Epidermal Growth Factor Receptor Kinase Inhibitors Synergize with TCDD to Induce *CYP1A1/1A2* in Human Breast Epithelial MCF10A Cells [S]

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ABSTRACT

CYP1A1 and *CYP1A2* are transcriptionally activated in the human normal breast epithelial cell line MCF10A following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Shifting MCF10A cultures to medium deficient in serum and epidermal growth factor (EGF) caused rapid reductions in the activated (i.e., phosphorylated) forms of extracellular regulated kinases (ERKs) and the epidermal growth factor receptor (EGFR). Shifting to serum/EGF-deficient medium also enhanced TCDD-mediated induction of cytochrome P450 (*CYP*)1A1. Treatment of cells cultured in complete medium with the EGFR inhibitors gefitinib (Iressa), AG1478, and CI-1033 resulted in concentration-dependent reductions of active EGFR and ERKs, and increased CYP1A1 mRNA content ~3- to 18-fold above basal level. EGFR inhibitors synergized with TCDD and resulted in transient CYP1A1 and CYP1A2 mRNA accumulations ~8-fold greater (maximum at 5 hours) than that achieved with only TCDD.

AG1478, gefitinib, and TCDD individually induced small increases (~1.2- to 2.5-fold) in CYP1A1 protein content but did not cause additive or synergistic accumulations of CYP1A1 protein when used in combination. The mitogen-activated protein kinase kinase inhibitor PD184352 inhibited ERK and EGFR activation in a concentration-dependent fashion without causing CYP1A1 mRNA accumulation. However, cotreatment with PD184352 potentiated TCDD-mediated *CYP1A1* induction. TCDD-mediated induction of *CYP1A1* in MCF7-TETon-EGFR cells, a MCF7 variant in which EGFR expression can be controlled, was not affected by the activity status of EGFR or ERKs. Hence, EGFR signaling mutes both basal and ligand-induced expression of two aryl hydrocarbon receptor–responsive P450s in MCF10A cultures. However, these effects are cell context–dependent. Furthermore, CYP1A1 mRNA and protein abundance are not closely coupled in MCF10A cultures.

Introduction

The aryl hydrocarbon receptor (AhR) is a ubiquitously expressed, ligand-activated cytosolic receptor. Its activating ligands include several natural products as well as environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls, and some polycyclic aromatic hydrocarbons (Denison et al., 2011). Upon ligand binding the activated AhR translocates to the nucleus, whereupon it complexes with the aryl hydrocarbon receptor nuclear translocator protein, binds to xenobiotic response elements (XREs) in the promoters of AhR responsive genes, and recruits coactivators or corepressor proteins that modulate the expression of target genes (Denison et al., 2011). Although early studies focused on AhR-mediated transcriptional activation of a subset of cytochrome P450s (CYPs), such as *CYP1A1* and *CYP1A2*, a large number of genes have been subsequently characterized whose transcription is regulated by the AhR. Although the effects of the AhR are generally attributed to its direct effects on the transcription of target genes, it has increasingly become clear that the liganded-AhR can influence a variety of processes by nontranscriptional mechanisms. The epidermal growth factor receptor (EGFR) is one such example.

The EGFR/ErbB1 is the founding member of the ErbB family. The family consists of EGFR, ErbB2/HER2, ErbB3, and ErbB4 receptors (Yarden and Sliwkowski, 2001). The EGFR, ErbB3 and ErbB4 bind a diverse array of activating ligands. Whereas ligand-bound EGFR and ErbB4 transmit their signaling as homodimers or as a heterodimer with another member of the ErbB family, ErbB2 and ErbB3 to be functionally active must form heterodimers with other ErbB members (Yarden and Sliwkowski, 2001, Nickerson et al., 2011). Receptor and ligand expression are tissue-specific, in many cases developmentally regulated, and can be influenced by exogenous factors as well as the physiologic/pathologic

ABBREVIATIONS: AG1478, Tyrphostin AG 1478 or N-(3-chlorophenyl)-6,7-dimethoxyquinazolin-4-amine; AG825, tyrphostin AG 825 or 4-hydroxy-3-methoxy-5-(benzothiazolylthiomethyl)-benzylidenecyanoacetamide; AhR, aryl hydrocarbon receptor; CI-1033, canertinib or N-(4-(3-chloro-4-fluorophenylamino)-7-(3-morpholinopropoxy)quinazolin-6-yl)acrylamide; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular regulated kinase; Iressa, gefitinib or ZD1839 or N-(3-chloro-4-fluoro-phenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine); MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; P450, cytochrome P450; PD184352, CI-1040 or 2-[(2-chloro-4-iodophenyl)amino]-N-(cyclopropylmethoxy)-3,4-difluorobenzamide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element.

state (e.g., tissue regeneration, wound healing, neoplasia) of the tissue (Nickerson et al., 2011).

Numerous studies indicate that AhR ligands can influence EGFR signaling. Initially, it was noted that polycyclic aromatic hydrocarbon pretreatment of cultured cells reduced the binding of exogenously added epidermal growth factor (EGF) to the plasma membrane (Ivanovic and Weinstein, 1982; Kärenlampi et al., 1983). Thereafter, studies appeared documenting the effects of gestational TCDD exposure on embryonic EGF levels in and during development of salivary gland (Kiukkonen et al., 2006), palate (Abbott and Birnbaum, 1990), and ureteric bud (Bryant et al., 1997). Subsequent studies employing in vivo and cell culture models demonstrated that TCDD could activate the EGFR via AhR-dependent processes [for review see Haarmann-Stemmann et al. (2009)]. These processes include both a transcriptional mechanism in which TCDD stimulates the transcription of EGFR-activating ligands (Patel et al., 2006), as well as a transcriptionally independent process in which Src kinase is released from cytoplasmic AhR-chaperone-Src complexes following the binding of TCDD. Subsequent phosphorylation of the EGFR by Src results in ligand-independent activation of the EGFR (Biscardi et al., 1999). Additional AhR-dependent, transcriptional-independent mechanisms for TCDD-dependent activation of the EGFR have been described (Haarmann-Stemmann et al., 2009).

Whereas AhR-mediated modulation of EGFR activation and signaling is fairly well documented, relatively little is published on the converse. What is known is derived from analyses of TCDD effects on developmental processes in transforming growth factor-α– or EGF-deficient mice (Bryant et al., 2001; Abbott et al., 2003), and the effects of exogenously added EGF on the mRNA and protein contents of AhR-responsive genes (e.g., primarily *CYP1A1/1A2*) in cultured cells (Bachleda et al., 2009; Sutter et al., 2009). The results of these latter studies suggest that the activated EGFR mutes AhR signaling. In the current study we determined if small-molecule inhibitors of the ErbB family modulate basal and TCDD-inducible expression of *CYP1A1* and *CYP1A2* in the normal human breast epithelial cell line MCF10A, and the human mammary carcinoma MCF7-TETon-EGFR cell line. We chose the former because it expresses both the AhR and EGFR, with the EGFR being activated by exogenously added EGF. We chose the latter because it has a functional AhR and offered the opportunity to regulate the expression of the EGFR (Boerner et al., 2005). Our studies indicate that inhibitors of the EGFR, but not ErbB2/HER2, markedly enhance both basal and AhR-mediated accumulation of CYP1A1/1A2 mRNA in MCF10A, but not MCF7-TETon-EGFR, cells. Hence, within the appropriate cellular context, EGFR signaling may function as a rheostat to control AhR signaling.

Materials and Methods

Materials. TCDD was obtained from Midwest Research Institute (Kansas City, MO). Epidermal growth factor, penicillin/streptomycin solution, horse serum, TRIzol, and trypsin/EDTA were purchased from Invitrogen/ThermoFisher Scientific (Waltham, MA). Dimethysulfoxide was obtained from SigmaMillipore (St. Louis, MO). Radioimmunoprecipitation assay lysis buffer, HALT Protease Inhibitor Cocktail, and HALT Phosphatase Inhibitor Cocktail were obtained from ThermoFisher Scientific. CI-1033 and PD184352 were originally obtained as gifts from Pfizer, and subsequently purchased from Selleckchem (Houston, TX). Gefitinib (Iressa) was originally obtained as a gift from AstraZeneca and subsequently purchased from Selleckchem. AG1478 and AG825 were purchased from Calbiochem (San Diego, CA).

Cell Culture. Human breast epithelial MCF10A cells were obtained from the Cell Lines Resource, Karmanos Cancer Center (Detroit, MI) and carried in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium supplemented with 10% horse serum and growth factors (including 10 ng/ml EGF) as described by Reiners et al. (1998). The origin of the MCF7-TETon-EGFR cell line has been described (Boerner et al., 2005). This cell line was maintained in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate,

100 μg/ml G418, 10 μg/ml puromycin, and 1% penicillin/streptomycin. For the experiments reported herein MCF-TETon-EGFR cells were shifted to DMEM ($\pm$ phenol red) supplemented with 10% charcoal-stripped fetal bovine serum, 10 ng/ml EGF, and $\pm$ 1 μg/ml doxycycline for 48 hours prior to TCDD addition.

Western Blot Analyses. The procedures used for the preparation of cell extracts and Western blot analyses have been described in detail (Reiners et al., 1998; Elliott et al., 2012), with the exception that lysates were prepared in radioimmunoprecipitation assay lysis buffer supplemented with Halt phosphatase and protease inhibitor cocktails. Murine monoclonal antibodies to phospho-EGFR (Tyr1068; dilution 1:2000), a rabbit monoclonal antibody to human GAPDH (product no. 14C10, dilution 1:4000), a panning rabbit polyclonal antibody to the EGFR (dilution 1:2000), and a panning rabbit polyclonal antibody to p44/42 MAPK (dilution 1:2000) were purchased from Cell Signaling Technology (Danvers, MA). Murine monoclonal antibodies to diphosphorylated p44/42 (dilution 1:20000) and β-actin (dilution 1:20000) were obtained from SigmaMillipore. Murine monoclonal antibodies to rat CYP1A1 (product no. P1A1PT, dilution 1:2000) were the gift of Detroit R&D (Detroit, MI). Sheep anti-mouse IgG horseradish peroxidase–linked and donkey anti-rabbit IgG horseradish peroxidase-linked antibodies were purchased from GE Healthcare (Piscataway, NJ), and diluted 1:10,000 to 1:40,000, depending on the primary antibody.

RNA Preparation and Northern Blot Analyses. The procedures used for RNA isolation, separation of RNAs on agarose/formaldehyde gels, and transfer to nitrocellulose membranes have been described by Joiakim et al. (2004), who also described the probes and hybridization conditions used for the detection of human CYP1A1, CYP1A2, and 7S mRNAs.

Quantitative Reverse-Transcription Polymerase Chain Reaction. At select time points following drug treatment, total RNA was extracted from cells and column purified using the PureLink RNA isolation kit (Life Technologies/ThermoFisher Scientific, Grand Island, NY). Complementary DNA was then synthesized from 1.5 μg of total RNA using the high-capacity complementary DNA reverse transcription kit (Life Technologies/ThermoFisher Scientific) according to manufacturer's instructions. Gene-specific primers to detect CYP1A1 mRNA (GenBank accession number NM_000499.3) and TATA binding protein mRNA (GenBank accession number NM_003194) were purchased from Integrated DNA Technologies (IDT; Coralville, IA). The sequences of primer pairs for detecting CYP1A1 mRNA are as follows: 5′ ccttccctgatccttgtgatcc 3′ (forward) and 5′ gatcttggaggtggctgaggta 3′ (reverse). Primer pairs to detect TATA binding protein mRNA were predesigned and purchased from IDT and used as the internal control. Quantitative determination of *CYP1A1* gene expression was performed with the StepOne Plus Real Time PCR machine (Applied Biosystems, Foster City, CA) using SYBR Green master mix (Life Technologies/ThermoFisher Scientific), 50 ng RNA equivalents, and 150 nM of diluted primers. The real-time PCR cycle conditions were as follows: PCR initial activation step at 95°C for 15 minutes and a total of 40 cycles for melting (95°C, 15 seconds) and annealing/extension (60°C, 1 minute). All assays were performed in triplicate (technical replicates) with three independent samples (biologic replicates). Fold-changes in CYP1A1 mRNA levels were then quantified using the comparative CT ($\Delta\Delta C_T$) method (User bulletin no. 2, Applied Biosystems) and are expressed relative to vehicle-treated controls.

Real-Time Reverse Transcription–Polymerase Chain Reaction of CYP1A1 Heterogenous Nuclear RNA. The procedures used for the isolation and analyses of CYP1A1 hnRNA have been described in detail (Elliott et al., 2012). For analyses, we used PCR primers that amplified a 120-base pair sequence corresponding to a region spanning the first exon-intron of human *CYP1A1*. Cycle threshold values were used to normalize CYP1A1 to GAPDH levels.

Electrophoretic Mobility Shift Assay. The procedures used for TCDD-mediated AhR transformation in rat liver extract, and electrophoretic mobility shift assay (EMSA) with a radiolabeled oligonucleotide containing a consensus XRE, are published (Reiners et al., 1998).

Statistical and Imaging Analyses. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered to be statistically significant. Western and northern blot images were quantified using ImageJ software (Schneider et al., 2012).

Results

EGFR Inhibitor Modulation of EGFR and Extracellular Regulated Kinase Phosphorylation. For our studies we employed small molecule kinase inhibitors that exhibit specificity for EGFR/ErbB1/HER1

(i.e., gefitinib and AG1478; Levitzki and Gazit, 1995; Arora and Scholar, 2005), for ErbB2/HER2 (i.e., AG825; Levitzki and Gazit, 1995), or for all members of the ErbB family (i.e., CI-1033; Arora and Scholar, 2005). As a screen for EGFR activation we monitored EGFR phosphorylation at tyrosine 1068, one of the three major autophosphorylation sites on the EGFR, and a nexus for recruitment of adaptor molecules that link the receptor to downstream signaling pathways such as Ras.

The EGFR inhibitors gefitinib and AG1478 (Fig. 1A), and the panning ErbB family inhibitor CI-1033 (Fig. 1B), caused concentration-dependent reductions in phosphorylated (i.e., activated) EGFR and extracellular regulated kinase (ERK) in MCF10A cultures. With each inhibitor the losses of phosphorylated EGFR and phosphorylated ERK paralleled one another over the range of tested concentrations. Interestingly, whereas loss of phosphorylated EGFR was paralleled by a reciprocal accumulation of total EGFR in gefitinib- or AG1478-treated cultures (Fig. 1A), loss of phosphorylated EGFR was paralleled by the loss of total EGFR in CI-1033-treated cultures (Fig. 1B).

EGFR Inhibitor Enhancement of *CYP1A1* Induction. Preliminary studies indicated that pretreatment with gefitinib dramatically enhanced the accumulation of CYP1A1 mRNA in TCDD-treated cultures. The extent to which this occurred necessitated the use of exceptionally short exposures (in Northern blot analyses) to avoid underestimating the magnitude of the enhancement (data not shown).

Pretreatment with gefitinib (Fig. 2, A and D), AG1478 (Fig. 2, B and D), or CI-1033 (Fig. 2C) all enhanced TCDD-mediated CYP1A1 mRNA accumulation within 5 hours of TCDD addition, as assessed by either Northern blot (Fig. 2, A–C) or real-time PCR (Fig. 2D) analyses. At the highest concentrations of gefitinib and AG1478 tested, CYP1A1 mRNA contents in cotreated cultures were ~8-fold greater than that measured in TCDD-only treated cultures. These effects most probably reflect enhanced transcription, since cotreatment with gefitinib increased CYP1A1 heterogeneous nuclear RNA levels ~7-fold within 2.5 hours of TCDD addition (Supplemental Fig. 1). Notably, the extent to which the three EGFR inhibitors enhanced TCDD-mediated *CYP1A1* induction correlated with the level to which the inhibitors suppressed
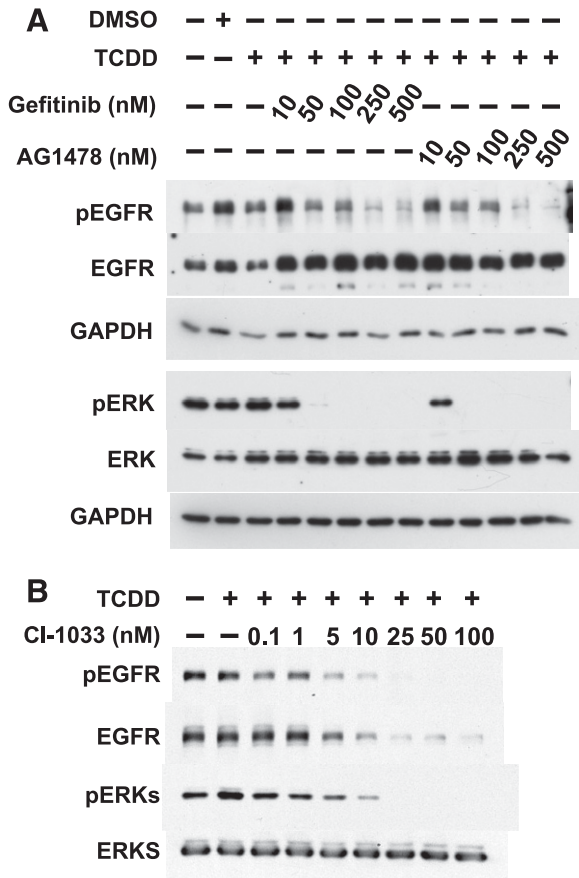

Fig. 1. EGFR kinase inhibitors modulate EGFR and ERK phosphorylation. Cultures of MCF10A cells were left untreated, or treated with dimethyl sulfoxide or 10 nM TCDD ± varied concentrations of (A) gefitinib or AG1478, or (B) CI-1033. The EGFR inhibitors were added 30 minutes prior to TCDD. Cultures were harvested for Western blot analyses 1 hour after dimethyl sulfoxide or TCDD treatment. Each lane contained 25 μg of protein. Similar results were obtained in two additional independent experiments.
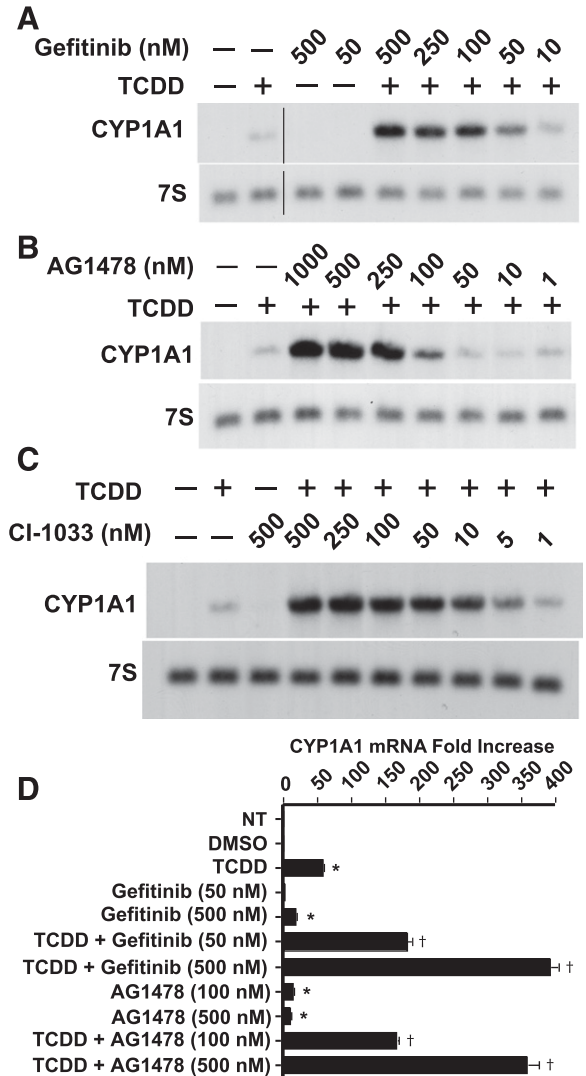

Fig. 2. EGFR kinase inhibitor enhancement of TCDD-mediated induction of *CYP1A1*. MCF10A cultures were left untreated (NT), or treated with 10 nM TCDD, varied concentrations of gefitinib (A,D) or AG1478 (B,D) or CI-1033 (C), or TCDD + gefitinib/AG1478/CI-1033 (A-D). EGFR inhibitors were added 30 minutes [15 minutes for studies in (D)] prior to TCDD addition. Cultures were harvested 5 hours after TCDD addition for mRNA isolation for Northern blot (A–C) or quantitative real-time PCR analyses of CYP1A1 mRNA. Results similar to those reported in panels (A)–(C) were obtained in two additional independent experiments. Data in (D) represent means ± S.D. (N = 3 independent samples). *Greater than NT or dimethyl sulfoxide–treated groups ($P < 0.05$). †Greater than NT, dimethyl sulfoxide–, or TCDD-treated groups ($P < 0.05$). The vertical line separating lanes in (A) represent the point at which the scan was cut and the gefitinib treatments were flipped so as to be consistent with the high dose to low dose orientation used in the other panels.

EGFR phosphorylation (compare Fig. 1A with Fig. 2 A, B, and D, and compare Fig. 1B with Fig. 2C).

Real time PCR analyses indicated that gefitinib and AG1478 alone, if used at concentrations sufficient to suppress EGFR activation, caused small but statistically significant accumulations of CYP1A1 mRNA (~3- to 18-fold above basal levels, Fig. 2D). CYP1A1 mRNA content, after combined TCDD and EGFR inhibitor treatment, greatly exceeded the theoretical mRNA content calculated by summing the effects of the individual agents (Fig. 2D). Hence it appears that TCDD and EGFR inhibitors synergize in their induction of *CYP1A1*.

Kinetic analyses indicated that CYP1A1 mRNA accumulation reached a steady state within ~3 hours of TCDD treatment, which was maintained for at least an additional 18 hours (Supplemental Fig. 2). Maximum accumulation of CYP1A1 mRNA following combinational TCDD plus gefitinib or AG1478 treatment occurred within 3–5 hours of TCDD addition. Thereafter, CYP1A1 mRNA content decreased until 13 hours post-treatment, at which time it was comparable to CYP1A1 mRNA content measured in cultures treated with only TCDD (Supplemental Fig. 2).

EGFR Inhibitor Effects on CYP1A1 Protein Levels. Although CYP1A1 mRNA contents increased ~60-fold following TCDD exposure, CYP1A1 protein contents were only marginally increased (~1.2- to 1.9-fold) at 6 hours (Fig. 3, A and B) and 11 hours (Fig. 3C) after treatment. The EGFR inhibitors gefitinib and AG1478 also increased CYP1A1 protein contents approximately 1.6- to 2.5-fold during the same time period (Fig. 3). No additive or synergistic effects on CYP1A1 protein content were observed following combinational treatment (Fig. 3). Results similar to those reported in Fig. 3 were obtained in two additional experiments spanning 6–23 hours of TCDD treatment.
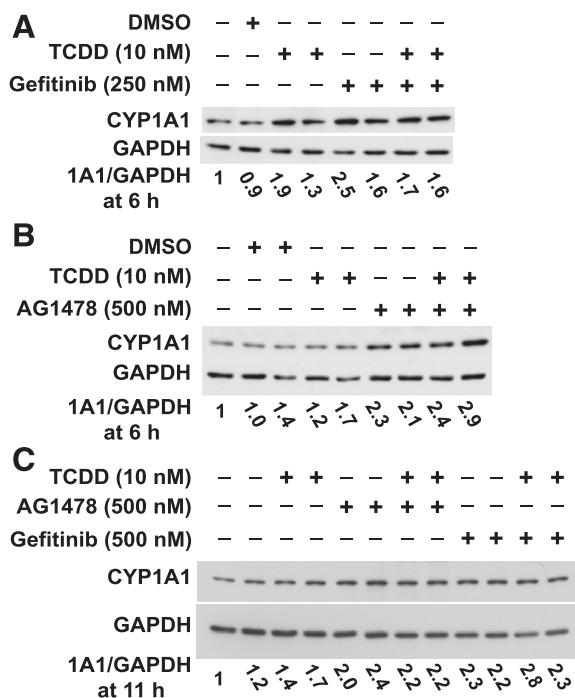

Fig. 3. Effects of EGFR inhibitors on CYP1A1 protein content. Subconfluent MCF10A cultures were treated with (A) 250 nM gefitinib or (B) 500 nM AG1478 for 30 minutes prior to the addition of 10 nM TCDD, and harvested 6 hours later, or (C) were treated with either 500 nM AG1478 or gefitinib for 30 minutes prior to the addition of 10 nM TCDD, and harvested 11 hours later. Control cultures were treated with dimethyl sulfoxide (A, B) or left untreated (C). Harvest times are relative to the time of TCDD addition. Western blots used 25 µg of protein per lane and were quantified using ImageJ software. All lanes represent extracts isolated from individual cultures, and each panel represents an independent experiment. Similar results were obtained in two additional independent experiments spanning 6–23 hours of TCDD treatment.

EGFR Inhibitor Enhancement of *CYP1A2* Induction. The activated AhR, in addition to effecting *CYP1A1* transcription, also induces *CYP1A2* transcription in MCF10A cells (Elliott et al., 2012). Pretreatment of MCF10A cultures with 500 nM gefitinib or AG1478, concentrations sufficient to suppress EGFR phosphorylation, dramatically increased the accumulation of CYP1A2 mRNA in TCDD-treated cultures (Fig. 4). Pretreatment with AG825, a structural analog of AG1478 that inhibits ErbB2, but not the EGFR (Levitzki and Gazit, 1995), had no effect on *CYP1A2* induction by TCDD (Fig. 4).

EGF Modulation of *CYP1A1* Induction. Shifting MCF10A cultures to serum- and EGF-deficient medium resulted in rapid reductions in total and phosphorylated/activated EGFR, which were paralleled by the loss of phosphorylated/activated ERK (Fig. 5A). Shifting cultures to serum- and EGF-deficient medium prior to TCDD addition also resulted in CYP1A1 mRNA accumulations comparable to those occurring in cultures maintained in complete medium, and cotreated with TCDD and gefitinib (Fig. 5B) or AG1478 (Fig. 5C). No additional CYP1A1 mRNA accumulation occurred if cultures were shifted to deficient medium prior to cotreatment with TCDD and either gefitinib (Fig. 5B) or AG1478 (Fig. 5C). Collectively, these studies suggest that the effects of the EGFR inhibitors on CYP1A1 mRNA content are mediated by their suppression of EGFR activity.

Modulation of *CYP1A1* Induction by PD184352. The EGFR activates several signaling pathways, one of which is the Raf-MEK-ERK pathway. Concentration-response analyses indicated a direct relationship between the extent to which EGFR inhibitors synergized with TCDD in the induction of *CYP1A1* and reduced activated ERK content. The use of MAPK kinase (MEK) inhibitors PD98059 and U0126 for investigation of AhR activation/signaling is confounded by the possibility that they may be AhR ligands, and function as agonists or antagonists (Reiners, et al., 1998; Andrieux et al., 2004; Chen et al., 2005). PD184352 is structurally unrelated to PD98059 and U0126, and is a potent MEK inhibitor (Frémin and Meloche, 2010). Electrophoretic mobility shift assays demonstrated that concentrations of PD184352 ≤ 50 µM did not transform rat liver AhR into an XRE-binding species, whereas concentrations >1 µM weakly suppressed TCDD-mediated transformation of rat liver AhR into an XRE-binding species (Supplemental Fig. 3). It is important to note that this latter activity occurs at a concentration far greater than that needed to suppress MEK activity in MCF10A cultures (Fig. 6A).

Exposure of MCF10A cultures to ≥25 nM PD184352 eliminated ERK activity in TCDD-treated cultures (Fig. 6A). At lower MEK inhibitor concentrations pERK content appeared to be elevated. This increase was not a consequence of the MEK inhibitor. Rather, TCDD alone significantly elevated pERK content within 2 hours of treatment (Fig. 6B). Other investigators have reported a similar result (Tan et al., 2002; Puga et al., 2009). Concentrations of PD184352 sufficient to
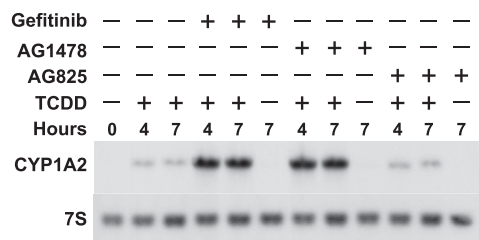

Fig. 4. Modulation of *CYP1A2* induction by EGFR and HER2 kinase inhibitors. MCF10A cultures were left untreated, or exposed to 10 nM TCDD ± 500 nM gefitinib, 500 nM AG1478, or 500 nM AG825. Inhibitors were added 1 hour prior to TCDD, and cultures were harvested for Northern blot analyses of CYP1A2 mRNA 4 or 7 hours after TCDD addition. Similar results were obtained in a second independent experiment.
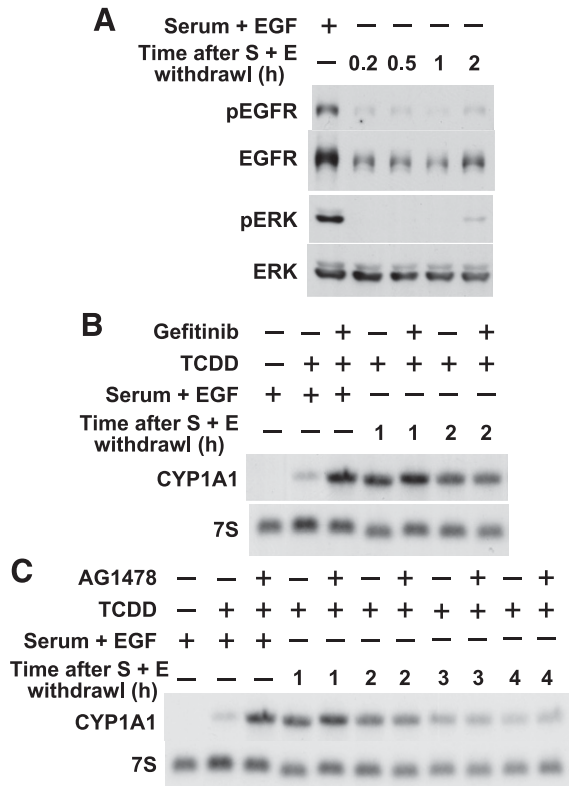
Fig. 5. EGF regulation of EGFR and ERK phosphorylation and *CYP1A1* induction. MCF10A cultures were maintained in complete medium or shifted to medium deficient in EGF and serum. (A) Cultures were shifted to deficient medium and harvested 0.2–2 hours later for analyses of EGFR and ERK by Western blot. (B, C) Cultures were maintained in complete medium, or shifted to deficient medium, and left untreated, or treated with 10 nM TCDD ± 500 nM gefitinib or 500 nM AG1478. Cultures were treated with gefitinib or AG1478 either 1 or 2 hours after the medium shift. TCDD was added 30 minutes after gefitinib or AG1478 addition. Cultures were harvested 5 hours later for analyses of CYP1A1 mRNA by Northern blot. Similar data were obtained in a minimum of two additional independent experiments.

potentiating effect of PD184352 on *CYP1A1* induction by TCDD (Reiners, unpublished data).

Effects of EGFR and MEK Inhibitors on *CYP1A1* Induction in MCF7 Derivatives. MCF7 cells express very little EGFR (Boerner et al., 2005). The MCF7-TETon-EGFR cell line is an MCF7 derivative that expresses a functional EGFR if cultured in the presence of doxycycline (Boerner et al., 2005). In the absence of doxycycline MCF7-TETon-EGFR cultures expressed activated ERK but no EGFR (Fig. 7A). ERK activities were suppressed by PD184352, but not by gefitinib or CI-1033 treatment (Fig. 7A). Neither PD184352 nor the two EGFR kinase inhibitors influenced the induction of *CYP1A1* by TCDD in MCF7-TETon-EGFR cells cultured in the absence of doxycycline (Fig. 7B).

In the presence of doxycycline, MCF7-TETon-EGFR cultures expressed the EGFR, and it was active, on the basis of analyses of Tyr1068 phosphorylation (Fig. 7A). Treatment with either gefitinib or CI-1033 suppressed EGFR phosphorylation, and reduced total EGFR protein, but had no effect on ERK phosphorylation (Fig. 7A). This lack of effect suggests that the activated EGFR is not coupled to the MAPK pathway in MCF7 cells. As expected, PD184352 suppressed ERK phosphorylation (Fig. 7A). None of the inhibitors influenced the induction of *CYP1A1* by TCDD in doxycycline-treated MCF7-TETon-EGFR cultures (Fig. 7B).

Discussion

The current study indicates that EGFR-mediated signaling modulates the induction of *CYP1A1* by TCDD in MCF10A cells. This statement is supported by the findings that the induction of *CYP1A1* by TCDD was enhanced either by pretreating cultures with small-molecule EGFR inhibitors or by shifting MCF10A cultures to EGF-deficient medium prior to TCDD addition. In the case of the EGFR inhibitor studies, concentration-response studies indicated an inverse relationship between EGFR activity and the level to which TCDD could transcriptionally activate *CYP1A1*. Since EGFR inhibitors also enhanced the induction of *CYP1A2* by TCDD, it appears that EGFR-mediated signaling dampens AhR function in MCF10A cells. Notably, because the ErbB2 inhibitor AG825 did not influence the induction of *CYP1A2* by TCDD, it is improbable that EGFR/ErbB2 heterodimers mediate the suppressive activity of the EGFR.

The dampening effect of the activated EGFR on ligand-activated AhR signaling has been noted by others. Specifically, Sutter et al. (2009) reported that the addition of EGF to confluent primary human keratinocyte cultures markedly suppressed the induction of *CYP1A1* by TCDD, as well as transcription initiated from reporter constructs
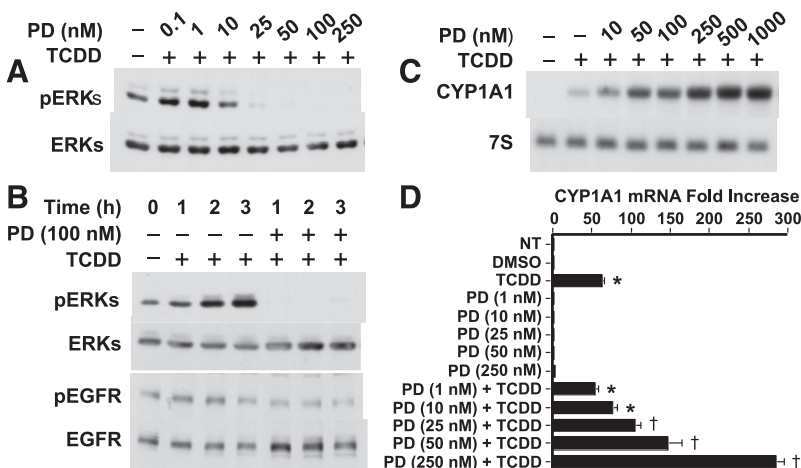
inhibit ERK phosphorylation also caused a minor reduction in EGFR Tyr1068 phosphorylation (Fig. 6B). Concentrations of PD184352 sufficient to inhibit ERK activity had little effect on basal CYP1A1 mRNA content (Fig. 6D). However, when used in combination with TCDD, concentrations of PD184352 sufficient to inhibit ERK dramatically enhanced the accumulation of CYP1A1 mRNA (Fig. 6, C and D). Cotreatment with gefitinib did not further enhance the

Fig. 6. MEK-inhibitor modulation of ERK phosphorylation and *CYP1A1* induction. (A) MCF10A cultures were treated with different concentrations of the MEK inhibitor PD184352 (PD) for 30 minutes prior to the addition of TCDD. Cultures were harvested for Western blot detection of ERKs 1 hour after addition of TCDD. (B) Treatments were similar to that described in (A) with the exception that cultures were harvested for analyses 1, 2, or 3 hours after TCDD treatment. Two additional independent variations of the studies reported in (A) and (B) were performed and yielded similar results. (C, D) MCF10A cultures were left untreated (NT), or treated with 10 nM TCDD ± varied concentrations of PD184352. PD184352 addition preceded TCDD addition by 15 minutes, and cultures were harvested for Northern blot (C) or real time PCR (D) analyses of CYP1A1 mRNA 5 hours after TCDD addition. The data reported in (D) represent means ± S.D. ($N = 3$ independent samples). *Greater than NT, dimethyl sulfoxide–, and PD-treated groups ($P < 0.05$). †Greater than NT, dimethyl sulfoxide–, PD-, or TCDD-treated groups ($P < 0.05$).
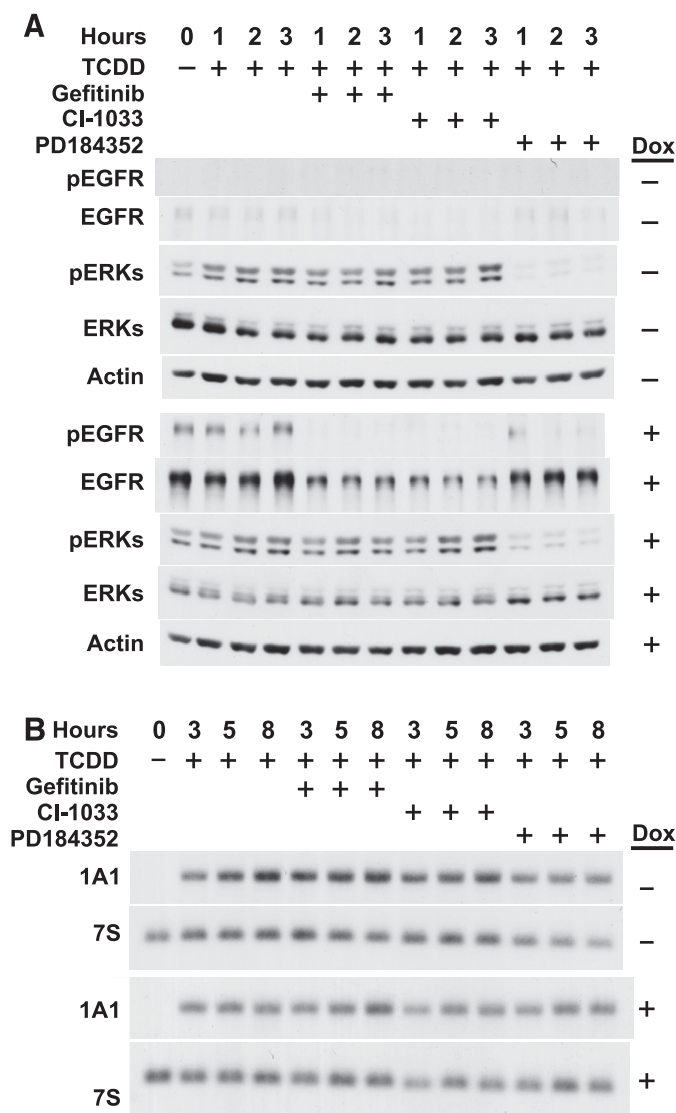
A

B

Fig. 7. Effects of EGFR expression on *CYP1A1* induction in MCF7-TETon-EGFR cultures. Cultures of MCF7-TETon-EGFR cells were cultured in the presence of 10 nM EGF ± doxycycline. Cultures were left untreated, or exposed to 15 nM TCDD, 250 nM gefitinib, 100 nM CI-1033, or 250 nM PD184352 ± TCDD. Inhibitors were added 0.5 hours before TCDD and cultures were harvested at the indicated times after TCDD addition for (A) Western blot analyses of EGFR and ERK, or (B) Northern blot analyses of CYP1A1 mRNA content. Western blot analyses used 25 μg of protein per lane. Results similar to those reported in the figure were obtained in two additional independent experiments.

containing promoters with XREs. Similarly, Bachleda et al. (2009) noted that the addition of EGF to cultured human hepatocytes suppressed TCDD-mediated induction of *CYP1A1* and *1A2*. In the case of the keratinocyte study, chromatin immunoprecipitation assays indicated that EGF supplementation did not disrupt *CYP1A1* promoter–AhR binding. Instead, EGF supplementation suppressed the recruitment of the coactivating protein p300 to the AhR-XRE complex (Sutter et al., 2009).

Signaling mediated through the EGFR may also modulate basal *CYP1A1* expression, as well as other genes that are transcriptionally regulated by the AhR. Concentrations of either gefitinib or AG1478 sufficient to inhibit the EGFR in MCF10A cells elevated basal CYP1A1 mRNA levels as much as 3- to 18-fold. Similarly, Alfieri et al. (2011) reported that both CYP1A1 and CYP1A2 mRNA levels were increased in a series of EGFR wild-type non–small cell lung carcinoma (NSCLC)

cell lines treated with gefitinib. The effects of gefitinib were presumably mediated by its interaction with the EGFR, since comparable results did not occur in NSCLC cell lines resistant to gefitinib owing to mutant EGFRs. Microarray analyses of a human glioblastoma cell line treated with camptothecin, an inhibitor of EGFR activation, also revealed an accumulation of mRNAs corresponding to AhR-responsive genes (Konkimalla and Efferth, 2010).

The EGFR serves as a platform and nexus for several signaling pathways, including the Ras/RAF/MEK/ERK pathway. ERK activity appears to be tightly coupled to EGFR activity in MCF10A cells, because pharmacological inhibition of the latter, or removal of EGF, was paralleled by reductions in phosphorylated ERK1 and 2. Interestingly, suppression of ERK activities with the MEK inhibitor PD184352 mimicked the ability of EGFR inhibitors to enhance the induction of *CYP1A1* by TCDD. Several studies have reported that treatment of cultured cells with U0126 alone (i.e., an MEK inhibitor) is sufficient to induce the accumulation of CYP1A1 mRNA (Andrieux et al., 2004; Chen et al., 2005; Bachleda and Dvorak, 2008) but also to modestly suppress *CYP1A1* induction when combined with AhR agonists (Andrieux et al., 2004; Chen et al., 2005). In agreement with this latter finding, nonpharmacological modulation of ERK activities in murine Hepa-1 cells indicated that ERKs enhanced TCDD-initiated AhR transcriptional responses (Tan et al., 2004). The current study does not rigorously address if ERKs are the EGFR downstream effectors responsible for dampening AhR function in the MCF10A cell line. However, as demonstrated in Fig. 7, PD184352-mediated suppression of ERK activity had no effect on *CYP1A1* induction in MCF7-TETon-EGFR cultures. Hence, in this latter cell line ERKs are not a regulator of AhR function. Collectively, our data and the existing literature suggest that the relationship between ERK activities and AhR function is most probably cell type– and context-dependent.

The physiologic significance of EGFR-mediated dampening of AhR signaling at this point is speculative but may be relevant to chemotherapy. A variety of therapeutics are metabolized by cytochrome P450s (P450s) (Patterson and Murray, 2002). For example, CYP1A1 and/or 1A2 are involved in the metabolic inactivation of tamoxifen (to a minor extent) and gefitinib (Crewe et al., 2002; Alfieri et al., 2011), and dacarbazine is activated by the same two P450s (Reid et al., 1999). Conditions that would suppress/dampen expression of these P450s (either basal or induced) in the target organ might increase the in vivo half-life of the former agents, at the same time decreasing the potency of the latter. Alternately, modulation of AhR activity may influence therapeutic responses by mechanisms independent of P450s. For example, the EGFR and its ligands are commonly overexpressed in many types of breast tumors, including those that are triple negative (LeJeune et al., 1993; Qi et al., 1994; Livasy et al., 2006; Burness et al., 2010; Foley et al., 2010). Recent studies suggest that AhR expression is also upregulated in breast cancer (Li et al., 2014). Interestingly, the AhR ligand raloxifene was recently reported to inhibit the growth of a triple negative breast cancer cell line via an AhR-dependent mechanism (O'Donnell, et al., 2014). A similar positive therapeutic outcome was achieved with the AhR ligand omeprazole in a triple negative breast cancer cell line (Jin et al., 2014). Possibly, suppression of EGFR signaling in breast tumors might enhance the therapeutic potency/efficacy of agents such as raloxifene and omeprazole, which work through an AhR-dependent mechanism.

An unexpected outcome of the current study was the absence of a quantitative relationship between CYP1A1 mRNA and protein content in MCF10A cultures. Although TCDD increased CYP1A1 mRNA content more than 50-fold, CYP1A1 protein content increased by less than 3-fold. Furthermore, although cotreatment with EGFR inhibitors and TCDD transiently increased CYP1A1 mRNA content by as much

as ~400-fold, CYP1A1 protein content remained similar to that achieved with TCDD alone. The basis for these findings is not known but is not unique to our study. Two groups have reported markedly discordant relationships between activity levels and CYP1A1 mRNA and protein contents following treatment of MCF10A cultures with TCDD (Chen et al., 2004) or benzo[*a*]pyrene (Kang et al., 2011). In the case of the mammary carcinoma cell line MCF-7, Divi et al. (2014) reported a 379-fold increase in CYP1A1 mRNA content but only an ~4.5-fold increase in CYP1A1 protein following exposure to benzo[*a*]pyrene. Indeed, several studies have reported that mRNA content is not always predictive of protein content for a variety of proteins (Anderson and Seilhamer, 1997; Chen et al., 2002; Lichtinghagen et al., 2002).

Authorship Contributions

Participated in research design: Joiakim, Mathieu, Shelp, Boerner, Reiners, Jr.

Conducted experiments: Joiakim, Mathieu, Shelp, and Boerner

Contributed new reagents or analytic tools: Boerner

Performed data analyses: Shelp, Reiners, Jr.

Wrote or contributed to the writing of the manuscript: Mathieu, Reiners, Jr.

References

Abbott BD and Birnbaum LS (1990) TCDD-induced altered expression of growth factors may have a role in producing cleft palate and enhancing the incidence of clefts after co-administration of retinoic acid and TCDD. *Toxicol Appl Pharmacol* 106:418–432.

Abbott BD, Buckalew AR, DeVito MJ, Ross D, Bryant PL, and Schmid JE (2003) EGF and TGF-α expression influence the developmental toxicity of TCDD: dose response and AhR phenotype in EGF, TGF-α, and EGF + TGF-α knockout mice. *Toxicol Sci* 71:84–95.

Alfieri RR, Galetti M, Tramonti S, Andreoli R, Mozzoni P, Cavazzoni A, Bonelli M, Fumarola C, La Monica S, and Galvani E, et al. (2011) Metabolism of the EGFR tyrosine kinase inhibitor gefitinib by cytochrome P450 1A1 enzyme in EGFR-wild type non small cell lung cancer cell lines. *Mol Cancer* 10:143 10.1186/1476-4598-10-143.

Anderson L and Seilhamer J (1997) A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18:533–537.

Andrieux L, Langouët S, Fautrel A, Ezan F, Krauser JA, Savouret JF, Guengerich FP, Baffet G, and Guillouzo A (2004) Aryl hydrocarbon receptor activation and cytochrome P450 1A induction by the mitogen-activated protein kinase inhibitor U0126 in hepatocytes. *Mol Pharmacol* 65:934–943.

Arora A and Scholar EM (2005) Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* 315:971–979.

Bachleda P and Dvorák Z (2008) Pharmacological inhibitors of JNK and ERK kinases SP600125 and U0126 are not appropriate tools for studies of drug metabolism because they activate aryl hydrocarbon receptor. *Gen Physiol Biophys* 27:143–145.

Bachleda P, Vrzal R, and Dvorák Z (2009) Activation of MAPKs influences the expression of drug-metabolizing enzymes in primary human hepatocytes. *Gen Physiol Biophys* 28:316–320.

Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH, and Parsons SJ (1999) c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J Biol Chem* 274:8335–8343.

Boerner JL, Biscardi JS, Silva CM, and Parsons SJ (2005) Transactivating agonists of the EGF receptor require Tyr 845 phosphorylation for induction of DNA synthesis. *Mol Carcinog* 44:262–273.

Bryant PL, Clark GC, Probst MR, and Abbott BD (1997) Effects of TCDD on Ah receptor, ARNT, EGF, and TGF-alpha expression in embryonic mouse urinary tract. *Teratology* 55:326–337.

Bryant PL, Schmid JE, Fenton SE, Buckalew AR, and Abbott BD (2001) Teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the expression of EGF and/or TGF-α. *Toxicol Sci* 62:103–114.

Burness ML, Grushko TA, and Olopade OI (2010) Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? *Cancer J* 16:23–32.

Chen G, Gharib TG, Huang C-C, Taylor JMG, Misek DE, Kardia SLR, Giordano TJ, Iannettoni MD, Orringer MB, and Hanash SM, et al. (2002) Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics* 1:304–313.

Chen S, Operaña T, Bonzo J, Nguyen N, and Tukey RH (2005) ERK kinase inhibition stabilizes the aryl hydrocarbon receptor: implications for transcriptional activation and protein degradation. *J Biol Chem* 280:4350–4359.

Chen ZH, Hurh Y-J, Na H-K, Kim J-H, Chun Y-J, Kim D-H, Kang K-S, Cho M-H, and Surh Y-J (2004) Resveratrol inhibits TCDD-induced expression of CYP1A1 and CYP1B1 and catechol estrogen-mediated oxidative DNA damage in cultured human mammary epithelial cells. *Carcinogenesis* 25:2005–2013.

Crewe HK, Notley LM, Wunsch RM, Lennard MS, and Gillam EMJ (2002) Metabolism of tamoxifen by recombinant human cytochrome P450 enzymes: formation of the 4-hydroxy, 4'-hydroxy and N-desmethyl metabolites and isomerization of trans-4-hydroxytamoxifen. *Drug Metab Dispos* 30:869–874.

Denison MS, Soshilov AA, He G, DeGroot DE, and Zhao B (2011) Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol Sci* 124:1–22.

Divi RL, Lindeman TLE, Shockley ME, Keshava C, Weston A, and Poirier MC (2014) Correlation between CYP1A1 transcript, protein level, enzyme activity and DNA adduct formation in normal human mammary epithelial cell strains exposed to benzo[a]pyrene. *Mutagenesis* 29:409–417.

Elliott A, Joiakim A, Mathieu PA, Duniec-Dmuchowski Z, Kocarek TA, and Reiners JJ, Jr (2012) p-Anilinoaniline enhancement of dioxin-induced CYP1A1 transcription and aryl hydrocarbon receptor occupancy of CYP1A1 promoter: role of the cell cycle. *Drug Metab Dispos* 40:1032–1040.

Foley J, Nickerson NK, Nam S, Allen KT, Gilmore JL, Nephew KP, and Riese DJ, 2nd (2010) EGFR signaling in breast cancer: bad to the bone. *Semin Cell Dev Biol* 21:951–960.

Frémin C and Meloche S (2010) From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. *J Hematol Oncol* 3:8 10.1186/1756-8722-3-8.

Haarmann-Stemmann T, Bothe H, and Abel J (2009) Growth factors, cytokines and their receptors as downstream targets of aryl hydrocarbon receptor (AhR) signaling pathways. *Biochem Pharmacol* 77:508–520.

Ivanovic V and Weinstein IB (1982) Benzo[a]pyrene and other inducers of cytochrome P₁-450 inhibit binding of epidermal growth factor to cell surface receptors. *Carcinogenesis* 3:505–510.

Jin U-H, Lee S-O, Pfent C, and Safe S (2014) The aryl hydrocarbon receptor ligand omeprazole inhibits breast cancer cell invasion and metastasis. *BMC Cancer* 14:498 10.1186/1471-2407-14-498.

Joiakim A, Mathieu PA, Elliott AA, and Reiners JJ, Jr (2004) Superinduction of CYP1A1 in MCF10A cultures by cycloheximide, anisomycin, and puromycin: a process independent of effects on protein translation and unrelated to suppression of aryl hydrocarbon receptor proteolysis by the proteasome. *Mol Pharmacol* 66:936–947.

Kang HJ, Hong YB, Kim HJ, Rodriguez OC, Nath RG, Tilli EM, Albanese C, Chung F-L, Kwon SH, and Bae I (2011) Detoxification: a novel function of BRCA1 in tumor suppression? *Toxicol Sci* 122:26–37.

Kärenlampi SO, Eisen HJ, Hankinson O, and Nebert DW (1983) Effects of cytochrome P1-450 inducers on the cell-surface receptors for epidermal growth factor, phorbol 12,13-dibutyrate, or insulin of cultured mouse hepatoma cells. *J Biol Chem* 258:10378–10383.

Kiukkonen A, Sahlberg C, Partanen AM, Alaluusua S, Pohjanvirta R, Tuomisto J, and Lukinmaa PL (2006) Interference by 2,3,7,8-tetrachlorodibenzo-p-dioxin with cultured mouse submandibular gland branching morphogenesis involves reduced epidermal growth factor receptor signaling. *Toxicol Appl Pharmacol* 212:200–211.

Konkimalla VB and Efferth T (2010) Inhibition of epidermal growth factor receptor-overexpressing cancer cells by camptothecin, 20-(N,N-diethyl) glycinate. *Biochem Pharmacol* 80:39–49.

LeJeune S, Leek R, Horak E, Plowman G, Greenall M, and Harris AL (1993) Amphiregulin, epidermal growth factor receptor, and estrogen receptor expression in human primary breast cancer. *Cancer Res* 53:3597–3602.

Levitzki A and Gazit A (1995) Tyrosine kinase inhibition: an approach to drug development. *Science* 267:1782–1788.

Li Z-D, Wang K, Yang X-W, Zhuang Z-G, Wang J-J, and Tong X-W (2014) Expression of aryl hydrocarbon receptor in relation to p53 status and clinicopathological parameters in breast cancer. *Int J Clin Exp Pathol* 7:7931–7937.

Lichtinghagen R, Musholt PB, Lein M, Römer A, Rudolph B, Kristiansen G, Hauptmann S, Schnorr D, Loening SA, and Jung K (2002) Different mRNA and protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 in benign and malignant prostate tissue. *Eur Urol* 42:398–406.

Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, and Perou CM (2006) Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. *Mod Pathol* 19:264–271.

Nickerson NK, Gilmore JL, Allen KT, Riese II DJ, Nephew KP, and Foley J (2011) EGFR-ligand signaling in breast cancer metastasis: Recurring developmental themes, in *Breast Cancer—Carcinogenesis, Cell Growth and Signaling Pathways* (Gunduz M and Gunduz E, eds) pp. 4–32, InTech, Rijeka, Croatia.

O'Donnell EF, Koch DC, Bisson WH, Jang HS, and Kolluri SK (2014) The aryl hydrocarbon receptor mediates raloxifene-induced apoptosis in estrogen receptor-negative hepatoma and breast cancer cells. *Cell Death Dis* 5:e1038.

Patel RD, Kim DJ, Peters JM, and Perdew GH (2006) The aryl hydrocarbon receptor directly regulates expression of the potent mitogen epiregulin. *Toxicol Sci* 89:75–82.

Patterson LH and Murray GI (2002) Tumour cytochrome P450 and drug activation. *Curr Pharm Des* 8:1335–1347.

Puga A, Ma C, and Marlowe JL (2009) The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochem Pharmacol* 77:713–722.

Qi CF, Liscia DS, Normanno N, Merlo G, Johnson GR, Gullick WJ, Ciardiello F, Saeki T, Brandt R, and Kim N, et al. (1994) Expression of transforming growth factor alpha, amphiregulin and cripto-1 in human breast carcinomas. *Br J Cancer* 69:903–910.

Reid JM, Kuffel MJ, Miller JK, Rios R, and Ames MM (1999) Metabolic activation of dacarbazine by human cytochromes P450: the role of CYP1A1, CYP1A2, and CYP2E1. *Clin Cancer Res* 5:2192–2197.

Reiners JJ, Jr, Lee JY, Clift RE, Dudley DT, and Myrand SP (1998) PD98059 is an equipotent antagonist of the aryl hydrocarbon receptor and inhibitor of mitogen-activated protein kinase kinase. *Mol Pharmacol* 53:438–445.

Schneider CA, Rasband WS, and Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675.

Sutter CH, Yin H, Li Y, Mammen JS, Bodreddigari S, Stevens G, Cole JA, and Sutter TR (2009) EGF receptor signaling blocks aryl hydrocarbon receptor-mediated transcription and cell differentiation in human epidermal keratinocytes. *Proc Natl Acad Sci USA* 106:4266–4271.

Tan Z, Chang X, Puga A, and Xia Y (2002) Activation of mitogen-activated protein kinases (MAPKs) by aromatic hydrocarbons: role in the regulation of aryl hydrocarbon receptor (AHR) function. *Biochem Pharmacol* 64:771–780.

Tan Z, Huang M, Puga A, and Xia Y (2004) A critical role for MAP kinases in the control of Ah receptor complex activity. *Toxicol Sci* 82:80–87.

Yarden Y and Sliwkowski MX (2001) Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2:127–137.

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