thead>
<tr>
<th>Treatment (dose, ( \mu )g/kg)</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Final tumor volume (mm³)</th>
<th>Final tumor weight (g)</th>
<th>Final tumor volume &lt;50% of initial volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>268 ± 5</td>
<td>275 ± 5</td>
<td>7730 ± 2530</td>
<td>16.0 ± 6</td>
<td>0/10</td>
</tr>
<tr>
<td>TAM (25 ( \mu )g/kg)</td>
<td>269 ± 8</td>
<td>271 ± 7</td>
<td>9330 ± 3140</td>
<td>13.2 ± 5</td>
<td>3/10</td>
</tr>
<tr>
<td>TAM (50 ( \mu )g/kg)</td>
<td>271 ± 9</td>
<td>267 ± 10</td>
<td>5060 ± 2920</td>
<td>6.8 ± 3</td>
<td>2/9</td>
</tr>
<tr>
<td>TAM (100 ( \mu )g/kg)</td>
<td>296 ± 13</td>
<td>295 ± 16</td>
<td>3050 ± 1180</td>
<td>4.9 ± 2</td>
<td>4/10</td>
</tr>
<tr>
<td>6-MCDF (25 ( \mu )g/kg)</td>
<td>273 ± 7</td>
<td>275 ± 8</td>
<td>5000 ± 1530</td>
<td>8.0 ± 4</td>
<td>0/9</td>
</tr>
<tr>
<td>6-MCDF (50 ( \mu )g/kg)</td>
<td>260 ± 5</td>
<td>265 ± 7</td>
<td>2710 ± 4300</td>
<td>3.7 ± 1</td>
<td>1/9</td>
</tr>
<tr>
<td>6-MCDF (100 ( \mu )g/kg)</td>
<td>277 ± 8</td>
<td>276 ± 11</td>
<td>1510 ± 540</td>
<td>2.3 ± 0.6</td>
<td>3/10</td>
</tr>
<tr>
<td>TAM (25 ( \mu )g/kg) + 6-MCDF (25 ( \mu )g/kg)</td>
<td>270 ± 5</td>
<td>258 ± 6</td>
<td>1390 ± 650</td>
<td>3.5 ± 2</td>
<td>4/10</td>
</tr>
<tr>
<td>TAM (50 ( \mu )g/kg) + 6-MCDF (50 ( \mu )g/kg)</td>
<td>280 ± 8</td>
<td>282 ± 10</td>
<td>2280 ± 640</td>
<td>4.2 ± 2</td>
<td>2/8</td>
</tr>
<tr>
<td>TAM (100 ( \mu )g/kg) + 6-MCDF (100 ( \mu )g/kg)</td>
<td>290 ± 6</td>
<td>277 ± 9</td>
<td>869 ± 347</td>
<td>0.96 ± 0.45</td>
<td>7/9</td>
</tr>
</tbody>
</table>

* After the first appearance of mammary tumors (100–200 mm³), female rats bearing DMBA-induced tumors were treated with TAM, 6-MCDF, or their combination for 20 days and sacrificed, and tumor and organ weights were determined as described in “Materials and Methods.” There were no significant differences in body weights or organ (uterus, liver, heart, spleen, and kidney) body weight ratios in any of the treatment groups (data not shown). Values are expressed as means ± SE.

* Significantly lower (P < 0.05) than observed in vehicle (corn oil)-treated animals.
Table 2. Effects of TAM, 6-MCDF, and their combination on body weight (BW) and organ weights and uterine and bone responses in ovariectomized rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial BW (g)</th>
<th>Control</th>
<th>TAM (400 μg/kg)</th>
<th>6-MCDF (800 μg/kg)</th>
<th>TAM (400 μg/kg) + 6-MCDF (800 μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>255 ± 3</td>
<td>261 ± 9</td>
<td>257 ± 4</td>
<td>244 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>295 ± 11</td>
<td>242 ± 6⁰</td>
<td>289 ± 7bc</td>
<td>236 ± 11bc</td>
<td></td>
</tr>
<tr>
<td>Uterine wet weight (g)</td>
<td>0.17 ± 0.015</td>
<td>0.21 ± 0.009⁰</td>
<td>0.13 ± 0.004⁰c</td>
<td>0.20 ± 0.009⁰b</td>
<td></td>
</tr>
<tr>
<td>Uterine wet weight (% BW)</td>
<td>0.058 ± 0.006</td>
<td>0.086 ± 0.004⁰</td>
<td>0.045 ± 0.002⁰c</td>
<td>0.085 ± 0.006⁰b</td>
<td></td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>11.7 ± 0.5</td>
<td>9.7 ± 0.4bc</td>
<td>11.0 ± 0.6</td>
<td>9.6 ± 0.7a</td>
<td></td>
</tr>
<tr>
<td>Liver weight (% BW)</td>
<td>3.95 ± 0.16</td>
<td>4.03 ± 0.15</td>
<td>3.78 ± 0.11</td>
<td>4.07 ± 0.20a</td>
<td></td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.06 ± 0.03</td>
<td>0.86 ± 0.04⁰</td>
<td>1.03 ± 0.04⁰c</td>
<td>0.87 ± 0.03⁰b</td>
<td></td>
</tr>
<tr>
<td>Heart weight (% BW)</td>
<td>0.360 ± 0.010</td>
<td>0.357 ± 0.023</td>
<td>0.356 ± 0.012</td>
<td>0.374 ± 0.023</td>
<td></td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.86 ± 0.07</td>
<td>0.61 ± 0.04⁰</td>
<td>0.76 ± 0.04⁰c</td>
<td>0.59 ± 0.04⁰b</td>
<td></td>
</tr>
<tr>
<td>Spleen weight (% BW)</td>
<td>0.269 ± 0.018</td>
<td>0.254 ± 0.018</td>
<td>0.265 ± 0.014</td>
<td>0.250 ± 0.004a</td>
<td></td>
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<tr>
<td>Kidney weight (g)</td>
<td>0.96 ± 0.03</td>
<td>0.89 ± 0.04</td>
<td>1.04 ± 0.07⁰c</td>
<td>0.86 ± 0.04⁰b</td>
<td></td>
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<tr>
<td>Kidney weight (% BW)</td>
<td>0.334 ± 0.010</td>
<td>0.369 ± 0.017</td>
<td>0.352 ± 0.015</td>
<td>0.357 ± 0.013</td>
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<tr>
<td>Femur length (mm)</td>
<td>35.6 ± 0.3</td>
<td>34.4 ± 0.2⁰</td>
<td>35.1 ± 0.2⁰e</td>
<td>34.9 ± 0.2⁰f</td>
<td></td>
</tr>
<tr>
<td>Femur length (% BW)</td>
<td>12.1 ± 0.4</td>
<td>14.2 ± 0.3⁰b</td>
<td>12.2 ± 0.3</td>
<td>15.0 ± 0.9⁰a</td>
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<tr>
<td>Femur wet weight (g)</td>
<td>1.02 ± 0.01</td>
<td>0.98 ± 0.03</td>
<td>0.98 ± 0.02</td>
<td>0.96 ± 0.02a</td>
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<tr>
<td>Femur wet weight (% BW)</td>
<td>0.35 ± 0.01</td>
<td>0.40 ± 0.01⁰</td>
<td>0.34 ± 0.01</td>
<td>0.41 ± 0.02b</td>
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</tr>
<tr>
<td>Femur dry weight (g)</td>
<td>0.53 ± 0.01</td>
<td>0.52 ± 0.02</td>
<td>0.53 ± 0.01</td>
<td>0.55 ± 0.01a</td>
<td></td>
</tr>
<tr>
<td>Femur dry weight (% BW)</td>
<td>0.18 ± 0.001</td>
<td>0.22 ± 0.005⁰</td>
<td>0.18 ± 0.003</td>
<td>0.24 ± 0.01b</td>
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<tr>
<td>Progesterone receptor binding (fmol/uterus)</td>
<td>765 ± 69</td>
<td>9062 ± 244⁰</td>
<td>441.5 ± 100⁰b</td>
<td>1875 ± 306⁰c</td>
<td></td>
</tr>
<tr>
<td>Peroxidase activity (absorbance/min/uterus)</td>
<td>1.00 ± 0.01</td>
<td>38.66 ± 0.32⁰</td>
<td>0.57 ± 0.01⁰c</td>
<td>19.55 ± 0.33⁰bc</td>
<td></td>
</tr>
</tbody>
</table>

a Ovariectomized female Sprague-Dawley rats were treated with TAM, 6-MCDF, and their combinations for 20 days, and body/organ weights, uterine responses, and bone weight and length were determined as described in “Materials and Methods.” Values are expressed as mean ± SE.

b Significantly different (P < 0.05) from control treatment.

c Significantly different (P < 0.05) from TAM treatment.

d Significantly different (P < 0.05) from 6-MCDF treatment.

Interaction of TAM and 6-MCDF on Uterine Responses and Bone Growth. The ovariectomized rat is a classical model for measuring the effects of SERMs such as TAM in uterus and bone (31). Interactions of TAM + 6-MCDF were determined in ovariectomized female Sprague Dawley rats using an estrogenic dose of TAM (400 μg/kg/day) for 20 days in combination with 6-MCDF at 800 μg/kg/day (Fig. 3 and Table 2) or 400 μg/kg (data not shown). TAM significantly induced uterine wet weight, peroxidase activity, and levels of progesterone receptor binding. 6-MCDF (800 μg/kg/day) significantly inhibited uterine peroxidase activity and did not induce wet weight or PR binding. In animals treated with TAM + 6-MCDF, minimal changes in uterine wet weight were observed compared with treatment with TAM alone; however, TAM + 6-MCDF (400 and 800 μg/kg/day) significantly inhibited uterine peroxidase activity, and TAM + 6-MCDF (800 μg/kg) inhibited PR binding (Fig. 3 and Table 2). These results show that 6-MCDF inhibits some TAM-induced uterine responses, and this is consistent with a previous study showing that a single dose of 43 mg/kg 6-MCDF completely inhibited E₂-induced uterine weight increase and PR induction in the immature rat (26). In the same ovariectomized female Sprague Dawley rats, treatment with TAM alone (400 μg/kg/day) resulted in significantly increased femur length and wet and dry weight. 6-MCDF (800 μg/kg/day) alone had no effect on bone growth, and the effects of treatment with TAM + 6-MCDF did not differ from those observed for treatment with TAM alone (Fig. 3B and Table 2). ER agonists inhibit weight gain in ovariectomized adult rats (31, 32), and in this study, TAM (with or without 6-MCDF) decreased rat body weight over the 21-day study (7% and 3%, respectively; Table 2). 6-MCDF had no effect on body weight compared with controls, and significant weight gain was observed in the two groups of rats over the 21-day study (12% and 16%, respectively; Table 2).

DISCUSSION

The remarkable efficacy of the combined therapy (TAM + 6-MCDF) is evident from the low-dose treatment (25 μg/kg/day) in which the compounds alone were inactive, but the compounds in combination inhibited mammary tumor growth. Although it is difficult to definitively characterize drug interactions in small studies, the application of two different test models (27–29) to our data indicates that the antitumorigenic effects of 6-MCDF and TAM at the 25 + 25 μg/kg dose level were synergistic and are additive at the 50 + 50 and 100 + 100 μg/kg doses for inhibition of rat mammary tumor growth. Additionally, 6-MCDF inhibited two TAM-induced markers of estrogenicity in the uterus without affecting the desirable ER agonist activity of TAM on bone growth (Fig. 3B and Table 2) or body weight, and this is consistent with other studies indicating that 6-MCDF blocks the uterotrophic effects of estrogen in a dose-dependent fashion (8, 26). These results demonstrate that SAhRMs acting through inhibitory Ah-R cross-talk pathways are not only potent antitumor agents but also markedly enhance the effects of TAM as an antiestrogen in the carcinogen-induced rat mammary tumor model. Thus, the use of 6-MCDF and other SAhRMs in combination with direct-acting antiestrogens such as TAM may lower the effective

50, or 25 μg/kg/day of each compound resulted in virtually complete inhibition of mammary tumor growth, and in some of the treatment groups, final tumor volumes were less than the initial volumes, indicating some growth stasis. Interactions were tested by comparing tumor volumes and by comparing the rate of tumor growth (slope of linear least squares fit). The fractional product method of Webb (27, 28) showed that the interactions of TAM + 6-MCDF at doses of 100 and 50 μg/kg are additive, and the interaction at 25 μg/kg is synergistic. This method was designed to compare the fractional inhibition of a response by two inhibitors with their effect in combination. The simplified isobologram method of Lowe (27, 29) distinguished a synergistic interaction at 25 μg/kg by comparing growth rates but was not robust enough to distinguish synergism from additivity for the interactions at 100 and 50 μg/kg. This analysis compares the effect of the addition of a second drug to the activity of the first drug against the effect of the addition of more of the first drug to itself. Body and organ (uterus, liver, heart, spleen, and kidneys) weights were unchanged in all treatment groups. No induction of hepatic CYP1A1-dependent EROD activity was observed in animals treated with 6-MCDF (alone or in combination), and this was consistent with previous studies showing that 6-MCDF induced EROD activity in rodents only at doses > 50 mg/kg (30).
dose of both compounds for treatment of human breast cancer while affording protection from SERM-induced estrogenic responses in the uterus.

Both AhR agonists and TAM inhibit the growth of DMBA-induced mammary tumors in rats (Fig. 2) and tumors in mice bearing MCF-7 cell xenografts (12, 25). In vitro, 6-MCDF and TAM alone also inhibit E$_2$-induced growth of MCF-7 cells and expression of E$_2$-regulated genes through inhibitory AhR-ER$_a$ cross-talk (for 6-MCDF; Ref. 8) and direct interactions with ER$_a$ (for TAM; Ref. 2). Mechanisms of action of 6-MCDF and TAM are complex and are likely to be gene specific (2).

Recent studies showed that treatment of MCF-7 cells with E$_2$ resulted in proteasome-dependent degradation of ER$_a$ and this response modulates the duration of hormone-induced activation of transcription (15, 33–35). Our laboratory has also shown that AhR agonists such as TCDD and 6-MCDF simultaneously induce proteasome-dependent degradation of the AhR and ER$_a$ in MCF-7 and T47D breast cancer cells (15). Moreover, in MCF-7 cells treated with E$_2$ + TCDD or 6-MCDF, dual activation of proteasome pathways resulted in even lower levels of immunoactive ER$_a$, and this may contribute to inhibitory AhR-ER$_a$ cross-talk by limiting cellular levels of ER$_a$.

Using MCF-7 cells as a model, we have investigated the effects of 4-OHTAM, 6-MCDF, and their combination on ER$_a$ levels. Because the growth of DMBA-induced mammary tumors and MCF-7 cell xenografts in rodent models is dependent on E$_2$, the effects of 4-OHTAM and 6-MCDF in combination with E$_2$ were also investigated. Fig. 1 shows that TAM, alone and in combination with E$_2$, increases levels of ER$_a$ protein in whole cell extracts, and this contrasts with the down-regulation of ER$_a$ observed after treatment with E$_2$ alone, 6-MCDF alone, or E$_2$ + 6-MCDF. These responses are blocked by proteasome inhibitors (Ref. 15; data not shown). It is paradoxical that 4-OHTAM increases ER$_a$ protein in MCF-7 cells (Fig. 1) but inhibits cell proliferation, and this has been observed previously in breast cancer cells (36–38). In contrast, TAM/4-OHTAM, which acts as an estrogen in the endometrium, decreases ER levels in this tissue (39–42). Cell context-dependent effects of TAM on ER$_a$ may be related to altered subcellular distribution or phosphorylation of ER$_a$, and this is currently being investigated. Additionally, it has been suggested that the conformational changes induced in ER by TAM that modulate coactivator binding and transcriptional activation may also affect recruitment of proteins necessary for proteasome-mediated degradation of ER$_a$ (36, 43). However, our results show that the combined effects of 6-MCDF + 4-OHTAM alone and in combination with E$_2$ result in significantly decreased ER$_a$ protein levels compared with those observed after treatment with 4-OHTAM alone. Additional studies are required to elucidate the degree to which TAM-induced ER stabilization or destabilization induces tissue-specific estrogenic and antiestrogenic responses and how the degradation of ER$_a$ in the presence of SARMs modulates these effects. These interactions may play a significant role in mediating the potent combined effects of 6-MCDF and TAM as inhibitors of mammary tumor growth in the DMBA-induced rat mammary tumor model.

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Tamoxifen-induced Antitumorigenic/Antiestrogenic Action Synergized by a Selective Aryl Hydrocarbon Receptor Modulator

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