Aryl hydrocarbon receptor activation of an antitumor aminoflavone: Basis of selective toxicity for MCF-7 breast tumor cells

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Abstract

Aminoflavone (4H-1-benzopyran-4-one, 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl; NSC 686288) demonstrates differential antiproliferative activity in the National Cancer Institute's anticancer drug screen. We demonstrate here that MCF-7 human breast cancer cells are sensitive to aminoflavone both *in vitro* and when grown *in vivo* as xenografts in athymic mice. As previous work has indicated that aminoflavone requires metabolic activation by cytochrome *P*450 1A1 (*CYP1A1*), we investigated the effect of aminoflavone on *CYP1A1* expression and on the aryl hydrocarbon receptor (AhR), a transcriptional regulator of *CYP1A1*. In aminoflavone-sensitive but not aminoflavone-resistant cells, the drug caused a 100-fold induction of *CYP1A1* mRNA and a corresponding increase in ethoxyresorufin-*O*-deethylase activity. An AhR-deficient variant of the MCF-7 breast carcinoma, AH^{R100}, with diminished *CYP1A1* inducibility, exhibits cellular resistance to aminoflavone and is refractory to *CYP1A1* mRNA induction by the drug. The increase in *CYP1A1* mRNA in the aminoflavone-sensitive MCF-7 breast tumor cell results from transcriptional activation of xenobiotic-responsive element (XRE) – controlled transcription. Aminoflavone treatment causes a translocation of the AhR from the cytoplasm to the nucleus with subsequent formation of AhR-XRE protein DNA complexes. In contrast to the aminoflavone-sensitive MCF-7 cells, the resistant cell lines (MDA-MB-435, PC-3, and AH^{R100}) demonstrated constitutive nuclear localization of AhR. Additionally, aminoflavone failed to induce ethoxyresorufin-*O*-deethylase activity, *CYP1A1* transcription, AhR-XRE complex formation, and apoptosis in aminoflavone-resistant cells. These results suggest that the cytotoxicity of aminoflavone in a sensitive breast tumor cell line is the result of the engagement of AhR-mediated signal transduction. [Mol Cancer Ther 2004;3(6):715 – 25]

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Introduction

Aminoflavone (4H-1-benzopyran-4-one, 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl; NSC 686288; Fig. 1) belongs to a family of novel amino-substituted flavone derivatives that have been shown to have noteworthy antiproliferative activity against MCF-7 breast tumor cells (1-3). MCF-7 cells are exquisitely sensitive to aminoflavone, while other breast tumor cell lines, such as MDA-MB-435, are highly resistant to the drug. In the present study, we have investigated the *in vitro* activity of aminoflavone in cells that are sensitive or resistant to aminoflavone *in vitro* as well as examine potential mechanisms for this selectivity. The antiproliferative activity of aminoflavone is dependent on the activation of the compound by cytochrome *P*450 1A1 (*CYP1A1*; ref. 4). We hypothesized therefore that the differential capacity to activate *CYP1A1* and cytochrome *P*450 1B1 (*CYP1B1*) might be the basis for the selective cytotoxicity of aminoflavone. Induction of *CYP1A1* activity is mediated by the aryl hydrocarbon receptor (AhR) signal transduction pathway (5-7). The AhR is a ligand-activated transcription factor, which binds carcinogens, including aryl and halogenated hydrocarbons, such as benzo(*a*)pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), respectively (8). Once activated, the receptor translocates from the cytosol to the nucleus, where it binds to another protein, the aryl hydrocarbon nuclear translocator. This heterodimer interacts with xenobiotic-responsive elements (XRE) present in several genes. The best-characterized molecular response to ligands of the AhR is the induction of the gene *CYP1A1* (9).

We report here that aminoflavone causes an increase in ethoxyresorufin-*O*-deethylase (EROD) activity and *CYP1A1* and *CYP1B1* transcription in sensitive MCF-7 breast tumor cells, which is accompanied by AhR translocation to the nucleus, an increase in XRE-driven luciferase activity, and induction of protein-DNA complexes on the XRE sequence of the *CYP1A1* promoter. MDA-MB-435 and PC-3 cells, which are resistant to aminoflavone, do not

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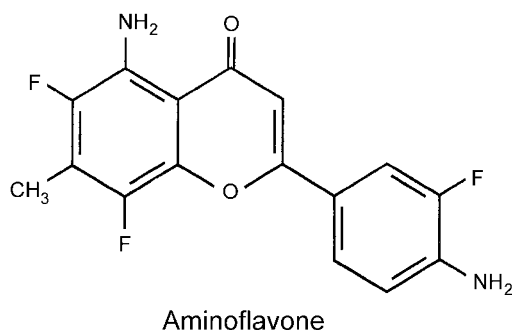

Figure 1. Structure of aminoflavone.

exhibit drug-induced EROD activity or induction of *CYP1A1* and *CYP1B1* gene expression. In aminoflavone-resistant cells, AhR was observed to be constitutively localized in the nucleus, insensitive to both induction of XRE-driven luciferase activity in transiently transfected cells and formation of protein-DNA complexes on the XRE sequence of *CYP1A1*. MCF-7-derived AhR-deficient cells, AH^{R100}, are insensitive to aminoflavone and also demonstrated a constitutive nuclear localization of their greatly diminished level of AhR. After aminoflavone exposure, EROD activity was not induced in these cells and there was no induction of *CYP1A1* or *CYP1B1* gene expression or formation of protein-DNA complexes on the XRE sequence. We also observed that, in drug-sensitive MCF-7 cells, aminoflavone exposure results in apoptosis, which was either not observed in the resistant cell lines or occurred at higher aminoflavone concentrations than required in aminoflavone-sensitive cells. These results suggest that differential engagement of AhR-related signaling in different tumor cell types may aid in further preclinical development and perhaps early clinical studies with aminoflavone.

Materials and Methods

Cell Culture

Aminoflavone was obtained from the Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan) and was dissolved in 100% DMSO to make a 100 mmol/L stock solution. MCF-7 (breast), MDA-MB-435 (breast), and PC-3 (prostate) cells were obtained from the National Cancer Institute Tumor Cell Repository (Frederick, MD). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). AhR-deficient MCF-7 (AH^{R100}) cells were generated from wild-type MCF-7 cells by continuous exposure for 6 to 9 months to 100 nmol/L benzo(*a*)pyrene (10). These cells exhibit a 100-fold higher resistance to benzo(*a*)pyrene than do the parent MCF-7 cells. The AH^{R100} cells had no detectable amounts of the AhR and normal concentrations of aryl hydrocarbon nuclear translocator (10).

Antiproliferative Properties of Aminoflavone

Cells grown in 75 cm^2 T-flasks were removed by trypsinization and seeded into 24-well plates at a concentration of 1×10^4 cells per well. Cells were allowed to grow for 48 hours at 37°C in a humidified atmosphere containing 5% CO_2. One replicate plate was processed as described below to obtain a cellular protein concentration prior to aminoflavone exposure, which can be used to assess whether aminoflavone treatment is antiproliferative or actually results in loss of cellular protein during the course of the assay (*in vitro* regression). Cells were treated with aminoflavone (10^{-10} to 10^{-5} mol/L) for an additional 72 hours. Experiments were terminated by the addition of 50% trichloroacetic acid to a final concentration of 10%, and protein was quantitated using the sulforhodamine B assay (11).

Cellular Accumulation and Covalent Binding of Aminoflavone by Human Tumor Cell Lines

Cell lines grown *in vitro* were used for covalent drug binding studies. Aminoflavone (specific activity 7.09 Ci/mmol) was kindly provided by the Research Triangle Institute (Research Triangle Park, NC) under contract to the Developmental Therapeutics Program, National Cancer Institute. Cells were exposed to 45 nmol/L aminoflavone for 16 hours at 37°C in growth medium in 12-well plates. Medium was removed and 2 mL of 100% methanol (4°C) were added to each well. The plate was placed at −20°C for 5 minutes and the wells were washed 15 times with 1.5 mL methanol (60°C) until noncovalently bound radioactivity was completely removed. Residual protein was solubilized with 1 N NaOH overnight at room temperature, and separate aliquots were used for radioactivity and protein (12) determinations.

In vivo Antitumor Properties of Aminoflavone

MCF-7 xenografts were established, maintained, and evaluated as described elsewhere (13). The animal studies were conducted under the USPHS guidelines with mice fed sterile feed and water *ad libitum* and housed in microisolator cages in an American Association for Accreditation of Laboratory Animal Care–accredited facility. For animal dosing, aminoflavone was prepared as a smooth suspension in saline containing 0.05% Tween 80. Each experiment contained a vehicle control group (n = 20) treated in parallel with the aminoflavone-treated groups. Aminoflavone was evaluated at three dose levels (n = 6 per dose) by each of two treatments routes (i.p. and i.v.). The *in vivo* treatment regimen was selected based on the tumor doubling time for MCF-7 tumor xenografts (<2.5 days; ref. 13). The treatment schedule selected for all evaluations was once-daily dosing for a total of 5 days (QD × 5), with the first treatment given on day 13 when the median tumor weights were 136 ± 10 mg. Data collection and analysis were described previously (13, 14). Tumor weights were determined by measuring the tumors in two dimensions and calculating the tumor weight from the formula for a prolate ellipsoid: Weight in mg = [(tumor length) × (tumor width in mm^2)] / 2. Median tumor weights were used to make comparisons among the treatment groups [(median tumor weight of treated) / (median tumor weight of control) × 100 (%T/C)]. This value achieved is defined as the optimal %T/C. The experiment was performed twice with similar results.

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Induction of Apoptosis by Aminoflavone

Cells were seeded into Nunc (Naperville, IL) chamber slides for 24 hours and treated for 24 hours with 1 to 1000 nmol/L aminoflavone. Growth medium containing aminoflavone was removed and medium containing 100 μg/mL 4′,6-diamidino-2-phenylindole was added to control and aminoflavone-exposed cells. Cells were stained for 10 minutes and examined under a Leitz Diaplan fluorescent microscope. Apoptotic cells exhibiting nuclear shrinkage/chromatin condensation were quantitated and expressed as a percentage of the total cells examined.

***CYP1A1* Activity in MCF-7, MDA-MB-435, PC-3, and AH^{R100} Cells**

Cells were plated in 24-well plates, allowed to grow to confluence, and treated with either 0.1% DMSO (control) or aminoflavone in 1 mL of growth medium at the indicated concentrations for 24 hours. Medium was removed and the cells were washed with PBS. *CYP1A1* enzyme activity was determined by EROD activity in intact cells as described elsewhere (15). The assay was carried out at 37°C using 5 mmol/L ethoxyresorufin in growth medium as the substrate and 1.5 mmol/L salicylamide to inhibit conjugating enzymes. The fluorescence of resorufin generated from the conversion of ethoxyresorufin by *CYP1A1* was measured every 10 minutes for 60 minutes using a Cyto Fluor II multiwell fluorescence plate reader (PerSeptive Biosystems, Framingham, MA), with excitation at 530 nm and emission at 590 nm. A standard curve was constructed using resorufin. Cell number was measured by direct counts (1.25×10^6 cells were contained per well); aminoflavone produced no changes in cell number during the treatment period. EROD activity was expressed by quantification of resorufin as picomoles per minute per 1.25×10^6 cells.

Real-time Reverse Transcription-PCR

Cells were exposed to 10^{-9} to 10^{-6} mol/L aminoflavone for 24 hours at 37°C in complete growth medium, trypsinized, and harvested by centrifugation at 300 × *g* for 10 minutes. Total RNA was isolated from cell pellets using the RNeasy mini kit (Qiagen, Valencia, CA) followed by reverse transcription using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA).

The evaluation of gene expressions was performed by real-time reverse transcription-PCR. For *CYP1A1* and *CYP1B1*, the primers and probes were GATTGGGCA-CATGCTGACC (*CYP1A1* forward), CTGTCAAGGAT-GAGCCAGCA (*CYP1B1* reverse), FAM-TGGGAAAGAA CCCGCACCTGGC-TAMRA; *CYP1A1* probe), TTTCGG CTGCCGCTACA (*CYP1B1* forward), ACTCTT CGTTGTGGCTGAGCA (*CYP1B1* reverse), and FAM-ACGACGACCCCGAGTTCCGTGAG-TAMRA (*CYP1B1* probe). For the endogenous control glyceraldehyde-3-phosphate dehydrogenase, human primers and probes were used (PE Biosystem, Foster City, CA). PCR consisted of 40 cycles. Data were analyzed using the comparative C_t method (User Bulletin 2, Perkin-Elmer, Norwalk, CT), and induction of *CYP1A1* and *CYP1B1* was expressed relative to untreated controls.

Transfection Experiments

Cells were plated in six-well plates at a concentration of 2×10^5 cells per well. Cells were transfected after 12 hours, using Lipofectamine (Invitrogen, Carlsbad, CA), with 0.5 mg *Renilla reniformis* luciferase (pRL-TK, Promega, Madison, WI), and 1.5 mg pTX.Dir [two XRE sequences extending from nucleotides −1026 to −999 relative to the transcription start site of the rat *CYP1A1* inserted in a vector containing the herpes simplex virus thymidine kinase promoter (HSV-TK) and the luciferase reporter gene (16)] or pT81 (same reporter plasmid without the XRE sequence, used as a negative control; ref. 17)]. Transfected cells were treated with 10 nmol/L TCDD or 1 nmol/L to 1 μmol/L aminoflavone after 24 hours as indicated in the figures. Control cells were transfected with pTX.Dir and treated with 0.1% DMSO. Luciferase activity was measured after a 9-hour treatment by the dual-luciferase assay system (Promega). Transfection efficiency was monitored by *R. reniformis* luciferase activity using the pRL-TK plasmid as an internal control.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were blocked with 1% bovine serum albumin for 1 hour prior to incubation with goat anti-human AhR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which was used at a dilution of 1:100. Goat IgG (Santa Cruz Biotechnology) was used to determine nonspecific background. Cells were subsequently stained with 0.4% 4′,6-diamidino-2-phenylindole for nuclear visualization.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to a previously published method (18). Synthetic oligonucleotides containing the AhR binding site of the human *CYP1A1* promoter (5′-CTCCGGTCCTTCTCACGCAACGCCTGGGCA-3′; Life Technologies) or consensus SP1 (Promega) were used as probes or competitors. End labeling was performed with T4 polynucleotide kinase and [^{32}P]γ-ATP. Nuclear extract (20 μg) was incubated in binding buffer [10 mmol/L Tris-HCl (pH 7.5)-4% glycerol-1 mmol/L $MgCl_2$-50 mmol/L NaCl-0.5 mmol/L EDTA-0.5 mmol/L DTT] containing 1 mg poly(deoxyinosinic-deoxycytidylic acid)-poly(deoxyinosinic-deoxycytidylic acid) in 15 mL reaction volume for 10 minutes at room temperature. Samples were incubated for 20 minutes at room temperature after addition of the DNA probe (100,000 counts per minute per reaction). The resulting protein-DNA complexes were separated from free DNA under nondenaturing conditions on a 6% polyacrylamide gel (Novex, San Diego, CA) under high ionic strength. Gels were dried and imaged by autoradiography.

Results

Antiproliferative Activity of Aminoflavone *In vitro* and *In vivo*

Aminoflavone produced differential cytotoxicity against the human tumor cell lines used in the present

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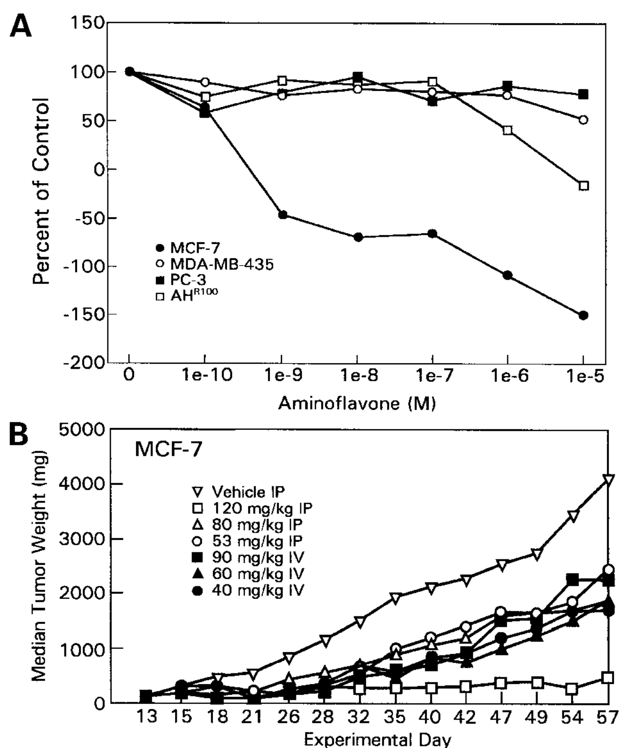

Figure 2. Cytotoxicity of aminoflavone *in vitro* and *in vivo*. **A,** selective cytotoxicity of aminoflavone to MCF-7 breast tumor cells. Cell lines were seeded into 24-well plates as described in Materials and Methods and allowed to grow for 48 hours. One replicate plate was processed as described below to determine protein content prior to aminoflavone exposure. Cells were treated with 10^{-10} to 10^{-5} mol/L aminoflavone for an additional 72 hours. Drug exposure was terminated by addition of trichloroacetic acid to a final concentration of 10%. Cell monolayers were stained with sulforhodamine B, and protein was determined spectrophotometrically (11). *Points,* mean ± SD (n = 10). SD was <5% for all drug concentrations and was omitted for purpose of graphical clarity. **B,** antitumor activity of aminoflavone against MCF-7 breast tumor xenografts. Treatments were given on a QD × 5 schedule beginning on day 13. There were 20 mice in the vehicle control group and 6 mice per dose of aminoflavone in the treated groups.

investigation (Fig. 2A; Table 1). Concentrations causing 50% growth inhibition (GI_{50}) values ranged from 0.13 nmol/L for MCF-7 breast tumor cells to >10,000 nmol/L for PC-3 prostate cells. MCF-7 breast cancer cells exhibited the greatest sensitivity to aminoflavone, which produced 100% growth inhibition at a drug concentration of 0.4 nmol/L. Exposure of MCF-7 breast tumor cells to aminoflavone concentrations of >0.4 nmol/L resulted in *in vitro* "regression" because cellular protein progressively decreased relative to non-aminoflavone-treated cells. The AhR-deficient variant of MCF-7 breast tumor cells, AH^{R100}, exhibited noteworthy resistant to aminoflavone. The GI_{50} of AH^{R100} cells is 4,500-fold higher than MCF-7 wild-type cells, suggesting that a fully functional AhR signaling pathway may be necessary for induction of cytotoxicity by aminoflavone.

Previous studies had demonstrated that microsomes can convert aminoflavone to multiple but yet to be definitively identified metabolite(s) (4), which bind to DNA, protein, and smaller molecular weight nucleophiles. In support of these findings, MCF-7 cells bind covalent metabolite(s) of aminoflavone to a 10-fold greater degree than MDA-MB-435 cells (15.2 vs. 1.2 dpm/μg protein; Table 1). However, binding of metabolite(s) cannot be the sole basis for cytotoxicity as both the resistant cell types, AH^{R100} and PC-3, exhibit even higher covalent binding of metabolite(s) (87 and 37 dpm/μg protein, respectively). This high level of covalent binding in the aminoflavone-resistant cells is currently being investigated. Apparently, interpretation of the covalent binding data may be influenced by the very low levels of binding in these cell lines and that residual noncovalently bound drug/metabolite(s) may be responsible for high values observed with AH^{R100} and PC-3 cells.

The MCF-7 breast carcinoma cell line was selected for evaluation of *in vivo* antitumor activity of aminoflavone based on *in vitro* studies. MCF-7 tumor growth was inhibited in athymic mice at all aminoflavone doses (Fig. 2B); The growth delay ranged from 50% to 231%, with an optimal %T/C of 15% achieved with an i.p. dose of 120 mg/kg QD × 5.

Aminoflavone Induces Apoptosis in Aminoflavone-Sensitive Breast Tumor Cells

We next investigated the ability of aminoflavone to induce apoptosis in the aminoflavone-sensitive MCF-7 breast tumor cells and aminoflavone-resistant cells. Aminoflavone treatment of MCF-7 cells using concentrations that produce inhibition of cellular growth (1 to 1000 nmol/L) resulted in apoptosis (Table 2); aminoflavone-resistant cell

Table 1. **Aminoflavone sensitivity and covalent binding in selected breast and prostate human tumor cell lines**

	GI_{50}* (nmol/L)	TGI† (nmol/L)	LC_{50}‡ (nmol/L)	Covalent Binding§ (dpm/μg Protein)
MCF-7	0.1	0.4	1.0	15.2 ± 1.7
AH^{R100}	600	5,000	>5,000	86.8 ± 8.3
MDA-MB-435	10,000	>10,000	>10,000	1.2 ± 0.2
PC-3	>10,000	>10,000	>10,000	37.1 ± 3.6

* GI_{50}, drug concentration that produces a 50% inhibition of cell growth relative to control.
† TGI, drug concentration that produces no cell growth during the treatment period.
‡ LC_{50}, drug concentration that results in 50% reduction in the cellular protein content during the content period relative to control.
§ Values represent mean ± SD for three separate experiments.

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Table 2. Induction of apoptosis by aminoflavone in aminoflavone-sensitive and aminoflavone-resistant cell lines

Cell Line	Aminoflavone (nmol/L)					
	0	0.1	1	10	100	1,000
MCF-7	0.3 ± 0.6*	10.7 ± 3.1	14.3 ± 2.1	18.3 ± 4.7	25.3 ± 3.8	41.0 ± 2.0
AH_{R100}	0	0	0.7 ± 1.2	1.7 ± 2.1	1.7 ± 0.6	2.3 ± 1.5
MDA-MB-435	0	0.3 ± 0.6	3.3 ± 1.5	5.0 ± 1.7	8.7 ± 3.2	8.7 ± 2.3
PC-3	0	0	1.0 ± 0	1.0 ± 0	1.0 ± 1.7	2.3 ± 1.5

NOTE: Cell lines were treated with 0 to 1,000 nmol/L aminoflavone for 24 hours as described in Materials and Methods, stained with 4′,6-diamidino-2-phenylindole, and examined under a Leitz Diaplan fluorescent microscope. Apoptotic cells were defined by a nuclear shrinkage and chromatin condensation.
*Values are percentage apoptotic cells (mean ± SD) derived from examination of three separate fields each containing 100 cells.

lines were much less susceptible to apoptosis and, when observed, occurred at much higher drug concentrations. These results are consistent with the observed loss in protein in MCF-7 cells (Fig. 2A). Additional studies (data not shown) indicated that aminoflavone produced cell cycle arrest in S phase, which was dependent on both the aminoflavone concentration and the length of exposure.

Aminoflavone Induces EROD Activity and *CYP1A1* and *CYP1B1* Transcription in MCF-7 Cells

Previous studies indicated that aminoflavone caused induction of *CYP1A1* protein in MCF-7 cells but that induction of the protein did not occur in insensitive cell lines (4). Relative induction of *CYP1A1* in intact MCF-7, MDA-MB-435, PC-3, and AH^{R100} cells treated with aminoflavone was assayed by measuring EROD activity. Incubation of the cells with aminoflavone for 24 hours caused a concentration-dependent increase in EROD activity over the range of concentrations examined in MCF-7 cells; however, aminoflavone had no effect on EROD activity in MDA-MB-435, PC-3, and AH^{R100} cells (Fig. 3A). Aminoflavone treatment of the sensitive MCF-7 breast tumor cells caused a remarkable induction of nearly 100-fold in the *CYP1A1* mRNA level with respect to the control (Fig. 3B). A smaller 10-fold increase of *CYP1B1* mRNA was observed in this cell line. However, in MDA-MB-435, PC-3, and AH^{R100} aminoflavone-resistant cells, the level of *CYP1A1* and *CYP1B1* after treatment remained similar to control. These data indicate that the increased level of enzyme activity caused by aminoflavone is mediated by enhanced gene expression of *CYP1A1* and *CYP1B1* in MCF-7 cells. We further examined transcriptional regulation using the transcriptional inhibitor actinomycin D. Preincubation with actinomycin D (5 μg/mL) for 1 hour abolished the increased levels of mRNA for *CYP1A1* and *CYP1B1* observed after treatment with aminoflavone (Fig. 3C). In this experiment, treatment with 100 nmol/L aminoflavone was decreased to 6 hours to avoid actinomycin D-induced cytotoxicity. Our results are consistent with transcriptional activation of *CYP1A1* and *CYP1B1* by aminoflavone.

Aminoflavone Induces Activation of *CYP1A*1-Related Promoter Sequences

The activity of *CYP1A1* and *CYP1B1* promoters is regulated by AhR, which forms a heterodimer with aryl hydrocarbon nuclear translocator. Binding of the complete dimer to XRE in the promoter region mediates transcription of AhR-responsive genes, including *CYP1A1* and *CYP1B*1. Thus, we sought to define whether aminoflavone activates the AhR signaling pathway differentially in aminoflavone-sensitive and aminoflavone-resistant cells, resulting in induction of *CYP1A1* transcription. MCF-7, MDA-MB-435, PC-3, and AH^{R100} cells were transfected with a XRE luciferase reporter construct (pTX.Dir), and as a control, the same reporter construct without XRE elements (pT81) was used. Cells were treated with 0.1% DMSO, 10 nmol/L TCDD, or 250 nmol/L aminoflavone. TCDD was used as a prototypic activator of *CYP1A1* transcription. As shown in Fig. 4A, in MCF-7 cells transfected with pTX.Dir, a 15-fold induction of luciferase activity was observed when cells were treated with TCDD, whereas aminoflavone caused a 7-fold induction. However, in MDA-MB-435 cells transfected with pTX.Dir, 250 nmol/L aminoflavone caused an increase of only 1.5 fold over the control. Similarly, when AH^{R100} cells were transfected with XRE luciferase and treated with 250 nmol/L aminoflavone, XRE luciferase activity was only 1.7-fold higher than control. PC-3 cells were 1.9-fold higher than control. The data for the effect of TCDD in pT81-transformed cells are illustrated in Fig. 4A. Similar results were obtained when cells were transfected with a fragment of mouse native *CYP1A1* promoter (inclusive of four dioxin-responsive elements upstream of luciferase; pGudLuc1.1; data not shown). No induction in luciferase activity was observed when cells transfected with pT81 were treated with 250 nmol/L aminoflavone or 10 nmol/L TCDD (Fig 4A). To determine the extent of XRE luciferase induction, pTX.Dir-transfected cells were treated with increasing concentrations of aminoflavone. In MCF-7 cells, increased luciferase activity was evident at 1 nmol/L aminoflavone with a maximum induction occurring between 100 and 250 nmol/L aminoflavone. In MDA-MB-435, PC-3, or AH^{R100} cells little increase in luciferase activity was observed and was independent of the concentration of aminoflavone (Fig. 4B). It is apparent that the response of MCF-7 cells to aminoflavone is biphasic, suggesting a mechanism in which increased concentration of an inactive metabolite, which binds to

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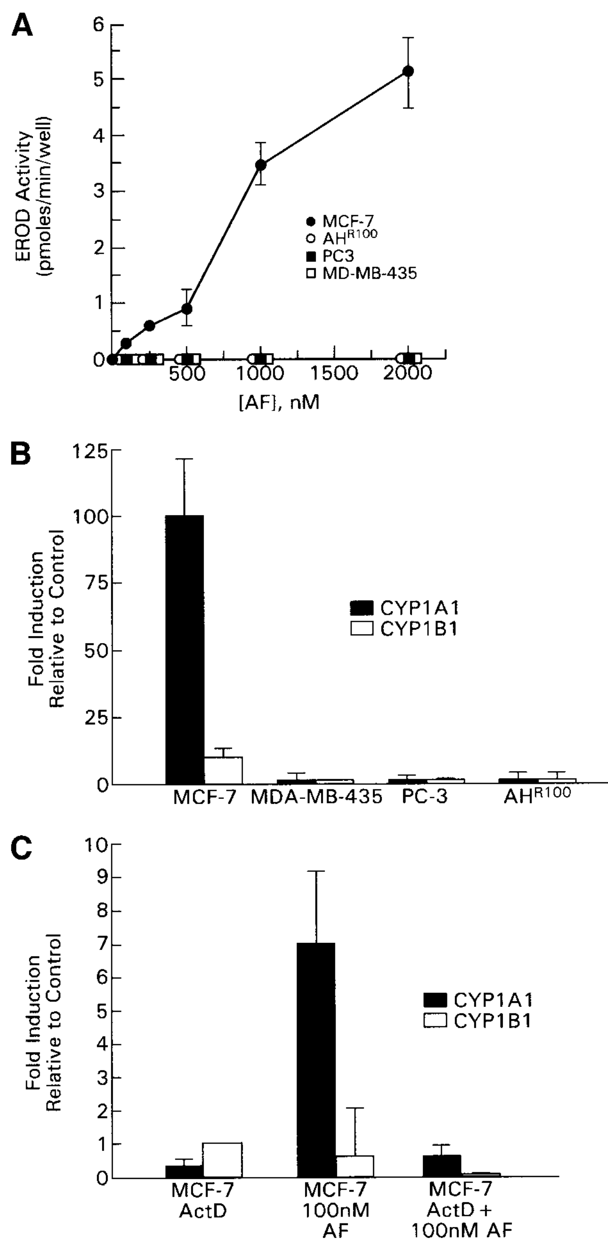

Figure 3. Aminoflavone induces *CYP1A1* activity in aminoflavone-sensitive MCF-7 cells but not in resistant cells. **A,** MCF-7, MDA-MB-435, PC-3, and AH^{R100} cells were incubated with aminoflavone for 24 hours and assayed for *CYP1A1* enzyme activity by the EROD assay as described in Materials and Methods. *Points,* mean (n = 4); *bars,* SD. **B,** aminoflavone induces *CYP1A1* and *CYP1B1* transcription in aminoflavone-sensitive MCF-7 cells. MCF-7, MDA-MB-435, PC-3, and AH^{R100} cells were treated with 100 nmol/L aminoflavone for 24 hours, RNA was isolated from control and treated cells, and *CYP1A1* and *CYP1B1* gene expression was measured by real-time reverse transcription-PCR as described in Materials and Methods. *Columns,* mean fold induction of treated cells relative to constitutive expression in control cells (n = 7 from two independent experiments); *bars,* SD. **C,** expression of *CYP1A1* and *CYP1B1* in MCF-7 cells pretreated with 5 μg/mL actinomycin D for 1 hour followed by no additional drug for 6 hours (*left*); MCF-7 with no actinomycin D and 100 nmol/L aminoflavone for 6 hours (*center*); or MCF-7 pretreated with 5 μg/mL actinomycin D followed by 100 nmol/L aminoflavone and actinomycin D for 6 hours (*right*).

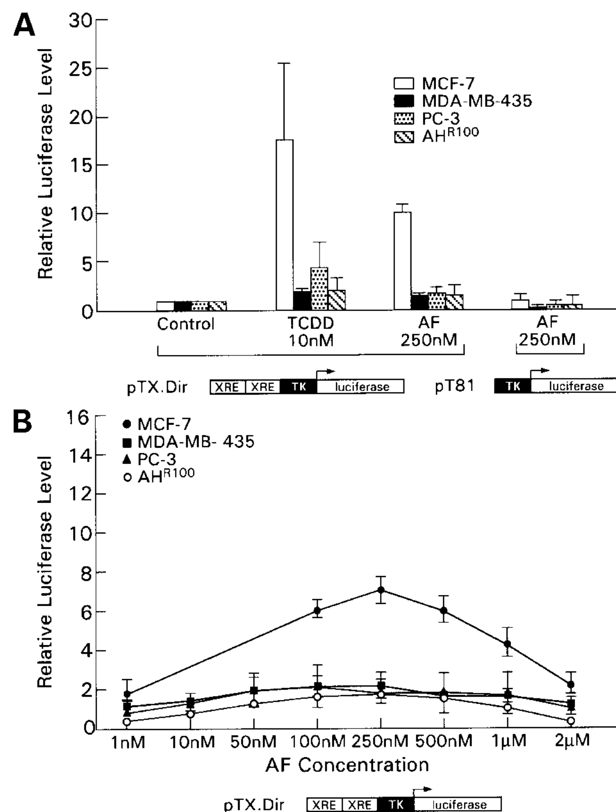

Figure 4. Aminoflavone induces binding to XRE sequences of *CYP1A1* in MCF-7 cells. **A,** MCF-7, MDA-MB-435, PC-3, and AH^{R100} cells were transfected with XRE luciferase (pTX.Dir) or pT81. Transfected cells were treated with DMSO, 10 nmol/L TCDD, or 250 nmol/L aminoflavone for 9 hours. XRE luciferase activity was determined by normalizing activity to the amount of *R. reniformis* luciferase activity. *Columns,* mean luciferase levels relative to the control; *bars,* SD. **B,** transfected cells were treated with the indicated concentrations of aminoflavone for 9 hours. Aminoflavone increased XRE-driven luciferase activity in a concentration-dependent manner in MCF-7 cells. The amount of luciferase was determined as described in **A**.

CYP1A1 and not DNA, results in a reduced capacity to generate toxic metabolites. These findings suggest that aminoflavone induces activation of promoter sequences known to respond to AhR-mediated signals.

Aminoflavone Induces AhR Translocation to the Nucleus in Aminoflavone-Sensitive MCF-7 Cells

We studied whether aminoflavone could activate the AhR signaling pathway with resulting AhR translocation from the cytoplasm to the nucleus. AhR was localized throughout the cytoplasm in non-aminoflavone-treated cells (Fig. 5A). However, after treatment with either 10 nmol/L TCDD or 1 μmol/L aminoflavone for 1 hour, AhR translocation to the nucleus occurred. In contrast, in vehicle-treated MDA-MB-435 cells, AhR is colocalized in both cytoplasm and nucleus; after treatment with 1 μmol/L aminoflavone or 10 nmol/L TCDD for 1 hour, AhR is present predominantly in the nucleus (Fig. 5B). A similar pattern of baseline AhR intracellular distribution is

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observed in resistant PC-3 cells (Fig. 5C). AH^{R100} cells, which express low levels of AhR and are resistant to aminoflavone, also exhibit constitutive nuclear localization of AhR (Fig. 5D). These results indicate that, in the aminoflavone-resistant cell lines, a substantial fraction of AhR resides in the nucleus prior to drug exposure and suggest that the regulation of AhR is different in aminoflavone-sensitive cells as compared with aminoflavone-resistant cells, consistent with results from the transfection studies illustrated in Fig. 4.

Aminoflavone Increases Protein-DNA Complexes Formed on XRE Elements

The effect of aminoflavone on ligand-induced formation of protein-DNA complexes on the XRE sequence was examined by the electrophoretic mobility shift assay. Extracts from cells treated with 0.1% DMSO, 10 nmol/L TCDD, or 250 nmol/L aminoflavone were incubated with labeled oligonucleotide corresponding to the XRE sequence from the human *CYP1A1* promoter. Similarly, extracts from TCDD-treated cells were used as positive controls. In MCF-7 cells (Fig. 6A), three protein-DNA complexes were found in nuclear extracts of control cells, although the band intensity of the highest apparent molecular weight complex was less than the others (*lane 1*). Treatment for 20 minutes with 100 nmol/L aminoflavone resulted in increased binding of the uppermost complex (*lane 3*), while treatment with 250 nmol/L aminoflavone for 20 minutes (*lane 4*) caused an 11-fold increase in the uppermost complex. This induction exceeded that afforded by 10 nmol/L TCDD treatment (*lane 2*). AH^{R100} cells exposed for 20 minutes to 250 nmol/L aminoflavone actually exhibited a decrease in XRE binding (*lane 6*). The specificity of the binding to XRE was examined by pretreating nuclear extracts from cells treated with 250 nmol/L aminoflavone for 20 minutes with 100× unlabeled XRE probe or AhR polyclonal antibody. The binding of the three complexes to labeled XRE sequences was greatly diminished in the presence of excess unlabeled XRE (*lane 9*) or pretreatment with AhR antibody (*lane 7*). These protein-DNA complexes do not disappear when nuclear extracts from aminoflavone-treated cells were preincubated with 100× unlabeled SP1 oligonucleotide (*lane 10*) or control antiserum (*lane 8*). Attempts to supershift the band with AhR antibody were unsuccessful. In PC-3 cells (Fig. 6B), constitutive expression of one protein-DNA complex with an intermediate molecular weight was found in the control

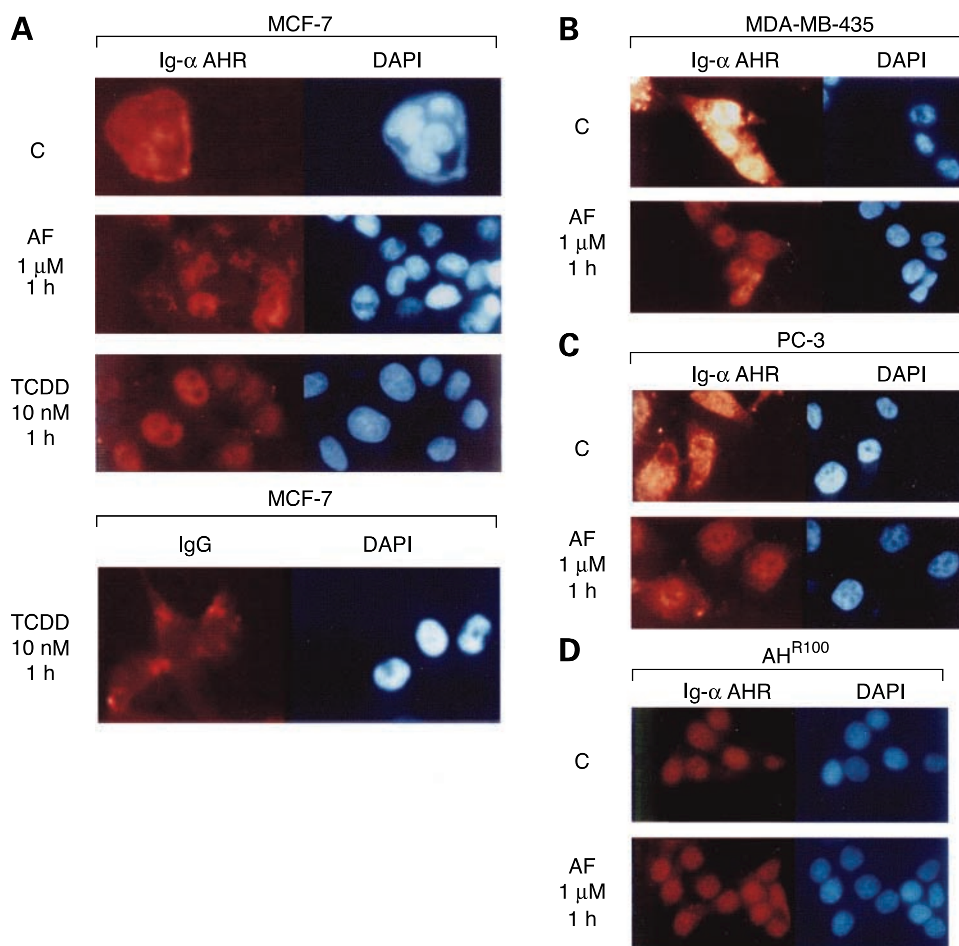

Figure 5. Aminoflavone induces translocation of the AhR to the nucleus in aminoflavone-sensitive MCF-7 cells. MCF-7 (**A**), MDA-MB-435 (**B**), PC-3 (**C**), and AH^{R100} (**D**) cells were grown on coverslips. The cells were treated with DMSO (**C**), 1 μmol/L aminoflavone, or 10 nmol/L TCDD for 1 hour. After fixation, the cells were double stained for AhR (*red*) and 4′,6-diamidino-2-phenylindole (*blue*) as described in Materials and Methods. Cells treated with 10 nmol/L TCDD were incubated with goat control serum and Cy3-conjugated rabbit antigoat IgG to determine nonspecific background. Stained cells were visualized on a Zeiss Axiovert microscope using a 63× objective, and images were captured with an Optronics CCD camera.

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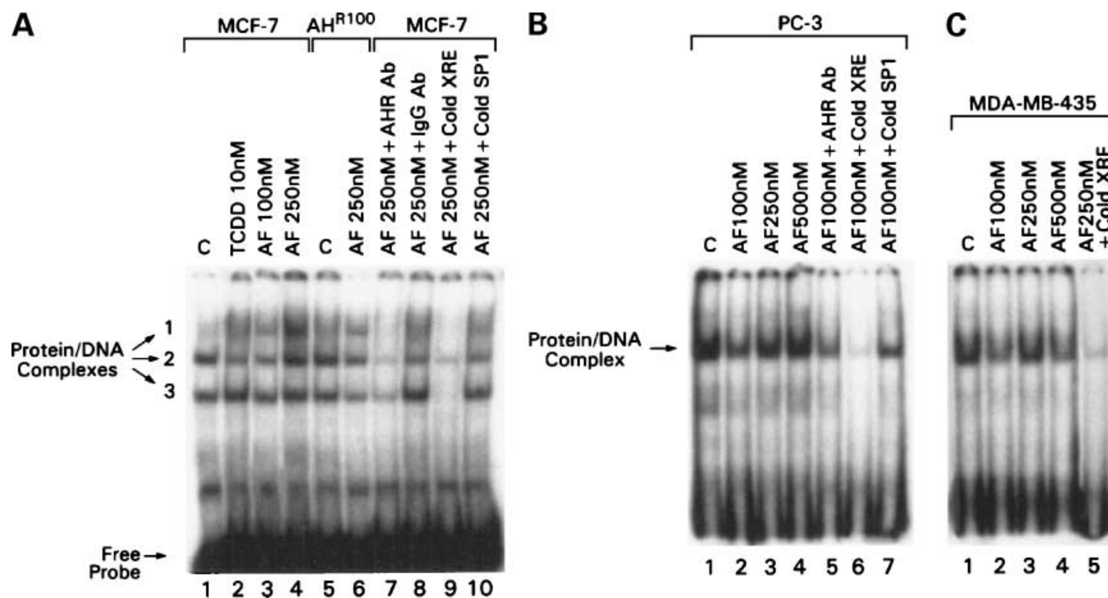

Figure 6. Aminoflavone induces protein-DNA complexes on the XRE sequence of the *CYP1A1* promoter in MCF-7 cells. **A,** nuclear extracts (20 μg) prepared from MCF-7 cells treated with DMSO (control, *C*; *lane 1*), TCDD (10 nmol/L, 1 hour; *lane 2*), or aminoflavone (100 to 250 nmol/L, 20 minutes; *lanes 3* and *4*) were incubated with labeled XRE sequence derived from the *CYP1A1* promoter for 10 minutes at room temperature. Free and bound DNA were separated as described in Materials and Methods. For competition, nuclear extracts from MCF-7 cells treated with aminoflavone (250 nmol/L, 20 minutes) were incubated with 4 μg anti-AhR antibody (*lane 7*) or 100-fold excess of unlabeled XRE oligonuclotide (*lane 9*). Protein-DNA complexes from AH^{R100} cells were resolved in the same gel. Nuclear extracts from these cells (20 μg) treated with DMSO (*lane 5*) or aminoflavone (250 nmol/L, 20 minutes; *lane 6*) were incubated with radioactive XRE and the complexes were resolved on a 6% polyacrylamide gel. Nuclear extracts (20 μg) from PC-3 (**B**) and MDA-MB-435 (**C**) cells treated with DMSO (control, *C*) or aminoflavone (100 to 500 nmol/L) for 20 minutes were incubated with labeled XRE, and free and bound DNA were separated as described above. Specificity of the binding was determined by incubation of nuclear extract from aminoflavone-treated cells (100 or 250 nmol/L for 1 hour, respectively) with 4 μg anti-AhR antibody or 100-fold excess of unlabeled XRE oligonucleotide.

(*lane 1*). This pattern did not change after treatment with 100 to 500 nmol/L aminoflavone (*lane 2-4*). This complex was competed by preincubation with cold XRE oligonucleotide (*lane 6*) or AhR antibody (*lane 5*) but not with cold SP1 oligonucleotide (*lane 7*). Similarly, in MDA-MB-435 cells (Fig. 6C), we observed one protein-DNA complex with an intermediate molecular weight in the control (*lane 1*). Treatment with 100 to 500 nmol/L aminoflavone for 20 minutes showed a similar pattern as the control (*lane 2-4*). The binding of the protein-DNA complex was abolished in the presence of unlabeled XRE (*lane 5*). These data confirm that aminoflavone is an AhR agonist that induces binding of AhR to the XRE sequence of the *CYP1A1* promoter, resulting in transcriptional activation of XRE-regulated genes in MCF-7; in MDA-MB-435 and PC-3 cells, a nuclear XRE binding complex was demonstrated to be constitutively present. In the aminoflavone-resistant cell lines, aminoflavone did not induce binding of AhR to XRE concordant with the absence of any discernible effect on *CYP1A1* and *CYP1B1* expression (Fig. 3).

Discussion

Aminoflavone has demonstrated activity against breast and renal tumor cell lines in the *in vitro* antitumor drug screen of the National Cancer Institute (19). Consequently, we have undertaken studies designed to explore the basis for this sensitivity to aminoflavone using the MCF-7 breast tumor cell and several aminoflavone-resistant variants. Treatment of MCF-7 wild-type cells with aminoflavone *in vitro* was accompanied by induction of apoptosis and an increase in *CYP1A1* and, to a lesser extent, *CYP1B1* enzyme activity and gene expression. In contrast, the aminoflavone-resistant cell lines did not exhibit apoptosis to the same extent as MCF-7 after treatment with aminoflavone, nor was there an increase in *CYP1A1* and *CYP1B1* enzyme activity and gene expression. Although AH^{R100} and PC-3 cells showed noteworthy covalent binding of aminoflavone metabolites (as did MCF-7 cells), aminoflavone treatment of these aminoflavone-resistant cell lines did not result in induction of *CYP1A1*, which suggests that selective activation of *CYP1A1*, and possibly *CYP1B1*, can generate a unique toxic metabolite of aminoflavone in sensitive cells, which would mediate enhanced sensitivity of MCF-7 cells to the drug. Accordingly, toxic metabolites produced by *CYP1A1* generated in situ would result in antitumor activity. It has been described in the literature that several chemotherapeutic prodrugs are metabolized in the liver by a cytochrome *P*450-catalyzed prodrug activation reaction that is required for therapeutic activity. Preclinical studies have shown that the chemosensitivity of these prodrugs can be dramatically increased by *P*450 gene transfer, which confers the capability to activate the prodrug directly within the target tissue. This *P*450-directed therapy enhances the therapeutic effect of *P*450-activated anticancer

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prodrugs without increasing host toxicity associated with systemic distribution of active drug metabolites formed in the liver (20). Previous studies described the *P*450 enzyme patterns in the National Cancer Institute human tumor cell line panel in response to different anticancer drugs. Many compounds, including aminoflavone, have been identified as anticancer agents, the activity of which can be altered via cytochrome *P*450 metabolism (21). Our results confirm that aminoflavone induces expression of *CYP1A1* and thus is itself responsible for the generation of the enzyme activity, which others (4) have shown can directly metabolize aminoflavone. This is unusual in that most of the known inducers of *CYP1A1* are carcinogens such as TCDD or benzo(*a*)pyrene; however, naturally occurring flavonoids are known inducers of *CYP1A1* (22), and aminoflavone is structurally related to these compounds. *CYP1B1* mRNA was likewise increased in MCF-7 cells treated with aminoflavone (Fig. 3B). The exact contribution of *CYP1A1* and *CYP1B1* to the activation of aminoflavone is unclear and will require a detailed analysis of the metabolites produced by each enzyme.

CYP1A1 and *CYP1B1* gene expression is regulated at the transcriptional level by the XRE, a promoter sequence that, when bound by ligand-activated AhR, causes an increase in gene transcription. We carried out two experiments to determine whether aminoflavone induced *CYP1A1* and *CYP1B1* expression at the transcriptional level. First, we treated cells with aminoflavone in the presence of the RNA polymerase II inhibitor actinomycin D, which blocks transcription. The increase in *CYP1A1* and *CYP1B1* mRNA in MCF-7 cells normally caused by aminoflavone was completely blocked by actinomycin D (Fig. 3C), demonstrating that *de novo* mRNA synthesis is required. Secondly, we investigated the effects of aminoflavone on XRE-controlled reporter transcription to determine whether the increase in *CYP1A1* and *CYP1B1* mRNA caused by aminoflavone was the result of transcriptional control. The prototypical *CYP1A1* inducer, TCDD, caused a profound increase in XRE-controlled transcription. Aminoflavone likewise caused a dose-dependent increase in XRE-controlled transcription (Fig. 4A) in MCF-7 cells. In contrast, aminoflavone-resistant cell lines exhibited no increase in XRE-mediated transcription on treatment with aminoflavone. On ligand binding, the cytoplasmic AhR translocates to the nucleus. We therefore investigated the effect of aminoflavone on AhR localization. As shown in Fig. 5, aminoflavone treatment of MCF-7 cells caused a translocation of the AhR from the cytoplasm to the nucleus of the cell similar to that observed with TCDD. Interestingly, in aminoflavone-resistant cell lines, the AhR was colocalized in the cytoplasm and nucleus, even in the absence of ligand. Aminoflavone treatment of these resistant cells produced a more complete translocation of AhR to the nucleus without activating *CYP1A1* or *CYP1B1* transcription. Thus, aminoflavone-resistant cell lines apparently have an altered AhR signaling pathway, which is accompanied by an inability of aminoflavone to induce *CYP1A1* and *CYP1B1* expression in these cells. To test this hypothesis, we performed electrophoretic mobility shift assay to determine the capacity of the activated AhR to bind to the XRE. As shown in Fig. 6, aminoflavone treatment of MCF-7 cells caused the appearance of several protein-DNA complexes. These complexes were demonstrated to contain the AhR because antibody to the AhR, but not preimmune serum, abolished their formation. Furthermore, an excess of unlabeled XRE, but not SP1 oligonucleotides, abolished the complex formulation.

The failure to observe activation of *CYP1A1*/*CYP1B1* transcription in aminoflavone-resistant cells, despite nuclear residence of the XRE binding complex, suggests that transcriptional control in these cells is quite complex. Our results (Fig. 6) indicate that little or no induction in the protein complexes binding to XRE occurs in the resistant cells. The failure to observe *CYP1A1*/*CYP1B1* induction may be due to altered degradation/turnover of the AhR in these cells because a previous report (23) indicated that turnover of the AhR and resulting *CYP1A1* transcriptional activity is regulated by the 26S proteosome. Specifically, TCDD induces ubiquitination of AhR resulting in increased turnover of the receptor. Inhibition of proteosomal degradation of the AhR results in increased nuclear AhR-aryl hydrocarbon nuclear translocator complex and results in the increased ability of TCDD to induce *CYP1A1* transcription. Thus, the increased nuclear localization of AhR in our resistant cells may be the result of altered proteosomal degradation of AhR. Whether this is due to reduced cellular proteosomal content/activity is not clear at this time and will require further investigation. Alternatively, the increased nuclear content of AhR in aminoflavone-resistant cells may be the result of altered AhR translocation. Several studies (24, 25) have documented the ability of AhR to shuttle between the nucleus and the cytoplasm using nuclear localization and export signals in the amino-terminal region. These studies demonstrated that inhibition of nuclear export of AhR by the antifungal antibiotic leptomycin B resulted in repressed transcriptional activation of *CYP1A1*. Thus, the aminoflavone-resistant cells may have an altered amino acid sequence within the amino terminus of the AhR, which results in reduced AhR nuclear export.

In the present work, we have also demonstrated that aminoflavone produces apoptosis in aminoflavone-sensitive MCF-7 breast tumor cells, which was not observed in the resistant cell lines. The molecular mechanisms by which the aryl hydrocarbons induce cell death remain unclear, but it has been demonstrated that deficiency of AhR-mediated signal transduction in cells, causing a lack of *CYP1A1* induction and a corresponding reduction in aryl hydrocarbon metabolism, protected the cells against 7,12-dimethylbenz(*a*)anthracene-induced apoptosis (10). The current results with aminoflavone are similar and suggest that aminoflavone acts analogously to the aryl hydrocarbons but at very low concentrations causing cell death. Our data are concordant with previous reports indicating that 7,12-dimethylbenz(*a*)anthracene, benzo(*a*)pyrene, and TCDD induce apoptosis in a variety of cells, including pre-B-cells (26), Heplace17 murine hepatoma cells (27), and mouse epidermis (28). MCF-7 cells do not express caspase-3,

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thought by some to be a critical component of the apoptosis cascade. However, aminoflavone treatment results in condensation and fragmentation of MCF-7 cell nuclei, which may occur via a mechanism independent of caspase-3 (29) because it was demonstrated previously that caspase-3 is not essential for DNA fragmentation in MCF-7 cells during apoptosis induced by some chemical agents (30).

We have demonstrated previously that AhR activation also mediates sensitivity of MCF-7 to the antitumor agent, 2-(4-amino-3-methylphenyl) benzothiazole (DF 203; ref. 31). *CYP1A1* is also essential for the metabolism of DF 203 and has a pivotal role in its anticancer activity in sensitive cell lines. DF 203 induced transcriptional activation of *CYP1A1* mediated through the activation of the AhR signaling pathway (31). However, although DF 203 and aminoflavone share certain mechanistic similarities, several differences between these two drugs were observed both *in vitro* and *in vivo*. For example, Colo-205, HCC-2998, and HCT-116 colon cancer cells were sensitive to DF 203, whereas aminoflavone, when evaluated against the same colon cancer cell lines, exhibited little activity against these colon tumor cell lines. Aminoflavone had demonstrable activity against K-562 leukemia and NCI-H460 non-small cell lung cancer cell lines, whereas DF 203 was inactive. Some cell lines such as the breast MDA-MB-435 and prostate PC-3 carcinoma cells were insensitive to both drugs. Studies in animals have demonstrated that DF 203 caused hepatobiliary and pulmonary toxicities (32); in contrast, aminoflavone caused primarily pulmonary toxicity and evidence of gastrointestinal toxicity (33). These results lead one to the conclusion that aminoflavone and DF 203, two structurally dissimilar compounds, share certain characteristics in their mechanism of action but are certainly not identical in their pattern of activity. This may relate to the observation that the AhR recruits several coactivators and corepressors including hypoxia inducible factor-1α, nuclear factor-κB, and the estrogen receptor (34-36), the distribution of which may vary according to tissue type.

In conclusion, these results suggest that aminoflavone activates the AhR signaling pathway leading to increase *CYP1A1* and *CYP1B1* gene expression in aminoflavone-sensitive MCF-7 cells but not in aminoflavone-resistant MDA-MB-435, PC-3, or MCF-7 AhR-deficient AH^{R100} cells. Although activation of the AhR signaling pathway by aminoflavone may be necessary for increases in *CYP1A1* and *CYP1B1* gene expression, additional metabolic conversions may be necessary to produce cytotoxicity. Characterization of AhR cellular pharmacology in model systems sensitive and resistant to aminoflavone may further aid in efforts to elucidate the basis for its selective cytotoxicity and will be important in both preclinical and early clinical development of the compound.

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Aryl hydrocarbon receptor activation of an antitumor aminoflavone: Basis of selective toxicity for MCF-7 breast tumor cells

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