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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

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ABSTRACT

Exposure of the human breast epithelial cell line MCF10A to ≥1 μg/ml cycloheximide (CHX)-induced accumulations of CYP1A1 mRNA 6-fold greater than that achieved with only 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Cotreatment with CHX and TCDD caused superinduction of *CYP1A1* with accumulations of CYP1A1 mRNA 30-fold greater than that achieved with only TCDD. Similar results were obtained with the protein translation inhibitors anisomycin (ANS) and puromycin (PUR). Intra- and interinhibitor comparisons of dose/concentration response curves demonstrated the absence of a quantitative relationship between [^{3}H]leucine incorporation and *CYP1A1* induction/superinduction. The inducing/superinducing activities of CHX were suppressed by coincubation with the aryl hydrocarbon receptor (AhR) antagonists α-naphthoflavone and 3′-methoxy-4′-nitroflavone (PD168641). Electrophoretic mobility shift assays demonstrated that nuclear extracts from CHX-treated and CHX + TCDD cotreated cultures formed ~58 and ~340% of the AhR/DNA complexes obtained with TCDD-treated cultures, respectively. In contrast, rat liver extracts did not form AhR/DNA complexes after in vitro transformation with CHX. AhR turnover in TCDD-treated hepatoma 1c1c7 cultures was suppressed by cotreatment with CHX. In contrast, CHX or ANS treatment of MCF10A cultures induced AhR loss and enhanced AhR loss in cultures cotreated with TCDD. Cotreatment with *N*-benzoyloxycarbonyl-(*Z*)-Leu-Leu-leucinal (MG132) but not leptomycin B suppressed AhR loss. Hence, in MCF10A cells, CHX is not an AhR agonist but can superinduce *CYP1A1* via an AhR-dependent mechanism; *CYP1A1* superinduction by translation inhibitors is neither quantitatively related to effects on protein synthesis nor due to a generalized prevention of AhR proteolysis, and proteasome-mediated degradation of the activated AhR can occur in the nucleus.

The aryl hydrocarbon receptor is a ligand-activated transcription factor. In the absence of ligand, the AhR is found primarily in the cytoplasm complexed with hsp90, ARA9/AIP/XAP2, and a 23-kDa co-chaperone protein (Ma and Whitlock, 1997; Kazlauskas et al., 1999). After binding ligand the AhR complex undergoes a conformational change resulting in translocation to the nucleus, complex dissociation, and subsequent heterodimerization with ARNT (Heid et al., 2000). Thereafter, AhR/ARNT complexes, in conjunction with other coactivating or corepressing proteins, interact with enhancer sequences in target genes designated dioxin-responsive elements (DREs) and either stimulate or suppress target gene transcription (Hankinson, 1995; Schmidt and Bradfield, 1996; Dong et al., 1997). Several genes involved in phase I metabolism (e.g., *CYP1A1*, *CYP1A2*, and *CYP1B1*) and phase II metabolism (e.g., *NQO1* and *ALDH4*) contain DRE sequences in their promoters and are transcriptionally activated by AhR agonists (Schmidt and Bradfield, 1996).

A variety of chemicals are AhR ligands (Denison et al., 2002). In general, they are small, aromatic, and planar; how-

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; AMC, 7-amino-4-methylcoumarin; ANS, anisomycin; ARNT, aryl hydrocarbon receptor nuclear translocator; CHX, cycloheximide; DRE, dioxin-responsive element; EMSA, electrophoretic mobility shift assay; HA14-1, ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate; JNK, Jun N-terminal kinase; LMB, leptomycin B; polyHEMA, poly(2-hydroxyethyl methacrylate); PD168641, 3′-methoxy-4′-nitroflavone; PBS, phosphate-buffered saline; PUR, puromycin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TNFα, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MG132, N-benzoyloxycarbonyl-(*Z*)-Leu-Leu-leucinal.

ever, they can differ markedly in how they affect AhR function. TCDD functions exclusively as an agonist and is a potent activator of the AhR. Conversely, 3′-methoxy-4-nitroflavone functions as an antagonist and suppresses AhR activation by agonists (Henry et al., 1999). In some cases, the function of an AhR ligand varies with its concentration. For example, α-naphthoflavone functions as an AhR antagonist when used at a concentration of 1 μM and as an agonist at concentrations >20 μM (Wilhelmsson et al., 1994).

Several studies suggest that the AhR can be activated by processes not involving AhR agonists. For example, omeprazole is not an AhR ligand; however, it is a potent AhR activator and inducer of *CYP1A1* in rat hepatoma H4IIE cells (Backlund et al., 1997). Similarly, the suspension culturing of cells that normally grow as adherent cultures activates the AhR and induces *CYP1A1* in several cell types (Sadek and Allen-Hoffmann, 1994a,b; Monk et al., 2001). Finally, the proteasome inhibitor MG132 induces AhR translocation and induction of *CYP1A2* in cultured fibroblasts (Santiago-Josefat et al., 2001). The transcriptional activations of *CYP1A1* and *CYP1A2* occurring in suspension-cultured and MG132-treated cells presumably required the AhR since induction did not occur in AhR- or ARNT-deficient cell lines (Sadek and Allen-Hoffmann, 1994b; Santiago-Josefat et al., 2001).

TCDD exposure often results in the proteolytic turnover of the AhR (Davarinos and Pollenz, 1999; Ma et al., 2000). This turnover can be suppressed by cotreatment with the proteasome inhibitor MG132 (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). In addition, AhR turnover in TCDD-exposed murine hepatoma 1c1c7 cultures can be suppressed by cotreatment with the protein translation inhibitor CHX (Ma et al., 2000). In addition to preventing AhR turnover, CHX cotreatment also potentiates the induction of *CYP1A1* by TCDD and suspension culturing (Ma et al., 2000; Monk et al., 2001). This potentiation is commonly referred to as "superinduction". It has been hypothesized that CHX suppresses the synthesis of a labile protein required for AhR proteolysis and in doing so inhibits AhR turnover and increases both the duration and amount of activated AhR available for *CYP1A1* transcription (Ma et al., 2000).

We previously reported that the induction of *CYP1A1* by TCDD is suppressed in cultures of the human breast epithelial cell line MCF10A cotreated with 12-*0*-tetradecanoylphorbol-13-acetate (TPA; Guo et al., 2001). Because CHX facilitates the TCDD-mediated induction of *CYP1A1* in some cell lines in which *CYP1A1* transcription is normally nonresponsive to TCDD (Arellano et al., 1993; Kress and Greenlee, 1997), we reasoned that CHX might reverse the effects of TPA. To our surprise, CHX was itself an effective inducer of *CYP1A1*. The current study was initiated to determine whether the effect of CHX was directly linked to the suppression of translation. As an approach, we used three inhibitors of protein translation having different mechanisms of action. In eucaryotes, anisomycin (ANS) and CHX suppress the peptidyl transferase and translocation steps in polypeptide synthesis, respectively. In contrast, puromycin (PUR) promotes the release of nascent polypeptide chains by competing with aminoacyl tRNA for binding to the large ribosomal subunit. In the course of our studies, we generated data that were contrary to a current model of how CHX causes *CYP1A1* superinduction. Specifically, we observed that cotreatment with protein translation inhibitors caused *CYP1A1* superinduction but coincidently potentiated AhR loss. Furthermore, neither the induction nor superinduction of *CYP1A1* by protein translation inhibitors quantitatively correlated with their abilities to suppress polypeptide synthesis.

Materials and Methods

Materials. TCDD was purchased from Midwest Research Institute (Kansas City, MO). Trypsin/EDTA, epidermal growth factor, penicillin/streptomycin solution, and horse serum were purchased from Invitrogen (Carlsbad, CA). Ac-DEVD-AMC was purchased from BD Biosciences PharMingen (San Diego, CA). Cycloheximide, leptomycin B, polyHEME, and puromycin dihydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Anisomycin, MG132, and recombinant tumor necrosis factor-α were purchased from Calbiochem (San Diego, CA). L-[3,4,5-^{3}H(N)]Leucine (170 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). PD168641 was the gift of Pfizer Global Research and Development (Ann Arbor, MI). HA14-1 was purchased from Ryan Scientific Inc. (Isle of Palms, SC).

Adherent and Suspension Cell Culture. The MCF10A cell line was obtained from the Cell Lines Resource, Karmanos Cancer Institute (Detroit, MI). Cells were maintained as attached cultures in supplemented Dulbecco's modified Eagles's medium/Ham's F-12 medium as described by Guo et al. (2001). The supplements consisted of human insulin (10 μg/ml), epidermal growth factor (10 μg/ml), cholera toxin (100 ng/ml), hydrocortisone (0.5 ng/ml), 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 5% horse serum. MCF10A cells were suspension cultured by plating on dishes that had been precoated with polyHEMA to prevent their attachment. The procedure described by Li et al. (1999) was used to prepare polyHEMA-coated dishes. The murine hepatoma cell line Hepa 1c1c7 was grown in α-minimum essential medium containing 5% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate.

All cell lines were grown at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2. Subconfluent cultures were treated with various concentrations of TCDD, CHX, or ANS dissolved/diluted in dimethyl sulfoxide (absolute volume of solvent ≤0.1% of medium volume). Puromycin was dissolved/diluted in sterile water.

RNA Preparation and Northern Blot Analyses. Cultures used for RNA isolation were washed twice with calcium and magnesium-free PBS at the time of harvest and stored at −80°C. Total cellular RNA was isolated using TRIzol reagent. RNA was resolved on 1.2% agarose/formaldehyde gels and transferred to nitrocellulose membranes as described by Reiners et al. (1997). The probes used for the detection of 7S and human CYP1A1 and NMO1 RNAs, and the conditions used for hybridization have been described in detail (Reiners et al., 1997). A 1.5-kilobase probe for human CYP1A2 was obtained from Oxford Biomedical Research (Oxford, MI). The conditions used for hybridization to CYP1A2 mRNA were similar to those used for CYP1A1 mRNA with the exception of the washing conditions. CYP1A2 blots were washed three times for 15 min each with 0.1× standard saline citrate and 0.1% SDS at 55°C. ^{32}P was detected by exposure to X-ray film, or quantified using a BioRad GS-525 Molecular Imager. Northern blot data were normalized by calculating CYP1A1 mRNA signal strength to 7S signal strength ratios. These ratios were then used to calculate "fold of TCDD response".

Extract Preparation. Sprague-Dawley rats were euthanized with pentobarbital. Livers were removed after in situ perfusion with HEG (25 mM Hepes, pH 7.6, 1.5 mM EDTA, and 10% glycerol), and subsequently homogenized in HEDG (HEG plus 1 mM dithiothreitol) supplemented with protease inhibitors as described by Gasiewicz and Bauman (1987). The homogenate was sequentially centrifuged at 10,000*g* for 10 min and 100,000*g* for 1 h. The resulting supernatant fluid was stored at −80°C.

MCF10A cells released from culture plates by trypsin/EDTA treatment were diluted with culture medium containing 10% fetal bovine serum, pelleted, and washed once with PBS. After centrifugation, nuclear extracts were prepared from the pellets by a procedure described by Osborn et al. (1989). The resulting supernatant fluid was stored at −80°C.

Electrophoretic Mobility Shift Assay. The conditions reported by Shen et al. (1991) were used for in vitro AhR transformation and EMSA. Nuclear extracts prepared from MCF10A cultures, and in vitro transformed rat liver extracts were used in the EMSA. Complementary oligonucleotides 5′-GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′ and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCG-3′ (the single-core DRE recognition sequence is underlined) were annealed and used to detect activated AhR/ARNT complexes. In supershift studies, AhR/oligo complexes were incubated with 2 μg of normal goat IgG or an affinity purified goat polyclonal IgG made to the carboxy terminus of Arnt 1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

[^{3}H]Leucine Incorporation. Cells grown in 35-mm culture dishes were washed twice with PBS and then refed with fresh medium containing L-[3,4,5-^{3}H(N)]leucine (1 μCi/ml culture medium). Inhibitors of protein translation were added immediately thereafter. The cultures were returned to the incubator and harvested 1 h later for analyses of [^{3}H]leucine incorporation into protein. The procedure used for the processing of labeled cells has been described in detail (Schöller et al., 1994). Radioactivity was detected by scintillation counting.

Western Blot Analyses. The conditions used for the preparation of MCF10A and 1c1c7 extracts, separation of proteins on SDS-polyacrylamide gels, and transfer to nitrocellulose were similar to those previously described for the identification of protein kinase C isoforms by Western blot analyses (Guo et al., 2001). A rabbit polyclonal immunoaffinity purified IgG made to amino acid resides 1–402 of the murine AhR obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA) was used as the primary antibody. A goat polyclonal immunoaffinity purified IgG made to the carboxy terminus of human IκBα was obtained from Santa Cruz Biotechnology Inc. A murine monoclonal antibody made to a keyhole limpet hemocyanin-conjugated β-actin N-terminal peptide was obtained from Sigma-Aldrich. AhR, IκBα, and β-actin immunoglobulin complexes were detected with species appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescense. AhR and IκBα Western blots were stripped before probing with β-actin antibodies. Chemiluminescence was recorded on X-ray film.

DEVDase Assay. Cultures were washed twice with PBS before being flooded with lysis buffer (solution A: 10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium fluoride, 10 mM sodium P_i, and 10 mM sodium PP_i). Cells in culture medium and PBS washes were pooled, pelleted, washed with PBS, and then repelleted by centrifugation. After ~3 to 10 min of incubation on ice, culture dishes were scraped, and the lysate was added to the cell pellet derived from the culture medium. The lysate was then transferred to a small tube and stored at −80°C. On the day of assay, lysates were sonicated for 1 s and centrifuged at 13,000*g* for 10 min. The procedure for assay of DEVDase in supernatant fluids, using Ac-DEVD-AMC as substrate, has been described in detail (Reiners and Clift, 1999). The only deviation from the published protocol was that assay mixtures were scaled for 96-well plates. Changes in fluorescence over time were converted into picomoles of product by comparison to a standard curve made with AMC. DEVDase-specific activities are reported as nanomoles of product per minute per milligram of protein. The bicinchoninic acid (BCA) assay, using BSA as a standard, was used to estimate protein concentrations.

Results

Suppression of [^{3}H]Leucine Incorporation by Inhibitors of Protein Translation. Cotreatment of MCF10A cultures with CHX and [^{3}H]leucine caused a dose-dependent suppression of [^{3}H]leucine incorporation into acid insoluble material (Fig. 1A). Maximum suppression occurred with doses of CHX ≥1 μg/ml. Little suppression was seen with doses ≤0.01 μg/ml. Cotreatment with the protein translation inhibitor ANS also suppressed [^{3}H]leucine incorporation in a concentration-dependent fashion over a 2 log range (Fig. 1B). Near maximal suppression was achieved with 1 μM ANS. The protein synthesis inhibitor PUR began to suppress [^{3}H]leucine incorporation at about 1 μg/ml (Fig. 1C). A dose of 20 μg/ml suppressed [^{3}H]leucine incorporation by ~95% (Fig. 1C).

It should be noted that CHX and PUR treatments are commonly reported in the literature as micrograms per milliliter, whereas ANS treatments are reported as nanomolar or micromolar. We have adhered to this practice in our studies. As a point of reference, 1 μg/ml CHX equals 3.55 μM, and 1 μg/ml puromycin equals 1.83 μM.

Cytotoxicity of Protein Translation Inhibitors. Visual monitoring of cultures by light microscopy, over a 20-h time period, indicated that some doses/concentrations of the three inhibitors induced profound morphological changes and/or were overtly cytotoxic. Indeed, inhibitors of protein translation have been used to intentionally induce apoptosis

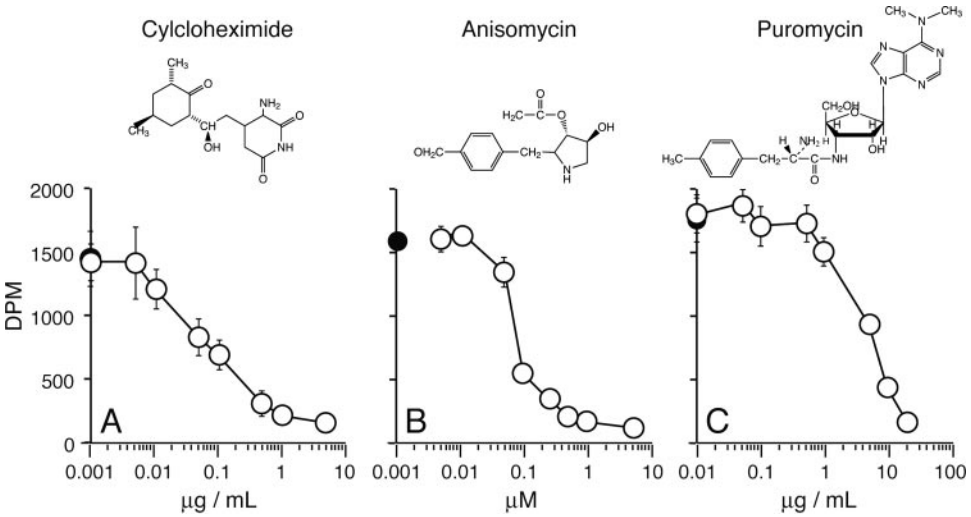

Fig. 1. Inhibition of protein synthesis in MCF10A cultures by inhibitors of protein translation. Cells grown in 35-mm culture dishes were washed twice with PBS and refed with fresh medium containing [^{3}H]leucine. Immediately thereafter, cultures were treated with various concentrations/doses of CHX (A), ANS (B), or PUR (C). Cultures were harvested ~60 min later. Data represent means ± S.D. of four plates per treatment group. ●, cultures treated with solvent; ○, translation inhibitors. Similar results were obtained in a second experiment.

in a variety of cell types. To monitor apoptosis, we assayed the ability of cell lysates to cleave the caspase-3/7 substrate Ac-DEVD-AMC (Fig. 2). DEVDase-specific activities were compared with those obtained with lysates prepared from cultures treated with the pro-apoptotic, nonpeptidic Bcl-2 pocket binding chemical HA14-1 (Wang et al., 2000). Microscopy suggested that virtually every cell in MCF10A cultures was undergoing apoptosis within 6 h of treatment with 25 μM HA14-1 (data not presented). The appearance of apoptotic cells in HA14-1-treated cultures was accompanied by dramatic increases in DEVDase-specific activities that peaked within 5 to 8 h (Fig. 2A).

CHX treatment did not markedly elevate DEVDase activities across the dose range of 0.05 to 5 μg/ml (Fig. 2A). The low level of DEVDase activation occurring in CHX-treated cultures was not accompanied by the appearance of cells having an apoptotic morphology; however, cell counting and light microscopy demonstrated that CHX became increasingly cytostatic as the dose exceeded 0.05 μg/ml (J. J. Reiners, Jr., unpublished data). Furthermore, whereas 1 μg/ml CHX exhibited weak cytotoxicity after 24 h of treatment ($\sim$85% of the cells were trypan blue impermeable versus 94% in the solvent control), 5 μg/ml was very cytotoxic (>50% of cells were trypan blue permeable).

Anisomycin was cytostatic to MCF10A cultures at $\geq$0.5 μM and increasingly cytotoxic at concentrations $\geq$1 μM. ANS cytotoxicity was associated with the accumulation of morphologically apoptotic cells and significant increases in DEVDase-specific activities within 6 h of treatment (Fig. 2B). Virtually every cell was dead within 20 h of treatment with 10 μM anisomycin.

Puromycin was cytostatic to MCF10A cultures at $\geq$0.5 μg/ml. Light microscopy and DEVDase measurements demonstrated that doses $\geq$5 μg/ml were pro-apoptotic. DEVDase activities were markedly elevated within 6 h of treatment with $\geq$5 μg/ml PUR and continued to increase with passing time until they approximated the values obtained with 25 μM HA14-1 (compare Fig. 2, A and C). Although 1 μg/ml PUR did not markedly elevate DEVDase activities (Fig. 2C), light microscopy and trypan blue staining suggested that at least 50% of the culture was nonviable within 20 h of treatment (J. J. Reiners, Jr., unpublished data).

Induction and Superinduction of *CYP1A1* by Inhibitors of Protein Translation. In our initial studies, we attempted to analyze the kinetics of *CYP1A1* induction and superinduction in cultures treated with concentrations/doses of CHX, ANS, and PUR that were not apoptotic but sufficient to suppress protein translation. Neither 1 μg/ml CHX nor 0.5 μM ANS were pro-apoptotic but suppressed [^{3}H]leucine incorporation by $\geq$90%. However, all doses of PUR capable of suppressing [^{3}H]leucine incorporation >10% were pro-apoptotic. Unable to avoid this complication, we chose a dose of PUR (5 μg/ml) capable of suppressing [^{3}H]leucine by at least 50%. All three protein translation inhibitors, at the examined doses, caused accumulations of CYP1A1 mRNA comparable with, if not greater than, that obtained with TCDD over the monitored 8-h period (Fig. 3A). Cotreatment of TCDD-exposed cultures with each of the inhibitors of protein translation also caused the superinduction of *CYP1A1* (Fig. 3A). Superinduction was obvious within 2 h of cotreatment and was maintained for several additional hours.

It should be noted that the weak TCDD-induced CYP1A1 mRNA signals observed in Fig. 3 and subsequent figures reflect shortened film exposure times. In general, mRNAs prepared from TCDD-treated MCF10A cultures yield strong CYP1A1 signals by Northern analyses when exposed to X-ray film for 18 to 24 h (Reiners et al., 1997; Guo et al., 2001). In superinduction protocols, film exposures were reduced to 2 to 8 h to maintain an appropriate perspective of the magnitude of the superinduction.

The suspension culturing of normally adherent cells often leads to accumulations of CYP1A1 mRNAs via an AhR-dependent process (Sadek and Allen-Hoffmann, 1994a,b; Monk et al., 2001). Although no dose of CHX used in the current study caused the release of MCF10A cells into the culture medium, doses $\geq$1 μg/ml caused a rapid but transient retraction/slight rounding of the cells. At issue is whether these phenotypic changes, which are indicative of altered cell-cell and cell-extracellular matrix interactions, are paralleled by the activation of processes normally initiated by suspension

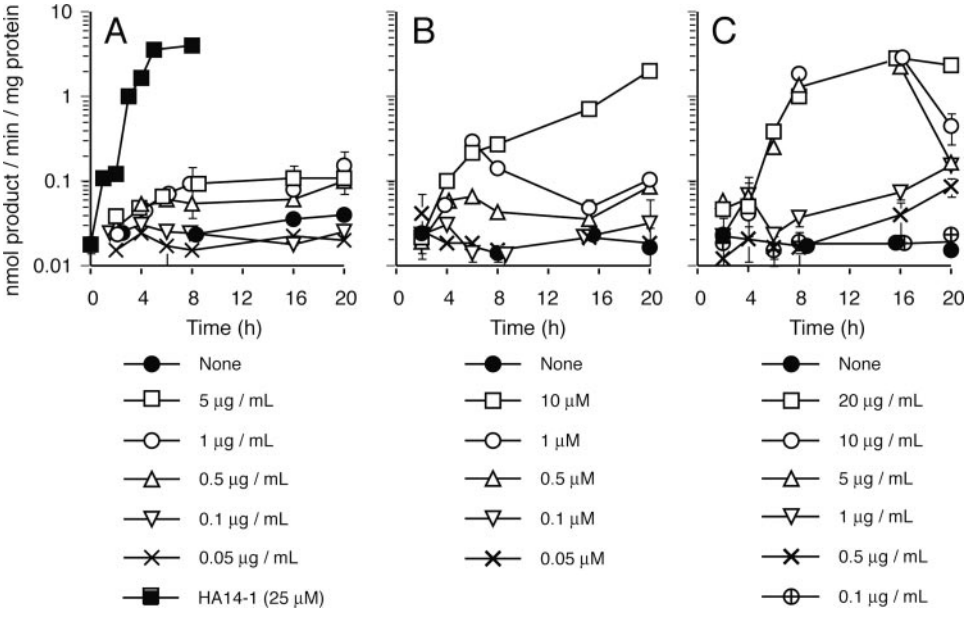

Fig. 2. Activation of DEVDase in MCF10A cultures by inhibitors of protein translation. Cultures were treated with nothing, HA14-1 or various doses/concentrations of CHX (A), ANS (B), or PUR (C) before being harvested for analyses of DEVDase activities. Treatments are described in the figure. Data represent means $\pm$ S.D. of triplicate determinations.

culturing that affect *CYP1A1* induction. Northern blot analyses demonstrated transient accumulations of CYP1A1 mRNAs following the suspension culturing of MCF10A cells. Accumulations were detectable within 2 h of suspension culturing, but undetectable 6 h later (A. Joiakim and J. J. Reiners, Jr., data not presented). The amount of CYP1A1 mRNA accumulated 2 to 4 h after suspension culturing, in either the absence or presence of TCDD, was markedly less than the amount detected following treatment of adherent cultures with only TCDD or CHX (Fig. 3B). However, a dramatic synergistic accumulation of CYP1A1 mRNA occurred when suspension cultures were cotreated with CHX (Fig. 3B). Hence, processes induced by suspension culturing can transcriptionally activate *CYP1A1* but do not duplicate the superinducing activities of CHX.

In Fig. 3, cultures were exposed to a fixed dose/concentration of protein translation inhibitor and harvested for analyses at various times post-treatment. In subsequent studies MCF10A cultures were treated with TCDD and/or various doses/concentrations of protein translation inhibitors and harvested 3/3.5 (Fig. 4) or 6/6.5 h (Fig. 5) after treatment. In Figs. 4 and 5, panel A depicts representative Northern blot data obtained in a single experiment. Panels B, C, and D represent composites derived from data generated in several experiments in which ratios of CYP1A1 mRNA and 7S RNA contents were normalized to the values determined for TCDD-treated cultures. All three inhibitors, by themselves, caused time and concentration/dose-dependent accumulations of CYP1A1 mRNA. At the highest concentrations/doses examined, at 3.5 and 6.5 h after treatment, all three inhibitors caused CYP1A1 mRNA accumulations 2- to 3-fold and 10- to 20-fold greater, respectively, than the contents measured in TCDD-treated cultures.

Dose/concentration analyses also demonstrated the *CYP1A1* superinducing activities of the three inhibitors of protein synthesis (Figs. 4 and 5). Within 3 and 6 h of TCDD addition at the highest concentrations/doses of inhibitors tested, CYP1A1 mRNA contents in cotreated cultures exceeded the CYP1A1 mRNA contents of TCDD-treated cultures by ~8- to 25-fold (Fig. 4, B–D), and 50- to 70-fold (Fig. 5, B–D), respectively. Both the 3 and 6 h data suggest that doses of puromycin ≥5 μg/ml were sufficient for maximum superinduction of *CYP1A1* in cotreatment protocols (Figs. 4D and 5D).

Induction and Superinduction of *CYP1A2* and *NMO1* by CHX. Both *CYP1A2* and *NMO1* contain DRE(s) in their promoters and are transcriptionally activated by TCDD via an AhR-dependent process. Basal levels of CYP1A2 mRNAs were not detected in MCF10A cultures by Northern blot

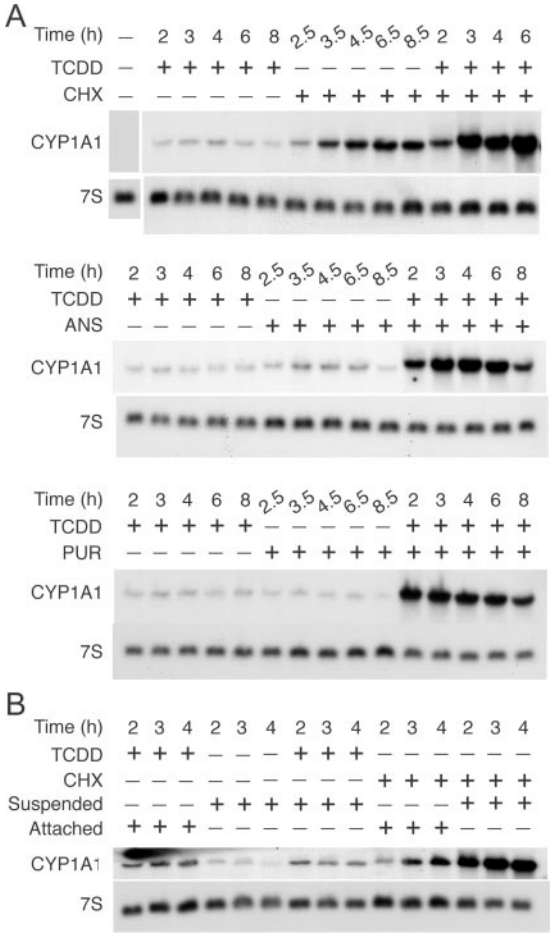

Fig. 3. Time-dependent induction/superinduction of *CYP1A1* by TCDD, suspension culturing, and inhibitors of protein translation. A, adherent MCF10A cultures were treated with dimethyl sulfoxide, 10 nM TCDD, 1 μg/ml CHX, 0.5 μM ANS, or 5 μg/ml PUR for various times before being harvested for subsequent analyses of CYP1A1 and 7S RNAs. The no treatment CYP1A1 signal depicted in the CHX panel is representative of Northern analyses of over 50 RNA preparations isolated from control MCF10A cultures and reflects results obtained after 2 days of film exposure. All of the other CYP1A1 blots reflect 2 to 8 h exposures. B, adherent and suspended MCF10A cultures were treated with nothing, 10 nM TCDD, and/or 1 μg/ml CHX for various times before being harvested. Suspension cultures were treated with agents within 5 min of the transfer of cells to polyHEME-coated dishes. In all cotreatment protocols, the inhibitors were added 30 min before TCDD, and harvest times are relative to TCDD addition.

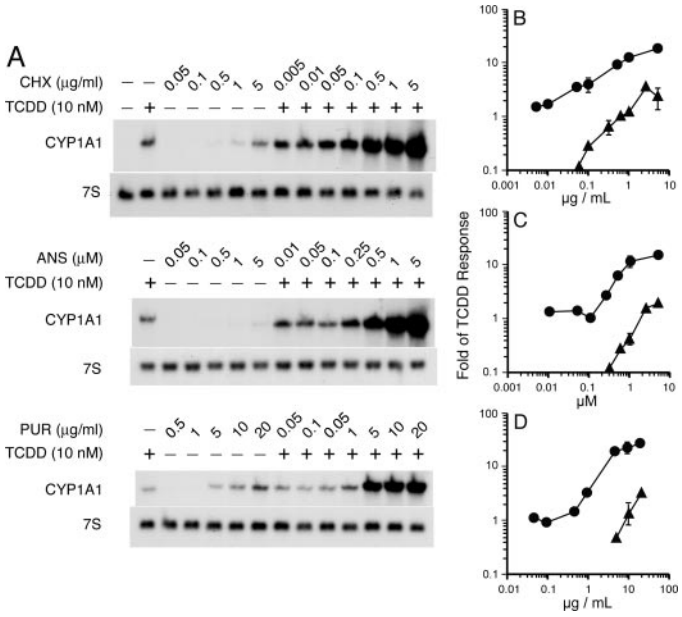

Fig. 4. Accumulation of CYP1A1 mRNA 3 h after exposure to TCDD and inhibitors of protein translation. A, cultures of MCF10A cells were treated with nothing, 10 nM TCDD, and/or various doses/concentrations of CHX, ANS, and PUR. The inhibitors were added 30 min before TCDD in cotreatment protocols. Cultures were harvested for the isolation of RNAs 3 h after the addition of TCDD and 3.5 h after treatment with only the protein translation inhibitors. Side panels are composite graphs of the effects of CHX (B), ANS (C), and PUR (D) derived from data in Figs. 3, 4A, and three additional experiments. Data represent means ± S.D. ▲, translation inhibitor alone; ●, translation inhibitor + TCDD.

analyses (Fig. 6A). If films were exposed for at least 4 days, a very weak CYP1A2 signal could be detected in TCDD-treated cultures (Fig. 6A). In contrast, CYP1A2 mRNAs were easily detected in CHX-treated cultures. Cotreatment with TCDD and CHX resulted in the synergistic accumulation of CYP1A2 mRNAs (Fig. 6A). The kinetics of CYP1A1 versus CYP1A2 mRNA accumulations following CHX and CHX + TCDD treatments were very similar (compare Figs. 3A and 6A).

We previously reported that *NMO1* is constitutively expressed in MCF10A-Neo cells, a derivative of the MCF10A line and that the induction of *NMO1* by TCDD is generally slower than the induction of *CYP1A1* (Reiners et al., 1997). Basal NMO1 mRNA contents were easily detected in MCF10A cultures (Fig. 6B). TCDD or CHX treatment resulted in NMO1 mRNA accumulations ~2- and 3-fold above basal levels, respectively (Fig. 6B). CHX and TCDD cotreatment resulted in NMO1 mRNA accumulations ~7-fold above basal levels (Fig. 6B).

In Vivo and in Vitro AhR Transformation and DNA Binding. The EMSA patterns observed following incubation of MCF10A nuclear extracts with a labeled DRE oligonucleotide were complex. In general, extracts from nontreated cells yielded two bands, one of which could be competed with a 50-fold excess of cold DRE oligo. Singular or combinational treatment of cells with TCDD and/or CHX resulted in a third slower migrating EMSA band. This band could be eliminated by coincubation of EMSA mixtures with cold DRE oligo, with antibodies made to the C-terminal region of ARNT (Fig. 7A), with the N-terminal region of the AhR (A. Joiakim, data not presented). Although coincubation with ARNT antibodies eliminated the third band, this loss was not quantitatively paralleled by the appearance of a supershifted product (Fig. 7A). In contrast, coincubation of lysates with control IgG did not disrupt TCDD-induced AhR/DNA complex formation (Fig. 7A, two additional experiments, one of which used a different control IgG).

The accumulation of CYP1A1 mRNAs in MCF10A cultures following TCDD treatment was paralleled by an enhanced ability of nuclear extracts to form AhR/DNA complexes as assessed by EMSA (Fig. 7B). Complexes were detectable within 1 to 3 h of treatment; however, there appeared to be some loss with the progression of time. Specifically, nuclear lysates isolated 3 h after TCDD exposure formed 47 ± 6% (mean ± S.D. of four experiments) of the AhR/DNA complexes formed after 1 h of TCDD treatment. Nuclear extracts isolated from cultures treated with 1 μg/ml CHX also formed AhR/DNA complexes during the same time period. The amount of complex formed after 1 h of CHX exposure was 58 ± 4% of the amount formed with lysates from TCDD-treated cultures (Fig. 7B, two additional experiments); however, the capacity of CHX-derived nuclear extracts to form AhR/DNA complexes decreased by ≤10% in the following 2 h. Cotreatment with TCDD and CHX synergistically increased the yield of AhR/DRE complexes (Fig. 7B). Specifically, nuclear lysates prepared 1 h after cotreatment generated 342 ± 55% (mean ± S.D., $n = 4$ experiments) of the AhR/DNA complex obtained with lysates from cultures treated with only TCDD for 1 h. Furthermore, the capacity to form complexes remained fairly stable over a 3-h time period (94 ± 5% of the 1 h response after 3 h of cotreatment, $n = 3$ experiments).

Although in vivo CHX treatment induced AhR/DNA complex formation, it was unable to promote complex formation when added directly to rat liver extract (Fig. 7C). Specifically,

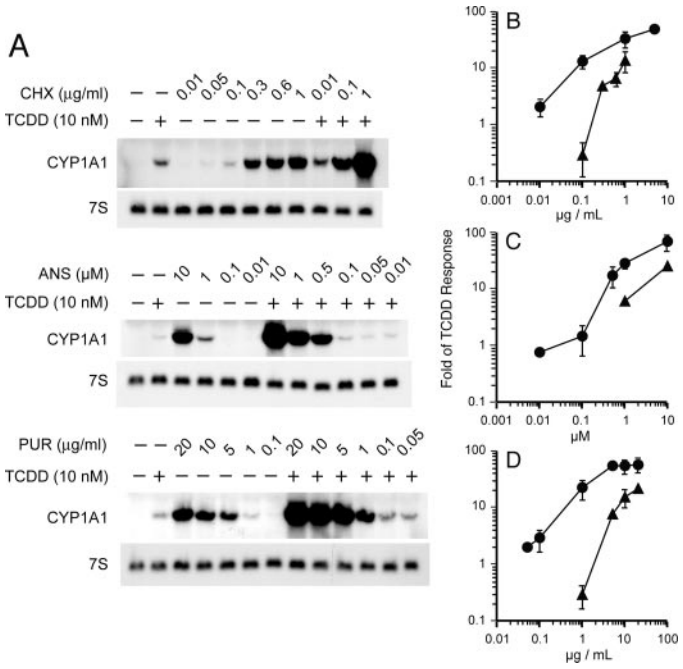

Fig. 5. Accumulation of CYP1A1 mRNA 6 h after exposure to TCDD and inhibitors of protein translation. A, cultures of MCF10A cells were treated with nothing, 10 nM TCDD, and/or various doses/concentrations of CHX, ANS, and PUR. The inhibitors were added 30 min before TCDD in cotreatment protocols. Cultures were harvested for the isolation of RNAs 6 h after the addition of TCDD and 6.5 h after treatment with only the protein translation inhibitors. Side panels are composite graphs of the effects of CHX (B), ANS (C), and PUR (D) derived from data in Figs. 3, 5A, and four additional experiments. Data represent means ± S.D. ▲, translation inhibitor alone; ●, translation inhibitor + TCDD.

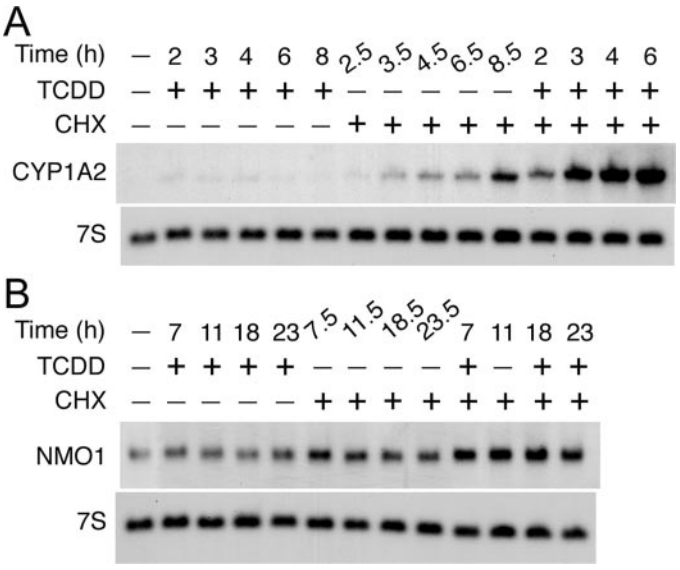

Fig. 6. Time-dependent induction/superinduction of *CYP1A2* and *NMO1* by TCDD and CHX. Adherent MCF10A cultures were treated with 10 nM TCDD and/or CHX for various times before being harvested for subsequent analyses of CYP1A2 (A) or NMO1 (B) mRNAs. Doses of CHX used in A and B were 1 μg/ml and 0.5 μg/ml, respectively. In cotreatment protocols, CHX was added 30 min before TCDD, and harvest times are relative to TCDD addition.

no complex was detected by EMSA when extracts were incubated with 0.5 to 5 μg/ml CHX. Furthermore, TCDD-mediated complex formation was not potentiated by the inclusion of CHX (Fig. 7C). Studies similar to those reported in Fig. 7C were also performed with ANS and PUR. In no case did protein translation inhibitors promote in vitro AhR/DNA complex formation as assessed by EMSA (A. Joiakim, unpublished data).

Although the studies reported in Fig. 7C argue that CHX is not an AhR agonist, we deemed it possible that an endogenous ligand might be generated in vivo as a consequence of CHX exposure. To test this possibility, MCF10A cultures were treated with CHX for 1 or 2 h before the preparation of cytosolic extracts. Extracts were subsequently mixed with rat liver extract and then analyzed by EMSA. These experiments did not yield data supporting the generation of an endogenous AhR ligand in CHX-treated MCF10A cultures (A. Joiakim, unpublished data). Variations of the protocol were also examined using shorter and longer incubation periods but provided no evidence supporting the generation of an endogenous ligand.

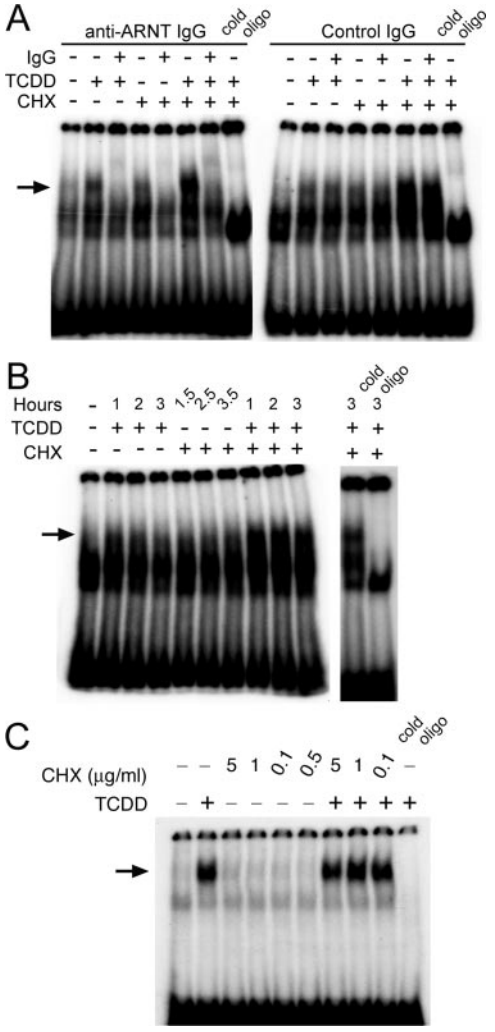

Fig. 7. Cycloheximide-induced AhR/DNA complex formation. A, MCF10A cultures were treated with 10 nM TCDD for 1 h, 1 μg/ml CHX for 90 min, or TCDD + CHX before the preparation of nuclear extracts, which were incubated with labeled DRE oligonucleotide and analyzed by EMSA. In some cases, oligo/AhR complexes were incubated with either control IgG or anti-ARNT IgG before electrophoresis. Similar results were obtained in three additional experiments. B, MCF10A cultures were treated with 10 nM TCDD, 1 μg/ml CHX, or a combination of the two agents for 1 to 3.5 h before the preparation of nuclear extracts and analyses by EMSA. Similar data were obtained in two additional experiments using different nuclear extracts. C, rat liver extracts were incubated with various doses of CHX in the absence or presence of 5 nM TCDD before being incubated with labeled DRE oligonucleotide and analyzed by EMSA. In all cases, the arrow represents TCDD and/or CHX-induced AhR/oligonucleotide complexes.

AhR Antagonist Suppression of *CYP1A1* Induction/Superinduction by CHX. AhR antagonists are often used to corroborate the role of the AhR in ligand-independent and -dependent induction of *CYP1A1*. The flavonoid αNF can function as either an AhR antagonist or agonist, depending upon the concentration (Wilhelmsson et al., 1994). No detectable accumulation of CYP1A1 mRNA occurred in MCF10A cultures treated solely with 1 μM αNF (Fig. 8A); however, cotreatment with 1 μM αNF suppressed the induction of *CYP1A1* mediated by either TCDD or CHX. *CYP1A1* superinduction was also effectively suppressed by cotreatment with αNF (Fig. 8A).

In contrast to αNF, PD168641 has been reported to function primarily as an AhR antagonist (Henry et al., 1999). Cotreatment of MCF10A cultures with 1 μM PD168641 completely suppressed the induction of *CYP1A1* by TCDD or CHX and the superinduction of *CYP1A1* by TCDD + CHX cotreatment (Fig. 8B).

AhR Turnover in TCDD- and CHX-Treated MCF10A Cultures. The AhR undergoes proteolysis in a variety of cell types following activation by TCDD (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). In 1c1c7 cells, this proteolysis can be suppressed by cotreatment with CHX (Ma et al.,

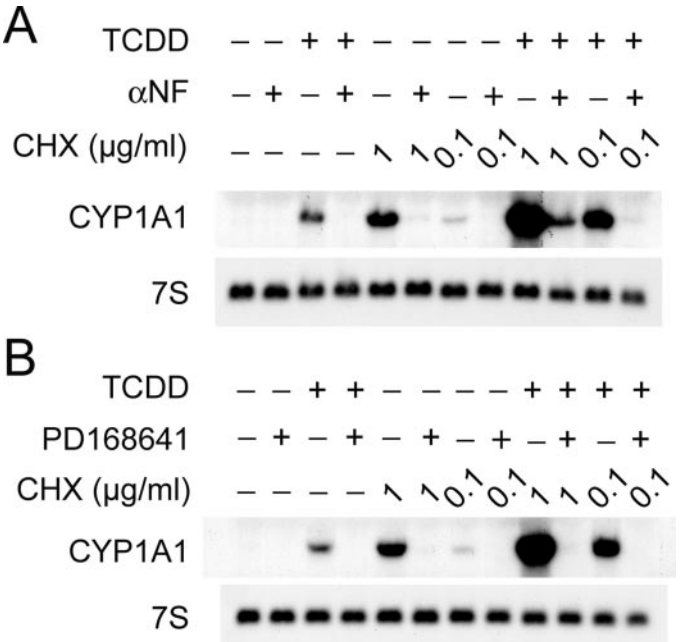

Fig. 8. Suppression of cycloheximide-mediated *CYP1A1* induction/superinduction by AhR antagonists. Cultures of MCF10A cells were treated with solvent, 10 nM TCDD, 1 μg/ml CHX, and 1 μM αNF (A), or 1 μM PD168641 (B), or combinations of the last four reagents. Cultures were treated with antagonists 5 and 15 min before the additions of CHX and TCDD, respectively. In CHX + TCDD cotreatment protocols, CHX was added 10 min before TCDD. Cultures were harvested for isolation of RNAs 6 h after the addition of TCDD (single and combined treatments), 6.25 h after only antagonist addition, and 6.16 h after the addition of only CHX.

2000). A time-dependent loss of the AhR occurred in MCF10A cultures following the addition of 10 nM TCDD (Fig. 9). This loss was obvious within 2 h of TCDD addition. Unexpectedly, AhR loss also occurred in CHX-treated cultures. The kinetics of AhR disappearance in CHX-treated cultures depended upon the CHX dose. Notable decreases in AhR content occurred within 4 and 6 h of treatment with 1 and 0.1 μg/ml CHX, respectively (Fig. 9). Cotreatment of MCF10A cultures with TCDD and $\geq$0.1 μg/ml CHX resulted in AhR losses greater than that observed following treatment with a single agent. This potentiation of AhR loss was obvious within 4 to 6 h of treatment.

Exposure of MCF10A cultures to a dose of ANS sufficient to inhibit [^{3}H]leucine incorporation by 90% and to induce *CYP1A1* also reduced AhR content and potentiated AhR loss when used in combination with TCDD (Fig. 9).

Effects of TCDD and CHX on AhR Content in 1c1c7 Cultures. Given the unanticipated finding that CHX cotreatment enhanced AhR loss in TCDD-treated MCF10A cultures, we thought it important to confirm the ability of CHX to suppress AhR turnover in 1c1c7 cultures. The protective effects of CHX in 1c1c7 cells reported by Ma et al. (2000) were achieved with 10 μg/ml CHX, a dose previously reported to be effective at suppressing protein translation in 1c1c7 cells (Lusska et al., 1992); however, the relative toxicity of this dose to 1c1c7 cells has not been reported.

A dose-response analysis of the effects of CHX on [^{3}H]leucine incorporation in 1c1c7 cells is presented in Fig. 10A. [^{3}H]Leucine incorporation was suppressed in a dose-dependent fashion. The curve is similar to that reported by Lusska et al. (1992). CHX was increasingly cytostatic as the dose increased above 0.1 μg/ml (J. J. Reiners, Jr., unpublished data). Doses $\geq$0.5 μg/ml provoked pronounced morphological changes and caused small increases in DEVDase-specific activities within 16 to 20 h of treatment (Fig. 10B). Although the changes in DEVDase activities were small compared with what occurred with the pro-apoptotic agent HA14-1, some apoptotic cells could be clearly seen by light microscopy within 20 h of treatment with 0.5 μg/ml. Within 20 h of treatment with 5 μg/ml CHX ~30% of the total culture population was trypan blue permeable (J. J. Reiners, Jr., unpublished data).

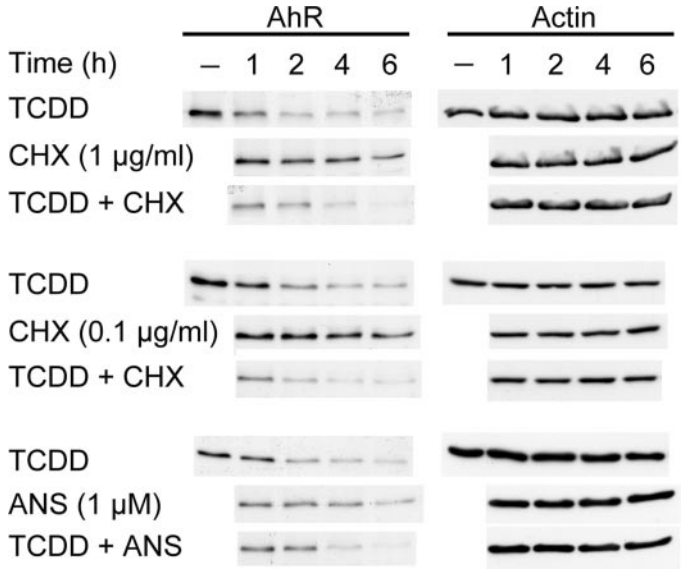

Fig. 9. Effects of cycloheximide and anisomycin on AhR content and TCDD-induced AhR loss. Cultures of MCF10A cells were treated with TCDD, CHX, ANS, or a combination of TCDD plus protein translation inhibitor. In cotreatment protocols, CHX or ANS were added 15 min before TCDD. Cultures were harvested 1, 2, 4, and 6 h after the addition of TCDD (single and cotreatment protocols), or 1.25, 2.25, 4.25, and 6.25 h after addition of just CHX or ANS. Western blot analyses of AhR and β-actin used 25 μg of whole cell extract per lane. Data are representative of a minimum of two additional independent experiments, one of which used 30 min of pretreatment with protein translation inhibitors.

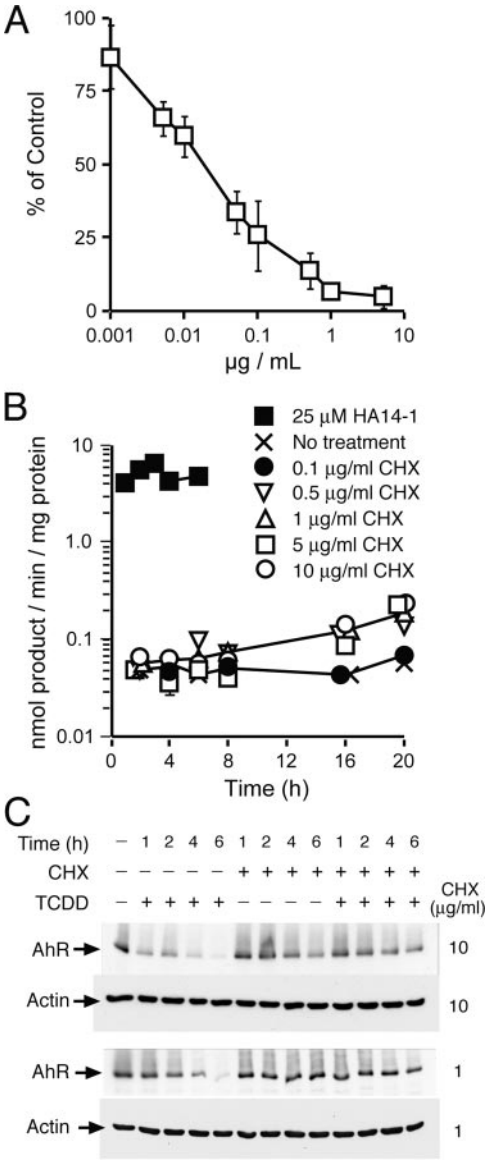

Fig. 10. Cycloheximide-mediated cytotoxicity, suppression of protein synthesis, and TCDD-dependent AhR turnover in 1c1c7 cells. A, cultures were coincubated with [^{3}H]leucine and various doses of CHX for 1 h before being processed for analyses of [^{3}H]leucine incorporation into protein. Data represent means $\pm$ S.D. of results obtained in a minimum of three independent experiments and are normalized to the values measured in cultures treated with solvent. B, cultures were incubated for various lengths of time with HA14-1 or various doses of CHX before harvest for analyses of DEVDase activities. Data represent means $\pm$ S.D. of triplicate analyses. Treatments are described in B. C, cultures of 1c1c7 cells were treated with TCDD (2 nM), CHX (1 or 10 μg/ml), or a combination of TCDD and CHX. In cotreatment protocols, the CHX was added 15 min before TCDD. Harvest times are relative to TCDD addition (single and cotreatment protocols). Western blot analyses of AhR and β-actin used 25 μg of whole cell extract per lane. Data are representative of two independent experiments.

Exposure of 1c1c7 cultures to 2 nM TCDD caused a rapid and pronounced loss of the AhR (Fig. 10C); however, unlike the results obtained with MCF10A cells, neither 1 nor 10 μg/ml CHX alone caused significant loss of the AhR. Furthermore, cotreatment with either 1 or 10 μg/ml CHX effectively suppressed AhR turnover in TCDD-treated 1c1c7 cultures (Fig. 10C).

MG132 Modulation of TCDD- and CHX-Mediated AhR Turnover. AhR turnover in TCDD-treated 1c1c7 cultures can be suppressed by cotreatment with the proteasome inhibitor MG132 (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). At issue is whether the proteasome is also responsible for mediating AhR loss in CHX-treated MCF10A cultures. To address this question, we first determined the concentrations of MG132 needed to inhibit TNFα-induced IκBα degradation, a well characterized proteasome-dependent process (Rodriguez et al., 1999). IκBα contents in MCF10A cells were dramatically reduced within 30 min of TNFα exposure but returned to pretreatment levels within 1 h of treatment due to resynthesis (Fig. 11A). Pretreatment of TNFα-exposed cultures with either 1 or 5 μM MG132 suppressed the disappearance of IκBα and led to comparable accumulations of phosphorylated IkBα, which in the absence of proteasome inhibitors is normally degraded by the proteasome (Fig. 11A). Exposure of MCF10A cultures to just 1 or 5 μM MG132 had no effect on IκBα content (Fig. 11A).

Treatment of MCF10A cultures with 5 μM MG132 before the addition of either TCDD or CHX effectively suppressed the disappearance of the AhR (Fig. 11B). Hence, AhR loss initiated by a dose of CHX sufficient to activate the receptor is mediated by the proteasome. Pretreatment with 1 to 10 μM MG132 also suppressed AhR disappearance induced by CHX plus TCDD cotreatment (Fig. 11C).

AhR Turnover in Leptomycin B-Treated Cultures. Previous studies with 1c1c7 cells have demonstrated that inhibition of the CRM1 nuclear export pathway with LMB suppresses TCDD-induced AhR turnover by trapping the receptor in the nucleus (Davarinos and Pollenz, 1999). To determine whether CHX- and TCDD-mediated AhR turnover in MCF10A cultures occurred exclusively in the cytosol, we first titered the concentration of LMB necessary to suppress TNFα-induced IκBα degradation. IκBα normally shuttles back and forth between the cytosol and nucleus. Its activation and subsequent degradation in the cytoplasm by the proteasome can be blocked by prior treatment with LMB, which traps it in the nucleus (Rodriguez et al., 1999). Pretreatment of MCF10A cultures with LMB for 2.5 h before TNFα addition suppressed IκBα degradation in a concentration-dependent fashion (Fig. 12A). Effective protection could be achieved with 25 nM LMB.

Pretreatment of MCF10A cultures with 25 nM LMB did not prevent AhR loss following exposure to CHX (Fig. 12B). Similarly, pretreatment with LMB offered little protection in TCDD-treated cultures (Fig. 12C). TCDD plus CHX cotreatment potentiated the loss of the AhR above what occurred in single treatment protocols (Fig. 12, B and C). LMB pretreatment of cultures cotreated with CHX and TCDD had little effect on AhR loss. In one experiment, a small protection was noted (Fig. 12B) whereas in two other experiments no protection was afforded by pretreatment with LMB (Fig. 12, C and D).

The LMB data reported in Fig. 12, B and C, suggest that AhR degradation can occur in the nucleus of MCF10A cells. This degradation of nuclear AhR could be suppressed by cotreatment with MG132 (Fig. 12D); however, no protection occurred in cultures cotreated with the cysteine protease inhibitor E64d, the aspartate protease inhibitor pepstatin A, or the calpain inhibitor *N*-acetyl-Leu-Leu-aldehyde (J. J. Reiners, Jr., unpublished data).

Discussion

The concept that *CYP1A1* superinduction by CHX is linked to suppressed protein synthesis is based primarily upon the observations that doses of CHX and PUR sufficient to suppress translation can superinduce *CYP1A1*. However, with the exception of a study by Lusska et al. (1992) using CHX, most studies used a single concentration of CHX or PUR capable of suppressing translation by ≥90%. This is the first study that we know of to use extensive dose-response studies to quantitatively relate *CYP1A1* superinduction with sup-

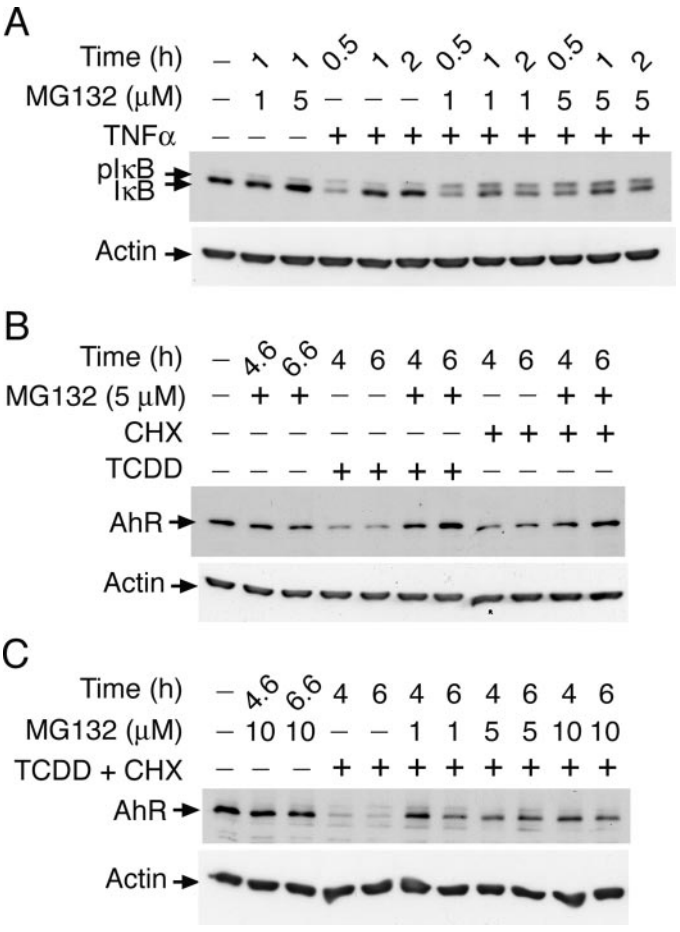

Fig. 11. Suppression of IκBα and AhR turnover by MG132. A, MCF10A cultures were treated with 2 pg/ml of TNF$\alpha\pm$ 1 or 5 μM MG132. In cotreatment protocols, MG132 was added 30 min before TNFα. Cultures treated with TNFα or TNFα + MG132 were harvested 0.5, 1, and 2 h after TNFα addition for analyses of IκBα by Western blot analyses. B, MCF10A cultures were treated with 1 μg/ml CHX or 10 nM TCDD for 4 or 6 h before being harvested for analyses of AhR contents. In cotreatment protocols, MG132 was added 35 min before CHX or TCDD. C, MCF10A cultures were treated with 1, 5, or 10 μM MG132 before the addition of 10 nM TCDD and 1 μg/ml CHX. MG132 was added 35 min before CHX, and CHX was added 5 min before TCDD. Cultures were harvested either 4 or 6 h after TCDD addition. Western blot analyses of AhR, IκB, and β-actin used 25 μg of whole cell extract per lane.

pression of protein translation by CHX, ANS, and PUR. Figure 13 represents a composite made from the data presented in Figs. 1 and 5 and depicts [^{3}H]leucine incorporation and *CYP1A1* induction/superinduction across a range of inhibitor doses/concentrations. Intercomparisons of the inhibitors indicate that comparable levels of [^{3}H]leucine suppression do not equate with comparable superinduced CYP1A1 mRNA contents. For example, CYP1A1 mRNA contents in cultures treated with concentrations of CHX, ANS, and PUR sufficient to suppress [^{3}H]leucine incorporation by 50% were ~12-, 1-, and 60-fold the TCDD control content, respectively. Analyses of dose-response data obtained with individual inhibitors also emphasize the absence of a quantitative relationship between *CYP1A1* superinduction and effects on translation. Specifically, CYP1A1 mRNA contents continued to increase markedly over a range of ANS concentrations (i.e., 0.5 to 5 μM) that all maximally suppressed translation (Fig. 13B). In contrast, maximum *CYP1A1* superinduction occurred with a dose of PUR sufficient to inhibit [^{3}H]leucine incorporation by only 55% (Fig. 13C). These data suggest that the superinduction of *CYP1A1* by translation inhibitors is not quantitatively related to the extent by which they suppress protein synthesis.

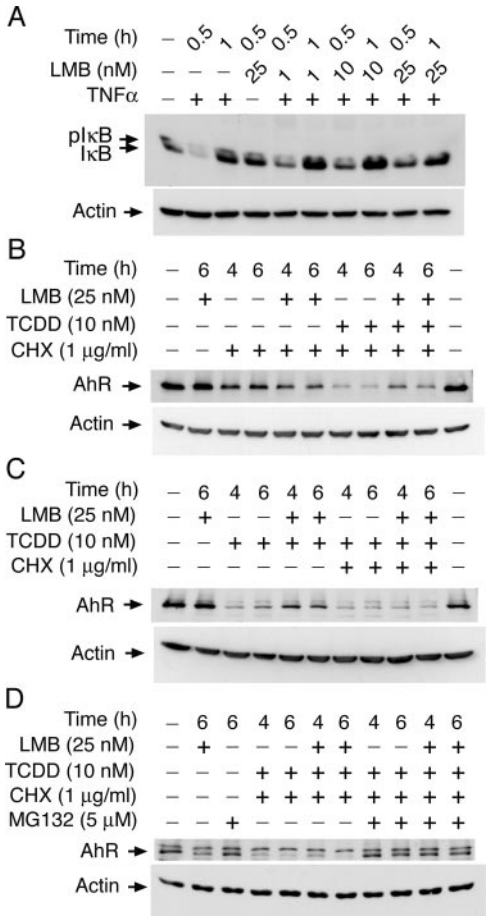

Fig. 12. Suppression of IκBα and AhR turnover by leptomycin B. A, MCF10A cultures were treated with various amounts of LMB or 2 pg/ml TNFα± LMB. In cotreatment protocols, LMB was added 90 min before TNFα and cultures were harvested 0.5 or 1 h after TNFα addition for analyses of IκBα by Western blot. B and C, MCF10A cultures were treated with CHX, LMB, TCDD, or combinations of the three agents. In cotreatment protocols, LMB and CHX were added 2.33 h and 0.75 h, respectively, before TCDD. Cultures were harvested 4 or 6 h after TCDD addition for analyses of AhR contents. D, MCF10A cultures were treated singularly or with combinations of LMB, TCDD, CHX, or MG132. In cotreatment protocols, LMB, MG132 and CHX were added 3, 1, and 0.5 h, respectively, before TCDD addition. Cultures were harvested 4 or 6 h after TCDD addition for analyses of AhR contents. All harvest times are relative to TNFα addition (A) or TCDD addition (B–D). Hence, the indicated harvest times for groups not including TNFα or TCDD underestimate real exposure times by the lengths of pretreatment. Western blot analyses of AhR, IκB, and β-actin used 25 μg of whole cell extract per lane.

The data summarized in Fig. 13 also demonstrate that CHX, ANS, and PUR induce *CYP1A1* in MCF10A cultures in the absence of any exogenous AhR ligand. We previously documented the existence of nuclear AhR in nontreated MCF10A cultures, which was presumably inactive (Reiners et al., 1997). *CYP1A1* induction in the current study only occurred with concentrations of inhibitors sufficient to suppress translation. It is unlikely that this induction reflects the suppressed synthesis of a labile protein capable of repressing *CYP1A1* transcription. Both inter- and intracomparisons of the dose-response curves obtained with the three inhibitors indicate the absence of a quantitative relationship between *CYP1A1* induction and effects on translation. Instead, induction of *CYP1A1* by the three translation inhibitors correlates more closely with their cytotoxicities. (compare Figs. 2 and 13, information in text).

The induction/superinduction of *CYP1A1* in MCF10A cultures by CHX appears to involve the AhR. First, induction/superinduction of *CYP1A1* by CHX could be blocked by the AhR antagonists αNF and PD168641 (Fig. 8). Second, two other members of the *Ah* battery (i.e., *CYP1A2* and *NMO1*) were also induced by CHX and superinduced by combined TCDD + CHX treatments. Third, CHX treatment resulted in AhR activation. Nuclear extracts isolated from CHX-treated cultures formed AhR/DNA complexes when assayed by EMSA with an oligonucleotide containing a DRE sequence (Fig. 7, A and B). It is unclear as to how protein translation inhibitors activate the AhR. None of the inhibitors have structural features characteristic of AhR ligands. They are not flat, planar, or particularly aromatic (Fig. 1). Furthermore, none of the inhibitors could transform the AhR in rat liver extract into a species capable of binding to DNA as analyzed by EMSA. Hence, it seems unlikely that the translation inhibitors are themselves AhR ligands. We were also unable to detect the presence of AhR agonists in the cytosolic fraction of CHX-treated cultures. Although our data suggest that endogenous AhR ligands are not generated as a consequence of CHX treatment, we cannot unequivocally eliminate this possibility.

So how do protein translation inhibitors activate the AhR? An unappreciated property of protein translation inhibitors is their ability to activate numerous kinases (i.e., JNK, p38, mTOR and p70S6 kinase; Barros et al., 1997; Sidhu and Omiecinski, 1998; Khaleghpour et al., 1999; Sah et al., 2003). Preliminary studies indicate that doses of CHX resulting in *CYP1A1* induction/superinduction rapidly activate p38, JNK1, and JNK2 in MCF10A cells (J. J. Reiners, Jr., unpublished data). These latter kinases may be relevant to the mechanism of *CYP1A1* induction by translation inhibitors. Specifically, ligand-independent induction of members of the *Ah* gene battery following suspension culturing or treatment of fibroblasts with MG132 must entail the activation of signaling pathways capable of AhR activation (Sadek and Allen-Hoffmann, 1994a,b; Santiago-Josefat et al., 2001). Both MG132 and the suspension culturing of

adherent cell types induce a stress response and JNK and p38 activation (Khwaja and Downward, 1997; Luss et al., 2002; Wu et al., 2002). JNK and/or p38 activation may also be germane to the mechanism by which protein translation inhibitors superinduce *CYP1A1*. CHX superinduces glucocorticoid-mediated transcription of a gene encoding the α epithelial sodium channel protein via a mechanism that can be suppressed by a p38 MAPK inhibitor (Itani et al., 2003). Similarly, Edwards and Mahadevan (1992) have shown that concentrations of ANS insufficient for suppression of translation but sufficient for JNK activation could superinduce the transcriptional activation of c-*fos* and c-*jun* by epidermal growth factor. We are currently examining the roles of JNK and p38 in *CYP1A1* induction/superinduction in MCF10A cells.

Nuclear lysates isolated from MCF10A cultures 1 h after TCDD and CHX cotreatment formed ~3.4-fold more AhR/DNA complex than TCDD-treated cultures as analyzed by EMSA. Furthermore, the capacity for AhR/DNA complex formation was fairly stable in cotreated cultures for at least an additional 2 h. In contrast, during the same time period, this capacity had declined by ~50% in TCDD-treated cultures. Hence, within 3 h of treatment, AhR/DNA complex formation was ~7-fold higher in cotreated cultures. It is conceivable that this difference in AhR/DNA complex content contributes to *CYP1A1* superinduction in MCF10A cultures.

The AhR undergoes proteolytic degradation by the proteasome in a variety of cell types following agonist-mediated activation (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). Ma et al. (2000) reported that cotreatment of 1c1c7 cells with CHX suppressed overall AhR loss following TCDD-mediated receptor activation and significantly enhanced AhR/DNA complex formation as analyzed by EMSA. Because EMSAs were performed at a single time point several h after treatment, it is unclear as to whether elevated AhR/DNA complex contents in cotreated cultures reflected differences in the stabilities or capacities for the formation of complexes or a combination of the two. Nevertheless, Ma et al. (2000) speculated that CHX-mediated *CYP1A1* superinduction was a consequence of having more activated AhR in the nucleus due to the suppressed synthesis of a putative labile protein termed "AhR degradation promoting factor", which presumably facilitated AhR proteolysis. Our EMSA data are consistent with one component of their superinduction model. Cotreated MCF10A cultures formed more AhR/DNA complexes than TCDD-treated cultures; however, unlike what was observed in 1c1c7 cultures, cotreatment did not suppress overall AhR loss in MCF10A cultures. Indeed, cotreatment of TCDD-exposed MCF10A cultures with CHX potentiated overall AhR loss. A similar effect occurred in cultures treated with a superinducing dose of ANS. Hence, superinduction of *CYP1A1* in MCF10A cultures by protein translation inhibitors occurred in spite of enhanced overall AhR loss.

We currently do not know why CHX prevented AhR loss in TCDD-CHX cotreated 1c1c7 cultures but potentiated AhR loss in MCF10A cells. Deletion analyses of the murine AhR indicate that ligand-initiated AhR turnover requires the transactivating domain of the receptor. Specifically, truncated AhR molecules lacking the transactivating domain did not undergo proteolysis in cultures treated with TCDD (Ma and Baldwin, 2000). To date, a limited number of polymorphisms have been described in the transactivating domain of the human AhR (for review, see Harper et al., 2002). The effects of these polymorphisms on AhR function are not fully known. Although speculative, it is conceivable that MCF10A cells express a variant AhR that is not responsive to the protective effects mediated by CHX in other cell lines. Alternatively, MCF10A cells may lack the cellular components/processes activated by CHX that afford protection to the activated AhR.

Two lines of evidence suggest that AhR proteolysis by the proteasome in 1c1c7 cells occurs in the cytosol. Specifically, trapping the activated AhR in the nucleus of 1c1c7 cells by either inhibiting the CRM1 transport system with LMB or mutational inactivation of the nuclear export signal sequence suppresses AhR turnover (Davarinos and Pollenz, 1999). In contrast, our LMB studies suggest that degradation of the activated AhR in MCF10A cells by the proteasome can occur in the nucleus. The basis for the differences in the two cell lines is not known; however, our observation is not unique. Two other laboratories have demonstrated proteasome-dependent degradation of nuclear AhRs (Song and Pollenz, 2002; Lees et al., 2003).

In summary, our studies demonstrate that the superinduction of *CYP1A1* in MCF10A cells by protein translation inhibitors is neither quantitatively related to effects on protein synthesis nor due to an overall inhibition of agonist-induced AhR proteolysis. In addition, AhR degradation following agonist activation does not require export to the cytosol.

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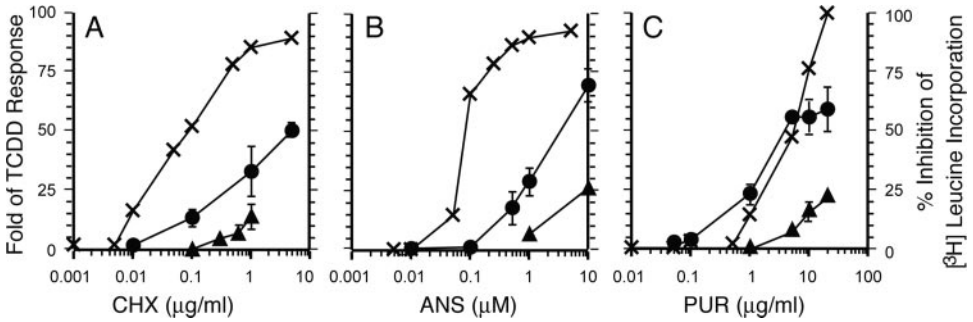

Fig. 13. Composite dose/concentration response graphs relating *CYP1A1* induction/superinduction and inhibition of [^{3}H]leucine incorporation by inhibitors of translation. MCF10A cultures were treated with various doses/concentrations of CHX (A), ANS (B), and PUR (C) either alone or in combination with TCDD. Cultures were harvested 6.5 h after addition of protein translation inhibitor or 6 h after addition of TCDD. In combination studies, the protein translation inhibitor was added 30 min before TCDD. X, [^{3}H] leucine incorporation; relative CYP1A1 mRNA content with translation inhibitor alone (▲) or translation inhibitor + TCDD (●). Panels represent a composite of data presented in Figs. 1 and 5, B–D.

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