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Divergent Mechanisms for Loss of Ah-Responsiveness in Benzo[*a*]pyrene- and Adriamycin^R-Resistant MCF-7 Cells

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ABSTRACT. The intracellular aryl hydrocarbon receptor (AhR) mediates signal transduction by environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo[*a*]pyrene by functioning as a ligand-activated transcription factor. We have investigated AhR signaling in sublines of the human breast cancer cell line MCF-7 selected for resistance to Adriamycin^R (Adr^R) and benzo[*a*]pyrene (BP^R). Previously we reported that Adr^R cells have a loss of estrogen receptor (ER) expression and are Ah-nonresponsive. Here we show that AhR mRNA and protein are expressed at normal levels in Adr^R cells, and the activated AhR complex is functionally capable of binding a xenobiotic responsive element. In MCF-7 cells AhR was depleted to 15% of normal levels after 4 hr TCDD treatment; however, 45% of AhR remained in Adr^R cells during this time course. In BP^R cells AhR mRNA levels were found to be decreased relative to wild-type cells, which led to decreased AhR protein levels and DNA-binding activity. Cellular ER content has been shown to correlate with Ah-responsiveness in human breast cancer cell lines. BP^R cells were found to be ER-positive, although chronic (BP^R cells) and acute (24 hr) exposure to benzo[*a*]pyrene led to significantly lower ER protein levels in MCF-7 cells. We conclude that loss of Ah-responsiveness occurs by different mechanisms in xenobiotic-resistant MCF-7 sublines: AhR mRNA is down-regulated in BP^R cells, whereas Adr^R cells are deficient in AhR signaling by a mechanism unrelated to AhR expression and activity. BIOCHEM PHARMACOL **57**;11:1253–1263, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. Ah receptor; Arnt; benzo[*a*]pyrene; Adriamycin^R; MCF-7 cells

The AhR† signaling pathway mediates the cytotoxic and genotoxic effects of several environmental contaminants (reviewed in [1, 2]). Dioxins and PAHs are able to induce the transcription of genes by binding to the AhR. In the absence of ligand, cytoplasmic AhR is complexed to a 90-kDa heat shock protein dimer and an immunophilin-like protein [3, 4]. Binding of ligand to AhR initiates a process leading to dissociation of chaperone proteins and heterodimerization with Arnt in the nucleus. This complex functions to transactivate a discrete set of genes possessing upstream xenobiotic responsive elements. Members of the AhR gene battery include CYP1A1, CYP1A2, CYP1B1, NADPH:quinone oxidoreductase, aldehyde dehydrogenase 3c, UDP-glucuronosyltransferase, and glutathione *S*-transferase Ya. Studies in various animal models indicate that dioxins are responsible for species-specific toxic syndromes including wasting, immunosuppression, teratogenesis, hyperkeratosis, and chloracne, as well as cancer [5]. PAHs such as BP can be metabolized by cytochrome P450 enzymes to reactive intermediates and are thus more carcinogenic than dioxins. It has been suggested that exposure to PAHs may lead to breast cancer since these lipophilic compounds can be stored and concentrated in the mammary fat pad [6, 7], and enzymes that activate PAHs are expressed in human mammary epithelial cells [8–11].

Mammary tumors, which are responsive initially to chemotherapeutic treatment, quickly progress to a hormone-independent and cytotoxic drug-resistant phenotype. *In vitro* models of xenobiotic resistance have been studied extensively by us [12–18] and others to elucidate the mechanisms by which cells cope with environmental stress and to gain insight for strategies to counteract chemotherapy resistance. MCF-7 human breast cancer cells are ideally suited for investigating drug resistance since many hormone, growth factor, and xenobiotic receptor signaling pathways have been studied extensively in this cell line. Previously we characterized MCF-7 cells selected for resis-

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† *Abbreviations*: Adr^R, Adriamycin^R-resistant; AhR, aryl hydrocarbon receptor; BP, benzo[*a*]pyrene; BPDE, (±)-*anti*-benzo[*a*]pyrene-*R*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide; BP^R, benzo[*a*]pyrene-resistant; CAT, chloramphenicol acetyltransferase; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; EROD, ethoxyresorufin O-deethylase; HSP, heat shock protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAH, polycyclic aromatic hydrocarbon; PAS, Per-Ah receptor/Arnt-Sim homology; RT-PCR, reverse transcription-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WT, wild-type; and XRE, xenobiotic responsive element.

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tance to Adriamycin^R. This subline was found to have increased glutathione *S*-transferase activity [12], overexpression of P-glycoprotein [19, 20], cross-resistance to BP [13] and radiation [15, 18], and loss of ER expression [13]. This study reports the isolation and characterization of a BP-resistant MCF-7 subline. Vickers *et al.* [21] have shown that a correlation exists between Ah-responsiveness and ER expression in human breast cancer cell lines. Our data show that BP^R cells retain the ER-positive status of the parental cell line. Therefore, these cell lines provide a model to compare AhR signaling in MCF-7 cells or derivatives thereof with AhR$^+$ER$^+$ (WT), AhR$^-$ER$^+$ (BP^R), and AhR$^-$ER$^-$ (Adr^R) phenotypes. To this end, we have analyzed AhR and Arnt for genetic mutation, changes in mRNA and protein expression, and DNA-binding activity. Results from this study showed that AhR mRNA was down-regulated in the BP^R subline. In contrast, AhR expression and activity were normal in Adr^R cells, but TCDD treatment did not deplete AhR protein to the extent observed in WT cells. These data suggest that AhR is protected from ligand-binding *in vivo* in Adr^R cells.

MATERIALS AND METHODS

Drugs and Reagents

TCDD and BPDE were purchased from the Midwest Research Institute; [^{3}H]TCDD from ChemSyn Laboratories; and BP from the Sigma Chemical Co. BP and BPDE were dissolved in dimethyl sulfoxide and TCDD in acetone. A 1200-bp fragment from a *Pst*I digestion of rat CYP1A1 cDNA (plasmid courtesy of Dr. Alan Anderson) was used as a probe for northern blot analysis. Human β-actin oligonucleotide probe was purchased from Clontech. Polyclonal anti-AhR antibodies were obtained from Dr. Allan Okey and Affinity BioReagents; polyclonal anti-Arnt antibody was a gift from Dr. Oliver Hankinson; monoclonal α-tubulin was purchased from Boehringer Mannheim; and monoclonal anti-ER (Ab-1, clone AER314) from NeoMarkers.

Cell Lines and Culture Conditions

MCF-7 wild-type cells and resistant sublines were maintained in RPMI medium supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, and 50 μg/mL streptomycin. The BP^R cell line was selected by exposing MCF-7 cells to increasing concentrations of BP. When cells were able to survive at a given level of drug, the concentration was increased in a stepwise manner until growth at 10 μM BP was achieved. Adr^R cells were described previously [13]. LS180 is a human colon adenocarcinoma cell line known to express high levels of AhR [22].

Northern Blot Analysis

Total RNA was isolated from cells in log growth phase using the RNAzol B (Tel-Test) method by the manufacturer's guidelines. Twenty-five micrograms of RNA for each sample was loaded onto a 1.1% agarose/formaldehyde/ethidium bromide gel. The samples were run at 100 V for 3 hr and a photograph of the gel was taken. The RNA was transferred to a Zeta-Probe membrane (Bio-Rad) by capillary transfer overnight and cross-linked with ultraviolet light. Oligonucleotide probes were radiolabeled with [α-^{32}P]dCTP by random primer extension (Oligo Labeling Kit; Pharmacia, Baie d'Urfe). Hybridization was performed in 40% formamide, 4x SSC, 4x Denhardt's reagent, 0.2 mg/mL salmon sperm DNA, 1.2% SDS, and 10% dextran sulphate at 42° overnight. Final high-stringency washes were carried out in 0.1x SSC/0.1% SDS at 65°. Membranes were reprobed for β-actin expression to standardize for loading differences.

Cytotoxicity Assay

Cells were plated out in 200 μL of medium at a concentration of 1000 cells per flat-bottomed well in 96-well microtiter plates and incubated overnight at 37° in an atmosphere of 5% CO_2. Two hundred microliters of medium containing drug dissolved in appropriate solvent was added to wells and incubated for a further 72 hr. Medium then was removed from each well and replaced by 150 μL of medium containing 10 mM 1,4-piperazinediethanesulfonic acid (pH 7.4) and 50 μL MTT (Sigma) at 2 mg/mL in PBS. Plates were then wrapped in aluminum foil and incubated for 4 hr at 37°. Medium was removed, and the formazan crystals that remained were dissolved in 200 μL dimethyl sulfoxide and 25 μL glycine buffer [0.1 M glycine, 0.1 M NaCl (pH 10.5)]. Quantitation of cell viability was performed by measuring the absorbance at a wavelength of 570 nm on a microtiter plate reader. The negative control (background) consisted of wells that were administered medium although no cells were seeded. For the positive control (100% viability) cells did not receive drug treatment.

Sequencing

The DNA-binding domains of AhR and Arnt [23] and the ligand-binding domain of AhR [24] were PCR-amplified (see Table 1 for primers) and purified from a 1.2% agarose gel using Wizard PCR Preps (Promega). These oligonucleotides were sequenced from both ends using the GibcoBRL dsDNA Cycle Sequencing System (Canada Life Technologies) with the same primers. Reaction products were resolved through 6% polyacrylamide gels containing 7 M urea at 50°. Gels were transferred to Whatman 3MM paper, dried, and autoradiographed.

Preparation of Cell Lysates

Cells in exponential phase growth were washed with PBS and detached from flasks by trypsinization [0.05% trypsin, 0.5 mM EDTA] and sedimented. All following steps were

TABLE 1. Sequence of oligonucleotides

Oligonucleotide*	Sequence†	Assay‡
U-AhR/DBD	GCAGTGGTCCCAGCCTACAC	Seq
L-AhR/DBD	GCAGGCTAGCCAAACGGTCC	
U-AhR/LBD	CATCTAAGCTTGCCGCCATGAATTTCCAAGGGAAG	Seq
L-AhR/LBD	AGTCCCTCGAGGTTAGGGATCCATTATGGCA	
U-Arnt/DBD	AGGTCGGATGATGAGCAGAGC	Seq
L-Arnt/DBD	ATGTGTTGCCAGTTCCCCGC	
U-β-Actin	CGTGATGGACTCCGGTGACGGGG	Q-PCR
L-β-Actin	GATGGAGTTGAAGGTAGTTTCGTG	
U-AhR	ATACTTCCACCTCAGTTGGC	Q-PCR
L-AhR	AAAGCAGGCGTGCATTAGAC	
U-Arnt	CGGAACAAGATGACAGCCTAC	Q-PCR
L-Arnt	ACAGAAAGCCATCTGCTGCC	
XRE	GATCTGGCTCTTCTCACGCAACTCCG	EMSA

*U, upper; L, lower; AhR, Ah receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; XRE, xenobiotic responsible element.

†All oligonucleotides are single-stranded, except for XRE, which is double-stranded.

‡Seq, sequencing; Q-PCR, semi-quantitative reverse transcribed-polymerase chain reaction; EMSA, electrophoretic mobility shift assay.

carried out on ice. Cell pellets were washed again in PBS and then resuspended in lysis buffer containing 25 mM HEPES (pH 7.4), 20 mM sodium molybdate, 5 mM EGTA, 3 mM $MgCl_2$, 5 μg/mL leupeptin, 10 μg/mL aprotinin, 0.5% NP-40, and 10% glycerol. Suspensions were sonicated for 10 sec and supplemented with phenylmethylsulfonyl fluoride (100 μM) and 150 U of DNase. The lysate was incubated for 4 min and then sonicated for an additional 10 sec. At this time, a sample was removed for protein determination by the Bradford assay (Bio-Rad), and the remainder of the sample was combined with an equal volume of 2x gel sample buffer [125 mM Tris (pH 6.8), 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, 0.005% bromophenol blue]. Samples were heated at 100° for 5 min and stored at −20°.

Gel Electrophoresis and Western Blotting

Protein samples were resolved through 7.5% polyacrylamide-sodium dodecyl sulfate gels and electrophoretically transferred to nitrocellulose by standard techniques. The blots were stained with Ponceau S to verify the efficiency of transfer and the gel loading. If areas of the gel did not transfer properly or if differences in gel loading were apparent, the blot was not used. Membranes were blocked overnight in BLOTTO buffer [50 mM Tris (pH 7.5), 0.2% Tween-20, 150 mM NaCl, 5% dry milk powder] at 4° and then washed 4 times in TTBS+ buffer [50 mM Tris, 0.5% Tween-20, 300 mM NaCl (pH 7.5)] for a total of 40 min. Immunohistochemical staining was carried out with 1:1000 anti-AhR antibody or 90 ng/mL anti-Arnt antibody in BLOTTO buffer for 2 hr at room temperature. Membranes were washed as previously and incubated with 1:10,000 horseradish peroxidase-conjugated secondary antibody in BLOTTO buffer for 1 hr at room temperature. Following another wash cycle, the blots were developed with the ECL kit (Amersham). In some cases the blot was stripped and reprobed with antibodies against α-tubulin to ensure that gel loading was equivalent from sample to sample.

Semi-quantitative RT-PCR Analysis

Samples of total RNA (see above) were subjected to DNase treatment and reprecipitated. Five micrograms of RNA was used to prime cDNA synthesis (Gibco/BRL Superscript preamplification system). The 50 μL reaction volume contained 100 nM primers, 200 μM dNTPs, 3 μCi [α-^{32}P]dCTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), cDNA, and 2.5 U of Taq polymerase (Pharmacia). After 4 min at 94°, PCR comprised 25 cycles at 94° for 1 min, 55° for 1 min, and 72° for 1 min, followed by a final round of extension at 72° for 10 min. Samples (10 μL of each PCR product) were subjected to 10% polyacrylamide gel electrophoresis in TBE buffer [90 mM Tris, 90 mM boric acid, 3 mM EDTA]. Gels were transferred to Whatman 3MM paper, dried, and autoradiographed. The primer sequences for β-actin, AhR [25, 26], and Arnt [25] are found in Table 1. To ensure that PCR amplification was linear, a series of PCR reactions were initially performed over a range of MCF-7 cDNA dilutions. These data were plotted in a graph of optical density units versus cDNA volume, and a suitable cDNA dilution was chosen for each set of primers such that PCR amplification fell within the linear range. Endogenous β-actin mRNA expression was used as an internal control to account for differences in RNA quantity and integrity between samples.

Preparation of Nuclear Extracts

Nuclear extracts were prepared according to Whitlock and Galeazzi [27], with minor changes. All steps were performed on ice or at 4°. Confluent cells were washed with PBS

(without Ca^{2+} or Mg^{2+}), harvested by scraping, and centrifuged at 165,000 *g* in a Sorvall RT6000B centrifuge. The cellular pellet was resuspended in 5 vol. of 10 mM HEPES (pH 7.5). After swelling for 10 min, the cells were collected by centrifugation as before and resuspended in 5 vol. of 3 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM HEPES (pH 7.5), and 0.01 mg/mL each of the protease inhibitors aprotinin, leupeptin, and pepstatin. Cells were again collected by centrifugation and resuspended in 2 vol. of the same buffer. Cells were broken with 15 strokes in a Dounce homogenizer using a tight pestle and immediately centrifuged at 15,000 *g*. The crude nuclear pellet was resuspended in 2 vol. of 0.1 M KCl, 25 mM HEPES (pH 7.5), 1 mM dithiothreitol, and protease inhibitors. Nuclei were lysed by adding 2 M KCl to a final concentration of 0.4 M and mixing gently for 30 min. The lysate was adjusted to 20% glycerol and centrifuged at high speed in an Eppendorf 5402 microcentrifuge for 30 min. The transparent supernatant was aliquoted in small volumes, snap-frozen on dry ice, and stored at −80°.

EMSA

Fifteen micrograms of nuclear extract protein was incubated with a [^{32}P] end-labeled oligonucleotide (Table 1) containing an internal XRE from the human *CYP1A1* gene [28] in the presence of 1 μg of poly(dI-dC)·poly(dI-dC). Binding reactions were carried out in 25 mM HEPES (pH 7.5), 1 mM dithiothreitol, 0.15 M KCl, and 10% glycerol in a volume of 30 μL at 20°. Samples were layered onto 5% polyacrylamide gels and electrophoresed in buffer consisting of 20 mM HEPES, 20 mM Tris, 1 mM EDTA (pH 8.0), at 4°. Gels were fixed in 10% (v/v) methanol and 10% (v/v) acetic acid, transferred to Whatman 3MM paper, dried, and autoradiographed.

Immunohistochemistry

Cells that had been plated the previous day on coverslips were fixed with 4% paraformaldehyde/PBS for 15 min at 37°. Membranes were made permeable with a 0.3% Triton X-100/PBS solution for 5 min followed by blocking for 30 min with 5% BSA/PBS. The specimens were incubated for 2 hr with AhR antibody (1:1000 dilution in 5% BSA/PBS) at 37°. The cells were washed 3 times with PBS. The secondary antibody consisted of rhodamine-conjugated rat anti-rabbit IgG (1:100 dilution in 5% BSA/PBS) and was applied for 1 hr at 37°. Following another wash cycle, the coverslips were fixed to slides with polyvinyl alcohol. AhR cellular localization was determined using a Zeiss LSM410 confocal microscope.

TCDD Uptake Studies

Transport of TCDD in wild-type and Adriamycin^R-resistant MCF-7 cells was determined according to the methods described by Schilsky *et al.* [29]. Briefly, MCF-7 wild-type and Adr^R cells were plated in T25 flasks in complete medium. When the cells were at 90–95% confluency, the medium was removed and replaced with 2 mL of 10 nM [^{3}H]TCDD in serum-free RPMI for various periods at 37°. Drug uptake was stopped by the rapid addition of 10 mL of ice-cold PBS to the flasks. The cells were washed three times with 5 mL PBS, and digested by the addition of 1 mL of 1 N NaOH. An aliquot of the lysate was taken for scintillation counting and another for protein assay. Results were determined as the mean of experiments performed in duplicate and expressed as picomoles TCDD per gram total cellular protein.

Steroid Receptor Assay

Cells were collected by scraping in PBS and centrifuged at 1500 rpm in a Sorvall RT6000B centrifuge at 4°. Cells were washed again in PBS and recentrifuged. The pellet was resuspended in 10 vol. phosphate-glycerol buffer [1 mM NaH_2PO_4, 4 mM Na_2HPO_4, 10 mM sodium molybdate, 0.1% monothioglycerol, 10% glycerol (pH 7.5)] and homogenized with a Polytron homogenizer. Extracts were incubated with 7.6 nM [^{3}H]estradiol (New England Nuclear Corp.) alone or in the presence of 100-fold excess of unlabeled estradiol (Sigma). Specific binding was determined using a dextran-coated charcoal assay procedure described previously [30].

Densitometry

Radiographic and ECL exposures were scanned into a Power Macintosh computer with a UMAX VistaS-12 scanner and Adobe Photoshop 3.0.5 software. Images were then quantified with the use of National Institutes of Health Image 1.61 software. Quantification was performed as follows. A rectangular tool was produced that surrounded the largest band of interest. The size of this tool was held constant for all measurements within a specific set of samples. The mean value of the intensity within the tool was then determined for (i) the band of interest, (ii) the area directly above the band, and (iii) the area directly below the band. The intensities of the area above and below the band of interest were then averaged and subtracted from the band of interest.

RESULTS

MCF-7 cells were cultured in escalating concentrations of benzo[*a*]pyrene over a 5-month period leading to the establishment of BP^R cells. This was the identical procedure used for selection of Adriamycin^R-resistant MCF-7 cells [12]. Induction of CYP1A1, an essential enzyme in the metabolism of BP to BPDE, was analyzed to determine the mechanism of resistance. As shown in Fig. 1, BP^R cells possessed a very limited capacity to transactivate *CYP1A1* gene expression in the presence of either BP or TCDD. Cytotoxicity studies based on the MTT assay showed that

the IC_{50} for BP was approximately 2 orders of magnitude greater for BP^R cells compared with WT cells (Fig. 2A). To rule out the possibility that resistance is associated with other mechanisms such as enhanced phase II detoxification, DNA repair, or efflux of BP out of the cell, MTT assays were carried out using the activated metabolite, BPDE (Fig. 2B). These experiments showed that WT and BP^R cells were equally sensitive to BPDE, indicating that resistance is mediated through decreased phase I activation of BP.

We analyzed components of the Ah receptor signaling pathway for alterations in genetic structure, expression, and activity. AhR and Arnt are members of a family of transcription factors containing basic, helix-loop-helix, and PAS motifs. The functional domains of the AhR and Arnt have been investigated by deletion, site-specific mutation, and chimeric protein analyses [31–34]. These studies suggest that residues within the basic region specify the DNA recognition site, whereas the ligand-binding domain of AhR overlaps with the PAS region. We sequenced the DNA-binding domains of AhR and Arnt and the ligand-binding domain of AhR. However, no mutations were detected in the benzo[*a*]pyrene- and Adriamycin^R-resistant MCF-7 cell lines.

Immunoblot analyses were performed to determine whether the deficiency in CYP1A1 induction could be attributed to decreased AhR and Arnt expression. As shown in Fig. 3, AhR protein levels in Adr^R cells are equivalent to those in WT cells, whereas BP^R cells have about a 4-fold decrease in expression. In addition, Adr^R cells have a 3-fold decrease in Arnt protein expression relative to WT and BP^R cells (Fig. 3). Semi-quantitative RT-PCR analysis was utilized to determine if the decrease in AhR expression found in BP^R cells was a result of lower AhR mRNA levels (Fig. 4). Indeed, AhR mRNA levels are decreased 3-fold in the BP^R subline, and Arnt levels are not altered. Therefore, benzo[*a*]pyrene-resistance is associated with down-regulation of AhR mRNA in this model.

Nuclear extracts were combined with a radiolabeled XRE to determine if the DNA-binding capability of activated AhR was decreased in the resistant sublines (Fig. 5). LS180 cells, which have previously been shown to overexpress AhR, were used as a control. The *arrow* in Fig. 5 indicates the AhR/Arnt complex, since this band was induced by TCDD (lane 3 vs lane 2), and anti-AhR or -Arnt antibodies completely inhibited the specific retardation of the XRE

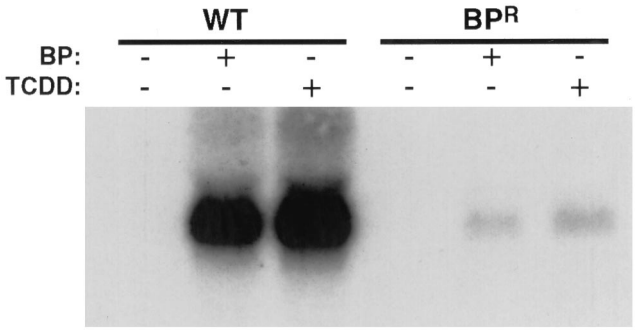
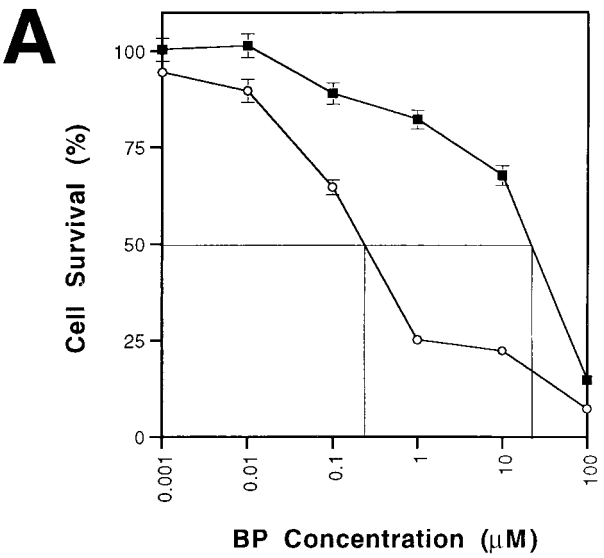


FIG. 1. CYP1A1 mRNA levels in MCF-7 WT and BP^R cell lines. Cells were treated with either solvent, 1 μM BP, or 100 nM TCDD for 24 hr prior to RNA isolation. Northern blot analysis was performed with 25 μg of RNA in each lane. Membranes were stripped and rehybridized with a β-actin probe to standardize for loading differences. These data are representative of three separate experiments.



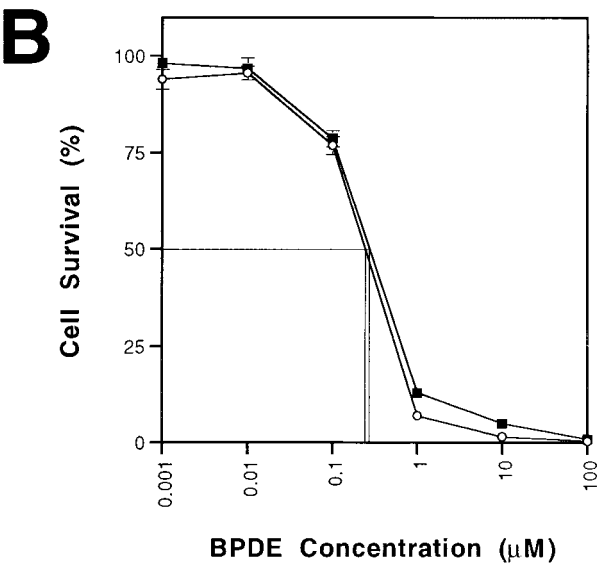


FIG. 2. Cytotoxicity studies. MCF-7 WT (○) and BP^R (■) cells were treated with BP or the activated metabolite, BPDE, for 72 hr in 96-well microtiter plates. Cell survival was determined by a colorimetric assay described in Materials and Methods, which measures the ability of living cells to reduce a dye to a formazan by-product. These data are representative of three experiments. Results are the average of 12 wells per drug concentration per cell line and are expressed as ± SEM.

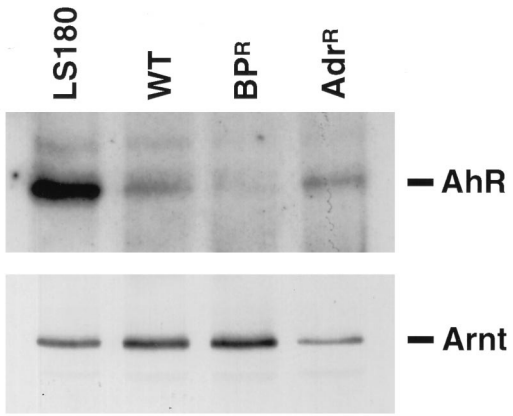


FIG. 3. Analysis of AhR and Arnt protein levels in MCF-7 WT and xenobiotic-resistant cell lines. The presence of AhR and Arnt in total cellular extracts were detected by immunoblot analyses. LS180 is a human colon adenocarcinoma cell line known to overexpress AhR. Amounts loaded: AhR, 100 μg protein/lane; Arnt, 20 μg protein/lane. Relative protein levels (as a percentage of wild-type) for BP^R and Adr^R cells are AhR protein, 28.0 ± 7.6 and 140.7 ± 41.6, respectively; Arnt protein, 120.4 ± 8.7 and 33.0 ± 0.3, respectively. Results are expressed as ± SEM for 3 determinations for each experiment.

(lanes 5 and 6, respectively), whereas a non-specific control antibody did not (anti-ER, lane 7). The epitope recognized by the anti-AhR antibody occurred within the DNA-binding domain (residues 13–31), whereas the anti-Arnt antibody did not obstruct DNA-binding or dimerization, which caused the AhR/Arnt/antibody complex to be super-shifted in the loading well (lane 6). In these experiments nuclear extracts from BP^R cells showed little affinity for the XRE, which is concordant with AhR expression in this cell line. Although Adr^R cells have no detectable increase in aryl hydrocarbon hydroxylase activity when exposed to TCDD [13, 35] and express a relatively low level of Arnt, nuclear proteins from this subline possessed a greater ability to bind a XRE compared with extracts prepared from WT cells. These data show that Arnt is not a limiting factor for AhR signaling in MCF-7 cells, and that loss of Ah-responsiveness in Adr^R cells is independent of AhR/Arnt expression and DNA-binding activity.

It is conceivable that MCF-7 cells selected for resistance to Adriamycin^R are transformed to an Ah^- phenotype through an alteration in the subcellular distribution of AhR. To address this question we investigated AhR localization by confocal microscopy. WT and Adr^R cells were plated out on coverslips. The following day cells were exposed to 1 nM TCDD for 0, 1, or 2.5 hr. As shown in Fig. 6, the pattern of AhR subcellular distribution was not parallel between the two cell lines. In WT cells (Fig. 6, panel 1A) outlines of the nuclei were clearly demarcated and AhR was visualized throughout the cytoplasm, whereas in Adr^R cells (panel 2A) AhR appeared in globular pockets toward the cell periphery. Treatment with ligand resulted in a rapid depletion of AhR in WT cells (panels 1A–1C). In contrast, TCDD had less of an effect on AhR expression in Adr^R cells (panels 2A–2C). Triplicate immunoblot experiments confirmed that a difference in the rate of AhR protein turnover existed between WT and Adr^R cells (data not shown). Quantitation of AhR expression by densitometry determined that 4 hr TCDD exposure (10 nM) depleted AhR to 15% of basal levels in WT cells, compared with 45% in Adr^R cells. These results prompted us to investigate TCDD transport in these cell lines. Surprisingly, our results indicated that the rate of TCDD uptake and the total intracellular level of TCDD in Adr^R cells were higher than in the parental MCF-7 cell line (Fig. 7).

Vickers *et al.* [21] noted that there is a correlation between Ah-responsiveness and ER expression in human breast cancer cell lines and suggested that there may be a common factor(s) that regulates both AhR and ER signal transduction pathways, although these remain unknown. To determine the ER status of the BP^R sublines, protein levels were quantitated by competitive binding of a radioligand. Figure 8 (*stippled* bars) shows a significant decrease ($P = 0.0437$) in the level of ER in BP^R cells compared with WT cells. Moreover, acute exposure (24 hr, *solid* bars) to BP or TCDD caused a significant down-regulation ($P = 0.0461$) of ER in WT cells, but had no effect on BP^R cells.

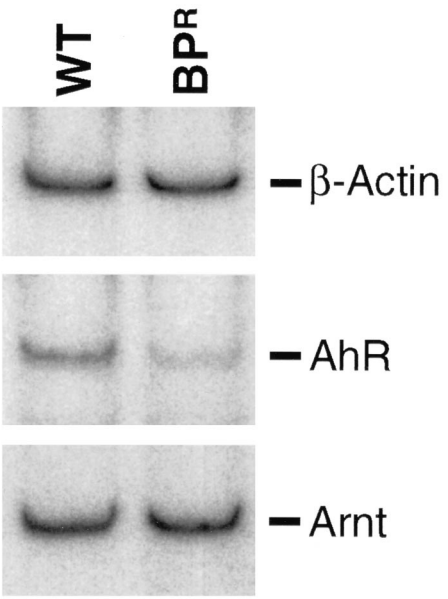


FIG. 4. Representative semi-quantitative RT-PCR analysis of Ah receptor and Arnt expression in MCF-7 WT and BP^R cell lines. Five micrograms of DNase-treated total RNA was reverse transcribed in a final volume of 20 μL. A volume of this cDNA (1/10 μL for AhR; 1/100 μL for Arnt; 1/10000 μL for β-actin) was chosen such that PCR amplification was linear for each gene of interest. Conditions for "hot PCR" and subsequent gel electrophoresis are described in Materials and Methods. β-actin expression was used as an internal control. Relative mRNA levels (as a percentage of wild-type) for BP^R cells are AhR mRNA, 30.1 ± 4.4; Arnt mRNA, 73.1 ± 10.4. These results are expressed as ± SEM for 2 experiments performed with different RNA preparations.

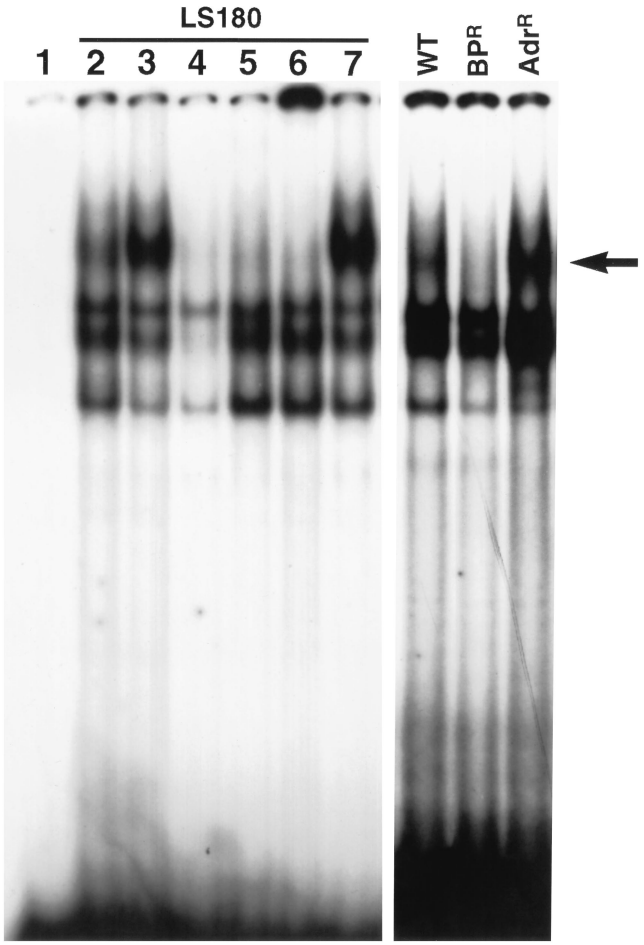


FIG. 5. EMSA using an oligonucleotide containing an internal XRE. *Arrow* indicates the Ah receptor/Arnt/XRE complex. Lane 1 was run with radiolabeled probe only. All remaining lanes were loaded with 15 μg nuclear protein that had been incubated with probe as described in Materials and Methods. Nuclear extracts were obtained after cells were treated with 10 nM TCDD in serum-free medium for 1 hr, except for lane 2 in which cells were treated with solvent only. Lane 3 represents the positive control treated with TCDD. Lane 4 was competed with 100-fold excess unlabeled XRE. Lanes 5–7 had additions of anti-Ah receptor, -Arnt, and -estrogen receptor antibodies, respectively. These data are representative of at least three separate experiments.

DISCUSSION

We have cultured MCF-7 cells in escalating concentrations of BP leading to the establishment of a resistant subline. Ah-responsiveness was characterized initially by quantitation of CYP1A1 mRNA levels. These studies showed that *CYP1A1* gene expression was severely repressed in the BP^R cell line. From cytotoxicity assays showing that WT and BP^R cells were equally sensitive to BPDE, we were able to demonstrate that resistance is mediated through decreased phase I activation of BP. Furthermore, our data strongly indicated that AhR signaling was inhibited in BP^R cells via down-regulation of AhR. This conclusion is drawn from experiments showing significantly lower AhR mRNA and protein by semi-quantitative RT-PCR, immunoblot, and band shift analyses. Previously, Moore *et al.* [36] isolated a BP-resistant MCF-7 clone and characterized loss of Ah-responsiveness by showing that these cells were less responsive to TCDD as determined by CYP1A1 mRNA induction, *CYP1A1* promoter-CAT activity, EROD activity, and XRE binding studies, and by showing that resistant cells metabolized BP at a slower rate. Basal levels of AhR and Arnt were not examined, although velocity sedimentation analysis after exposure to [^{3}H]TCDD for 2 hr indicated that AhR levels were about 50% higher in the BP^R clone [36]. In a subsequent study this group reported that, relative to WT cells, there was a significant reduction (by about 40%) of AhR mRNA levels in BP-resistant T47D cells, whereas Arnt mRNA levels were unchanged [37]. Therefore, it is possible that down-regulation of AhR at the level of transcription is a common mechanism by which cells adapt to prolonged BP exposure.

AhR and ER signal transduction pathways interact with one another on at least two levels. Firstly, TCDD and other AhR agonists suppress a broad range of estrogen-induced responses in laboratory animals [38, 39] and mammalian cells in culture [40–42]. Secondly, Ah-responsiveness has been shown to correlate with ER expression in a panel of human breast cancer cell lines [21]. For example, MCF-7 cells are ER^+ and Ah-responsive, whereas MDA-MB-231 cells do not express ER and are Ah-nonresponsive. In addition, transient transfection of an ER expression vector converts MDA-MB-231 cells to an Ah^+ phenotype [43]. We quantitated ER protein levels to determine whether there was correlation between ER status and Ah-responsiveness in the BP-resistant MCF-7 subline. We found BP^R cells to be ER^+, although expression was reduced by 33% compared with WT cells. Down-regulation of cellular ER content may be one mechanism by which AhR agonists function as antiestrogens. *In vivo* studies have shown that 2-day TCDD exposure decreases hepatic and uterine ER in murine and rat models by up to 42% [44–46]. To determine whether short-term exposure also decreases ER *in vitro*, we exposed MCF-7 cells to TCDD and BP for 24 hr. These experiments showed a 25% reduction of ER expression in WT cells, but ligand did not alter ER expression in the BP^R cell line. It is not known at the present time whether this level of ER suppression is associated with the antiestrogenic nature of AhR agonists, or is sufficient to affect Ah-responsiveness.

$Adriamycin^R$-resistant MCF-7 cells are cross-resistant to benzo[*a*]pyrene [13, 35]. Aryl hydrocarbon hydroxylase activity is undetectable in this cell line, and TCDD failed to induce either endogenous *CYP1A1* expression or a transfected construct containing the normal mouse *CYP1A1* promoter fused to a CAT gene [35]. These results indicated that the defect was due to an alteration in AhR signaling and not a mutation in the regulatory region of the *CYP1A1* gene. We found no significant differences in AhR expression between WT and Adr^R cells. One possibility is that AhR activity is down-regulated in Adr^R cells by a post-translational mechanism. For example, phosphoryla-

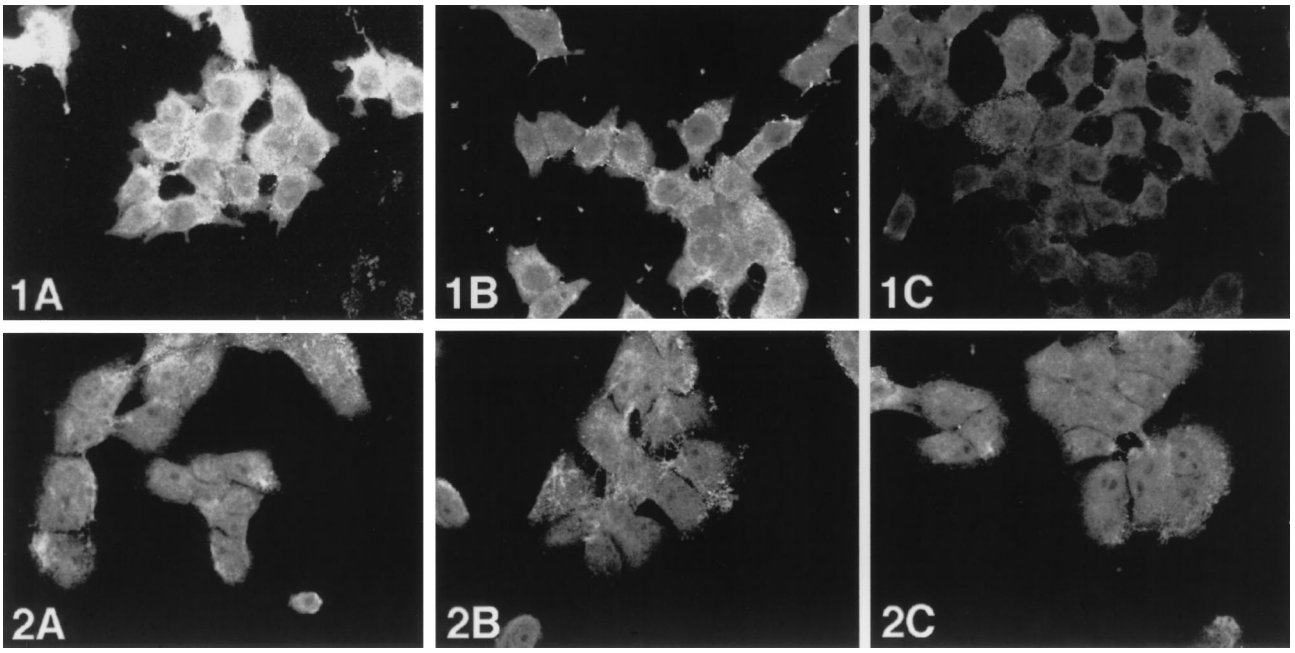


FIG. 6. Effect of TCDD exposure on Ah receptor localization in (1) wild-type and (2) Adriamycin^R-resistant MCF-7 cell lines. Cells plated the previous day on coverslips were incubated with 10 nM TCDD for (A) 0, (B) 1, or (C) 2.5 hr followed by immunohistochemical labeling of Ah receptor as described in Materials and Methods. Fluorescence was visualized by confocal microscopy. This experiment was repeated with similar results.

tion of residues within the AhR DNA-binding domain may be a critical determinant for receptor signaling [47–49]. Our results show that the transformed AhR complex was able to bind a XRE in electrophoretic mobility shift assays. Thus, AhR DNA-binding activity does not appear to be aberrant in Adr^R cells. These data are in agreement with previous studies, which have indicated that AhR expression or XRE binding may not be predictive of Ah-responsiveness [21, 50, 51].

TCDD induces a rapid loss of AhR in cultured cell lines [52–54]. In mouse hepatoma cells (Hepa-1), it has been shown that less than 20% of the total cellular AhR content is present after 6 hr treatment with TCDD, and this effect is prolonged for at least 3 days [55, 56]. Our experiments show that AhR is depleted to 15% of normal levels in WT cells after 4 hr TCDD exposure. In contrast, 45% of AhR remained in Adr^R cells over this time-course. P-glycoprotein has been reported to act as an energy-dependent drug efflux pump for a wide range of xenobiotics, including benzo[*a*]pyrene [57]. However, drug uptake studies showed that P-glycoprotein overexpression does not decrease the level of intracellular TCDD in Adr^R cells. Okey and co-workers [55] have shown that a decrease in AhR protein following exposure to TCDD is not accompanied by a decrease in mRNA. It is plausible that AhR becomes more vulnerable to proteolytic cleavage during receptor activation. The HSP90-binding domain colocalizes with the ligand-binding domain of AhR and steroid hormone receptors. In the process of ligand activation, HSP90 becomes disassociated from the receptors. Furthermore, the presence of ligand depletes cellular receptor levels, which is accompanied by a change in the half-life of the receptor proteins. This same effect occurs when cells are treated with the HSP90 inhibitor geldanamycin [58]. For example, a 75% depletion in AhR was found to occur within 1 hr of exposure to geldanamycin in HeLa and Hepa 1c1c7 cells

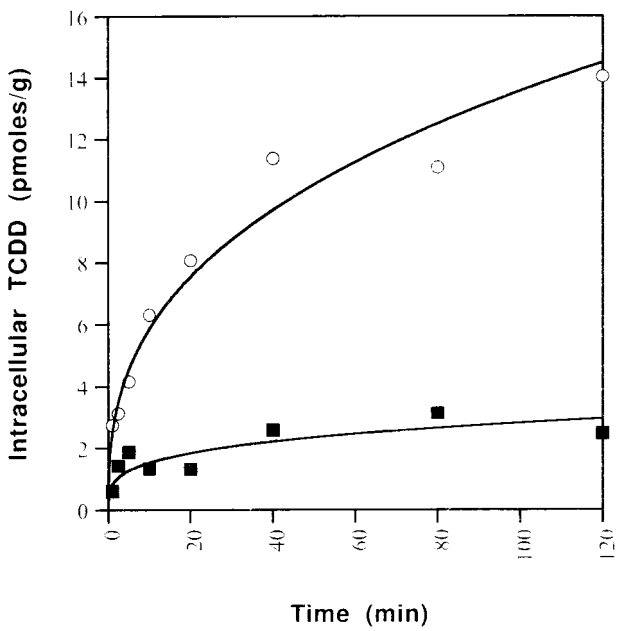


FIG. 7. TCDD uptake in MCF-7 wild-type (○) and Adriamycin^R-resistant (■) cell lines. Cells growing in T25 flasks were exposed to 10 nM [^{3}H]TCDD (specific activity 22.2 Ci/mmol) in serum-free RPMI medium. Following incubation at 37° for the designated times, the cells were washed and the total intracellular counts were determined as described under Materials and Methods. These data are representative of two experiments, each performed in duplicate.

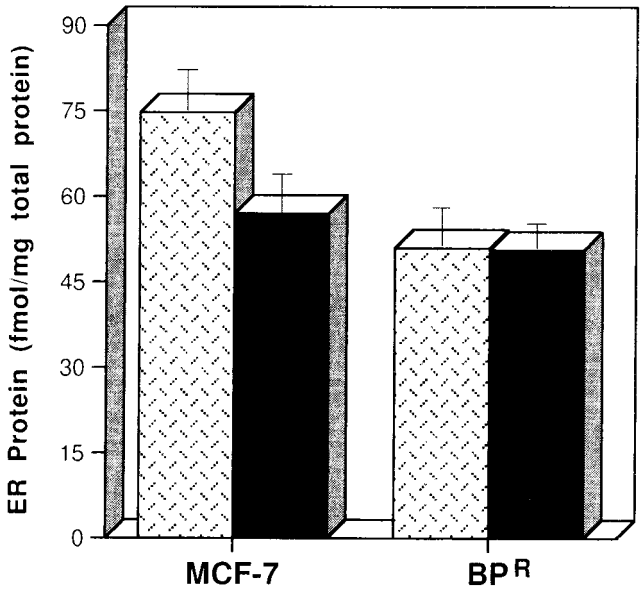


FIG. 8. Determination of ER levels in MCF-7 wild-type and BP^R cell lines. Cells were treated with solvent (*stippled* bars) or agonist (1 μM BP or 100 nM TCDD; *solid* bars) for 24 hr. Aliquots of total cellular extract were incubated for 4 hr at 4° with [^{3}H]estradiol either alone or in the presence of 100-fold excess of unlabeled estradiol as described in Materials and Methods. Specific binding was determined using a dextran-coated charcoal assay procedure. Results shown are the arithmetic mean of 3 experiments and are expressed as ± SEM.

[59]. In the case of the glucocorticoid receptor, there is evidence that receptor degradation following geldanamycin treatment involves the ubiquitin-proteasome pathway [60].

The DNA binding activity of AhR from cell extracts may not accurately reflect the ability of AhR to bind DNA *in vivo*. For example, we have shown by confocal microscopy that the pattern of AhR subcellular distribution is altered in the Adr^R subline. This in turn may contribute to loss of Ah-responsiveness if AhR co-localizes with a high concentration of inhibitory factors such as HSP90. Members of the HSP90 family are cytosolic proteins that have been shown to interact with unliganded or liganded receptors by a dynamic process, and to inhibit their native DNA binding activity [61, 62]. Based on results showing that AhR expression and DNA-binding activity are similar in WT and Adr^R cells, but rates of AhR protein turnover and CYP1A1 transactivation are decreased in the latter, our data suggest that AhR may be protected from ligand-binding in Adr^R cells.

In summary, these studies have established the following conclusions: (1) BP-resistance is associated with down-regulation of AhR mRNA in MCF-7 cells; (2) the Ah^- phenotype of BP-resistant MCF-7 cells is not associated with a loss of ER, although a 33% reduction of expression was found; (3) despite an Ah^- phenotype, AhR expression and DNA-binding activity are normal in $Adriamycin^R$-resistant MCF-7 cells; and (4) TCDD-induced AhR depletion occurs about 3-fold less efficiently in Adr^R cells compared with the parental cell line.

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