The Impact of Fish Oil on the Chemopreventive Efficacy of Tamoxifen against Development of N\textsuperscript{-}Methyl-N\textsuperscript{-}Nitrosourea–Induced Rat Mammary Carcinogenesis

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Abstract

The antiestrogen tamoxifen reduces breast cancer incidence in high-risk women but is unable to inhibit the development of hormone-independent tumors. Omega-3 polyunsaturated fatty acids (n-3 PUFA), known ligands of the peroxisome proliferator activated receptor-\(\gamma\) (PPAR\(\gamma\)), generally exert tumor-suppressive effects. Based on the known crosstalk between the estrogen and the PPAR\(\gamma\) receptors, we tested the hypothesis that the combination of tamoxifen with n-3 PUFA results in a superior antitumor action over the individual interventions. In this study, we report for the first time that the combination of a fish oil diet rich in n-3 PUFA and tamoxifen seemed to inhibit N\textsuperscript{-}methyl-N\textsuperscript{-}nitrosourea–induced mammary carcinogenesis, tumor multiplicity, and volume to a greater extent than the individual interventions. The potential superiority of the combination was particularly evident at a suboptimal dose of tamoxifen, which, by itself, was unable to significantly decrease tumor development. Because activation of PPAR\(\gamma\) is known to inhibit oxidative stress, we examined the effects of our interventions on circulating and tumor levels of glutathione, a major intracellular antioxidant. Our results indicate that reduction in the level of oxidative stress may be a potential mechanism by which the n-3 PUFA–rich diet potentiated the tumor-suppressive effect of tamoxifen. Our interventions were well tolerated without evidence of toxicity. Combined administration of tamoxifen and n-3 PUFA is a promising new approach to breast cancer prevention. Because of its safety, this combination can quickly be translated to the clinic if its superiority can be supported by future studies. Cancer Prev Res; 3(3); 322–30. ©2010 AACR.

Introduction

The antiestrogens tamoxifen and raloxifene have been shown to be effective chemopreventive agents in women at high risk of having breast cancer (1, 2). However, the clinical impact of these drugs is limited by their inability to prevent hormone-independent tumors for which there are currently no effective chemopreventive interventions. Furthermore, their use is associated with side effects such as thromboembolic events, which, although rare, are significant when considering that these drugs are given to normal women for breast cancer prevention.

In established breast cancer, it is well known that there is positive bidirectional crosstalk between the estrogen receptor and various growth factor signaling pathways, which can eventually lead to tumor progression and hormone resistance (3). Concomitant targeting of the estrogen receptor and interacting oncogenic pathways has indeed been shown to have a superior antitumor action in preclinical breast cancer models, preventing or delaying the development of antiestrogen resistance (4). Based on this experimental data, this combined treatment strategy is currently being tested in therapeutic clinical trials (4).

The experiments reported in this article use an established rodent model of mammary carcinogenesis (5) to test the novel hypothesis that similar mechanisms may be operating during tumor development and that a superior tumor-preventive effect, possibly to include prevention of hormone-independent tumors, may be achieved by a combined biological approach targeting the estrogen receptor and other interacting cell signaling pathways. Specifically, we tested the combination of different doses of tamoxifen with a diet rich in omega-3 polyunsaturated fatty acids (n-3 PUFA), known ligands of the peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\); ref. 6), which are known to exert protective effects against some common cancer types.
cancers, including breast cancer (7–9). The rationale for this combined strategy is based on the documented crosstalk between the estrogen receptor and the PPARγ receptor in breast cancer cells (10, 11). Although estrogen receptor activation has been shown to suppress PPARγ-mediated transcription, estrogen receptor antagonists such as ICI182780 and 4-hydroxy-tamoxifen have been shown to stimulate PPARγ transactivation and to potentiate the antiproliferative activity of a PPARγ agonist in breast cancer cells (10). Activation of PPARγ has recently been shown to inhibit oxidative stress (12, 13), thus raising the possibility that this could be a mechanism of cooperativity between tamoxifen and n-3 PUFA in suppressing mammary carcinogenesis. Modification of oxidative stress has indeed been proposed as one of the mechanisms by which n-3 PUFA exert their antitumor action (14–16). As an initial step to investigate whether this mechanism may be operating in our system, we tested the effects of fish oil and tamoxifen on circulating and tumor levels of glutathione (GSH), a major intracellular antioxidant.

In addition to its possible superior antitumor action, this combination strategy is attractive from a safety standpoint because n-3 PUFA lack significant toxicity and may have added health benefits such as reduction in cardiovascular risk (17). Furthermore, because of the anticipated synergism between PPARγ activation and antiestrogen administration, we may be able to use lower, and hence potentially less toxic, doses of tamoxifen and retain the chemopreventive efficacy.

Materials and Methods

Reagents
1-Methyl-1-nitrosourea (MNU) was obtained from the National Cancer Institute Chemical Repository. Tamoxifen was purchased from Sigma. The Ki67 antibody was obtained from DAKOCytomation, and the cleaved caspase-3 antibody was purchased from Cell Signaling Technology.

Diets
Modified AIN-76A diets were designed by TestDiet. All dietary ingredients were obtained from TestDiet except for Virginia Prime Gold Fish Oil (Omega Protein). The diets were prepared weekly in our laboratory, flushed with nitrogen, and stored at 4°C until use. The high-fat corn oil and high-fat fish oil diet were isocaloric (4.54 kcal/g), whereas the low-fat corn oil was not (3.84 kcal/g). The compositions of the diets are shown in Supplementary Table S1. The calculated n-3 to n-6 ratio was 0.005 in the 20% corn oil diet and 2.3 in the 17% fish oil + 3% corn oil diet.

Experimental model and design
Female Sprague-Dawley rats with 10 female pups were purchased from Charles River Laboratories International, Inc. Body weights were obtained at the day of weaning (day 20). At 21 d of age, groups of rats were matched for weight (n = 35-39 per group), injected i.p. with 50 mg MNU/kg body weight, and randomly assigned to the following experimental interventions for the next 8 wk: (1) 5% corn oil; (2) 20% corn oil; (3) 20% corn oil + 50 μg/kg tamoxifen, s.c., 5 d/wk; (4) 20% corn oil + 100 μg/kg tamoxifen, s.c., 5 d/wk; (5) fish oil (17% fish oil, 3% corn oil); (6) fish oil + 50 μg/kg tamoxifen, s.c., 5 d/wk; (7) fish oil + 100 μg/kg tamoxifen, s.c., 5 d/wk. These doses of tamoxifen were selected based on previous chemopreventive studies with this drug in a related MNU rat mammary tumor experimental system (18). Rats were grouped three per cage and fed ad libitum with the indicated modified AIN-76A purified powdered diets. The diets were changed every 2 to 3 d.

Palpable tumors started to develop by week 5, and their incidence was recorded weekly until the time of sacrifice at week 8. At week 8 after MNU injection, rats were euthanized through CO2 asphyxiation in a numerical order while under anesthesia. All rats were necropsied, and their livers were removed, weighed, and subjected to histologic analysis according to previously published criteria (5). All images were obtained with an Olympus BX51 microscope and a DP71 digital camera using MicroSuite Basic 2.6 imaging software.

The two abdominal inguinal mammary fat pads were carefully excised from each rat and spread onto clean 50 × 75 mm prelabeled microscope slides (Brain Research Laboratories) for whole-mount preparation. The slides were left out for 4 to 5 min to allow the mammary fat pad to tack on, and then prepared as previously described (5). The mammary fat pads were processed for routine histologic analysis as previously reported (5).

To ensure lack of toxicity from our interventions, liver enzymes were measured in selected rats per group. In addition, their livers were removed, weighed, and subjected to histologic analysis according to previously published criteria (19).

Immunohistochemistry and counts of apoptotic bodies
Immunohistochemistry for Ki67 and cleaved caspase-3 was done as previously described by us (20) using a monoclonal rat anti-mouse antibody for Ki67 and a rabbit polyclonal antibody for cleaved caspase-3. Labeling was detected with EnVision + horseradish peroxidase-labeled polymer, visualized with 3,3′-diaminobenzidine, and counterstained with Mayerʹs hematoxylin (DAKO-Cytomation). Scoring for Ki67 was done by one of us (R.B.), blinded to the treatment, using the image analysis...
software in Adobe Photoshop CS3, which recorded the number of positively stained cells in three random fields (400×) per sample. Scoring for cleaved caspase-3 (too low for adequate detection by this software) was blindly done by one of us (T.C.) by counting the number of positively stained cells in three replicate 200× fields. The number of apoptotic bodies was similarly counted by T.C. in three replicate 400× fields per tumor.

Analysis of liver enzymes and GSH

For liver enzymes, serum samples (n = 10/group) were centrifuged, aliquoted, and stored at −70°C until assayed. The liver enzymes aspartate aminotransferase and alanine aminotransferase were measured using a Cobas Mira Plus automated analyzer (Roche). Free and protein-bound GSH levels were analyzed in metaphosphoric acid extracts of tissues, and red blood cells were analyzed by an enzymatic recycling method as described previously (21).

Food consumption

To assess food consumption, 15 randomly selected rats per group (three rats per cage) were monitored on 5 consecutive days at weeks 7 and 8 after MNU injection. Food consumption per cage was calculated by subtracting the food leftover from that added every 2 or 3 d and divided by 3 (the number of rats per cage) to reflect consumption per rat per day. Spillage was also monitored and considered to be negligible.

Analysis of plasma fatty acids

Plasma fatty acids were esterified and analyzed by gas chromatography equipped with flame ionization detectors (22). The identification of fatty acid methyl esters was accomplished by comparison with the relative retention time of standards. Quantification was based on internal standard calculations compared with calibration analyses with authentic standards.

Statistical analysis

χ² and Cochran-Mantel-Haenszel tests were used to compare the experimental groups with respect to binary (presence of tumor) and count (number of tumors) outcomes, respectively. Analysis of variance was used to compare groups with respect to continuous outcomes. All P values were corrected for multiple comparisons. All analyses were done using SAS version 9.1 (SAS, Inc.).

Results

Dietary influence on plasma fatty acid profile

To verify that our dietary manipulations produced the expected changes in circulating fatty acids, we measured
plasma fatty acid levels in rats fed with diets containing 5% corn oil, 20% corn oil, or 17% fish oil + 3% corn oil. The results reported in Supplementary Table S2 show that the high fish oil diet significantly increased the level of circulating n-3 PUFA and the n-3 to n-6 ratio from 0.1 to 4.4, consistent with the difference in ratio observed in the experimental diets.

Treatment effects on the incidence, multiplicity, and volume of palpable lesions

As can be seen in Fig. 1, 81% of 20% corn oil–fed rats developed palpable tumors by 8 weeks following MNU administration. Neither the low-fat diet (5% corn oil) nor the n-3 PUFA–rich fish oil diet significantly affected mammary carcinogenesis by week 8 after MNU, although the results reported in Supplementary Table S2 show that the high fish oil diet significantly increased the level of circulating n-3 PUFA and the n-3 to n-6 ratio from 0.1 to 4.4, consistent with the difference in ratio observed in the experimental diets.

Table 1. Treatment effects on tumor multiplicity and volume

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>No. of rats</th>
<th>No. of tumors/rat (mean ± SE)</th>
<th>Tumor volume/rat (mm³; mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 5</td>
<td>Week 6</td>
</tr>
<tr>
<td>1, 5% CO</td>
<td>35</td>
<td>0.06 ± 0.04</td>
<td>0.37 ± 0.1</td>
</tr>
<tr>
<td>2, 20% CO</td>
<td>36</td>
<td>0.11 ± 0.05</td>
<td>0.47 ± 0.1</td>
</tr>
<tr>
<td>3, 20% CO + 50 μg/kg tamoxifen</td>
<td>39</td>
<td>0.08 ± 0.04</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>4, 20% CO + 100 μg/kg tamoxifen</td>
<td>39</td>
<td>0</td>
<td>0.15 ± 0.06*</td>
</tr>
<tr>
<td>5, 17% FO</td>
<td>36</td>
<td>0.03 ± 0.03</td>
<td>0.19 ± 0.1</td>
</tr>
<tr>
<td>6, 17% FO + 50 μg/kg tamoxifen</td>
<td>36</td>
<td>0</td>
<td>0.08 ± 0.05†</td>
</tr>
<tr>
<td>7, 17% FO + 100 μg/kg tamoxifen</td>
<td>35</td>
<td>0</td>
<td>0†</td>
</tr>
</tbody>
</table>

NOTE: P values are from statistical comparisons to control (20% corn oil).
Abbreviations: CO, corn oil; FO, fish oil.
*P < 0.05.
†P < 0.01.
‡P < 0.001.

Table 2. Treatment effects on the incidence and multiplicity of palpable adenocarcinomas with and without DCIS according to histologic subtypes

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>No. of tumors/rat (mean ± SE)</th>
<th>Tumor volume/rat (mm³; mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats (%)</td>
<td>No. of tumors (mean ± SE)</td>
</tr>
<tr>
<td>1 5% CO (n = 35)</td>
<td>27 (57)</td>
<td>0.77 ± 0.15</td>
</tr>
<tr>
<td>2 20% CO (n = 36)</td>
<td>32 (64)</td>
<td>0.89 ± 0.14</td>
</tr>
<tr>
<td>3 20% CO + 50 μg/kg tamoxifen (n = 39)</td>
<td>27 (49)</td>
<td>0.69 ± 0.14</td>
</tr>
<tr>
<td>4 20% CO + 100 μg/kg tamoxifen (n = 39)</td>
<td>23 (44)</td>
<td>0.59 ± 0.13</td>
</tr>
<tr>
<td>5 17% FO (n = 36)</td>
<td>28 (50)</td>
<td>0.78 ± 0.18</td>
</tr>
<tr>
<td>6 17% FO + 50 μg/kg tamoxifen (n = 36)</td>
<td>16 (33)</td>
<td>0.44 ± 0.12</td>
</tr>
<tr>
<td>7 17% FO + 100 μg/kg tamoxifen (n = 35)</td>
<td>16 (31)</td>
<td>0.46 ± 0.14</td>
</tr>
</tbody>
</table>

NOTE: Some rats exhibited more than one histologic subtype. P values are from statistical comparisons to control (20% corn oil).
*They also include one tubular adenocarcinoma (group 2), one comedo adenocarcinoma (group 1), one tubular adenocarcinoma (group 3), two comedo adenocarcinomas (group 4), and one tubular adenocarcinoma (group 6).
†P < 0.05.
‡P < 0.01.
§P < 0.001.
the latter had a transient inhibitory effect at earlier time points, which was significant at week 6 ($P < 0.05$; Fig. 1A). Tamoxifen administration in the presence of 20% corn oil diet inhibited mammary carcinogenesis, although its effect was only significant at week 7 at the higher dose of 100 μg/kg ($P = 0.01$ versus 20% corn oil, Fig. 1B). The combination of the fish oil–rich diet with the lower dose of tamoxifen of 50 μg/kg significantly inhibited carcinogenesis at week 6 ($P < 0.01$), week 7 ($P = 0.01$), and marginally ($P = 0.06$) at week 8 (Fig. 1C). At termination, this combination had reduced tumor development by 35%, a reduction of borderline statistical significance. In contrast, tamoxifen alone at this dose did not affect mammary carcinogenesis significantly at any time points (Fig. 1C). However, the effect of the combination treatment was not found to be statistically different from that of tamoxifen alone when compared directly. Remarkably, the combination of the higher dose of tamoxifen (100 μg/kg) with fish oil–rich diet profoundly inhibited mammary carcinogenesis at week 6 ($P < 0.001$), week 7 ($P < 0.001$), and week 8 ($P = 0.002$; Fig. 1D). Overall, this combination reduced tumor formation by 47% by the end of the experiment. In contrast, the inhibitory effect of tamoxifen at this dose on mammary carcinogenesis was only significant at week 7 ($P = 0.01$; Fig. 1D). However, direct comparison of the effects of the combination with that of tamoxifen alone was not statistically different.

Table 1 illustrates the effects of our intervention on the multiplicity and volumes of the palpable lesions. Neither the low-fat diet nor the fish oil–rich diet significantly affected tumor multiplicity. Starting at week 6, the combination of fish oil–rich diet and 50 μg/kg tamoxifen significantly reduced tumor multiplicity (group 6), whereas the individual interventions (groups 3 and 5) did not. Increasing the dose of tamoxifen to 100 μg/kg reduced tumor multiplicity (group 4). However, the combination of this dose of the drug with a fish oil–rich diet (group 7) exerted a more marked effect, reducing the incidence of cribriform tumors by 52% ($P < 0.05$); the incidence and multiplicity of papillary tumors by 73% and 83%, respectively ($P < 0.01$ for both); and the incidence and multiplicity of all tumors combined by 47% and 65%, respectively ($P < 0.01$ and $P < 0.001$, respectively). However, direct comparisons of the effects of each combination with those of the corresponding doses of tamoxifen alone did not reveal statistically significant differences.

The combination of fish oil–rich diet with 100 μg/kg tamoxifen was the only intervention able to cause a significant reduction in the tumor volume, whereas the effect of 50 μg/kg tamoxifen plus fish oil–rich diet did not reach statistical significance ($P = 0.06$). When non–tumor-bearing rats were excluded from the analysis, no differences in tumor volume were observed across groups (data not shown).

Supplementary Table S3 reports the histopathology of the palpable lesions. The vast majority were invasive adenocarcinoma, 14% of which with an associated ductal carcinoma in situ (DCIS) component. Next, we analyzed the effects of our treatments on the incidence and multiplicity of invasive adenocarcinomas (with and without DCIS) subdivided according to histologic subtypes (Table 2). The low-fat diet (5% corn oil) and the fish oil–rich diet by themselves did not affect tumor incidence or multiplicity. The combination of the fish oil–rich diet and 50 μg/kg tamoxifen significantly reduced total tumor multiplicity by ~50% (group 6), whereas the individual interventions did not (groups 3 and 5). Administration of 100 μg/kg tamoxifen to corn oil–fed rats significantly ($P < 0.05$) reduced multiplicity of papillary and total number of adenocarcinomas (group 4). However, the combination of 100 μg/kg tamoxifen with the fish oil–rich diet (group 7) exerted a more marked effect, reducing the incidence of cribriform tumors by 52% ($P < 0.05$); the incidence and multiplicity of papillary tumors by 73% and 83%, respectively ($P < 0.01$ for both); and the incidence and multiplicity of all tumors combined by 47% and 65% ($P < 0.01$ and $P < 0.001$, respectively). However, direct comparisons of the effects of each combination with those of the corresponding doses of tamoxifen alone did not reveal statistically significant differences.

### Table 3. Treatment effects on the incidence and multiplicity of benign and preinvasive mammary lesions

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>No. of rats</th>
<th>Intraductal proliferation</th>
<th>Moderate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lesions/rat (mean ± SE)</td>
<td>No. of rats (mean ± SE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. of lesions (%)</td>
<td>No. of rats (%)</td>
</tr>
<tr>
<td>1, 20% CO</td>
<td>36</td>
<td>81 (81%)</td>
<td>2.25 ± 0.33</td>
</tr>
<tr>
<td>2, 20% CO + 100 μg/kg tamoxifen</td>
<td>39</td>
<td>110 (90%)</td>
<td>2.82 ± 0.34</td>
</tr>
<tr>
<td>3, 17% FO</td>
<td>36</td>
<td>129 (100%)</td>
<td>3.58 ± 0.34*</td>
</tr>
<tr>
<td>4, 17% FO + 100 μg/kg tamoxifen</td>
<td>35</td>
<td>99 (86%)</td>
<td>2.83 ± 0.45</td>
</tr>
</tbody>
</table>

(Continued on the following page)
The combination of fish oil—

The data showed that compared with control (group 1), scored the premalignant and preinvasive lesions. Again, adenocarcinomas in the same groups of rats in which we

incidence and multiplicity of palpable and nonpalpable

Table S5 illustrates the treatment effects on the

mas induced by the combination treatment. Supplementa-

observed greater inhibition of mammary adenocarci-

an effect that was statistically significant in the presence of

more, and surprisingly, it increased the incidence of DCIS,

in Table 3, tamoxifen administration caused an increase in the incidence and number of mild and moderate intraductal proliferations, which approached statistical sig-

nificance for the latter. Because tamoxifen decreased the

number of adenocarcinomas (Table 2), these findings

suggest that the drug blocked mammary carcinogenesis

at a stage distal to hyperplasia. The fish oil—

rich diet by itself (group 3) did not significantly re-

duce mammary carcinogenesis. However, the effect of

the combination was not statistically superior to that of
tamoxifen alone when compared directly.

Table 3. Treatment effects on the incidence and multiplicity of benign and preinvasive mammary lesions (Cont’d)

<table>
<thead>
<tr>
<th>Moderate</th>
<th>Florid</th>
<th>DCIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions/rat (mean ± SE)</td>
<td>No. of lesions</td>
<td>No. of rats (%)</td>
</tr>
<tr>
<td>0.25 ± 0.09</td>
<td>5</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>0.67 ± 0.13</td>
<td>5</td>
<td>5 (13%)</td>
</tr>
<tr>
<td>0.75 ± 0.16</td>
<td>4</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>0.80 ± 0.13†</td>
<td>8</td>
<td>7 (20%)</td>
</tr>
</tbody>
</table>

*P < 0.05 versus group 1.
†P < 0.05 versus group 2.
‡P < 0.01 versus group 1.

surprising because, as reported above, tumor volumes in tumor-bearing rats did not differ across experimental groups.

Treatment effects on the incidence and multiplicity of benign and preinvasive mammary lesions

Next, we performed histologic analysis of the inguinal mammary fat pads to examine the effect of tamoxifen (100 μg/kg) and fish oil–rich diet individually and in combination on the incidence and multiplicity of benign and preinvasive mammary lesions. As can be seen in Table 3, tamoxifen administration caused an increase in the incidence and number of mild and moderate intraductal proliferations, which approached statistical significance for the latter. Because tamoxifen decreased the number of adenocarcinomas (Table 2), these findings suggest that the drug blocked mammary carcinogenesis at a stage distal to hyperplasia. The fish oil–rich diet significantly increased the incidence and multiplicity of mild and moderate intraductal proliferations. Furthermore, and surprisingly, it increased the incidence of DCIS, an effect that was statistically significant in the presence of tamoxifen. This finding is difficult to reconcile with the observed greater inhibition of mammary adenocarcinomas induced by the combination treatment. Supplementary Table S5 illustrates the treatment effects on the incidence and multiplicity of palpable and nonpalpable adenocarcinomas in the same groups of rats in which we scored the premalignant and preinvasive lesions. Again, the data showed that compared with control (group 1), the combination of fish oil–rich diet and 100 μg/kg tamoxifen (group 4) exerted a greater tumor-suppressive effect than tamoxifen alone (group 2), whereas the fish oil–rich diet by itself (group 3) did not significantly reduce mammary carcinogenesis. However, the effect of the combination was not statistically superior to that of tamoxifen alone when compared directly.

Treatment effects on circulating and tumor levels of free and bound GSH

Because PPARγ activation is known to inhibit oxidative stress (12, 13), which, in turn, can affect carcinogenesis (14–16), we examined the effects of our treatments on circulating and tumor GSH levels as indicators of the level of oxidative stress. As can be seen in Table 4, a significant increase in red blood cell levels of both free and protein-bound GSH was observed in all groups of rats injected with MNU compared with rats not given the car-

cinogen. As the time interval between MNU administration and GSH measurements was 8 weeks, this finding is likely to represent an adaptive response to the increased burden of oxidative stress induced by MNU. Furthermore, GSH levels were similarly increased in tumor-bearing and non–tumor-bearing MNU-injected rats (data not shown), indicating that the increase was not just a consequence of tumor burden. We also observed that the level of protein-bound GSH in the fish oil–fed rats with and without tamoxifen (groups F, G, and H) was significantly lower (P = 0.0005) than the level of protein-bound GSH in the corresponding groups of corn oil–fed rats (groups C, D, and E). This finding suggests that fish oil may reduce the level of oxidative stress, resulting in decreased oxidation of GSH and consequent reduction in protein-bound GSH. To further pursue this observation, we measured free and protein-bound GSH in the tu-
mors of two groups of rats fed with either corn oil (group D) or fish oil (group G; both treated with the same dose of tamoxifen, 50 μg/kg). As can be seen in Table 4, tu-
mors from fish oil–fed rats had an ~60% reduction in both free and bound GSH, further supporting the notion that the fish oil–rich diet reduced the level of oxidative stress. Tamoxifen administration, on the other hand, did not influence the red blood cell levels of free and protein-bound GSH in the presence of either corn oil–rich (group D and E versus C) or fish oil–rich (groups G and H versus F) diet.
Treatment effects on body weight, food consumption, and liver parameters

Supplementary Fig. S1 shows the effects of our interventions on body weight gain. As can be seen, tamoxifen administration suppressed body weight gain, an effect that was significant starting at week 5 (tamoxifen 100 μg/kg) or week 6 (tamoxifen 50 μg/kg). A suppression of body weight gain in female rodents exposed to tamoxifen without any other evidence of toxicity has been reported in numerous studies (23–25). To further document the lack of toxicity, we evaluated treatment effects on a variety of liver parameters. As can be seen in Supplementary Table S6, tamoxifen did not cause any significant changes in liver enzymes or histology. Fish oil–fed rats, whether receiving tamoxifen or not, more frequently exhibited mild periportal hepatitis. This finding is not likely to be biologically significant because liver enzymes were not affected. Tamoxifen has been shown to have an anorectogenic effect leading to a reduction in food consumption (26). In our experiments, we observed that tamoxifen significantly reduced food consumption only in fish oil–fed rats, although it suppressed body weight gain to a similar degree in fish oil– and corn oil–fed rats (Supplementary Table S7). These results indicate that tamoxifen-induced inhibition of weight gain may not entirely be due to its anorectogenic effect, as also suggested by other investigators (27).

Discussion

Breast cancer prevention remains the best approach to reduce breast cancer mortality. To be applicable to a healthy population of women, a prevention strategy needs to be safe and easy to implement. Furthermore, to be optimally effective, it is necessary to use a multitargeted approach as breast cancer development requires the coordinated activation of multiple cellular mechanisms as a result of complex interactions between genetic aberrations and environmental factors.

In these experiments, we tested a novel approach to breast cancer prevention by combining the antiestrogen tamoxifen with an n-3 PUFA–rich diet. The rationale for this strategy is based on the known crosstalk between the estrogen receptor and the PPARγ pathways (10, 11), leading to enhanced breast cancer–suppressive effects when they are concomitantly inhibited by antiestrogens and activated by PPARγ agonists (such as n-3 PUFA), respectively (10). We report for the first time that the concomitant administration of tamoxifen and n-3 PUFA significantly reduced tumor incidence and size in rats exposed to tamoxifen and n-3 PUFA compared to rats receiving tamoxifen alone.

Table 4. Treatment effects on free and bound GSH levels in the tumors and red blood cells

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Red blood cells (mmol/g of packed cells)*</th>
<th>Tumors (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>Bound</td>
</tr>
<tr>
<td>A 5% CO, no MNU</td>
<td>1.75 ± 0.16†</td>
<td>0.32 ± 0.04‡</td>
</tr>
<tr>
<td>B 5% CO</td>
<td>2.95 ± 0.46</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>C 20% CO</td>
<td>2.75 ± 0.64</td>
<td>0.48 ± 0.08</td>
</tr>
<tr>
<td>D 20% CO + 50 μg/kg tamoxifen</td>
<td>2.93 ± 0.68</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>E 20% CO + 100 μg/kg tamoxifen</td>
<td>2.87 ± 0.53</td>
<td>0.46 ± 0.07</td>
</tr>
<tr>
<td>F 17% FO</td>
<td>2.94 ± 0.74</td>
<td>0.40 ± 0.09</td>
</tr>
<tr>
<td>G 17% FO + 50 μg/kg tamoxifen</td>
<td>2.91 ± 0.59</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>H 17% FO + 100 μg/kg tamoxifen</td>
<td>2.78 ± 0.41</td>
<td>0.39 ± 0.06</td>
</tr>
</tbody>
</table>

NOTE: Red blood cells and tumor levels of free and bound GSH were measured in randomly selected rats from the indicated experimental groups plus in rats that were not injected with MNU. Data represent mean ± SE. Numbers in parentheses indicate the number of observations.

*Results were identical if data are expressed as mmol/g hemoglobin.
†P = 0.001 versus the rest of the groups.
‡P = 0.02 versus the rest of the groups.
§P = 0.01 versus G.
bination of tamoxifen and an n-3 PUFA–rich diet seems to inhibit mammary tumor formation, multiplicity, and volume to a greater extent than the individual interventions. Although the chemopreventive effects of the combination were not statistically different from those of tamoxifen alone when compared directly, the protection from mammary carcinogenesis offered by tamoxifen and fish oil was consistently superior to that of tamoxifen alone when compared with control. Furthermore, the superiority of the combination was particularly evident at the lower dose of tamoxifen (50 μg/kg), which, by itself, did not have a statistically significant tumor-suppressive effect on any of the above parameters.

Under our experimental conditions, administration of a n-3 PUFA–rich diet alone did not decrease mammary tumorigenesis and actually increased the number of hyperplastic and DCIS lesions. n-3 PUFA have been shown in general to have tumor-protective effects, including in breast cancer (7, 8). However, this effect is not universal and, in some cases, fish oil (rich in n-3 PUFA) has been shown to either have no tumor-suppressive effect (28, 29) or to actually enhance tumorigenesis (30, 31). Such discrepancies may in part be explained by varying ratios of n-3 to n-6 PUFA present in the diet, which may be more important than the total quantity of n-3 PUFA themselves. This seems to be the case in the MNU mammary tumor model system in which the inhibitory effect of n-3 PUFA on carcinogenesis was apparent only when the n-3 to n-6 ratio ranged from 1:1 to 1:2 (32, 33). In contrast, the n-3 to n-6 ratio in our fish oil–rich diet was 2.3, which may account for its lack of chemopreventive effect when used by itself. Remarkably, however, such diet, although ineffective on its own, increased the chemopreventive effect of tamoxifen, thus pointing to the potential value of this combination. It will be important in future experiments to test diets containing more favorable ratios of n-3 to n-6 PUFA in combination with antiestrogens to fully explore the merit of this combined strategy in breast cancer prevention. Of interest, we found that tamoxifen administration also increased the number of hyperplastic lesions, although its effect was less pronounced than that of fish oil and did not include stimulation of DCIS. To the best of our knowledge, the effect of tamoxifen on preneoplastic mammary lesions has not been previously investigated. Our findings suggest that, at least in the MNU mammary model, tamoxifen may reduce the development of breast cancer by blocking the transition from hyperplasia to carcinoma. As reported in numerous other studies (23–25), tamoxifen significantly suppressed weight gain, an effect that could not entirely be explained by a reduction in food consumption (at least in corn oil–fed rats). The reduction in weight gain may be related to other effects of tamoxifen on body composition, adipose tissue, and energy expenditure reported in rats (27). We did not observe any obvious toxicity from tamoxifen as also supported by the lack of alterations in liver histology and liver enzymes induced by the drug. The effect of tamoxifen on body weight in humans is controversial. Of interest, data from two clinical trials indicate that at least obese women treated with tamoxifen had lower body mass index than those not receiving the drug (34, 35).

There is a need to pursue an in-depth study to understand the mechanism of interaction between tamoxifen and fish oil leading to inhibition of mammary carcinogenesis. Along this line, we investigated the effects of our interventions on blood and tumor GSH levels to explore whether a reduction in the level of oxidative stress may be involved in their antitumor action. Our results indicate that the fish oil–rich diet significantly reduced the increased level of oxidative stress induced by MNU administration. It is conceivable that this reduction may have contributed to the added antitumor action of fish oil–rich diet in the presence of tamoxifen. A major mechanism by which n-3 PUFA are believed to exert their antitumor action is through inhibition of proliferation and stimulation of apoptosis (9). We found that the fish oil–rich diet either by itself or added to tamoxifen did not affect tumor expression of Ki67 and cleaved caspase-3 and the number of apoptotic bodies. However, because these measurements were done in tumors whose development and growth was not affected by our interventions, they do not provide information on the mechanism of inhibition of carcinogenesis by the combination of tamoxifen and fish oil. More insight can be obtained by measuring these parameters in the hyperplastic lesions; this will be the object of future studies.

In conclusion, our data provide support for the first time for a novel breast cancer chemoprevention strategy that includes a combination of n-3 PUFA and antiestrogens. This strategy offers promise to improve breast cancer prevention beyond the benefits of antiestrogens based on the expected favorable interactions at the cellular level between these two interventions leading to tumor suppression. The safety of this approach derives from the known health benefits of n-3 PUFA, and the potential use of lower than conventional doses of antiestrogens makes this strategy easily translatable to the clinics. However, further studies are needed to identify optimal concentrations of antiestrogens and optimal ratios of n-3 to n-6 PUFA to be used in the combination and to define the specific cellular mechanisms mediating their effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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