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The p53 Inhibitor Pifithrin-α is a Potent Agonist of the Aryl Hydrocarbon Receptor

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Running Title: Pifithrin-α activation of AhR

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Abbreviations

AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; PAS, Period, ARNT, Single-minded homology domain; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzo-*p*-furan.

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ABSTRACT

The tumor suppressor protein, p53, is currently a target of emerging drug therapies directed towards neurodegenerative diseases, such as Alzheimer's and Parkinson's and side effects associated with cancer treatments. Of this group of drugs, the best characterized is pifithrin-α, a small molecule that inhibits p53-dependent apoptosis through an undetermined mechanism. In this study, we have used a number of molecular approaches to test the hypothesis that pifithrin-α acts as an AhR agonist and in this manner, inhibits the actions of p53. Towards this end, we have found that pifithrin-α is a potent AhR agonist as determined by its ability to bind the AhR, induce formation of its DNA binding complex, activate reporter activity and upregulate the classic AhR target gene, CYP1A1. However, examination of its ability to inhibit p53-mediated gene activation and apoptosis revealed that these actions occurred via an AhR-independent manner. The significance of this study is based on the fact that activation of the AhR is typically associated with an increase in phase I and phase II metabolizing enzymes and adverse biological events such as tumor promotion that may contribute to untoward effects of pifithrin α. Hence, this work will aid in the future design of more specific members of this important class of p53 inhibitors for use in a clinical setting.

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The tumor suppressor protein, p53, is a transcription factor that functions as a cellular gatekeeper and is often deregulated in human tumors (Hofseth 2004). While lack of functional p53 expression is associated with the development of cancers, its upregulation of the intrinsic apoptotic pathway is implicated in the cell death that occurs during the progression of a number of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Waldmeier 2003) and during chemo- and radiotherapies in the normal tissue surrounding the tumors (Gudkov and Komarova, 2003).

Given the therapeutic potential of p53 inhibitors, a chemical screen was employed to identify pifithrin-α ([2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-*p*-tolyethanone] hydrobromide) as an effective inhibitor of p53-mediated gene activation and apoptosis that was capable of protecting mice from lethal genotoxic stress elicited by gamma irradiation (Komarov et al., 1999). Further developments in the design of p53 inhibitors have identified a series of pifithrin-α analogues that display potent neuroprotective effects and show promise in their potential as therapeutic agents to be used to reduce or prevent neurodegeneration and protect the cancer patient from the dehabilitating effects that occur during current chemo- and radiotherapies (Zhu et al., 2002). In addition to its promise as a clinical tool, pifithrin-α has also proven to be effective in the laboratory using a variety of cell types and apoptotic inducing agents to characterize p53-mediated events (Chramostova et al., 2004; Kaji et al., 2003; Lorenzo et al., 2000; Schafer et al., 2003; Wang et al., 2004; Zhu et al., 2002).

An important consideration of all clinical and laboratory tools, is the specificity with which the therapeutic agent interacts with its intended target. With this in mind, we noted the structural similaries between pifithrin-α and ligands of the aryl hydrocarbon

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receptor (AhR, Fig. 1), as well as recent observations that ligand activation of the AhR can inhibit apoptosis (Schrenk et al., 2004) and senescence (Ray and Swanson, 2003; Ray and Swanson 2004), two p53-mediated events, and questioned whether pifithrin-α may act as an AhR agonist.

The AhR is best characterized as a transcriptional activator of phase I and phase II metabolizing enzymes (Rushmore and Kong, 2002). This basic helix-loop-helix PAS protein is a ligand-activated receptor that resides in the cytoplasm as part of a chaperone complex that includes two HSP90 molecules, the immunophilin-like protein ARA9/XAP2/AIP, and the co-chaperone p23 (reviewed in Denison and Nagy, 2003). Once activated, this complex translocates into the nucleus and dissociates, allowing the AhR to dimerize with its DNA-binding partner, ARNT. Gene regulation that ensues following the recognition of the AhR/ARNT heterodimer to its DNA recognition elements (DREs, TNGCGTG) has been best characterized using the target gene CYP1A1 (Whitlock 1999). In addition to drug/xenobiotic metabolism, the AhR is increasingly implicated in roles that include crosstalk with other nuclear transcription factors such as the estrogen receptor (Safe and Wormke, 2003) and NF-κβ (Tian et al., 2002), regulation of the cell cycle (Puga et al., 2002), senescence (Ray and Swanson, 2004) and embryonic processes such as the development of the hepatic vascular architecture (Lahvis et al., 2004). Thus, ligand-induced activation of the AhR/ARNT pathway has the potential to alter myriad events that have important toxicological and pharmacological endpoints.

In this report, we have characterized pifithrin-α as a potent AhR agonist. However, the ability of pifithrin-α to inhibit p53 gene activation and p53-mediated apoptosis does not appear to require its interaction with the AhR. Thus, while the AhR

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does not appear to be involved in the desired effects of pifthrin-α, it is likely to initiate many possible side effects, including alterations in drug metabolism that may be associated with the clinical use of this drug.

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METHODS

Chemicals

TCDD and TCDF were obtained from Dr. Stephen Safe (Texas A&M, College Station, TX). MNF (3'-methoxy-4'-nitroflavone) were gifts from Dr. Stephen H. Safe (Texas A & M University, College Station, TX) and Dr. Thomas A. Gaseiwicz (University of Rochester, Rochester, New York). High grade DMSO (>99.9% purity) was purchased from AMRESCO Inc. (Solon, OH). β-naphthoflavone was purchased from Sigma (St. Louis, MO). Pifithrin-α was purchased from both Sigma and Tocris Cookson Inc. (Ellisville, MO). Pifithrin-α from the two companies induced similar luciferase activities when analyzed in the *CYP1A1*-luc/HepG2 cells. [^{3}H]-TCDD was obtained from ChemSyn Laboratories (Lenexa, KS). Apigenin, kaempferol and all other chemicals were obtained from Sigma (St. Louis, MO).

Cell culture and treatment

Hepa-1 (*i.e.*, Hepa-1c1c7) and the AhR- and ARNT-deficient Hepa-1c1c7 cell lines, AhR-D and ARNT-D (also referred to as LA-I and LA-II), were generated by Dr. James P. Whitlock, Jr. (Stanford University) as previously described (Miller et al., 1983). The human hepatoma cell line, HepG2, was obtained from Dr. Christopher A. Bradfield (University of Wisconsin, Madison). The HaCaT cell line was obtained from Dr. Mitch Denning (Loyola University). All cells were maintained in Dulbecco's Modified Eagle's Media with glucose and glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin (Invitrogen Life Technologies Corp., Carlsbad, CA) at 37 °C and 5% CO_2.

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Oligonucleotides

The oligonucleotides that contained either the consensus DRE (Swanson et al., 1995) or mutated sequence were purchased from Integrated DNA Technologies (Coralville, IA) and are: HIS17, TCGAGCTGGGGGCATTGCGTGACATAC; HIS18, TCGAGGTATGTCACGCAATGCCCCCAGC; HIS 108, TCGAGCTGGGGGCATTGATTGACATAC; and HIS 109, TCGAGGTATGCAATCAATGCCCCCAGC.

Plasmids

The conDRE/Luc and mutDRE/Luc were generated via inserting two copies of the corresponding annealed oligonucleotides, HIS 17/18 or HIS 108/109, into the pGL3-Promoter vector (Promega, Madison, WI). The luciferase reporter plasmid that contains the human *CYP1A1* gene promoter (-1612 to +292), pLUC1A1, was obtained from Dr. Robert Tukey (University of California, San Diego). The human and murine AhR plasmids, phuAhR and pmuAhR, and human ARNT plasmid, phuARNT, were obtained from Dr. Christopher A. Bradfield (Dolwick et al., 1993a; Dolwick et al., 1993b). The plasmids bearing the wild-type and mutated forms of p53 were obtained from Dr. Dan Tai (College of Pharmacy, University of Kentucky). The luciferase plasmid containing p53 response elements (pp53-TA-luc) was obtained from Clontech.

Real Time PCR

The level of CYP1A1 mRNA was measured by real-time PCR following reverse-transcription of mRNA. Cells were plated for 48 hours and then treated for 4 hours with

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the appropriate drug(s). Total RNA was collected using Trizol reagent (Invitrogen Corp., Carlsbad, CA). One ug of RNA was primed with random hexamers to synthesize cDNA using the Omniscript RT kit (Qiagen, Valencia, CA) as per the manufacturer's guidelines. Real-time PCR amplification was carried out using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA) and its associated Brilliant SYBR Green QPCR master mix (Stratagene, La Jolla,CA) using 1/80th of the RT reaction as template. After an initial 10 min. at 95°C cycling parameters were as follows: 95° for 30 sec., 55°C for 1 min., 72°C for 1 min for 40 cycles. Cycle threshold (CT) values were assigned using the manufacturer's defaults and background fluorescence was corrected for by the use of a supplied reference dye. Sample loading was controlled by normalizing all values to GAPDH. Specificity of the CYP1A1 and GAPDH primer pairs was confirmed by the use of disassociation (melting curve) profiles available with this system. Primer sequences can be supplied upon request.

Western blot analysis

Western blot analysis was performed as previously described (Ray and Swanson, 2003). Total cellular extracts were prepared from cells by homogenization in F-buffer (10 mM Tris, 50 mM NaCl, 30 mM sodium pyrophasphate, 50 mM NaF, 5 µM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton X-100, 1 mM PMSF, 5 units/ml α2-macroglobulin, 2.5 units/ml pepstatin A, 2.5 units/ml leupeptin, 150 µM benzamidine, 2.8 µg/ml aprotinin; pH 7.05) in a Kontes Duall 1 mL Tissue Grinder (Fisher, Pittsburg, PA). Homogenates were centrifuged at 14,000 RPM at 4 ºC for 10 min, the supernatant removed, and protein concentrations were determined using BCA Protein Assay Reagents (Pierce, Rockford,

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IL). Sample buffer was added to the aliquots (50 µg total protein) and applied to a 10% SDS-polyacrylamide gel and subjected to western blotting procedures using the rabbit anti-CYP1A1 antibody (H-70, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-p21 antibody (556431, BD Biosciences Pharmingen, San Diego, CA), mouse anti-p53 antibody (3076, Abcam, Cambridge, MA), or rabbit anti-β-actin antibody (A-2668, Sigma) as primary antibodies and the corresponding anti-species IgG-HRP (Sigma) as the secondary antibodies.

Transient transfections

Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After an overnight incubation, the cells were treated with the indicated chemicals for either 18 or 24 hr. The Firefly and *Renilla* luciferase activities were determined with a TR 717 Microplate Luminometer from Applied Biosystem (Foster City, CA) using either the Luciferase Assay System kit or the Dual-Glo Luciferase kit from Promega according to the manufacturer's protocol.

Electromobility shift assay

The electromobility shift assays (EMSA) were performed as previously described (Heid et al., 2000). Nuclear lysates were prepared from HepG2 cells that had been treated for 1 h with the indicated compounds using the NucBuster Protein Extraction Kit from EMD Biosciences Novagen (San Diego, CA). Samples were 6 µg each and the consensus DRE (annealed HIS 17/18) was used as the probe. For super-shift analysis, the appropriate samples were incubated for 10 min at room temperature with 2 µg of mouse

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anti-AhR (RPT1, Abcam), 0.2 µg of goat anti-ARNT (sc-8076, Santa Cruz Biotechnology), or 2 µg of anti-rabbit IgG (Sigma) following the addition of the probe.

In vitro synthesized AhR and ARNT were synthesized using the pmuAhR and phuARNT plasmids and the TNT Coupled Reticulocyte Lysate System from Promega according to the manufacturer's protocol. The AhR and ARNT proteins (2.5 µl of each reaction) were incubated in MENG (25 mM MOPS, 1 mM EDTA, 3.8 mM NaN_3, 10% glycerol, pH 7.5) buffer containing the indicated ligands at a final volume of 16.5 µl. The mixtures were incubated at 30 ºC for 2 h. The KCl concentration was adjusted to 120 mM, 45 ng poly dIdC was added, and the mixture was incubated for 15 min at room temperature. The EMSA samples were then analyzed via gel separation as described above.

Ligand binding assays

The ligand binding competition assays were performed essentially as previously described (Denison et al., 1986). Cytosolic cell extracts from Hepa-1 cells were generated by resuspension of the cell pellets in HEDG buffer (25 mM Hepes, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, pH 7.5) containing 0.4 mM leupeptin, 4 mg/ml aprotinin, and 0.3 mM PMSF, homogenization, and centrifugation at 100,000x*g* for 45 min. Aliquots of the supernatant (120 µg) were incubated at room temperature for 2 h with the indicated concentrations of pifithrin-α in the presence of 3 nM [^{3}H]-TCDD in HEDG buffer. After incubation on ice with hydroxyapatite for 30 min, HEDG buffer with 0.5% Tween 80 was added. The samples were centrifuged, washed twice, resuspended in 0.2 ml scintillation fluid, and subjected to scintillation counting.

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Nonspecific binding was determined using a 150 fold molar excess of TCDF and subtracted from the total binding to obtain the specific binding. The specific binding is reported relative to [^{3}H]-TCDD alone.

Analysis of apoptosis

The analysis of apoptosis was conducted using the Cell Death Detection ELISA ^{PLUS} kit from Roche (Indianapolis, IN) according to the manufacturer's protocol. After washing twice with PBS, the cells were treated with 30 J/m^2 ultraviolet light (254 nm) using an FLX-20M ultraviolet light source from Enprotech (New York City, NY). Complete media that contained either 0.1% DMSO or 10 μM pifithrin-α was then added. After a 24 hr incubation, apoptosis was determined by measuring POD enzymatic activity via spectrophotometric determination. The data was analyzed using one-way ANOVA and Tukey's Multiple Comparison Test analyses using the GraphPad Prism 3.0 software.

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RESULTS

Pifithrin-α induces CYP1A1 protein levels and promoter activity

A classic marker of activation of the AhR pathway is upregulation of CYP1A1, a xenobiotic metabolizing enzyme that contains dioxin response elements (DREs) recognized by the AhR and its DNA binding partner, ARNT (aryl hydrocarbon receptor nuclear translocator)(Whitlock 1999). Thus, as a first test of whether pifithrin-α may function as an AhR agonist, we questioned whether it was capable of upregulating CYP1A1. As shown in Figure 2A, increasing doses of either TCDD, the prototypical AhR agonist, or pifithrin-α resulted in corresponding increases in the CYP1A1 mRNA levels. However, analysis of the EC_{50} values generated from these experiments revealed that the potency of pifithrin-α is considerably less than that of TCDD (i.e., 1.1×10^{-6} versus 8.7×10^{-11} for pifithrin-α and TCDD, respectively). Further, a 24 h treatment of either murine (Hepa-1) or human (HepG2) hepatoma cells with 10 μM pifithrin-α was sufficient to induce CYP1A1 protein expression to a level comparable to that induced with 1 nM TCDD.

As a first step in determining the role of the AhR in eliciting the actions of pifithrin-α, we determined whether cotreatment with known AhR antagonists (Lu et al 1995; Henry et al 1999; Allen et al., 2001; Zhang et al 2003) would inhibit the ability of pifithrin-α to induce CYP1A1 mRNA levels. As shown in Figure 3, the AhR antagonists, MNF, apigenin and kaempferol inhibited induction of CYP1A1 mRNA levels by both TCDD and pifithrin-α. Given that some actions of the AhR are thought to occur in an ARNT- independent manner, we also questioned whether induction of CYP1A1 mRNA required ARNT (Fig 3B). The idea that the ability of pifithrin-α to

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regulate CYP1A1 mRNA levels requires formation of the AhR/ARNT heterodimer is supported by its induction of CYP1A1 mRNA in the wild-type Hepa-1 cells, but not in those lacking expression of ARNT. Similarly, a role of the AhR/ARNT DNA response element (DRE) in eliciting these actions of pifithrin-α is indicated by the ability of pifithrin-α to induce reporter activity of constructs regulated by either the CYP1A1 promoter that contains multiple DREs or a consensus DRE, but not by that containing mutated DREs (Fig 3C).

Pifithrin-α induces DNA-binding of the AhR/ARNT heterodimer

Our next objective was to determine whether the ability of pifithrin-α to activate gene transactivation was associated with an increase in the formation of the AhR/ARNT DNA binding complex. Towards this end, we analyzed DNA binding of nuclear extracts prepared from HepG2 cells incubated with DMSO, TCDD, or pifithrin-α (Fig. 4A). Using the consensus DRE as the probe (conDRE), treatment with either TCDD (Fig. 4A, Lane 2) or pifithrin-α (Fig. 4A, Lane 8) was found to result in an increase in the formation of a DNA/protein complex. Specificity of this complex was determined by competitive displacement with unlabelled oligonucleotides that contained the consensus DRE (Fig. 4A, Lanes 3 and 9), but not with that containing a mutated DRE (Fig. 4A, Lanes 4 and 10). The presence of both the AhR and ARNT proteins in the protein/DNA binding complexes induced by either TCDD or pifithrin-α was demonstrated using super-shift analysis. Incubation with either the anti-AhR antibody (Fig. 4A, Lanes 5 and 11) or anti-ARNT antibody (Fig. 4A, Lanes 6 and 12), but not the nonspecific IgG antibody

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(Fig. 4A, Lanes 7 and 13), shifted the formation of the respective DNA binding complexes.

We next questioned whether pifithrin-α is able to directly activate DNA-binding by AhR and ARNT by performing additional EMSA analysis using *in vitro* transcribed and translated AhR and ARNT (Fig. 4B). Incubation of the murine AhR protein with pifithrin-α, β-napthoflavone, or TCDD induced formation of the AhR/ARNT DNA binding complex. As we have previously observed (Swanson et al., unpublished results), β-napthoflavone appears to be a more potent AhR agonist in this assay as compared to TCDD, presumably due to the high lipophilic nature of TCDD that may allow it to be sequestered by the rabbit reticulocyte lysate.

Pifithrin-α competitively displaces [^{3}H]-TCDD specific binding

In order to determine whether pifithrin-α directly activated the AhR via an interaction with its ligand-binding domain, we performed ligand-binding assays using cytosolic extracts prepared from Hepa-1 cells. As shown in Figure 5, increasing concentrations of pifithrin-α decreased the specific binding of [^{3}H]-TCDD. The relative binding affinity of pifithrin-α to AhR was determined to be 1.56×10^{-7} M.

Pifithrin-α inhibits p53-dependent gene regulation and apoptosis in an AhR-independent manner

We then hypothesized that the ability of pifithrin-α to inhibit p53-mediated gene regulation and p53-mediated apoptosis requires the AhR. Towards this end, we first performed reporter assays using a luciferase reporter that is regulated by p53-response

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elements. To ensure that the observed effects were specific to activation of p53, the values obtained from the cells transfected by the wild-type p53 expression plasmid were normalized to those transfected with that containing a mutated form of p53. Our initial data performed using varying concentrations of pifithrin-α indicated that a concentration of 1 X 10^{-5} was optimal for inhibiting p53-regulated reporter activity (data not shown). As shown in Figure 6A, pifithrin-α, but not TCDD or the AhR antagonist MNF, inhibited p53-mediated reporter activity. Further, cotreatment with both MNF and pifithrin-α yielded results similar to that of pifithrin-α alone, indicating that the AhR does not play a role in this action of pifithrin-α. Additional experiments performed in a cell line that lacks expression of the AhR (AhR-D) indicated that the ability of pifithrin-α to inhibit p53-mediated induction of p21 levels following exposure to ultraviolet light was not compromised by the absence of the AhR (Fig 6B).

Finally, we sought to determine whether the ability of pifithrin-α to inhibit p53-dependent apoptosis requires the AhR/ARNT signaling pathway (Fig 6C). Treatment with ultraviolet light resulted in an approximately 10-fold increase in apoptosis in the Hepa-1 cells. The addition of pifithrin-α immediately following exposure to ultraviolet light inhibited the induction of apoptosis within all three cells lines in a manner that was significantly enhanced within the AhR-D and ARNT-D cells, 55% and 48%, respectively, as compared to 33% in wild-type Hepa-1 cells.

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DISCUSSION

In this study, we report that pifithrin-α is a potent AhR agonist that is capable of upregulating AhR target genes, such as *CYP1A1*. We also report that pifithrin-α inhibits the p53 pathway via an AhR-independent mechanism. The AhR signaling pathway has been associated with many events including alterations in cell viability, oxidative stress, and crosstalk with other transcription factors such as nuclear factor kappaB, retinoblastoma protein and the estrogen receptor (Nebert et al., 2000; Puga et al., 2002; Tian et al., 2002; Carlson and Perdew 2002). Thus, pifithrin-α, via its activation of the AhR, has the potential of impacting on a number of p53-independent pathways.

Like many drugs in the early stages of development, two problems currently hinder further progress of this exciting new class of therapeutics; 1) the unknown mechanism(s) by which pifithrin-α and its analogues inhibit p53 and 2) the uncertainty as to whether the actions of these small molecules are specific to p53 inhibition. From the data available thus far, it appears that the inhibitory actions of pifithrin-α occur at a step subsequent to nuclear translocation of p53 (Murphy et al., 2004). A more controversial issue pertains to the specificity of the actions of pifithrin-α. Although it was previously found that the actions of pifithrin-α include suppression of the heat shock transcription factor and glucocorticoid signaling pathways (Komarova et al., 2003), these findings have been recently challenged by others (Murphy et al., 2004) who have failed to detect inhibition of either GR-mediated gene induction or the function of the chaperone machinery by pifithrin-α. In fact, because pifithrin-α has been shown to alter glucocorticoid signaling as well as the heat shock response (Komarova et al., 2003), it is possible that pifithrin-activation of AhR may play a role in mediating these effects of

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pifithrin-α. An additional putative role that the AhR may play in the actions of pifithrin-α is in the ability of pifithrin-α to induce NF-κβ activity in neurons (Culmsee et al 2003) and is based on the observations that in some cell types, ligand activation of the AhR can enhance the NFκβ pathway (Sulentic et al 2004).

With respect to the potency of pifithrin-α as an AhR agonist (i.e., an EC_{50} of 1 X 10^{-6} M), pifithrin-α is to be considered a moderate AhR agonist that would exert activities similar to that of the indole-derived pigment, indigo, but less than that of agonists such as TCDD and ITE (Denison and Nagy, 2003; Song et al., 2002). The concentrations of pifithrin-α used in the current study to induce the CYP1A1 protein levels and *CYP1A1* promoter activities (≤ 10 µM, Fig. 2 and 3), induce formation of the AhR/ARNT DNA binding complex (Fig. 4), and displace TCDD specific binding (Fig. 5), are similar to those doses typically used to inhibit p53-associated events, i.e., 10-30 µM (Komarov et al., 1999; Wang et al., 1999; Chramostova et al., 2004). Thus, in studies in which pifithrin-α is commonly used to inhibit p53, it can be expected that the AhR/ARNT pathway will also be up-regulated.

Another aspect that should be considered is whether the presence of the AhR signaling pathway may decrease the efficacy of pifithrin-α by enhancing its clearance via an increase in its metabolism. This possibility is supported by the data shown in Figure 6C. Here, the ability of pifithrin-α to inhibit ultraviolet light-induced apoptosis was significantly greater in cells that lacked either the AhR or ARNT as compared to that observed in the parental cell line. At this time, it is not know whether drug/xenobiotic metabolizing genes that are regulated by the AhR pathway, such as CYP1A1 or CYP1B1, are capable of metabolizing pifithrin-α.

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In summary, we have demonstrated that pifithrin-α activates the AhR signaling pathway, a pathway that mediates many clinically relevant effects including tumor promotion and altered responses to drugs and xenobiotics through changes in metabolism. Future studies performed using pifithrin-α and new drugs formulated to similarly inhibit p53 should take this effect into consideration within experimental design and data interpretation.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Comparison of pifithrin-α to previously characterized AhR agonists. Pifithrin-α is structurally similar to previously characterized AhR ligands including β-naphthoflavone, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), and omeprazole, but not the AhR agonists 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and indolo[3,2b]carbazole (ICZ)(Dension and Nagy, 2003; Guengerich et al., 2004; Song et al., 2002).

Figure 2. Pifithrin-α induces the mRNA and protein levels of CYP1A1. A) Induction of CYP1A1 mRNA levels by TCDD and Pifithrin-α. HepG2 cells were incubated with varying doses of either TCDD or pifithrin-α. After 4 hrs, mRNA was isolated and analyzed by real time PCR as described in Materials and Methods. The EC_{50} values were determined using the Prism software. The 95% CI were 1.53×10^{-11} to 5.02×10^{-10} M (TCDD) and 2.8×10^{-7} to 4.0×10^{-6} M (pifithrin-α). **B) Induction of CYP1A1 protein levels by TCDD and pifithrin-α.** HepG2 and Hepa-1 cells were incubated with DMSO, 1 nM TCDD, or 10 μM pifithrin-α for 24 h. Total cellular extracts were prepared and analyzed for CYP1A1 and actin expression using western blot analysis as described in Materials and Methods. The results shown are representative of two independent experiments performed each in triplicate.

Figure 3. Role of the AhR/ARNT and DRE in the induction of CYP1A1 by pifithrin-α. A) Antagonists of the AhR block the ability of both TCDD and pifithrin-α to induce CYP1A1 mRNA. The induction of CYP1A1 mRNA levels was

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determined as described in Figure 2A except that the HepG2 cells were incubated with either 1 nM TCDD or 1 μM pifithrin-α in the absence or presence of MNF (10 μM), apigenin (10 μM) or kaempferol (10 μM). **B) Induction of CYP1A1 mRNA levels by pifithrin-α requires ARNT.** Either the wild-type Hepa-1 (left) or those lacking ARNT (right) were incubated with DMSO, TCDD (1 nM) or pifithrin-α (1 μM) and CYP1A1 mRNA was determined as described in Figure 2 and Materials and Methods. **C) Induction of the CYP1A1 promoter by pifithrin-α requires the DRE.** HepG2 cells were transiently transfected with luciferase constructs containing the *CYP1A1* promoter (pLUC1A1), two copies of the consensus DRE (conDRE/Luc), or two copies of the mutated DRE (mtDRE/Luc). After a 24 h incubation with either 10 μM pifithrin-α or DMSO, the cells were harvested and analyzed for luciferase activities and *Renilla* activities. The values are reported as fold change relative to DMSO treatment group and represent the mean ± SE of three experiments performed in duplicate.

Figure 4. Pifithrin-α induces DNA binding of AhR/ARNT complexes in cultured cells and in vitro. A) EMSA was conducted using nuclear extracts prepared from HepG2 cells treated with DMSO (Lane 1), 1 nM TCDD (Lanes 2-7), or 10 μM pifithrin-α (Lanes 8-13) for 1 h at 37 °C. Aliquots were incubated with [^{32}P]-labeled DRE oligonucleotide in the presence or absence of unlabeled consensus DRE (conDRE; Lanes 3 and 9) or mutant DRE (mutDRE; Lanes 4 and 10). Super-shift analysis was conducted using anti-AhR (Lanes 5 and 11), anti-ARNT (Lanes 6 and 12), or nonspecific IgG (Lanes 7 and 13) antibodies. **B)** EMSA was conducted using *in vitro* synthesized AhR and ARNT. Reticulolysate expressed AhR and ARNT were incubated with DMSO, 10

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μM pifithrin-α, 1 μM β-naphthoflavone, or 1 nM TCDD prior to EMSA. The data are representative of two independent experiments.

Figure 5. Pifithrin-α specifically competes with [^{3}H]-TCDD binding. Cytosolic extracts prepared from Hepa-1 cells were incubated with 3 nM [^{3}H]-TCDD and the indicated concentrations of pifithrin-α for 2 h at room temperature. The specific binding was separated from the nonspecific binding using hydroxyapatite as described in Materials and Methods. The ordinate is Bx/Bo, specifically bound radioligand in the presence of a given amount of competitor (Bx) divided by specifically bound radioligand in the absence of competitor. The values represent the mean ± SE of four experiments and are represented as percent of that obtained using [^{3}H]-TCDD alone.

Figure 6. The ability of pifithrin-α to inhibit p53 transactivation and ultraviolet light-induced apoptosis is independent of the AhR. A) Pifithrin-α, but not TCDD or MNF inhibits p53 transactivation. Human keratinocytes that lack functional expression of p53 (HaCaT) were transiently transfected with a 53-regulated luciferase reporter plasmid and either the wild-type p53 or mutated p53 expression plasmids. After 24 hours, the cells were treated with the indicated chemicals and incubated for an additional 18 hours. The cells were harvested and luciferase and Renilla activities were determined. The values represent the means ± S.E. of three independent experiments. **B) Impact of pifithrin-α on p53 and p21 expression in cells that vary in functional AhR and ARNT.** Wild-type Hepa-1 or AhR-D cells were harvested 8 h after treatment with 30 J/m^2 ultraviolet light and either 10 μM pifithrin-α or DMSO. Protein expression of

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p21 and p53 were analyzed via western blot analysis as described within Materials and Methods. The results shown are representative of two independent experiments performed each in duplicate. **C) Impact of pifithrin-α on apoptosis in cells that vary in functional AhR and ARNT.** The cells were treated as in B) and apoptosis was measured via nucleasomal fragmentation analysis at 24 h after ultraviolet light treatment. The values represent the O.D. measured for the ultraviolet light + pifithrin-α treated samples normalized to each of the ultraviolet light+DMSO treated samples and represent the mean ± SE of five independent experiments conducted in triplicate. Statistical analysis determined that the level of apoptosis within the ultraviolet light+ pifithrin-α treated AhR-D and ARNT-D cells was significantly different from the respective ultraviolet light +DMSO treated cells (* = $p < 0.01$).

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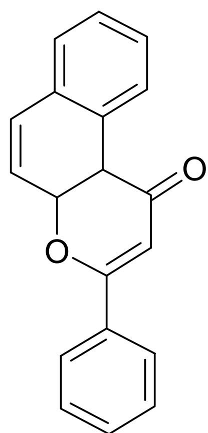
Figure 1



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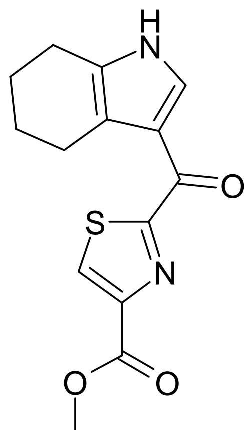

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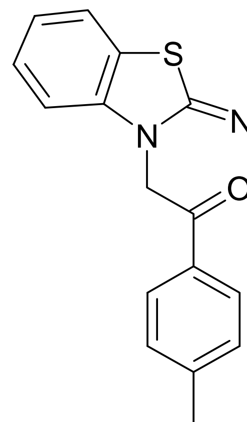

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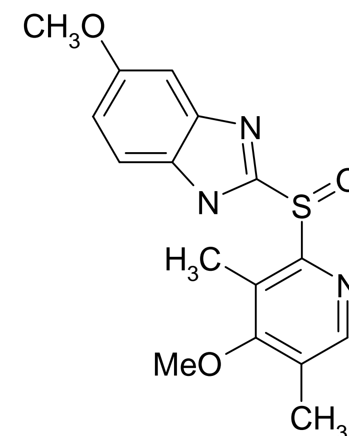
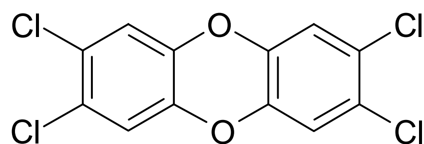
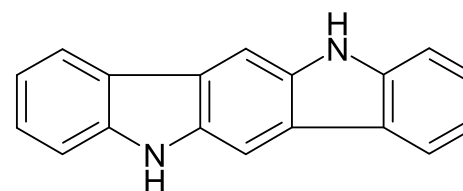


omeprazole



2,3,7,8-tetrachlorodibenzo-*p*-dioxin



indolo[3,2-b]carbazole

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Figure 2

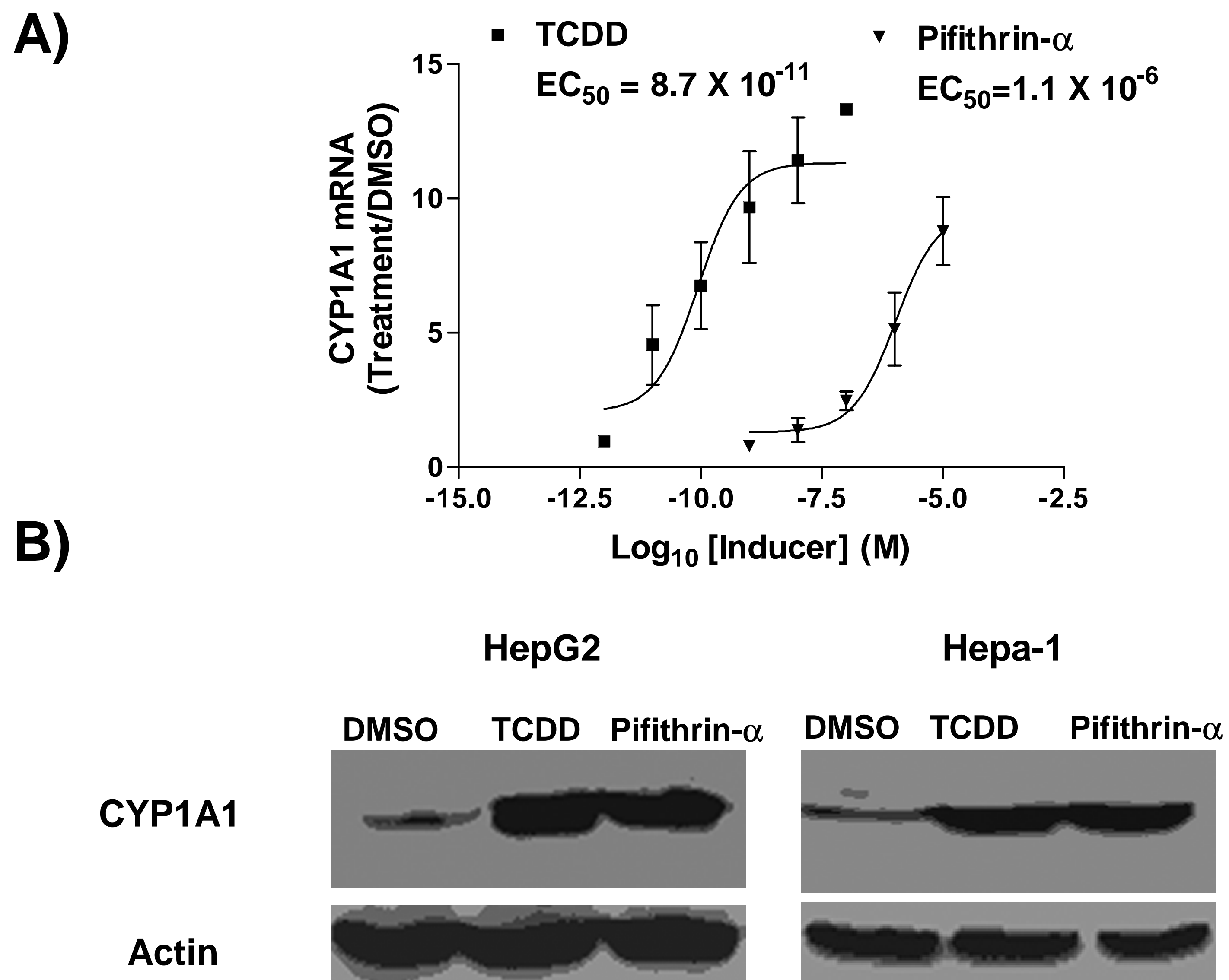

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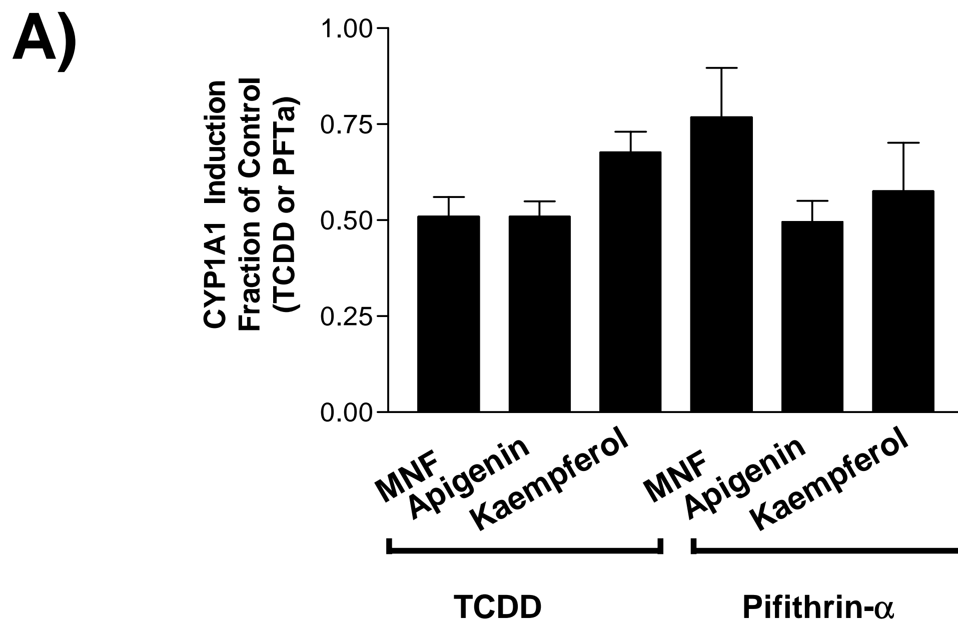
Figure 3 (A and B)



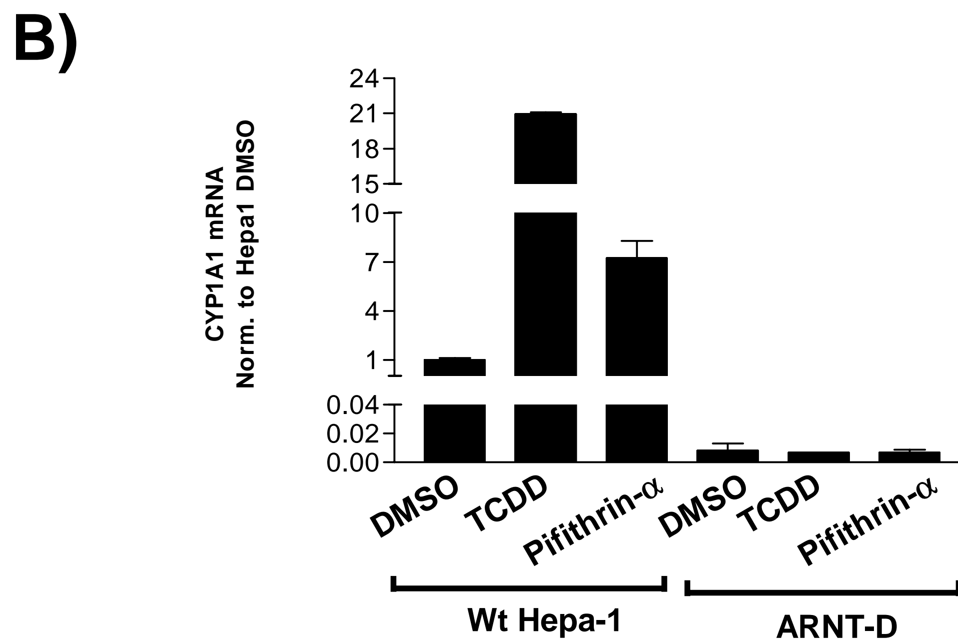


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Figure 3C

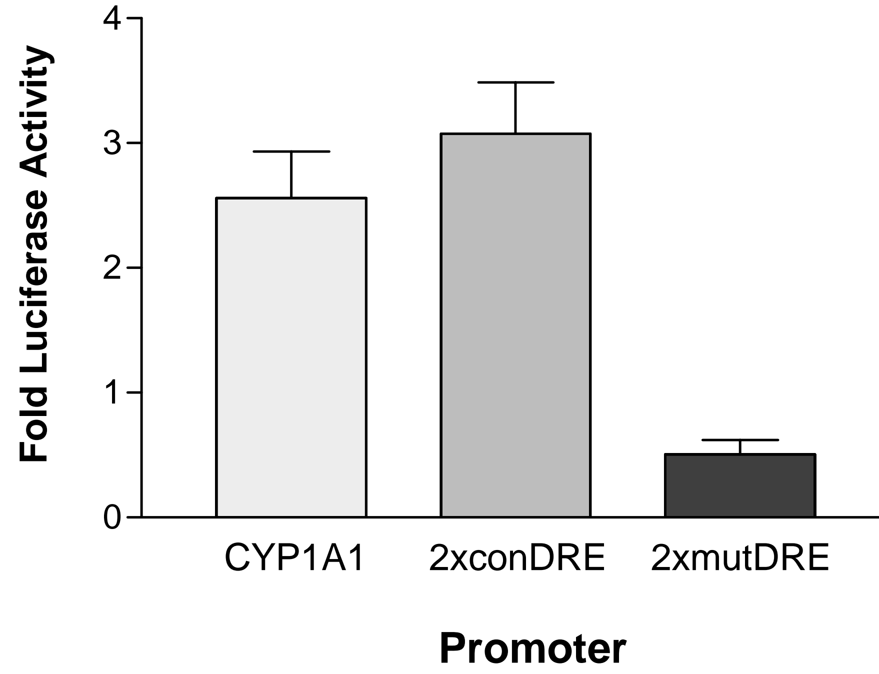

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Figure 4A

A)

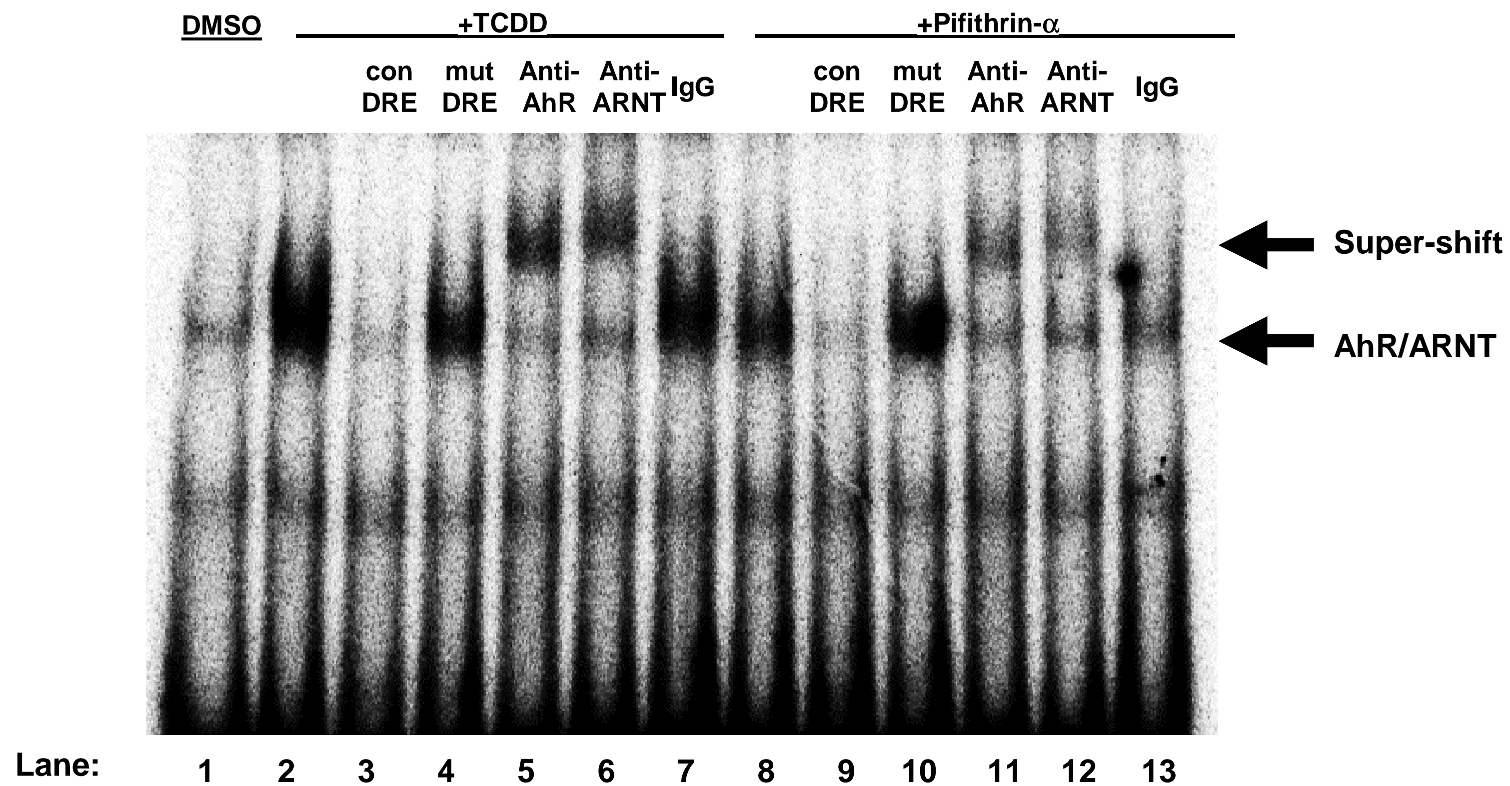

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Figure 4B

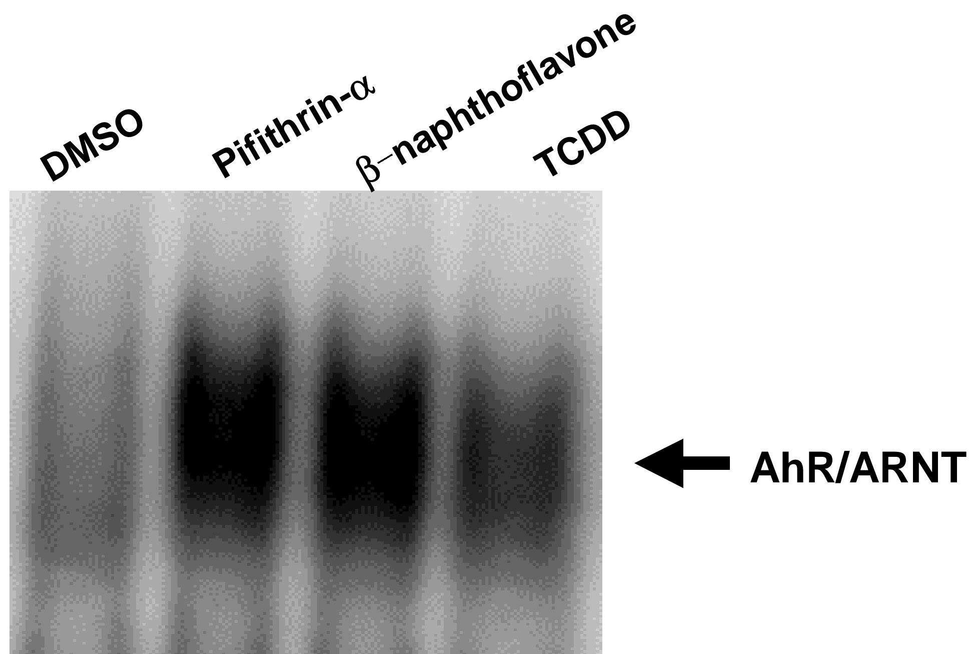

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Figure 5

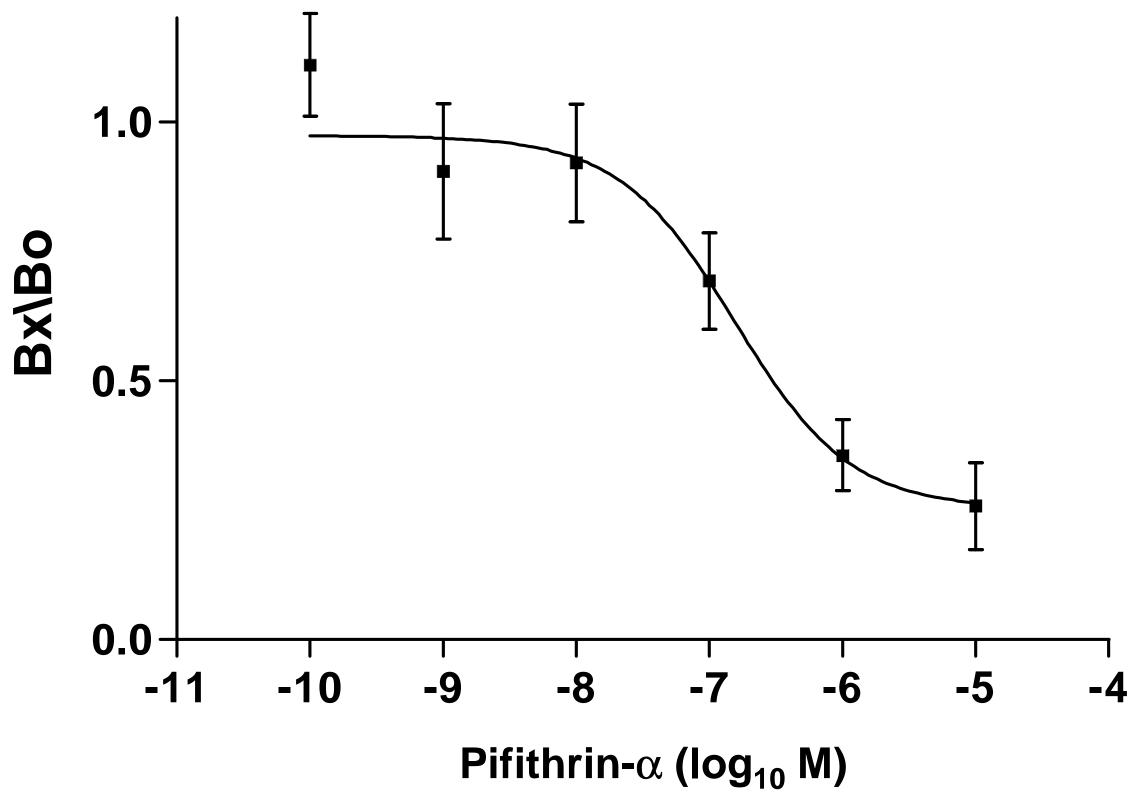

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Figure 6A

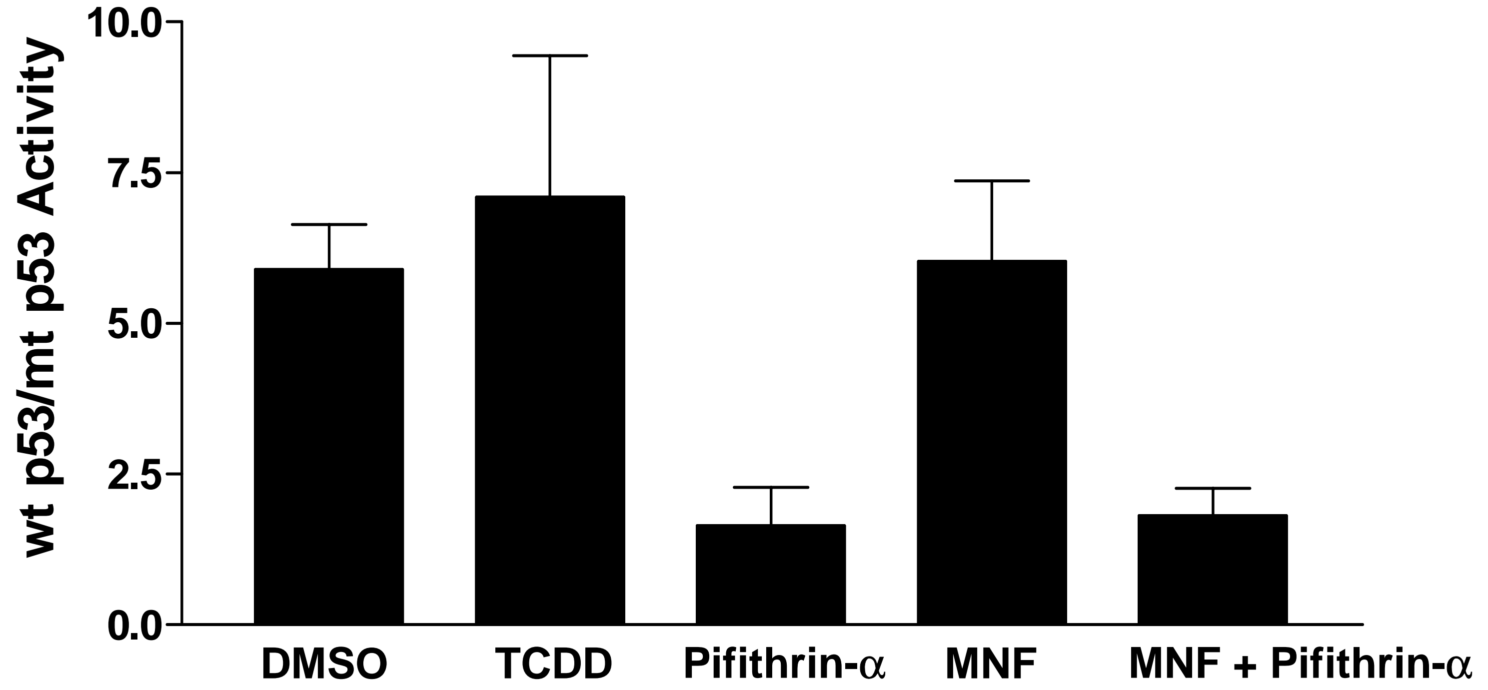

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Figure 6B

B)

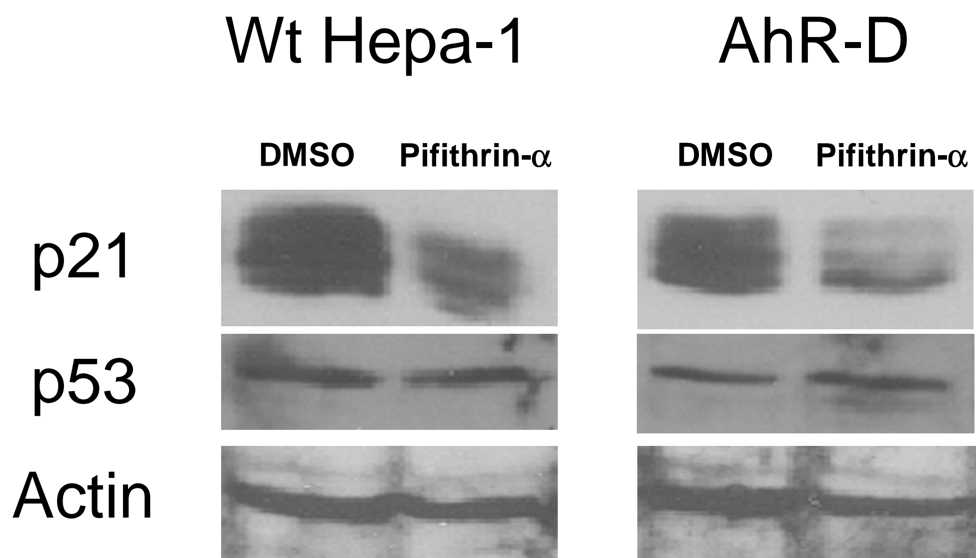

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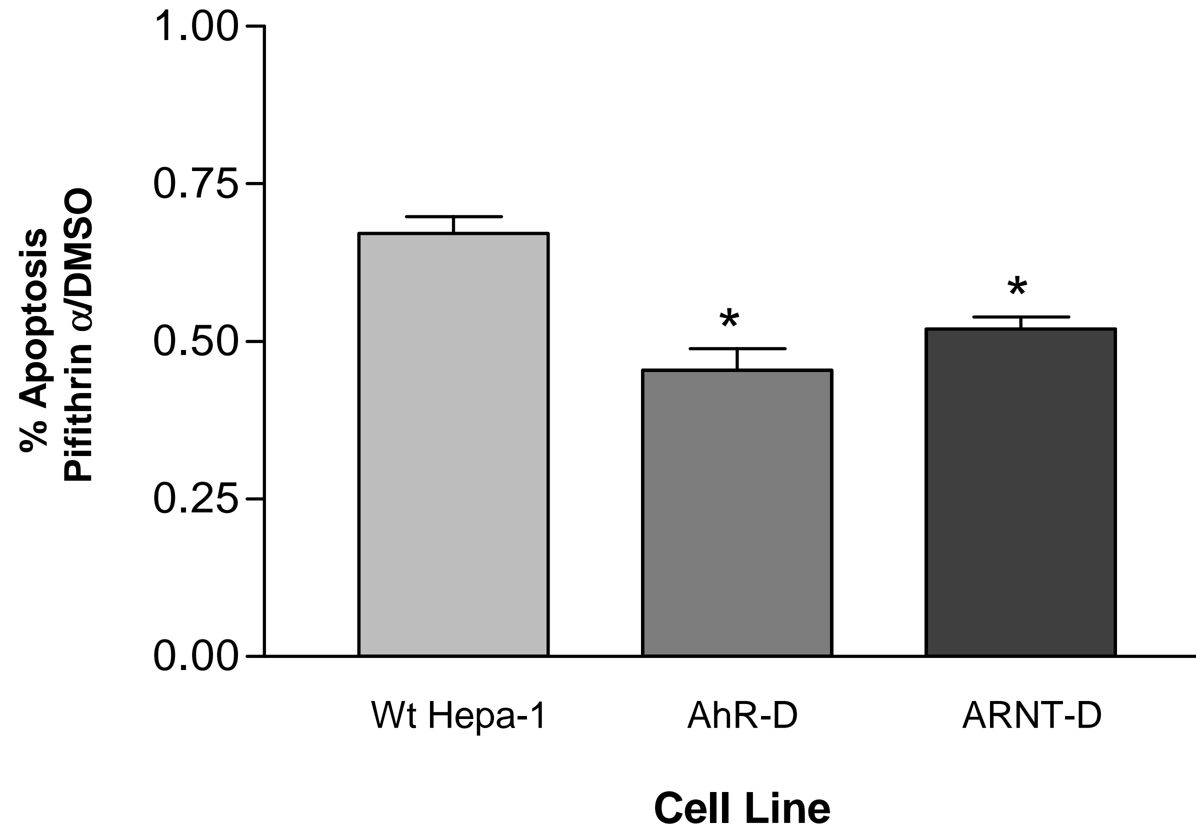
Figure 6C



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