Drug-Induced Expression of Nonsteroidal Anti-Inflammatory Drug-Activated Gene/Macrophage Inhibitory Cytokine-1/Prostate-Derived Factor, a Putative Tumor Suppressor, Inhibits Tumor Growth

Jeanelle M. Martinez, Tina Sali, Ryuji Okazaki, Colleen Anna, Melinda Hollingshead, Curtis Hose, Anne Monks, Nigel J. Walker, Seung Joon Baek, and Thomas E. Eling

Laboratories of Molecular Toxicology (J.M.M.) and Toxicology Operations Branch (N.J.W.) and Molecular Carcinogenesis (T.S., R.O., C.A., S.J.B., T.E.E.) National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina; SAIC-Frederick, Inc., Screening Technologies Branch, Laboratory of Functional Genomics, National Cancer Institute, Frederick, Maryland (M.H., C.H., A.M.); National Decontamination Team, Office of Solid Waste and Emergency Response, Environmental Protection Agency, Cincinnati, Ohio (J.M.M.); Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee (S.J.B.); and Department of Radiation Biology and Health, School of Medicine, University of Occupational and Environmental Health Kitakyushu, Japan (R.O.)

Received December 16, 2005; accepted May 15, 2006

ABSTRACT

A common in vitro response for many chemopreventive and anti-tumor agents, including some cyclooxygenase inhibitors, is the increased expression of nonsteroidal anti-inflammatory drug-activated gene (NAG)-1/macrophage inhibitory cytokine (MIC)-1/prostate-derived factor (PDF). The experimental anticancer drug 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F203) was a potent inducer of NAG-1 expression, and in MCF-7 cells, it inhibited cell growth and induced apoptosis. NAG-1 small interfering RNA blocked NAG-1 expression and 5F203-induced apoptosis in MCF-7 cells, indicating that NAG-1 may mediate the apoptosis and anticancer activity. One mechanism by which 5F203 increases NAG-1 expression is by increasing the stability of NAG-1 mRNA, dependent of de novo protein synthesis. Extracellular signal-regulated kinase (ERK) 1/2 phosphorylation was increased by 5F203, and inhibition of ERK1/2 phosphorylation abolished the induction of NAG-1 protein expression and increased the stability of NAG-1 mRNA. Thus, 5F203 regulates NAG-1 expression by a unique mechanism compared with other drugs. A mouse orthotopic mammary tumor model was used to determine whether 5F203 increased NAG-1 expression in vivo and suppressed tumor growth. Treatment of the mice with Phortress, the prodrug of 5F203, increased the in vivo expression of NAG-1 as measured by real-time reverse transcription-polymerase chain reaction from RNA obtained by needle biopsy, and the expression correlated with a reduction of tumor volume. These results confirm that NAG-1 suppresses tumor growth, and its in vivo expression can be controlled by treating mice with anticancer drugs, such as Phortress. Drugs that target NAG-1 could lead to a unique strategy for the development of chemotherapeutic and chemopreventive agents.
regulated by Cox inhibitors, independently of Cox inhibition, is the nonsteroidal anti-inflammatory drug-activated gene (NAG-1) (Baek et al., 2001b). NAG-1 is a member of the transforming growth factor (TGF)-β (TGF-β1) superfamily and has proapoptotic and antitumorogenic activities. NAG-1 is also known as macrophage inhibitory cytokine (MIC)-1, growth/differentiation factor-15, prostate-derived factor (PDF), and placental TGF-β (Baek and Eling, 2006). Not only is NAG-1 regulated by Cox inhibitors but also by an increasing number of chemopreventive and antitumorogenic chemicals. Transcriptional regulation of NAG-1 is complex, and the promoter sequence has many different cis- and trans-acting promoter elements (Baek et al., 2001a). In addition, NAG-1 seems to be a potentially important downstream target of three tumor suppressor genes. NAG-1 expression is regulated by the tumor suppressor genes p53 (Baek et al., 2002a, 2004a) and early growth response (EGR) gene-1 (Baek et al., 2004b). Recently, we have identified NAG-1 as a novel negative downstream target of the phosphatidylinositol 3-kinase/protein kinase B (AKT)/glycogen synthase kinase 3β pathway (Yamaguchi et al., 2004). Thus, NAG-1 seems to be a central and possibly key protein regulated by these tumor suppressor pathways.

The mechanism by which this protein acts to suppress tumor growth is not clear. Compelling evidence in the literature supports the hypothesis that NAG-1 regulates apoptosis in a number of cells, including human colorectal and breast cells. Since most of the data on the biological activity and expression are based on in vitro experiments, it is important to confirm the activity in vivo and to confirm that drug-targeted, NAG-1 up-regulation in an intact animal can be correlated with inhibition of tumor development. A potent drug with characterized anticancer activity in vivo would be useful to obtain these results. One candidate drug is 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5P203). 5P203 is the active moiety of Phortress (NSC 710305), a drug that is in the early phase 1 clinical studies in cancer patients. The antitumor activity of this drug is based on the growth inhibitory and cytoidal activity in specific tumor cell lines and on the inhibition of a variety of xenografts, including MCF-7 (Bradshaw et al., 2002). The mechanism for the antitumor activity is not clearly known, but the drug does offer the advantage of not being a Cox inhibitor and thus is devoid of toxic side effects associated with this class of drugs. MCF-7 breast cancer cells are sensitive to the antiproliferative activity of 5P203, and cDNA microarray analysis revealed NAG-1 as the most highly 5P203-induced gene in the MCF-7 cell line (Monks et al., 2003). If the activity of this potent antitumorogenic drug is dependent on NAG-1, it may be a useful model to show that the increase in NAG-1 expression in vivo can be correlated with the in situ NAG-1 expression and reduced tumor growth.

The goals of this study were to 1) determine whether NAG-1 expression is increased by 5P203 and ascertain the regulatory mechanism underlying the increase, 2) determine whether the biological activity of 5P203 is dependent on NAG-1, 3) determine whether NAG-1 inhibits MCF-7 tumor development in mouse xenographs, and 4) demonstrate in an animal model whether 5P203 can exert its antitumorogenic activity by targeting the up-regulation of NAG-1 in vivo.

Materials and Methods

Chemicals and Treatment. 5P203 and Phortress, the 1-lysyl- amide prodrug, are developed by The Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). For Phortress treatment of mice, stock solutions were prepared in DMSO at a concentration of 40 mM, aliquotted, and kept frozen at −80°C until required. For treatment of nude mice with Phortress, the drug was administered by i.p. three times a day for 4 days, and then the mice rested for 10 days, after which the treatment schedule was repeated.

Cell Culture Conditions. The MCF-7 cells, and HCT-116 cells were acquired from American Type Culture Collection (Manassas, VA). HCT-116 cells null for p53 were obtained from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD).

Cell Proliferation. On day 0, 50,000 cells were plated in a 96-well tissue culture plate (Nuncelon; NUNC A/S, Roskilde, Denmark). Cells were incubated for various indicated times. On day 1, 50 μl of medium was added for further incubations up to 72 h. The number of viable cells was determined daily by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) nonradioactive cell proliferation assay (Promega, Madison, WI). In brief, 25 μl of freshly prepared MTS mixture was added, and after 1 h absorbance at 490 nm was measured. Each experiment was repeated at least three times.

Cycloheximide Treatment. Cells grown to 60 to 80% confluency were washed with PBS, and then treated with serum-free medium containing DMSO plus or minus 5 μg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO). After 30 min, 5P203 or vehicle was added directly to the medium. Cells were harvested 24 h after the addition of 500 nM 5P203 and analyzed by real-time PCR, as described below.

MCF-7 Orthotopic Tumors. In nude mice, a 1- to 1.5-cm ventral midline incision was made through the skin in the abdominal region, and a flap of skin was bluntly dissected from the abdominal wall, exposing the ventral aspect of the number 4 mammary gland. A syringe with a 27-gauge needle was used to make the injection of 7 × 10⁶ MCF-7 breast cancer cells in 0.1 ml of PBS. Mice were given 3 mg/kg estradiol every 7 days subcutaneously throughout the experiment. The mice were treated with Phortress starting on day 20. Control mice were treated with the vehicle. Growth curves for xenograft tumors were determined by external measurements from two dimensions. Tumor measurements began when the size was more than 3 mm in diameter (around 14 days after injection). Tumor volume or weight was determined, respectively, by the equations V = [(L + W)/2] × L × W × 0.5236 and weight (in milligrams) = [length] × [width] × [weight] / 2.

Ectopic Expression of NAG-1 in MCF-7 Cells. MCF-7 cells lines were engineered to overexpress NAG-1 or empty vector. The stable cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 250 μg/ml G418 (Invitrogen, Carlsbad, CA). Log-phase NAG-1 was resuspended in PBS and then injected into mice at a concentration of 1 × 10⁷ cells in 50 μl as described above.

In Vivo Analysis of NAG-1. Samples of the mouse tumors were obtained by fine needle aspirates from the mammary fat pad of nude mice 6 and 24 h after 15- or 10-mg/kg treatment with Phortress. RNA samples were isolated using a QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA). NAG-1 expression in the samples was measured using Applied Biosystems (Foster City, CA) TaqMan real-time PCR. glyceraldehyde-3-phosphate dehydrogenase was used as the endogenous control.

Western Blot Analysis. Anti-human-NAG-1 antibody that recognizes both the precursor and secreted forms of NAG-1 (Baek et al., 2001b) developed in this laboratory was used. Actin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ERK1/2 and phosphorylated ERK1/2 was obtained from Cell Signaling Technology Inc. (Beverly, MA). Phospho-GSK-3β, AKT, and
phospho-AKT (Ser473) antibodies were purchased from Cell Signaling Technology Inc. Cells were grown to 60 to 80% confluence in 10-cm plates followed by 24 h of additional growth in the absence of serum. Cells were treated with indicated compounds, and total cell lysates were isolated using 0.1 M Tris, pH 8.0, containing protease inhibitors (Sigma-Aldrich). After sonication of samples, proteins (30 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred for 1 h to nitrocellulose membrane (Whatman Schleicher and Schuell, Keene, NH). The blots were blocked overnight with 5% skim milk in Tris-buffered saline/0.05% Tween, and probed with each antibody for 2 h at room temperature. After washing with Tris-buffered saline/0.05% Tween, the blots were treated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed several times. Protein bands were detected by the enhanced chemiluminescence system (GE Healthcare, Arlington Heights, IL).

**NAG-1 Small Interfering RNA and Apoptosis.** The NAG-1 siRNA construct and NAG-1 scrambled sequence siRNA construct were gifts from Jim Lambert (Department of Pathology, University of Colorado Health Sciences Center, Denver, CO). The oligonucleotides used for generating NAG-1 siRNA were 5′-GGGACCCCTCAAGCTTGAGTGCAACTCTGGCATTTTTTG-3′ and 5′-AATTCTAAAGGGACTTCAGAGCTGTTGCTAGGG-3′. Cells at 60 to 80% confluence were transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) with NAG-1 scrambled NAG-1 siRNA (25 nM/well) or with the scrambled NAG-1 siRNA (2 μg/well) overnight. Medium was removed, and the cells were washed with PBS and then treated with 100 to 500 nM 5F203 or vehicle in medium containing reduced serum (1%) for up to 48 h. Cells were harvested at 24 h and analyzed by Western blot as described above and harvested at 48 h for apoptosis analysis. For apoptosis, cells were washed with PBS, trypsinized, collected, washed in ice-cold PBS, and fixed by the slow addition of 75% ethanol to a total of 5 ml and stored at 4°C overnight. Fixed cells were pelleted and then washed with PBS and stained with 20 μg/ml propidium iodide and 1 mg/ml RNase in PBS for 20 min. Cells were examined by flow cytometry using Becton Dickinson FAC Sort and CellQuest software (BD Biosciences, San Jose, CA). Apoptosis was measured by the level of subdiploid DNA in cells.

**NAG-1 Promoter Activity.** The NAG-1 promoter constructs were prepared as described previously (Baek et al., 2001a). Specific constructs included the following promoter regions: −133/−70, −966/+1, −966/−70, −5700/−70, and −133/−70 with NAG-1 intron. HCT-116 or MCF-7 cells were cotransfected with the NAG-1 promoter cloned into the pGL3 basic vector (BD Biosciences) or into the luciferase vector and with the pRL-null vector (Promega) to control for transfection efficiency. Lipofectamine (Invitrogen) was used to transfect the cells for 5 h. Medium containing serum was then added, and cells were incubated at 37°C overnight. To treat cells, serum-free medium containing either 5F203, negative control (DMSO), or positive control (sulindac sulfide or troglitazone) was added. After 24 h of drug treatment, cells were lysed using passive lysis buffer (Promega), and β-galactosidase activity was measured using a luminometer and normalized using the β-galactosidase/pRL null ratio for comparison of the various samples. After 24 h of transfection, luciferase activity was determined and normalized to the pRL-null luciferase activity with a dual luciferase assay kit (Promega).

**RNA Stability in the Presence of Actinomycin D.** MCF-7 or HCT-116 cells were plated into six-well plates at 2 × 105 cells per well, grown to 60 to 80% confluence, and then treated with either DMSO or 5F203 in serum-free medium. After 24 h, 5 μg/ml actinomycin D (Sigma-Aldrich) was added for the indicated length of time. Cells were lysed in QIAGEN RNA isolation buffer, and RNA was purified using the QIAGEN RNeasy kit. Total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase, and real-time PCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Actin was used as the endogenous control. A negative control containing all the reagents and cDNA in the absence of the reverse transcriptase enzyme (no reverse transcription control) was routinely performed. Primers used for NAG-1 were 5′-GGGACCCCTCAAGCTTGAGTGCAACTCTGAGGGTCCCTTTTTG-3′ and 5′-CTCTGAGGAGCTGTGCTGTTGCTAGGACACCTCTTCAGACCTAAGCTTGAGG-3′; the oligonucleotides used for generating the control siRNA containing a scrambled sequence were 5′-GGGACCCCTCAAGCTTGAGTGCAACTCTGAGGGTCCCTTTTTG-3′ and 5′-AATTCTAAAGGGACTTCAGAGCTGTTGCTAGGG-3′. Cells were grown to 60 to 80% confluence were transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) with NAG-1 scrambled NAG-1 siRNA (25 nM/well) or with the scrambled NAG-1 siRNA (2 μg/well) overnight. Medium was removed, and the cells were washed with PBS and then treated with 100 to 500 nM 5F203 or vehicle in medium containing reduced serum (1%) for up to 48 h. Cells were harvested at 24 h and analyzed by Western blot as described above and harvested at 48 h for apoptosis analysis. For apoptosis, cells were washed with PBS, trypsinized, collected, washed in ice-cold PBS, and fixed by the slow addition of 75% ethanol to a total of 5 ml and stored at 4°C overnight. Fixed cells were pelleted and then washed with PBS and stained with 20 μg/ml propidium iodide and 1 mg/ml RNase in PBS for 20 min. Cells were examined by flow cytometry using Becton Dickinson FAC Sort and CellQuest software (BD Biosciences, San Jose, CA). Apoptosis was measured by the level of subdiploid DNA in cells.

**Results**

5F203 Induces NAG-1 Expression. The human colon adenocarcinoma HCT-116 cell line has been used by this laboratory as a model system to investigate the regulation of NAG-1 expression (Baek et al., 2001b) and to compare NAG-1 induction by chemopreventive chemicals. Therefore, initial studies in this well defined model focused on the response of NAG-1 to 5F203. Robust induction of NAG-1 was observed following treatment with 5F203, and this induction was dependent on incubation time and the drug concentration (Fig. 1, A and B). The time course of NAG-1 protein expression was similar to the course observed previously (Monks et al., 2003) in that the highest expression was observed after 24 h (Fig. 1A). An increase in NAG-1 expression was observed at 10 nM 5F203, the lowest concentration tested. Thus, 5F203 was more potent than any other drug we have tested in its ability to increase NAG-1 expression in HCT-116 cells (Baek et al., 2001b).

To examine whether 5F203 induction of NAG-1 expression was dependent on de novo synthesis of the protein, cells were pretreated with the translational inhibitor cycloheximide for 30 min. As shown in Table 1, 5F203-induced NAG-1 mRNA expression was inhibited by the presence of cycloheximide, suggesting that the up-regulation of NAG-1 expression by 5F203 requires, in part, de novo protein synthesis of regulatory intermediate proteins as observed for other drugs (Baek et al., 2005). Phase I cancer clinical studies using the prodrug form of 5F203 (Phortress) are based, in part, on inhibition of tumor growth with the breast cancer cell lines in the nude mouse xenograft model and other biochemical data (Bradshaw and Westwell, 2004). Thus, it was important to determine whether 5F203 increased NAG-1 expression in human breast cancer cells. Two human tumor cell lines were used: the MCF-7 cells, which are responsive to 5F203, and MDA-MB-453, which are nonresponsive to 5F203 (Bradshaw et al., 2002). To measure the expression of NAG-1 protein in MCF-7 cells, cells were incubated with 1 μM 5F203 for up to 96 h (Fig. 1C). A time-dependent increase in NAG-1 protein expression was observed in the MCF-7 cells (Fig. 1C). 5F203 was also a potent NAG-1 inducer in MCF-7 cells with induction observed at as low as 10 nM, and maximal induction...
with 100 to 500 nM (Fig. 1D). In addition, NAG-1 induction by 5F203 treatment was observed in the human prostate cancer cells LNCaP and PC3, but we did not observe an increase in the expression of NAG-1 in MDA-MB-435 cells (data not shown). To determine the optimal concentration of 5F203 to alter cell growth of MCF-7, cell proliferation was measured after treatment with 5F203, which was a potent inhibitor of cell proliferation (Table 2). Both a time- and concentration-dependent inhibition of cell growth was observed, which was similar to the time and concentration dependence of NAG-1 induction. The growth of MDA-MB-435 cells has been shown not to be inhibited by 5F203 (Bradshaw et al., 2002).

**Effect of 5F203 on NAG-1 Transcription.** NAG-1 has been reported to be regulated by many chemicals at the transcriptional level (Li et al., 2000; Baek et al., 2001a,b, 2002a, 2004a,b, 2005) and some of which are dependent on de novo protein synthesis (Newman et al., 2003; Baek et al., 2005). Regulation of NAG-1 at the transcriptional level was examined with various promoter constructs designed with different regions of the 5-kilobase 5′ flanking region and inserted into luciferase and β-galactosidase reporting plasmids in HCT-116 and MCF-7 cells. However, despite incubation with several concentrations of 5F203, we could not detect any drug-induced increase in the promoter activity. Furthermore, incubation with 5F203 also inhibited the basal activity of the promoters. The 5F203 seems to inhibit luciferase and β-galactosidase activities or is toxic to the promoter constructs; thus, this approach cannot be used to investigate the transcriptional regulation (data not shown).

The expression of NAG-1 is regulated by antitumorigenic chemicals at the transcriptional level and is mediated by tumor suppressor gene p53, by EGR-1, and by GSK-3β/akt, three pathways linked to tumor growth. HCT-116 and MCF-7 cells were incubated with either 1 μM 5F203 or 30 μM sulindac sulfide, and the expression of EGR-1 was measured by Western analysis. As shown in Fig. 2A, for HCT-116 cells, sulindac sulfide, a positive control (Baek et al., 2005), increased the expression of EGR-1, but 5F203 was ineffective at increasing the expression of EGR-1. Similar results were observed in MCF-7 cells (data not shown). These results indicate that 5F203 does not increase NAG-1 by altering EGR-1 expression.

To determine whether the increase in NAG-1 by 5F203 was dependent on p53, we treated HCT-116 cells that were made null for p53 with 5F203. As shown in Fig. 2B, these cells did not express p53 protein, but 5F203 effectively increased the expression of NAG-1. Thus, 5F203 increased the expression of NAG-1 in both p53 wild-type and p53 null cells. Furthermore, the forced expression of p53 into the HCT116 null cells did not alter the response to 5F203 (data not shown). Because NAG-1 expression is also regulated downstream of AKT, we incubated MCF-7 cells with 1 μM 5F203, and the cell lysates were analyzed for GSK-3β and phosphorylated GSK-3β, AKT, and phosphorylated AKT expression (Fig. 2C). However, we did not observe changes in the expression of these proteins after treatment with 5F203. Thus, 5F203 did not seem to alter the expression of NAG-1 by established transcriptional mechanisms examined, but we cannot rule out other transcriptional mechanisms.

**5F203 Stimulates ERK Phosphorylation.** The effect of 5F203 on the phosphorylation of several regulatory proteins was examined in HCT-116 and MCF-7 cells. 5F203 did not alter p38 expression or p38 phosphorylation (data not shown), but it did alter the phosphorylation of ERK1/2 in HCT-116 cells (Fig. 3A). The expression of total ERK1/2 was not altered by 5F203. We next determined whether the expression of NAG-1 was dependent on ERK1/2 phosphoryla-
tion. MCF-7 and HCT-116 cells were incubated with 5F203 in the presence and absence of PD98059, an inhibitor of ERK1/2 phosphorylation. As shown in Fig. 3B, the inhibitor PD98059 suppressed the 5F203 induction of NAG-1 protein. Similar results were obtained with the ERK inhibitor U0216 (data not shown).

**Effect of 5F203 on RNA Stability.** Because 5F203 apparently does not affect NAG-1 expression by the known transcriptional factors, we suspected that 5F203 may affect NAG-1 expression at the post-transcriptional level. 5F203 could act post-transcriptionally by increasing mRNA stability. MCF-7 cells were treated with 5F203 or vehicle for 24 h, and then transcription was inhibited by the addition of actinomycin. RNA was harvested at different time points after RNA transcription was halted, and levels of NAG-1 mRNA were determined by real-time RT-PCR. Incubation with 5F203 increased the half-life of NAG-1 mRNA in the MCF-7 cell line from $\frac{1}{2}$ to $\frac{1}{4}$ min (Fig. 4). This increase in NAG-1 mRNA was subsequently confirmed by Northern analysis in the HCT-116 cell line (data not shown). Thus, 5F203 increased the expression of NAG-1 protein via a post-transcriptional mechanism, in turn increasing mRNA stability.

We next examined the effect of the ERK inhibitor on 5F203-mediated increases in the half-life of NAG-1 mRNA. Inhibition of ERK phosphorylation inhibited the 5F203-dependent increase in the half-life of mRNA from $\frac{1}{2}$ to $\frac{1}{4}$ min (Fig. 4), but it did not alter the mRNA stability in the absence of 5F203 ($\approx 16$ min). These results suggest that ERK1/2 kinase may play an important role in the 5F203-induced NAG-1 expression.

### Table 2

**Effect of 5F203 on growth of MCF-7 cells**

Concentration-dependent decrease in viable cells after 24-, 48-, and 72-h treatment with 5F203 as determined by MTS assay. MCF-7 cells were treated with vehicle or different concentrations of 5F203 for several days. Cell growth was measured as described under Materials and Methods. The measurement at each time, day, and dose was calculated as a percentage of the vehicle control value for that time and day. Kruskal-Wallis ANOVA was used to determine differences among hours for each dose level (Conover, 1971). Where significant differences were found by the Kruskal-Wallis ANOVA, two-sided Mann-Whitney U-tests were used to identify pairs of hours that differed significantly (Conover, 1971). Values are percentage of controls, mean ± S.E., n = 3 unless otherwise specified.

<table>
<thead>
<tr>
<th>Hours</th>
<th>1 nM</th>
<th>10 nM</th>
<th>100 nM</th>
<th>1 μM</th>
<th>10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>92.1 ± 2.0 (n = 2)</td>
<td>78.3 ± 2.7</td>
<td>71.1 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.4 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.7 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>93.7 ± 12.5 (n = 2)</td>
<td>66.5 ± 7.8</td>
<td>49.6 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.8 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.7 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>72</td>
<td>87.9 ± 9.3 (n = 2)</td>
<td>60.4 ± 9.3</td>
<td>38.6 ± 3.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>36.2 ± 1.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>36.7 ± 1.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Kruskal-Wallis p value

0.95 | 0.66 | 0.022 | 0.002 | 0.001

<sup>a</sup> Differs from 24 h at p < 0.05.<br>
<sup>b</sup> Differs from 48 h at p < 0.05.
5F203 Induction of Apoptosis Is Blocked by NAG-1 siRNA. The controlled expression of NAG-1 in MCF-7 and HCT-116 cells induces apoptosis, and it may, in part, be responsible for the anticancer activity of NAG-1 as measured in mouse xenograph models (Baek et al., 2001b). To link the antitumorigenicity of 5F203 to NAG-1 proapoptotic activity, we transfected MCF-7 cells with NAG-1 siRNA or scrambled NAG-1 siRNA (vector) and then incubated cells with 100 to 500 nM 5F203 and measured apoptosis and NAG-1 expression (Fig. 5). A large induction of NAG-1 was observed at 24 h in cells transfected with the scrambled siRNA and treated with 5F203. In contrast, cells transfected with NAG-1 siRNA and then treated with 5F203 showed minimal induction of NAG-1 protein (Fig. 5A). Incubation of the scrambled siRNA-transfected cells with 5F203 increased apoptosis compared with control, DMSO-treated cells (Fig. 5B). In contrast, cells transfected with NAG-1 siRNA and then treated with 5F203 showed a significantly reduced apoptosis. These data support the conclusion that 5F203-induced apoptosis in MCF-7 cells is mediated, in part, by the increased expression of NAG-1.

5F203 Blocks Growth of Tumors in Vivo by Induction of NAG-1. The antitumorigenic properties of 5F203 on MCF-7 tumors in vivo may be dependent, in part, on NAG-1 expression. Nude mice injected with MCF-7 cells into the mammary fat pad were injected with 5F203 in the form of its L-lysylamide prodrug Phortress. We measured the size of orthotopic mammary tumors and used real-time PCR to measure the levels of NAG-1 in the tumors. The median mammary tumor weight was effectively decreased by up to 75% (Fig. 6A) following treatment of the mice with Phortress. To relate the inhibition of tumor growth with NAG-1 expression, samples of the tumors were removed by needle biopsy at 6 and 24 h after initiation of drug treatment, and the in situ NAG-1 expression was measured by real-time RT-PCR. Phortress caused an apparent dose-dependent increase in the expression of NAG-1 mRNA in the tumors at both 6 and 24 h after drug treatment (Fig. 6B). A statistically significant difference (p < 0.05) was observed at the 15-mg/kg dose at both times but not at 10 mg/kg compared with the untreated mice, although the dose dependence is apparent on inspection of the data. In addition an inverse relationship was observed between the size of the tumor and the expression of NAG-1 RNA (data not shown). To confirm that NAG-1 expression in MCF-7 cells can directly inhibit tumor growth in an orthotopic tumor model, MCF-7 cells were developed that overexpress NAG-1. Antisense vectors were not used since MCF-7 cells express very low levels of NAG-1. Overexpression of NAG-1 in the developed cells (Fig. 6C) was confirmed using Western blot analysis with a pool of transfected cells since it was not possible to isolate individual clones due to the apoptosis induced by NAG-1 expression. The NAG-1-expressing cells were injected into the mammary fat pad of nude mice, and tumor growth was measured as shown in Fig. 6C. The overexpression of NAG-1 reduced the size of the orthotopic tumor by approximately 50%. These findings indicate that an increase in the in situ NAG-1 expression occurs in the orthotopic mammary tumor as a result of treatment of the
nude mice with Phortress. These results support the hypothesis that the in vivo induction of NAG-1 mediates the inhibition of tumor growth by Phortress in the mouse orthotopic mammary model.

Discussion

A common biological response of many chemopreventive agents is the increased expression of the protein NAG-1/MIC-1/PDF, a member of the TGF-β superfamily (Baek et al., 2002a,b, 2004a, 2005; Bottone et al., 2002; Wilson et al., 2003; Lee et al., 2005). Considerable evidence obtained from in vitro experiments and biochemical data indicated that this protein has a proapoptotic activity. The antitumor activity is based solely on the inhibition of tumor growth of human colorectal cells in a nude mouse xenograft model (Baek et al., 2001b). In this report, we have confirmed the antitumor activity of NAG-1 and extended these findings to inhibition of mammary tumors formed from human breast cancer cells. The pharmacological agent 5F203 has well documented anticancer activity and has lead to the development of the prodrug Phortress that is entering phase 1 clinical trials in the United Kingdom (Bradshaw et al., 2002). Of the drugs and chemicals this laboratory has so far tested in a colon carcinoma cell culture model system, 5F203 is by far the most potent drug to induce NAG-1 in vitro. Thus, 5F203 was selected as a model drug to determine whether it is possible for a drug to alter the in vivo expression of NAG-1 and then to test for a correlation of NAG-1 expression with reduction of tumor growth. The mechanisms responsible for the anticancer activity of 5F203 are not fully understood, and most likely several possible mechanisms are responsible for the activity.

Induction of NAG-1 protein by 5F203 has been confirmed in several cell lines, including the MCF-7 breast cancer cell line, which has been used to establish the anticancer activity of 5F203 in mouse models of cancer (Bradshaw et al., 2002; Monks et al., 2003). 5F203 inhibits cell growth of MCF-7 cells and stimulates apoptosis of MCF-7 cells in culture. The controlled expression of NAG-1 in these cells stimulates apoptosis in vitro, and MCF-7 cells engineered to express NAG-1 form smaller tumors than the vector control cells. In support of the role of NAG-1 as a key proapoptotic stimuli induced by 5F203, siRNA for NAG-1 blocks the transcription of NAG-1 and leads to inhibition of 5F203-induced apoptosis and NAG-1 protein expression. Furthermore, the correlation of NAG-1 expression with the reduction of tumor growth by 5F203 treatment is supported by studies with MDA-435 cells. Mammary tumors derived from MDA-MB-435 cells in the mouse orthotopic model do not respond to treatment with the prodrug Phortress (Bradshaw and Westwell, 2004), nor does 5F203, siRNA for NAG-1 blocks the transcription of NAG-1 and leads to inhibition of 5F203-induced apoptosis and NAG-1 protein expression.

Treatment of mice with the prodrug Phortress inhibited growth of MCF-7-derived tumors in the mouse mammary cells overexpressing human NAG-1 or vector only were implanted in the mouse mammary fat pad, and the volume of the tumor was measured as described under Materials and Methods. Using one-sided p values, the groups are significantly different for all of the weeks (p < 0.05) as determined by two-sample t tests. n = 4 mice per group. The data are reported as means ± S.D.
breast cancer model and increased the in situ expression of NAG-1 RNA in the tumors. In addition, there seems to be an inverse relationship between tumor size and in vivo NAG-1 expression. Treatment of mice with 5F203 increased the expression of NAG-1 in situ and inhibited tumor growth. The results clearly support the hypothesis that the antitumorigenic effect of 5F203 is mediated, in part, by the induction of NAG-1. Modlich et al. (2004) recently examined gene expression in biopsies from primary breast cancer before and after neoadjuvant chemotherapy, which is associated with improved survival. Two genes p21 and NAG-1 were up-regulated most prominently in the post-treatment biopsy samples. Thus, an increase in the expression of NAG-1 is observed after drug treatment in both a mouse tumor model and in patients with primary cancer. These findings raise the possibility for the development of other chemopreventive or anticancer chemicals that act by increasing the expression of NAG-1 in the tumor.

Regulation of NAG-1 seems to be complex, involving both transcriptional and post-transcriptional mechanisms. NAG-1 is regulated via a number of different cis- and trans-acting promoter elements present in the promoter sequence, and most of the chemicals and drugs we have studied increase NAG-1 by transcriptional mechanisms. In contrast, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid, a retinoid X receptor agonist, increased NAG-1 expression by a post-transcriptional mechanism that altered NAG-1 mRNA stability, and for this retinoid the increase in mRNA stability was regulated independently of ERK1/2 (Newman et al., 2003). Although we cannot rule out a transcriptional mechanism without additional data, we show here that the increased NAG-1 protein expression from 5F203 treatment is mediated by increasing the stability of NAG-1 mRNA. In contrast to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid, the increased stability is partially dependent on ERK1/2. 5F203-induced ERK1/2 phosphorylation and NAG-1 expression can be effectively inhibited by an ERK1/2 inhibitor. The ERK pathway is also linked to post-transcriptional regulation. Indeed, the ERK pathway controls tumor necrosis factor-α, interleukins, and β-adrenergic receptor expressions at the post-transcriptional levels (Rutault et al., 2001). The 3′-untranslated region of NAG-1 mRNA contains four AU-rich elements that could determine the stability of NAG-1 mRNA. Other evidence points to the mitogen-activated protein kinase pathway as a regulator of mRNA stability (Headley et al., 2004). The suppression of 5F203 induction of NAG-1 by cycloheximide suggests the de novo synthesis of proteins is responsible for the stabilization of the mRNA. This finding was unexpected and points to a novel mechanism for the regulation of NAG-1 by this drug. The results illustrate the complex nature of the regulation of NAG-1 expression and a mechanism that is not fully understood.

In summary, we have presented evidence demonstrating that the expression of NAG-1 attenuates tumor growth, supporting NAG-1 as an important mediator for the activity of 5F203, an antitumorigenic drug in clinical trials for cancer. 5F203 increases the expression by a novel post-transcriptional mechanism that mediates an increase in the stability of NAG-1 mRNA, resulting in an increase in protein expression. The in vivo expression of NAG-1 is increased in the mammary tumors by drug treatment, and this increased expression correlates with reduced mammary tumor growth. We propose that NAG-1 is a key modulator in tumor suppression by a number of chemopreventive and antitumorigenic chemicals and may provide a common pathway to the antitumor activity of these structurally and chemically diverse xenobiotics.

Acknowledgments
We thank Drs. Darryl Zeldin and Wayne Glasgow for critical review and comments of this manuscript; Dr. Carl Bortner for assistance in the analysis of apoptosis by flow cytometry; and Dr. Jim Lambert for providing the NAG-1 siRNA constructs. We also thank Kali Chryseyrgis for assistance with protein analyses, Dr. Grace Kissling for statistical analyses, and Misty Bailey (University of Tennessee, Knoxville, TN) for critical reading of manuscript.

References

Address correspondence to: Dr. Thomas E. Eling, Laboratory of Molecular Carcinogenesis, 111 T.W. Alexander Dr., P.O. Box 12233, MD E4-09, Research Triangle Park, NC 27709. E-mail: eling@niehs.nih.gov