Abstract  

Purpose: The methylnitrosourea (MNU)-induced mammary cancer model in rats is similar to estrogen receptor–positive breast cancer in women. In prevention studies using this model, tumor incidence and multiplicity were typically primary end points. The ability of various agents administered for a short period to modulate cell proliferation [proliferation index (PI)] and apoptosis [apoptotic index (AI)] in mammary cancers was compared with their efficacy in long-term prevention and therapy studies.

Experimental Design: Rats were injected with MNU to induce mammary cancers. For the prevention studies, agents were administered by gavage or in the diet beginning 5 days after MNU. For proliferation (PI) and apoptosis (AI) experiments, animals with a palpable mammary cancer were treated with the agents for only 4 to 7 days. PI was determined following 5-bromo-deoxyuridine labeling whereas AI was determined using the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay. Therapeutic efficacy was evaluated by measuring cancer size over a 6-week period.

Results: Treatments with differing chemopreventive efficacy and mechanism(s) of action were examined: (a) hormonal treatments [tamoxifen, vorozole (an aromatase inhibitor), and ovariectomy]; (b) retinoid X receptor agonists (targetretin, 9-cis retinoic acid, and UAB30); (c) inducers of drug-metabolizing enzymes (indole-3-carbinol, 5,6 benzoflavone, and diindoyl methane); (d) agents that alter signal transduction (R115777, a farnesyltransferase inhibitor); Iressa (an epidermal growth factor receptor inhibitor); sulindac and celecoxib (cyclooxygenase 1/2 and cyclooxygenase 2 inhibitors); and (e) diverse agents including meclizine, vitamin C, and sodium phenylbutyrate. Correlations between inhibition of PI, increase of AI, and chemopreventive efficacy were observed. Although most agents with moderate or low preventive efficacy suppressed PI, they minimally affected AI.

Conclusions: The data confirmed that the short-term effects of various agents on cell proliferation and apoptosis in small mammary cancers can predict their preventive/therapeutic efficacy. Thus, these biomarkers can be used to help determine the efficacy of compounds in phase II clinical prevention trials.

The methylnitrosourea (MNU) model of mammary carcinogenesis in rats was developed ~30 years ago by Gulliano et al. (1). The resulting mammary tumors are primarily carcinomas (1, 2) and show many of the histopathologic and biological characteristics of estrogen receptor–positive breast cancer in women (3). In this model, almost all animals develop multiple mammary tumors after a relatively short latency (3–4 months). In addition, the morphology, origin (terminal end buds or terminal ductal lobular units), and preinvasive stages (hyperplasia, ductal carcinoma in situ) of these tumors seem to be similar to human breast cancer (2, 3). More recently, studies on gene expression have shown that MNU-induced mammary cancers are similar in gene expression to that of well-differentiated ductal carcinomas in women (4). This model has been extensively used as a preclinical in vivo model for assessment of the efficacy of agents that can prevent and/or treat breast cancer. This model is highly responsive to a number of regimens that are effective in altering human breast cancer, including selective estrogen receptor modulators, aromatase inhibitors, pregnancy, and ovariectomy (5–10). Another class of agents that has proved strikingly effective in this model, although they do not seem to be mediated via the hormonal axis, are the retinoid X receptor (RXR) agonists that can modulate cell growth by receptor-dependent or receptor-independent pathways (6, 7, 11, 12). Unlike human breast
Materials and Methods

**Chemicals.** Preventive/therapeutic agents used were as follows: tamoxifen, sulindac, sodium phenylbutyrate, meclizine, indole-3-carbinol, 5,6-benzoflavone, and vitamin C (Sigma Chemical Co.); diindoylmethane (National Cancer Institute Chemical Repository); and targetgin, celecoxib, IUB30, Iressa, and 9-cis retinoic acid (9cRA), which were made by custom synthesis and confirmed by verified analytical techniques. The farnesyltransferase inhibitor R115777 and vorozole were supplied by Johnson and Johnson Pharmaceuticals. The carcinogen MNU was obtained from Ash Stevens, Inc.. MNU was dissolved in sterile acidified saline (pH 5.0) and injected i.v. (50 mg/kg body weight) via the jugular vein when the animals were 50 days of age. Control animals at the same age received saline. Teklad mash (4%) diet and Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. The agents that were evaluated and the doses used, as well as the duration of exposure, are shown in Table 2. Agents were incorporated into the diet by mixing with Teklad (4%) mash diet using a liquid-solid blender (Patterson-Kelly Co.). 5,6-Benzoflavone, sulindac, celecoxib, meclizine, sodium phenylbutyrate, and vitamin C were mixed directly into the diet (i.e., no vehicle was required). To ensure stability, the retinoids (9cRA, IUB30, and targetgin) were initially dissolved in 12 g ethanol, 19 g triptocain, 0.05 mL TenoX 20, and 0.05 mL 0.1-α-tocopherol per kilogram of diet, and then added to the diet. To ensure homogeneity of tamoxifen in the diet (because very low dose levels were used), the agent was initially dissolved in 12 g ethanol and 19 g triptocain before adding to the diet. Vorozole, the farnesytransferase inhibitor 115777, Iressa, indole-3-carbinol, and diindoylmethane were administered by gavage, seven times a week, in a volume of 0.5 mL/gavage. The vehicle for vorozole and Iressa was 10% ethanol: 90% polyethylene glycol 400; for 115777, the vehicle was 0.1 N HCl/40% B-cyclodextrin (50:50, v/v); and for indole-3-carbinol and diindoylmethane, the vehicle was 10% degassed ethanol:90% corn oil.

**Prevention studies.** Chemoprevention studies were done as previously described (8, 13, 14). In brief, female Sprague-Dawley rats were obtained at 28 days of age and housed in polycarbonate cages (five per cage). At 50 days of age, the rats received one injection of MNU (50 mg/kg body weight) via the jugular vein. Each group had 15 rats.

**Table 1. Preventive/therapeutic agents and their mechanism of action**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone related</td>
<td>Antiestrogen/estrogen</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>Vorozole</td>
<td>Removal of estrogen source</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td></td>
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<tr>
<td>Targeted agents</td>
<td></td>
</tr>
<tr>
<td>R-115777</td>
<td>Farnesyltransferase inhibitor</td>
</tr>
<tr>
<td>Iressa</td>
<td>Epidermal growth factor receptor inhibitor</td>
</tr>
<tr>
<td>RXR agonists</td>
<td></td>
</tr>
<tr>
<td>9cRA</td>
<td>Retinoic acid receptor/RXR agonist</td>
</tr>
<tr>
<td>UAB30</td>
<td>RXR agonist</td>
</tr>
<tr>
<td>Targetgin</td>
<td>RXR agonist</td>
</tr>
<tr>
<td>Phase I/II inducers</td>
<td></td>
</tr>
<tr>
<td>5,6-Benzoflavone</td>
<td>Synthetic phase I/II inducer</td>
</tr>
<tr>
<td>Indole-3-carbinal</td>
<td>Plant derived phase I/II inducer</td>
</tr>
<tr>
<td>Diindoylmethane</td>
<td>Metabolite of indole-3-carbonil</td>
</tr>
<tr>
<td>COX 1/2 inhibitors</td>
<td></td>
</tr>
<tr>
<td>Sulindac</td>
<td>COX 1/2 inhibitor</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>COX 2 inhibitor</td>
</tr>
<tr>
<td>Various</td>
<td></td>
</tr>
<tr>
<td>Meclizine</td>
<td>H2 antagonist</td>
</tr>
<tr>
<td>Sodium phenylbutyrate</td>
<td>Histone deacetylase inhibitor</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Antioxidant</td>
</tr>
</tbody>
</table>

In identifying potential biomarkers in cancer prevention/therapeutic studies, there are a wide variety of general approaches which might be used: (a) identifying biomarkers using a rather wide platform (e.g., gene array or proteomic methodologies), (b) examining modulation of certain candidate genes or proteins that are typically associated with tumorigenesis (e.g., oncogenes, tumor suppressor genes, and cell cycle or apoptosis-related genes); and (c) measurement of more generalized biomarkers such as alterations in cell proliferation [proliferation index (PI)] and apoptosis [apoptotic index (AI)]. The appeal of these more general biomarkers is that they seem to be end points that are modulated by multiple agents. Therefore, irrespective of the specific mechanism of action of any preventive/therapeutic agent, the target cells will stop proliferating and die either by apoptosis or by a nonapoptotic pathway (17–20). Both cell proliferation and apoptosis have been used clinically for assessment of tumor prognosis because there is a relationship between PI and malignancy in many tumors (including breast cancer) and for analysis of response of cancer cells to clinical interventions (21, 22). In addition, a variety of clinical investigators using aromatase inhibitors or selective estrogen receptor modulators have used PI as a biomarker of potential efficacy (23–26).

In examining a more limited number of agents in the MNU mammary model, we have shown that highly effective agents affect both tumor cell proliferation and apoptosis (12, 14, 19, 27). Recently, we found that tamoxifen, inhibitors of estrogen synthesis, and certain retinoids can also induce senescent phenotype in tumor cells, an additional cellular mechanism of tumor suppression (28, 29). Although there are specific markers of senescence (28–30), cells that become senescent are in terminal proliferative arrest and will also affect the PI.

In this study, we examined a wide variety of agents (Table 1; Fig. 1A and B) for their ability to modulate PI and AI in small palpable mammary tumors. The agents were given for a period of 4 to 7 days at different doses to obtain dose-dependent alterations in the biomarkers and to determine the preferential cellular mechanism of response at the different dose levels. The reason for the relatively short treatment period is that after longer exposure with highly effective agents, significant disintegration of tumor parenchyma occurs. For biomarker validation in the short-term treatment protocols, the values of PI and AI were correlated with the efficacy of the same agents to suppress tumor growth in long-term prevention studies. We hypothesized that highly efficacious preventive agents would affect both PI and AI, whereas moderate or low efficacious agents would suppress cell proliferation but have minimal effect on apoptosis. Data supporting this hypothesis are presented.
The agents were administered beginning 5 days after MNU and continued throughout the duration of the study (18-20 weeks after MNU treatment). Rats were palpated twice per week for the development of mammary tumors. The efficacy of preventive agents was assessed by comparing the incidence and multiplicity of cancers between control and treated groups. Statistical analyses of these variables were done as previously described (9, 13, 14).

**Therapy studies.** For the therapeutic studies, female Sprague-Dawley rats were obtained at 28 days of age, housed in polycarbonate cages, and at 50 days of age received one injection of MNU (50 mg/kg body weight) via the jugular vein. When animals developed their first palpable mammary cancer, they were randomized into various groups. Tumor size was determined with calipers before initiation of treatment and twice weekly during the course of treatment. The largest diameter of the tumor was measured and this value was multiplied by the perpendicular diameter (size expressed as square millimeter). The animals with palpable cancers (10-12 rats per group) were treated with the individual agents for 5 to 6 weeks. An agent was considered to be (a) inactive (-), if >50% of tumors increased in size by 100%; (b) stable disease (+), if <30% of tumors increased in size by 100%; (c)
partially responsive (++, if <20% of tumors increased in size by 100% and
>25% of tumors decreased in size by >50%; (d) moderate response
(+++), if >70% of tumors increased in size by >50%; and (e) large
response (+++++), if >70% of tumors decreased in size by >75%.

Biomarker studies. For these studies, rats bearing small (100-
250 mm3) palpable mammary cancers were treated with the agents
for a period of 4 to 7 days. Two hours before sacrifice, the animals were
injected i.p. with 5-bromodeoxyuridine (BrdUrd), 100 mg/kg body
weight in saline, for labeling of proliferative cells (12, 14, 19, 27). After
sacrifice of the rats by CO2 asphyxiation, the mammary cancers were
injected with 5-bromodeoxyuridine (BrdUrd), 100 mg/kg body
weight in saline, for labeling of proliferative cells (12, 14, 19, 27). After
sacrifice of the rats by CO2 asphyxiation, the mammary cancers were
removed and fixed overnight in 10% formalin for histologic classifi-
cation and for proliferation and apoptosis measurements.

Cell proliferation. Proliferating cells in mammary cancers were
labeled in vivo by BrdUrd as previously described (9, 12, 14, 27). The
cells labeled with BrdUrd were identified using an anti-BrdUrd
monoclonal antibody (Becton Dickinson) and ABC kit. More than
1,000 cells were randomly scored from each tumor at two sectioning
levels 100 mm apart and the percent of BrdUrd-labeled cells (BrdUrd
labeling index) was evaluated. Student’s t test was used to compare
treated versus control values.

Apoptosis. Apoptotic cells were identified by the terminal deoxy-
ribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)
method (31) as previously described (12, 14, 19). The ApoTag in situ
hybridization detection kit was used for detection of cells in apoptosis
(Oncor Co.). The top sections of each slide, which were incubated
without digoxigenin-dUTP, were used as a negative control. Mammary
glands of rats taken 6 days after ovariectomy (when the number of
apoptotic cells was high) were used as positive controls. Tissue sections
were counterstained by methyl green for visualization of tumor
morphology. From each tumor, >1,500 cells were evaluated for the
presence of apoptotic cells. Student’s t test was used to compare treated
versus control values.

Results

Structurally and mechanistically varied group of agents
examined for their anticancer efficacy. In this study, the efficacy
of various agents to prevent mammary carcinogenesis and to
inhibit growth of established tumors were compared with their
potential to inhibit PI and increase AI in tumor cells. The
different agents, their structures, and their proposed mode(s)
of action are presented in Table 1 and Fig. 1A and B. As shown,
this represents a structurally varied group of agents with
the ability to induce apoptosis in a variety of mechanisms of action.
Three of the treatments (tamoxifen, vorozole, and ovariectomy) affect the hormonal
axis, and all were highly effective in preventing MNU-induced
mammary cancers (Table 2). Among the retinoids, 9cRA interacts with both retinoic acid receptors and RXRs, whereas
UAB30 and targetin are relatively specific RXR agonists. Two
targeted “therapeutic” agents, R115777 (a farnesyl transferase
inhibitor) and Iressa (an epidermal growth factor receptor
inhibitor), were examined, as well as the efficacy of two

### Table 2. Effects of agents on preventive/therapeutic efficacy, proliferation, and apoptosis

<table>
<thead>
<tr>
<th>Agent examined</th>
<th>Dose</th>
<th>Inhibition of cancer multiplicity</th>
<th>Decrease of PI*</th>
<th>Increase of AI†</th>
<th>AI/PI ratio ‡</th>
<th>Therapeutic efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>2.5 mg/kg diet</td>
<td>100∥</td>
<td>80∥</td>
<td>4.0∥</td>
<td>1.3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1.0 mg/kg diet</td>
<td>80∥</td>
<td>50∥</td>
<td>2.0</td>
<td>0.3</td>
<td>+</td>
</tr>
<tr>
<td>Vorozole</td>
<td>1.25 mg/kg body weight/d</td>
<td>94∥</td>
<td>92∥</td>
<td>5.0∥</td>
<td>3.0</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>0.32 mg/kg body weight/d</td>
<td>85∥</td>
<td>70∥</td>
<td>4.0∥</td>
<td>1.0</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>0.08 mg/kg body weight/d</td>
<td>48∥</td>
<td>65∥</td>
<td>3.0</td>
<td>0.6</td>
<td>+</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>0</td>
<td>100∥</td>
<td>94∥</td>
<td>4.0∥</td>
<td>4.1</td>
<td>++++</td>
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<td>Targeted agents</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R115777</td>
<td>40 mg/kg body weight/d</td>
<td>73∥</td>
<td>79∥</td>
<td>4.5∥</td>
<td>1.7</td>
<td>+++</td>
</tr>
<tr>
<td>Iressa</td>
<td>10 mg/kg body weight/d</td>
<td>95∥</td>
<td>95∥</td>
<td>4.0∥</td>
<td>3.1</td>
<td>+++</td>
</tr>
<tr>
<td>RXR agonists</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9cRA</td>
<td>50 mg/kg diet</td>
<td>45∥</td>
<td>55∥</td>
<td>2.0∥</td>
<td>0.34</td>
<td>+</td>
</tr>
<tr>
<td>UAB30</td>
<td>200 mg/kg diet</td>
<td>58∥</td>
<td>60∥</td>
<td>1.3</td>
<td>0.20</td>
<td>+</td>
</tr>
<tr>
<td>Targetin</td>
<td>150 mg/kg diet</td>
<td>80∥</td>
<td>83∥</td>
<td>3.3∥</td>
<td>0.90</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>15 mg/kg diet</td>
<td>38∥</td>
<td>35</td>
<td>1.6</td>
<td>0.15</td>
<td>+</td>
</tr>
<tr>
<td>Phase I/II inducers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,6-benzoflavone</td>
<td>1,650 mg/kg diet</td>
<td>53∥</td>
<td>32</td>
<td>1.2</td>
<td>0.13</td>
<td>-</td>
</tr>
<tr>
<td>Indole-3-carbinol</td>
<td>180 mg/kg body weight/d</td>
<td>38∥</td>
<td>42∥</td>
<td>1.5</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>Dinodiymethane</td>
<td>180 mg/kg body weight/d</td>
<td>8</td>
<td>12</td>
<td>1.1</td>
<td>0.10</td>
<td>ND</td>
</tr>
<tr>
<td>Nonsteroidal anti-inflammatory drugs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulindac</td>
<td>800 mg/kg diet</td>
<td>9</td>
<td>6</td>
<td>1.2</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>1,500 mg/kg diet</td>
<td>24</td>
<td>30</td>
<td>1.2</td>
<td>0.1</td>
<td>+</td>
</tr>
<tr>
<td>Various</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meclizine</td>
<td>1,000 mg/kg diet</td>
<td>48∥</td>
<td>50∥</td>
<td>2.5∥</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Sodium phenylbutyrate</td>
<td>4,000 mg/kg diet</td>
<td>14</td>
<td>05</td>
<td>0</td>
<td>0.06</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>165 mg/kg diet</td>
<td>3</td>
<td>15</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Data based on comparison of proliferation and apoptosis in 10 to 12 treated cancers versus control cancers. Rats bearing small palpable
tumors were examined after 4 to 7 d of continual treatment.

Abbreviation: ND, not determined.

* Data compared with the mean values of BrdUrd-labeled cells in control tumors. Control PI = 12.8%.

† Data compared with the mean values of apoptotic cells (TUNEL assay) in control tumors. Control AI = 0.6%.

‡ Ratio of percentage apoptotic cells to percentage proliferative cells.

*p < 0.01. 

*p < 0.05.
cyclooxygenase (COX) inhibitors (sulindac, which affects both COX 1 and COX 2, and celecoxib, which preferentially affects COX-2). In addition, there were three agents (5,6-benzoflavone, indole-3-carbinol, and diindoylmethane) that induce various drug-metabolizing enzymes. Finally, a less specific and structurally varied group of agents was examined, which included meclizine (an H2 antagonist), vitamin C (an antioxidant), and sodium phenylbutyrate (a histone deacetylase inhibitor). The efficacy of these agents to inhibit mammary carcinogenesis or to suppress the growth of established mammary tumors has been examined by numerous investigators. However, all the prevention and therapeutic data presented in Table 2 were generated in our laboratories and doses of agents were the same in the prevention, biomarker,
and therapeutic studies. Table 2 shows the overall effects of the various agents on PI and AI, as well as the effects in both preventive and therapeutic studies. As shown, the strikingly effective preventive agents included tamoxifen (as a representative of selective estrogen receptor modulators), vorozole, R115777, Iressa, and targretin. A dose-dependent increase in the efficacy of tamoxifen and vorozole to inhibit mammary carcinogenesis was also observed with increasing doses of these agents. UAB30, 9cRA, meclizine, 5,6 benzoflavone, and targretin at nontoxic doses were also effective, suppressing tumor multiplicity by 35% to 60%. Finally, the anti-inflammatory agents (nonsteroidal anti-inflammatory drugs) sodium phenylbutyrate, vitamin C, and diiodomethane were not significantly effective (reducing tumor multiplicity by <35%).

**Preventive agents differentially affect PI and AI in mammary cancers.** It was hypothesized that if an agent was an effective inhibitor of mammary carcinogenesis, it would suppress cell proliferation and/or induce apoptosis in established mammary cancers following short-term exposure. To test this, animals were treated with MNU and palpated twice a week until they developed a small palpable cancer. Cancer-bearing rats were randomized, treated for 4 or 7 days with the agents, and given BrdUrd 2 h before sacrifice. The percentages of proliferating cells and apoptotic cells were then determined (Table 2). The average percentage of labeled cells (PI) in 48 control rats (different experiments) was 12.8 ± 3.5% (Table 2). In these same animals, the AI was 0.6 ± 0.2%. In small mammary tumors, proliferating and apoptotic cells were almost randomly distributed, whereas in larger tumors necrotic areas occurred and most BrdUrd-labeled cells were identified close to the basal membrane or the surrounding connective tissues. In the same areas, apoptotic cells were rare (Fig. 2B). Our studies on cell proliferation and apoptosis were, therefore, conducted on relatively small mammary cancers. We also determined a potential correlation between PI and AI values in control tumors. These biomarkers were compared in all the control mammary cancers and a correlation analysis was done. However, no significant correlation between PI and AI was found ($R^2 = 0.207$ and $P = 0.154$), suggesting that tumors with high values of PI do not necessarily have low values of AI or vice versa.

Individual PI and AI values for controls and a variety of agents are shown in Fig. 3A and B. In the case of the highly effective agents (vorozole, Iressa, and targretin), a disintegration in tumor parenchyma (Fig. 2C and D) and a striking decrease in PI were observed (Fig. 2E and F, control versus treated animals, respectively). In the animals treated for 7 days with R115777, in addition to a decrease in PI and an increase in AI, multinuclear cells (Fig. 2C) were observed, suggesting cell death by mitotic catastrophe. These alterations in tumor architecture were large in the animals treated with vorozole, R115777, and Iressa, indicating not only preventive but therapeutic efficacy as well. In some tumors of animals treated with vorozole, detachment of tumor cells from the surrounding basal membrane and accumulation of apoptotic and necrotic cells were found, suggesting that aromatase inhibitors affect tumor cells, the basal membrane, and the cells in the surrounding stroma. The farnesyl transferase inhibitor R115777, in addition to causing a dramatic decrease in PI, sharply increased apoptotic cells (Fig. 2G and H, single apoptotic cell versus high number of apoptotic cells, respectively). In the case of agents that were moderately effective, the results are more heterogeneous. With regards to AI, even the highly effective agents increased the AI to only 3% (4- to 6-fold increase) and most of the moderately effective agents failed to yield a significant increase in AI (Table 2).

**Efficacy of agents to suppress mammary carcinogenesis correlates with their potential to inhibit cell proliferation and induce apoptosis.** The effects of representative agents on both PI and AI are shown in Fig. 3A and B. As can be seen, effective agents strongly decreased the PI. In contrast, only the most effective agents increased the AI significantly. A 2-fold increase in the AI was required to achieve statistical significance, which is due in part to the relatively low baseline levels of apoptosis. To further characterize the relationship between the efficacies of chemopreventive agents to inhibit mammary carcinogenesis and...
modulate cell proliferation and apoptosis in mammary tumors, correlation analysis between the values of tumor multiplicity, PI, and AI were done (Fig. 4). A striking correlation was found between the decrease in PI and inhibition of tumor multiplicity (Fig. 4A; \( R^2 = 0.911, P < 0.0001 \)). The correlation between AI and tumor multiplicity was good (\( R^2 = 0.721, P < 0.05 \)) but not as great as for PI (Fig. 4B). Thus, many of the agents that were partially effective in preventing tumor multiplicity (50-30%) had limited effects on apoptosis. When a combined AI/PI index (Fig. 4C) was examined, an imperfect relationship between this measurement and preventive efficacy (\( R^2 = 0.534, P < 0.05 \)) was similarly observed. However, agents with AI/PI indexes that were high (>0.8) tended to have highly significant preventive activity and even therapeutic activity in this model (see Table 2). This is not surprising because therapeutic activity determined by caliper measurements should reflect both strong decreases in PI as well as increases in AI.

**Discussion**

This article presents comprehensive data correlating efficacy of a wide variety of chemopreventive/therapeutic agents with their potential to suppress cell proliferation and to induce apoptosis in a short-term in vivo assay. Over the last several years, our laboratories have developed this assay for testing the efficacy of more than 20 preventive/therapeutic agents, some of them currently used in clinical trials for prevention and treatment of breast cancer (12, 19, 31). To further assess the feasibility of PI and AI as potential surrogate end points to predict preventive/therapeutic efficacy, agents with different mechanisms of action and at various dose levels were examined. PI and AI were selected because they are the major cellular mechanisms that modulate tumor growth or regression. They are also the final downstream cellular events affected by alterations in thousands of genes/proteins that have been altered in expression by various agents.

For preclinical and clinical cancer prevention and therapy studies, it is important that biomarkers be identified that reflect the efficacy of various agents at the tissue level. These biomarkers may not only indirectly reflect the accumulation and pharmacokinetics of the agent in the target organ or tissue but may also serve as surrogate end points in clinical trials (23–25, 32). Of particular interest is the development of a short-term in vivo assay (4-7 days of treatment) that can be used to screen for new agents preclinically, and perhaps clinically. The advantage of this assay is that (in addition to the short time of getting information about the potential efficacy of an agent) it may require <5% of the amount of an agent that is normally needed for a standard in vivo chemopreventive test. We previously used these end points to screen a variety of RXR receptor agonists and readily distinguished highly effective from less effective analogues (31).

![Fig. 4. A, relative decrease in cancer multiplicity versus relative decrease of PI as estimated by BrdUrd labeling. Linear correlation, \( P < 0.0001 \). B, relative decrease in cancer multiplicity versus relative increase in AI. Linear correlation, \( P < 0.05 \). C, correlation between decrease in tumor multiplicity and AI/PI values (\( P < 0.05 \)).](image-url)
The short-term effects (4-7 days exposure) of agents on proliferation and apoptosis ([Fig. 3A and B]) were compared with longer-term studies of the same agents in preventive (18 weeks) or therapeutic (6 weeks) regimens (Table 2). Thus, the short-term biomarker studies were obtained separately from the long-term prevention and therapy studies. Briefly, relatively small (100-250 mm²) palpable cancers were used for modulation of the biomarkers because they are composed predominantly of cancer cells and have no necrotic areas. The use of small cancers and short-term biomarkers is similar to what one would want to use in human presurgical studies with antitumor agents (23–25). Furthermore, our laboratories have examined the feasibility of such an approach in a number of preclinical studies (12, 14, 19, 27, 31). When examining alterations in small palpable mammary cancers, one should note that these tumors are diploid (as evaluated by flow cytometry) and not as genetically advanced as most human malignant tumors. Most human breast cancers (>75%) include DNA-aneuploid cell clones, which differ in their proliferative activity and probably in their sensitivity to preventive/anticancer agents (33).

From the results presented in Table 2 and Fig. 3A, there is a strong correlation between the ability of agents to inhibit proliferation in small mammary cancers and their ability to prevent formation of mammary cancers following long-term exposure. However, when trying to differentiate marginally effective agents from moderately effective agents, it is probably not so strong. The results with the individual animals show that highly effective agents can cause a striking and relatively homogeneous decrease in proliferation. However, even in those cases where tumor size is greatly decreased, the PI is infrequently reduced by >90%. This is noted because certain human studies have proposed a PI <1.0% in treated tumors to be considered positive (32). Given that the PI in human tumors may be >15%, final PI of <1% reflect a truly profound decrease and may be too high a hurdle to define agents that are likely to be active. The correlation between chemopreventive efficacy and PI was also examined. In contrast to the excellent correlation with PI, the correlation with AI was not as strong because compounds with moderate activity (30–60% decrease in tumor multiplicity; e.g., UIAB30 and 5,6-benzoflavone) failed to achieve significant apoptotic activity. Of interest, even a highly effective dose of tamoxifen (1.0 mg/kg diet), which greatly reduced tumor multiplicity (almost 80%) in the prevention assay, caused a limited, albeit significant, increase in AI. Determination of alterations in AI is more difficult because of the relatively low control AI levels. Nevertheless, most of the highly effective treatments (e.g., R115777, vorozole, targetin, ovariectomy, and iressa) that were also therapeutic in this model strongly increased the AIs (12, 14, 19).

The maximal values of AI (2-3%), although a 4- to 6-fold increase, were not very high even in agents that were profoundly effective. However, it should be noted that apoptosis is a short-term event. It takes only 20 to 40 min for a cell to die by apoptosis. Therefore, even the low values of AI suggest an increase in tumor cell death (17). Some of the agents with moderate preventive efficacy preferentially altered proliferating cells (PI) and had limited effect on apoptosis (AI). In contrast, removal of estrogen by either an aromatase inhibitor or by ovariectomy strongly increased the AI, decreased the PI, and resulted in a relatively high percentage of cells that seemed to be necrotic (19). The profound effects on tumor cell necrosis/apoptosis elicited by vorozole and ovariectomy presumably reflect the striking dependency of these tumors on continued estrogenic stimulation. We did not see significant numbers of necrotic cells with any of the nonhormonal agents, suggesting tumor growth inhibition by mechanisms that do not involve increased necrotic cell death.

The AI/PI ratio versus long-term chemopreventive efficacy was also examined. These results, like the AI, do not show as strong a correlation with chemopreventive efficacy as the PI. However, this additional variable shows that doses of an agent that have an AI/PI ratio >0.8 are effective therapeutic agents in the MNU mammary cancer model. This would inherently seem reasonable because a moderate to strong effect on AI combined with substantial inhibition of proliferation should result in regression of tumors. The major difference between breast cancer in women and chemically induced cancers in rats is that, in the latter, the responses are often much more rapid, leading to tumor disintegration and regression within a period of 2 to 3 weeks. Thus, vorozole at higher doses may profoundly decrease tumor size within weeks in the rat model, whereas clinical changes in humans with aromatase inhibitors may take many months. There have been recent reports in humans using aromatase inhibitors, selective estrogen receptor modulators, or epidermal growth factor receptor inhibitors that show that one can readily follow altered proliferation in early-stage tumors in either a presurgical or neoadjuvant setting (23–26, 34, 35).

Thus, this short-term assay can not only identify generally effective agents but also seems to mimic clinical results determined pathologically.

In a slightly more speculative mode, the question arises whether these studies might have been done in histologically normal “at risk” cells or in xenografts. The basic measurements of proliferation and apoptosis should be amenable to the widest range of models, but all models have some limitations. Using mammary tissue, with our BrdUrd labeling method, it was found that <1% of the cells were labeled and <5% of the cells were epithelial cells. This leads to practical problems in examining any agent where the inhibition is less than profound. It will similarly be quite difficult to collect a sufficient number of mammary epithelial cells to do such counts in a clinical setting. Cells from fine needle aspirates or even biopsy cores of human breast may not yield enough epithelial cells. A second question is perhaps more philosophical in nature. If one is trying to develop drugs for phase III prevention trials (with 3–5 years of treatment), the question arises whether “normal epithelium,” as contrasted with cells from lesions, such as used here, is even the proper target cells.

We are, however, attempting to discern whether this is feasible using different PI measurements (e.g., proliferating cell nuclear antigen or Ki67). The problem with trying to do similar studies in xenografts is that these cells are much more advanced genetically and phenotypically than most early lesions, thus making it impossible to carry out true prevention studies.

In summary, the present studies confirm that by using MNU-induced mammary cancers, one can predict the preventive efficacy of a variety of agents based on short-term modulation of PI and AI. It also seems that these indices will discern highly effective agents from marginally or minimally
effective agents. These studies offer support for clinical trials in a presurgical or neoadjuvant setting which attempt to identify potential effective agents for phase III trials. Some agents may preferentially suppress cell proliferation, whereas others may affect proliferation as well as enhance apoptotic cell death. This approach will also be advantageous for screening numerous agents in a preclinical setting because the assay requires limited time to complete and limited amounts of the proposed agent.

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References

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