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Aryl Hydrocarbon Receptor Mediates Sensitivity of MCF-7 Breast Cancer Cells to Antitumor Agent 2-(4-Amino-3-methylphenyl) Benzothiazole

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ABSTRACT

2-(4-Amino-3-methylphenyl) benzothiazole (NSC 674495; DF 203) demonstrates drug uptake and metabolism by tumor cells sensitive to the antiproliferative activity of the drug [*J Med Chem* 1999;42:4172–4184]. In insensitive cells, little metabolism occurs. Because CYP1A1 can metabolize DF 203, the aryl hydrocarbon receptor (AhR) may mediate drug action. We demonstrate here that DF 203 increases *CYP1A1* and *CYP1B1* transcription in sensitive MCF-7 cells, accompanied by AhR translocation to the nucleus, increase in xenobiotic-responsive element (XRE)-driven luciferase activity, and induction of protein/DNA complexes on the XRE sequence of the *CYP1A1* promoter. MDA-MB-435 and PC3 cells, resistant to DF 203, did not show drug-induced *CYP1A1* and *CYP1B1* gene expression. AhR was observed to be constitutively localized in the nucleus, with no induction of XRE-driven luciferase activity in transiently transfected cells and weak or no induction of protein/DNA complexes on the XRE sequence of *CYP1A1*. Taken together, these data elucidate a novel basis for antitumor drug action: induction in sensitive cells of a metabolizing system for the drug itself. These results suggest that clarification of the basis for differential engagement of AhR-related signaling in different tumor cell types may aid in further preclinical development and perhaps early clinical studies.

2-(4-Amino-3-methylphenyl) benzothiazole (NSC 674495 or DF 203) has emerged from the empirical anticancer drug screening program of the National Cancer Institute as an agent with markedly differential activity in distinct cancer cell types (Shi et al., 1996; Bradshaw et al., 1998b). For instance, both MCF-7 and T-47 D breast carcinoma cells were exquisitely sensitive and renal TK-10 or ovarian IGROV1 cells had intermediate sensitivity, whereas other cell lines, including breast MDA-MB-435, ovarian SK-OV-3, and renal CAKI I, were insensitive. It has been postulated that metabolism may underlie the selective profile of anticancer activity of DF 203 because drug uptake and biotransformation were observed only in those cell lines that are sensitive. In contrast, little or no metabolism occurred in the insensitive cell lines (Chua et al., 1999; Kashiyama et al., 1999). DF 203 has exhibited in vivo antitumor activity in breast and ovarian models (Bradshaw et al., 1998a).

CYP1A1 seems to be essential for the metabolism of DF 203 and may have a pivotal role in its anticancer activity. Induction of CYP1A1 and CYP1B1 activity and protein levels after treatment with the drug has been reported only in sensitive cell lines (Chua et al., 2000). Current evidence supports the subsequent generation of DNA damage from metabolites but only in sensitive cells (Stevens et al., 2001).

Induction of CYP1A1 activity is known to be mediated by the aryl hydrocarbon receptor (AhR) signal transduction pathway (Hankinson, 1995; Schmidt and Bradfield, 1996; Whitlock, 1999). The AhR is a ligand-activated transcription factor with a basic helix-loop-helix / periodicity/Arnt/simple-minded domain structure (Poland and Knutson, 1982; Whitlock, 1999). AhR mediates most of the biological responses to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which was characterized as a prototypic ligand for this receptor (Fernández-Salguero et al., 1996). The biological effects of TCDD include induction of drug-metabolizing enzymes, and toxic effects such as tumor promotion (Poland and Glover, 1980). AhR activation provokes a multistep, ligand-induced signal transduction process. For

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ABBREVIATIONS: DF 203, 2-(4-amino-3-methylphenyl) benzothiazole (NSC 674495); AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Arnt, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic-responsive element; DMSO, dimethyl sulfoxide; TK, thymidine kinase; RT-PCR, reverse transcription-polymerase chain reaction; ER, estrogen receptor.

instance, binding of the ligand to AhR triggers the dissociation of AhR from associated cytoplasmic proteins, including 90-kDa heat-shock protein (Ma and Whitlock, 1997). Subsequently, the activated AhR translocates into the nucleus, dimerizes with Arnt, another basic helix-loop-helix/periodicity/Arnt/simple-minded transcription factor, and activates the transcription of target genes by binding to specific enhancer sequences [xenobiotic-responsive elements (XREs)] in the regulatory region of genes such as *CYP1A1*. To address the mechanism of the selective metabolism of DF 203, activation of the AhR pathway was analyzed in sensitive and resistant cell types. We found that activation of this pathway in sensitive cells does occur in DF 203-sensitive cell types and does not occur in resistant cells examined here.

Materials and Methods

Drug and Cell Culture. DF 203 was synthesized by the Cancer Research Campaign UK laboratories at the University of Nottingham and the Drug Synthesis and Chemistry Branch, National Cancer Institute. The compound was dissolved in DMSO to make a 100 mM stock concentration. MCF-7, MDA-MB-435, or PC3 cells were obtained from the National Cancer Institute Repository at National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD. Cells were grown in RPMI 1640 medium plus 10% fetal bovine serum (Invitrogen, Carlsbad, CA).

Immunofluorescence. Cells were fixed with 3.7% formaldehyde and permeabilized with Triton X-100 (0.2%). Before incubation with antibodies, cells were blocked with 1% bovine serum albumin for 1 h. AhR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:100. To determine nonspecific background, goat IgG antibody (Santa Cruz Biotechnology) was used. Cells were subsequently stained with 0.4% 4,6-diamidino-2-phenylindole.

Transfections. Cells were plated at 2×10^5/well in a six-well plate. After 12 h, the cells were transfected using LipofectAMINE (Invitrogen), with 0.5 μg of *Renilla reniformis* luciferase (pRL-TK) (Promega, Madison, WI) and 1.5 μg of 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 (TK) promoter and the luciferase reporter gene] (Berghard et al., 1993) or pT81 (same reporter plasmid without the XRE sequence, used as a negative control) (Nordeen, 1988). After 24 h, transfected cells were treated with 10 nM TCDD or 1 nM to 1 μM DF 203 as indicated in the figures. Control cells were transfected with pTX.Dir and treated with DMSO (0.1%). After a 9-h treatment, luciferase activity was measured by the Dual-Luciferase Assay System (Promega) following the manufacturer's instructions. Transfection efficiency was monitored by *R. reniformis* luciferase activity with the pRL-TK plasmid as an internal control.

Western Blot Analysis. Purified AhR antibody was used at 1 μg/ml. Western blot analysis was carried out as reported previously (Singh et al., 1996), and proteins that interact with the antibody were detected by an enhanced chemiluminescence Western analysis detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

RT-PCR. The evaluation of gene expression was performed by RT-PCR. For *CYP1A1* and *CYP1B1*, the primers and probes were as follows: CYP1A1 forward, GATTGGGCACATGCTGACC; CYP1A1 reverse, CTGTCAAGGATGAGCCAGCA; CYP1A1 probe, FAM-TGGGAAAGAACCCGCACCTGGC-TAMRA); CYP1B1 forward, TTTCGGCTGCCGCTACA; CYP1B1 reverse, ACTCTTCGTTGTGGCTGAGCA; and CYP1B1 probe, FAM-ACGACGACCCCGAGTTCCGTGAG-TAMRA. For the endogenous control glyceraldehyde-3-phosphate dehydrogenase, human primers and probes were used (Applied Biosystems, Foster City, CA). Fifteen polymerase chain reaction cycles were done. RNA was isolated using RNeasy 96 kit and QIAvac vacuum manifold (QIAGEN, Valencia, CA).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared following a method described previously (Andrew and Faller, 1991). Synthetic oligonucleotides containing the AhR-binding site of human the *CYP1A1* promoter (5′ CTC CGG TCC TTC TCA CGC AAC GCC TGG GCA 3′) (Invitrogen) or consensus AP2 (Promega) were used as probes or competitors. End-labeling was performed with T4 polynucleotide kinase and [γ-^{32}P]ATP. Nuclear extract (20 μg) was incubated in binding buffer [10 mM Tris-HCl pH 7.5, 4% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 1 μg poly[dI-dC] · poly[dI-dC] in a 15-μl reaction volume, 10 min at room temperature. After addition of the DNA probe (100,000 cpm/reaction), samples were incubated for 20 min at room temperature. The resulting DNA/protein 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

DF 203 Induces *CYP1A1* and *CYP1B1* Transcription. Previous studies have described that DF 203 caused induction of CYP1A1 protein in MCF-7 cells but no induction was observed in insensitive cell lines such as MDA-MB-435 or PC3 (Chua et al., 2000). To confirm that DF 203 may affect *CYP1A1* and *CYP1B1* gene expression and provide a basis for assaying AhR function, MCF-7, MDA-MB-435, and PC3 cells were treated with the compound (1 μM) for 24 h and mRNA levels for these two genes were measured by RT-PCR. DF 203 caused an increase in both cytochrome mRNA levels in MCF-7 cells. Treatment with the drug caused a remarkable induction of approximately 100-fold in the CYP1A1 mRNA level with respect to the control (Fig. 1A). A smaller increase of CYP1B1 mRNA (10-fold) was observed in this cell line. However, in MDA-MB-435 and PC3 cells, the level of CYP1A1 and CYP1B1 after treatment remained similar to control (Fig. 1A).

We then confirmed that DF 203 action was through de novo transcription of CYP1A1 and CYP1B1 mRNA levels in MCF-7 cells. Actinomycin D (5 μg/ml) for 1 h abolished the increase in mRNA for CYP1A1 and CYP1B1 (Fig. 1B). These results are consistent with transcriptional activation of CYP1A1 and CYP1B1 induced by DF 203 (1 μM for 6 h).

DF 203 Induces Activation of *CYP1A1*-Related Promoter Sequences. The *CYP1A1* and *CYP1B1* promoters are regulated by AhR, which forms a heterodimer with Arnt. Binding of the complete dimer to XREs in the promoter region mediates transcription of AhR-responsive genes, including *CYP1A1* and *CYP1B1*. Thus, we sought to define whether DF 203 activates the AhR pathway, to cause *CYP1A1* transcription. MCF-7, MDA-MB-435, and PC3 cells were transfected with an XRE-luciferase reporter construct (pTX.Dir), and as a control, the same reporter construct without XRE elements (pT81) was used. Cells were then treated with 0.1% DMSO, 10 nM TCDD, or 1 μM DF 203. TCDD was used as a prototypic compound activator of *CYP1A1* transcription. As shown in Fig. 2A, in MCF-7 cells transfected with pTX.Dir, a 15-fold induction of luciferase activity was observed when cells were treated with TCDD, whereas DF 203 caused a 10-fold induction. However, in MDA-MB-435 cells transfected with pTX.Dir, DF 203 (1 μM) caused an increase of only 1.5-fold over the control. Similarly, in PC3 cells treated with DF 203, XRE-luciferase activity was 1.7-fold higher than control. No induction in luciferase activ-

ity was observed when cells transfected with pT81 were treated with DF 203 (1 μM) or TCDD (10 nM). Similar results were obtained when cells were transfected with a fragment of mouse native *CYP1A1* promoter (inclusive of four dioxin-responsive elements) (pGudLuc1.1) (data not shown) (Garrison et al. 1996). These findings clearly demonstrate that DF 203 induces activation of promoter sequences known to respond to AhR-mediated signals. This is in accord with the interaction of protein complexes induced by treatment with DF 203 through the XRE sequence of the *CYP1A1* promoter.

To determine the extent of XRE-luciferase induction, pTX.-Dir-transfected cells were treated with increasing doses of DF 203 (0–2 μM). In MCF-7 cells, increased luciferase activity was found from 1 nM DF 203, reaching a maximum induction in a range between 500 nM and 1 μM DF 203. In MDA-MB-435 or PC3 cells, little increase in luciferase activity was found independently of the concentration of DF 203 used (Fig. 2B). This dose-response relationship was concordant with the previously published (Bradshaw et al., 1998b; Chua et al., 1999) capacity of DF 203 to inhibit proliferation of MCF-7 cells in culture.

When pTX.Dir-transfected cells were pretreated with an antagonist of AhR, α-naphthoflavone, before the treatment with DF 203, luciferase activity was reduced by approximately 25%; pretreatment with α-naphthoflavone before treatment with TCDD reduced luciferase activity by 80% (data not shown). These results corroborate the likelihood that DF 203 may be a potent ligand for AhR. Recent ligand binding studies to AhR have indeed demonstrated that DF 203 is a very good AhR ligand, with an affinity comparable with TCDD and ED_{50} values in the nanomolar range (D. Bell, personal communication).

DF 203 Induces AhR Translocation in MCF-7 Cells. The results shown in Fig. 3 are consistent with the idea that DF 203 might be an agonist ligand for the AhR, inducing both translocation of AhR to the nucleus with subsequent binding to XRE sequences and transcriptional activation of target genes, including *CYP1A1* or *CYP1B1*. To assess this possibility, we studied whether DF 203 could activate the AhR pathway with resulting AhR translocation from cytoplasm to nucleus.

In control, vehicle-treated MCF-7 cells, AhR is localized exclusively in the cytoplasm (Fig. 3). However, after treatment with either 10 nM TCDD or 1 μM DF 203 for 1 h, AhR translocates completely to the nucleus. Surprisingly, and in contrast, in vehicle-treated MDA-MB-435 cells, AhR is localized in the cytoplasm and nucleus and, after treatment with 1 μM DF 203 or 10 nM TCDD for 1 h, AhR is also present predominantly in the nucleus. A similar pattern of baseline AhR intracellular distribution is observed in resistant PC3 as well as MDA-MB-435 cells. These results indicate that in the insensitive cell lines a substantial fraction of AhR is already in the nucleus before exposure to drug. These results suggest that the regulation of AhR is distinctive in DF 203-sensitive cells compared with drug-resistant cell lines, consistent with results from the transfection studies in Fig. 2.

DF 203 Induces Nuclear Localization of Immunoreactive AhR in MCF-7 Cells. To confirm and extend the immunofluorescence studies, the effect of both TCDD (10 nM) and DF 203 (1 μM) on the subcellular distribution of immu-

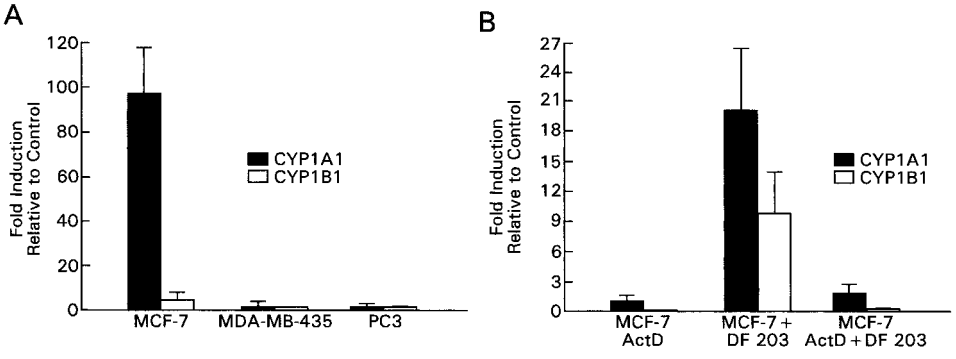

Fig. 1. DF 203 induces *CYP1A1* and *CYP1B1* transcription in sensitive (MCF-7) cells. A, MCF-7, MDA-MB-435, and PC3 cells were treated with DF 203 (1 μM) for 24 h, RNA was isolated from control and treated samples, and *CYP1A1* and *CYP1B1* gene expression was measured by RT-PCR as described under *Materials and Methods*. Data are shown as fold induction of treated cells relative to constitutive expression in control cells (±S.D.; n = 7 samples from two independent experiments). B, expression of *CYP1A1* and *CYP1B1* in MCF-7 cells pretreated with 5 μg/ml actinomycin D for 1 h followed by no additional drug for 6 h (left columns), MCF-7 with no actinomycin D and DF 203 (1 μM) for 6 h (center columns), or MCF-7 pretreated with 5 μg/ml actimomycin D followed by DF 203 (1 μM) + maintained actinomycin D for 6 h (right columns).

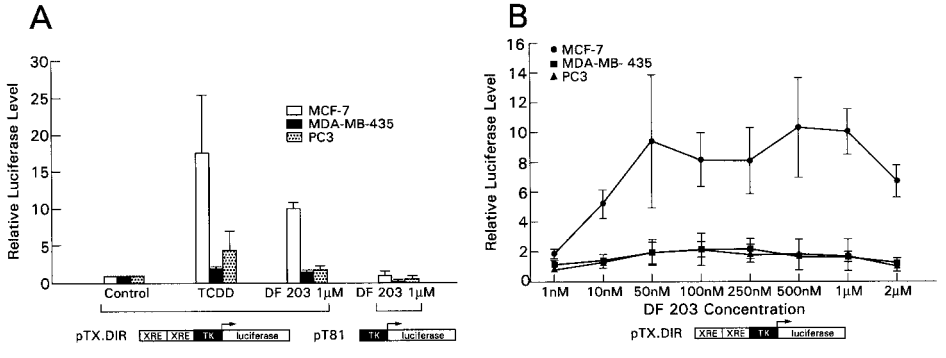

Fig. 2. DF 203 induces binding to the XRE sequence of CYP1A1. A, MCF-7, MDA-MB-435, and PC3 cells were transfected with XRE-luciferase vector (pTX.Dir.) or pT81. Transfected cells were treated with DMSO, 10 nM TCDD, or 1 μM DF 203 for 9 h. After this treatment the amount of luciferase was determined normalizing to the amount of *R. reniformis* luciferase. The values are expressed as luciferase levels relative to the control. B, transfected cells were treated with the indicated concentrations of DF 203 for 9 h. DF 203 increased XRE-driven luciferase activity in a dose-dependent manner in MCF-7 cells. The amount of luciferase was determined as described in A.

noreactive AhR protein was also investigated. As demonstrated in Fig. 4, in MCF-7 cells treated with DMSO only, the cytoplasmic fraction contained relatively high levels of the AhR protein compared with the nuclear fraction, confirming the visual impression from immunofluorescence studies. In contrast, after treatment with an agonist of AhR, 20 μM β-naphthoflavone, or 1 μM DF 203, immunoreactive AhR protein was almost exclusively localized in the nuclear fraction. In MDA-MB-435 cells, immunoreactive AhR protein was localized in nuclear and cytoplasmic fraction before and after 1-h treatment with 20 μM β-naphthoflavone or 1 μM DF 203. These results confirm that in a benzothiazole-insensitive cell line such as MDA-MB-435, AhR seems to be present constitutively in the nucleus.

DF 203 Increases Protein/DNA Complexes Formed on XRE Elements. Ligand-induced formation of protein/DNA complexes on the XRE sequence was also confirmed by electrophoretic mobility shift assay. Briefly, nuclear extracts from cells treated with 0.1% DMSO, 10 nM TCDD, or 1 μM DF 203 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, three protein/DNA complexes were found in nuclear extracts of control cells, although the intensity of the three complexes was very low (Fig. 5A, lane 1). Interestingly, DF 203 (1 μM) treatment for 5 min (Fig. 5A, lane 3) caused 11-fold induction of the binding capacity of the three complexes. This induction was comparable with 10 nM TCDD (1 h) treatment (Fig. 5A, lane 2). The specificity of the binding to XRE was examined by pretreating nuclear extract from cells treated with DF 203 (1 μM) for 5 min, with 100× unlabeled XRE probe or AhR polyclonal antibody. The binding of the highest and lowest molecular mass complexes to labeled XRE sequences was greatly diminished in the presence of excess unlabeled XRE (Fig. 5A, lane 6) or pretreatment with AhR antibody (Fig. 5A, lane 5), suggesting that only these complexes may be directly AhR related. The identity of the other proteins binding to XRE remains to be elucidated. Attempts to supershift the band with the AhR antibody were unsuccessful. These protein/DNA complexes do not disappear when nuclear extracts from DF 203-treated cells were incubated with 100× unlabeled AP2 oligonucleotide (data not shown).

Similarly, in MDA-MB-435 cells, we observed three protein/DNA complexes in the control (Fig. 5B, lane 1). Treatment with TCDD (10 nM) caused increased expression of the three complexes after overnight treatment (Fig. 5B, lane 2). Exposure to TCDD (10 nM) for 1 h did not increase these protein/DNA complexes. Treatment with 1 μM DF 203 for 5 min showed a similar pattern as the control (Fig. 5B, lane 3),

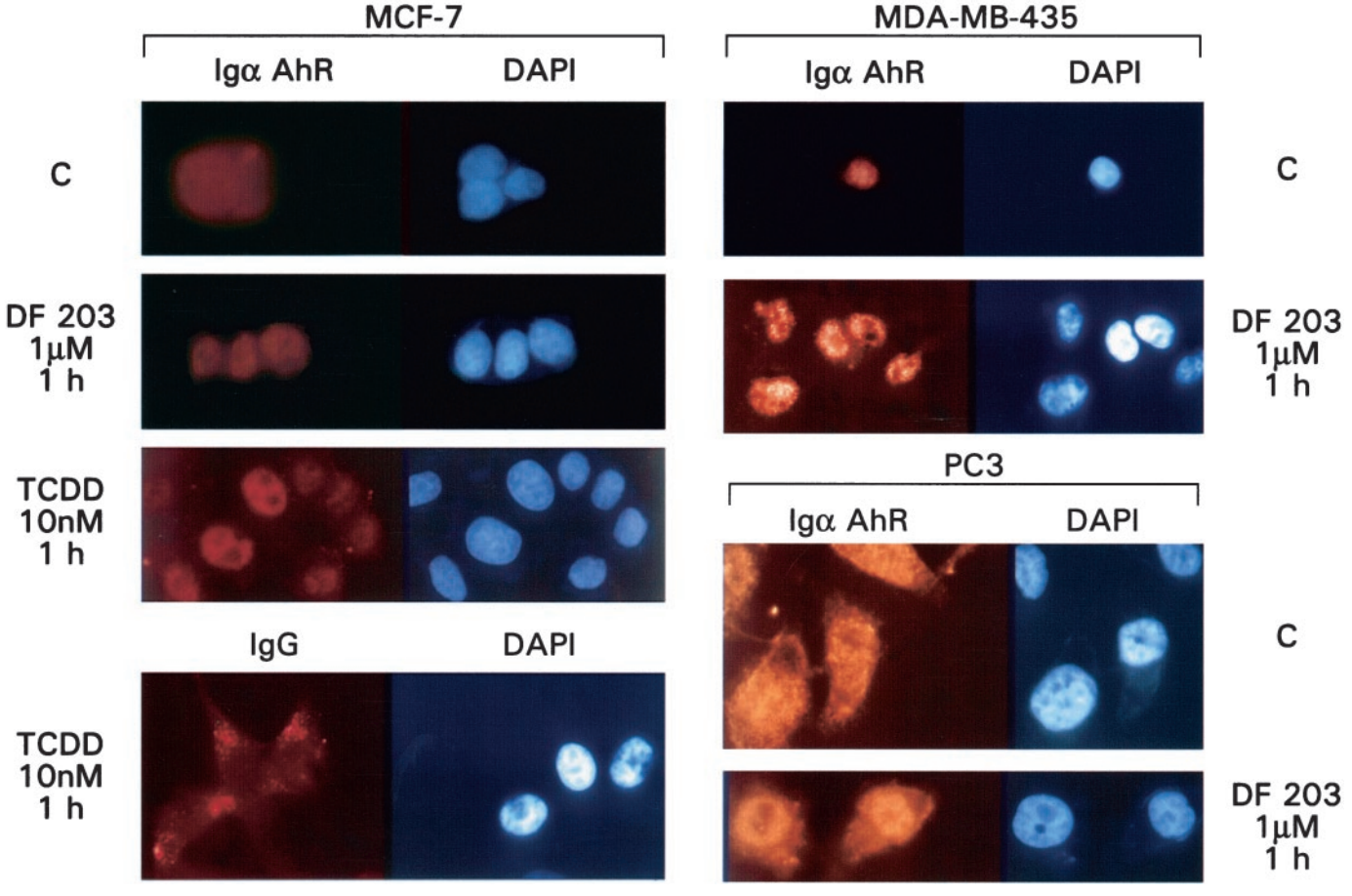

Fig. 3. DF 203 causes translocation of AhR to the nucleus in MCF-7 cells. MCF-7, MDA-MB-435, and PC3 cells were grown on coverslips. The cells were treated with DMSO (C), 1 μM DF 203, or 10 nM TCDD for 1 h. After fixation, the cells were double-stained for AhR (red) and 4,6-diamidino-2-phenylindole (blue) as described under *Materials and Methods*. To determine nonspecific background, cells treated with TCDD (10 nM) were incubated with goat IgG control antibody and the Cy3-conjugated rabbit anti-goat IgG. Stained cells were then visualized on a Zeiss Axiovert microscope using a 63× objective and appropriate images were captured with an Optronics charge-coupled device camera.

particularly with respect to the uppermost XRE-interacting complex. After 30-min treatment with DF 203, 10-fold induction of the bottom complexes (Fig. 5B, lanes 2 and 3) was found with respect to the control (Fig. 5B, lane 4) but without an increase in the uppermost XRE-interacting complex. The binding of the highest and lowest molecular mass protein/DNA complexes induced by TCDD was abolished in the presence of excess unlabeled XRE (Fig. 5B, lane 5), but only the higher molecular mass complex (Fig. 5B, lane 1) was diminished with pretreatment with AhR antibody (Fig. 5B, lane 6). The induction of the upper band (Fig. 5B, lane 1) after treatment with DF 203 was weak compared with the induction of the upper band observed in MCF-7 cells. Preincubation of nuclear extracts from TCDD-treated MDA-MB-435 cells with 100× unlabeled AP2 oligonucleotide did not abolish protein/DNA complexes formation (data not shown).

In PC3 cells, constitutive expression of three protein/DNA complexes was found in the control (Fig. 5C, lane 1). This pattern did not change after treatment with TCDD (10 nM; 1 h) (Fig. 5C, lane 6) or DF 203 (1 μM; 15 min, 30 min, or 1 h) (Fig. 5C, lanes 2–4).

These data confirm that DF 203 is an AhR agonist that induces binding of AhR to the XRE sequence of the *CYP1A1* promoter, causing transcriptional activation in MCF-7 of XRE-regulated genes; MDA-MB-435 cells treated with DF 203 (30 min) undergo a minimal activation of binding of AhR to XRE. In PC3 cells, nuclear XRE-binding complexes were demonstrated to be constitutively present, even in the control. In both PC3 and MDA-MB-435 cells, binding of AhR to XRE was not accompanied by induction of *CYP1A1* gene expression (Fig. 1).

Discussion

Previous studies have demonstrated clearly that different cell types in vitro may be exquisitely sensitive or markedly resistant to the drug (Shi et al., 1996; Bradshaw et al., 1998b; Chua et al., 1999). This suggests that definition of "sensitive"

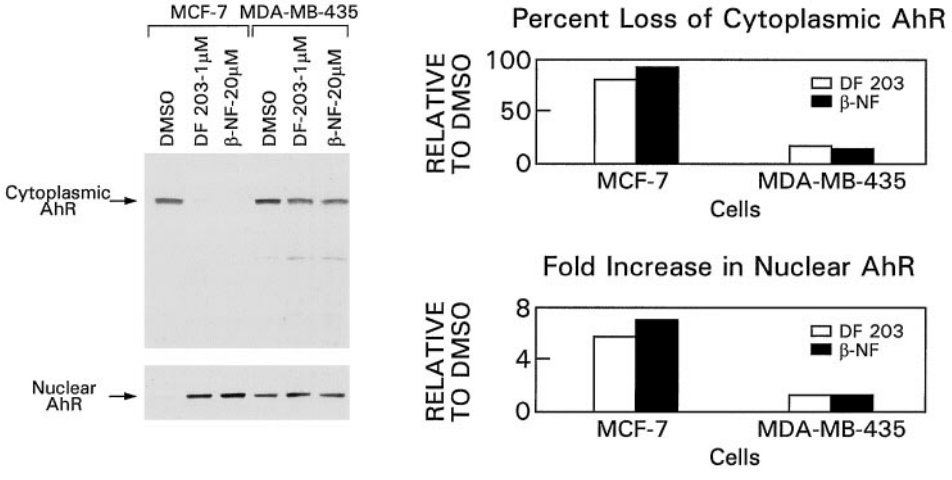

Fig. 4. DF 203 causes an increase in immunoreactive nuclear AhR in MCF-7 cells. MCF-7 and MDA-MB-435 were treated with 0.1% DMSO, 1 μM DF 203, or 20 μM β-naphthoflavone for 1 h. Determination of immunoreactive AhR in cytoplasmic and nuclear fractions was performed by Western blot as described under *Materials and Methods*. β-NF, β-naphthoflavone.

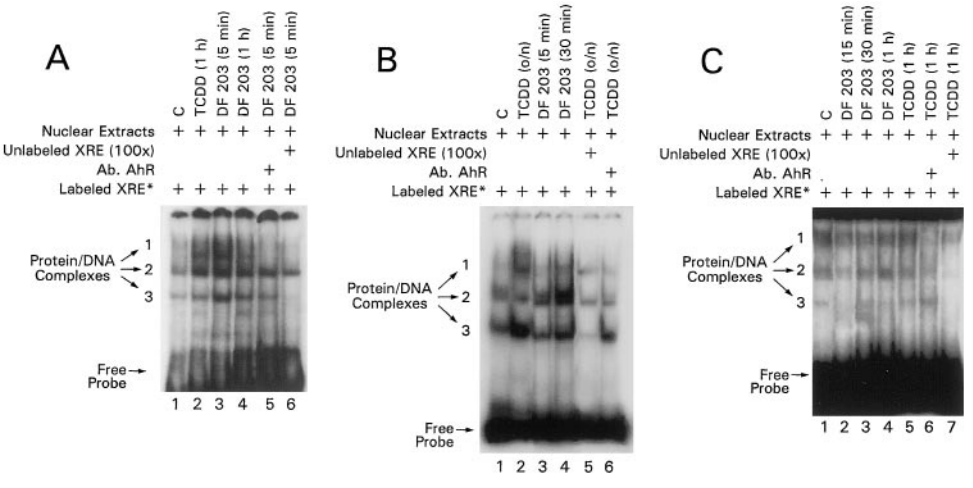

Fig. 5. DF 203 induces protein/DNA complexes on the XRE sequence of the *CYP1A1* promoter. A, nuclear extracts (20 μg) from MCF-7 cells treated with DMSO (C) (lane 1), 10 nM TCDD (1 h; lane 2), 1 μM DF 203 (5 min; lane 3), or 1 μM DF 203 (30 min; lane 4) were incubated with labeled XRE sequence of *CYP1A1* promoter for 10 min at room temperature. Free and bound DNA were separated as described under *Materials and Methods*. For competition, nuclear extracts from MCF-7 cells treated with DF 203 (1 μM; 5 min) were incubated with 4 μg of anti-AhR antibody (lane 5) or 100-fold excess of unlabeled XRE oligonucleotide (lane 6) B, nuclear extracts (20 μg) from MDA-MB-435 cells treated with DMSO (C), 10 nM TCDD (overnight), or 1 μM DF 203 for 5 or 30 min 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 10 nM TCDD (overnight) with 4 μg of anti-AhR antibody or 100-fold excess of unlabeled XRE oligonucleotide. C, nuclear extracts (20 μg) from PC3 cells treated with DMSO (C), 10 nM TCDD (1 h) or 1 μM DF 203 for 15 or 30 min were incubated with labeled XRE, and free and bound DNA were separated. Competition was performed by incubation of nuclear extracts from cells treated with 10 nM TCDD for 1 h, with anti-AhR antibody or 100-fold excess of unlabeled XRE.

potentially responding tumor phenotypes would be important in early clinical trials and allow a more accurate assessment of therapeutic index. Moreover, patients with tumors displaying intrinsic resistance to benzothiazoles would be spared unnecessary toxicity.

Activation of phase I enzymes, such as CYP1A1 and CYP1B1, usually occurs as a response in cells to promote detoxification of toxic agents such as benzo[*a*]pyrene, a polycyclic aromatic hydrocarbon. It was also described that the environmental pollutant TCDD, which is a potent agonist of AhR, induces *CYP1A1* and modulates *CYP1B1* expression in MCF-7 cells. TCDD is a nongenotoxic AhR ligand that has an antiestrogenic and thus antitumorigenic effect in rodent uterus and mammary cells and human breast cancer cells (Ramamoorthy et al., 1999). In contrast, other AhR agonists such as benzo[*a*]pyrene induce CYP1A1, which generates genotoxic metabolites causing DNA damage resulting in G_1 cell cycle arrest (Vaziri and Faller, 1997). The potential for useful antitumor activity on the part of benzo[*a*]pyrene remains to be defined. Evaluation of selected benzothiazoles has revealed their ability to cause potent growth inhibition of ER+ MCF-7 and ER− MDA-MB-468 breast cancer cells in vitro and tumor growth inhibition of ER+ MCF-7 and BO and ER− (MT-1 and MT-3) human mammary xenografts (Shi et al., 1996) as well as certain ovarian carcinoma models in vivo (Bradshaw et al., 1998a). When MCF-7 and MDA-MB-435 xenografts were transplanted in opposite flanks of the same mouse, only the growth of MCF-7 tumors were inhibited, indicating that the antitumor activity is selective in vivo (T. Bradshaw, personal communication).

In this study, we demonstrated that the antitumor agent DF 203 induces CYP1A1 and CYP1B1 mRNA levels, with evidence for activation through the AhR signaling pathway. Because DF 203 is known to be metabolized by CYP1A1 by a pathway that in sensitive cell types leads to induction of DNA damage (Stevens et al., 2001), cell growth inhibition, and evidence of antitumor effect in vivo, our data are consistent with a novel mechanism in which an antitumor agent acquires its cytotoxic potential by activating its own metabolism in susceptible cell types. In that regard, DF 203 may be regarded as a prodrug whose basis for efficacy is selective induction of CYP1A1 to produce an "active" species, which ultimately causes DNA damage and growth inhibition. This is unusual in that most of the known inducers of CYP1A1 are carcinogens such as TCDD or benzo[*a*]pyrene.

In MCF-7 cells, there is another cytochrome P450, CYP1B1, whose expression is regulated in a manner similar to CYP1A1. CYP1B1 may also be important in the activation of DF 203, because CYP1B1 shares overlapping substrate specificities with CYP1A1 (Shimada et al., 1997); for example, CYP1B1 also carries out ethoxyresorufin *O*-deethylase activity, albeit at much lower specific activity than CYP1A1 (Doostdar et al., 2000). CYP1B1 mRNA was likewise increased in MCF-7 cells treated with DF 203. Although it is unclear whether a primary metabolite produced by CYP1A1 itself or downstream "secondary" metabolites may be primarily responsible for DNA damage, further studies will attempt to define the nature of the CYP1A1-related metabolites in an effort to clarify this issue.

An additional basis for differential sensitivity to DF 203 could be differential capacity of the AhR in different cell types to regulate the expression of *CYP1A1*. In that regard, the finding that in DF 203-resistant cells there is constitutive nuclear localization of the AhR may indicate that differentially expressed aspects of AhR receptor functions, including translocation, pairing with nuclear factors, *trans*-activation of gene transcription, and degradation, could underlie selective cytotoxicity of the agent. For example, in sensitive cell lines, translocation of AhR to the nucleus and binding of AhR to XRE sequences seem to occur only after treatment with the drug. Because AhR contains a nuclear localization signal and a nuclear export signal (Ikuta et al., 1998, 2000), one basis for differential cytotoxicity would be differential function of the nuclear localization signal sequence.

Alternatively, our results show that in the insensitive cell lines, there is little or no induction in the protein complexes binding to XRE. This can clearly be the cause of low expression of *CYP1A1* and *CYP1B1* in insensitive cell lines. In this regard, a more complete understanding of DF 203 action must take into account the fact that AhR recruits a battery of coactivators and corepressors that may be different according to tissue type. Because breast and ovarian tumors have been reported to be responsive to estrogen-receptor signaling events, whether "cross talk" between estrogen receptor and AhR in these tumors contributes to selective sensitivity to DF 203 of these models must also be further investigated. Previous studies have shown that MCF-7 and MDA-MB-435 express wild-type AhR. However, a low-molecular-mass variant form of Arnt has been detected in the MDA-MB-435 and other ER-cell lines (Wilson et al., 1997). Future studies will elucidate whether the presence of the Arnt variant makes MDA-MB-435 cells insensitive to DF 203.

Finally, AhR is present in cytoplasm and nucleus of insensitive cells before and after treatment with DF 203, indicating that degradation may not occur efficiently in DF 203-insensitive cells. Others have shown that degradation of AhR is necessary for proper activation of the pathway. This process involves ubiquitination of the AhR protein (Ma and Baldwin, 2000) and insensitivity in MDA-MB-435 and PC3 cells may be caused by altered capacity of ubiquitination of the receptor or absence of the ubiquitin ligase machinery.

Pretreatment with actinomycin D abolishes the increase in CYP1A1 and CYP1B1 mRNA levels, and DF 203 induces *CYP1A1* promoter-driven luciferase activity. Thus, we conclude that the benzothiazole induces "de novo" synthesis of CYP1A1 and CYP1B1 mRNA. Thus, mutations in the *CYP1A1* promoter in insensitive cell lines may also lead to lack of activation of this cytochrome. Eight polymorphisms of the human *CYP1A1* gene have been reported (Goth-Goldstein et al., 2000), and the differential capacity of DF 203 to act at polymorphic *CYP1A1* loci remains to be defined.

In conclusion, we have found that the new antitumor benzothiazole DF 203 activates the AhR pathway in sensitive MCF-7 cells, but not in resistant MDA-MB-435 or PC3 cells. Activation of AhR pathway by DF 203 may be necessary but not sufficient, because additional metabolizing systems may also need to be present for the induction of efficient cytotoxicity.

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