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Development of an Aryl Hydrocarbon Receptor Antagonist Using the Proteolysis-Targeting Chimeric Molecules Approach: A Potential Tool for Chemoprevention

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ABSTRACT

Activation of the aryl hydrocarbon receptor (AHR) by agonists and environmental contaminants like dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) leads to many adverse biological effects, including tumor promotion. With this in mind, we propose that agents that block the AHR pathway may be therapeutically beneficial, particularly by exhibiting chemopreventive activities. In our current research, we have focused on the development of an AHR antagonist using a chemical genetic approach called PROTACS (PROteolysis-TArgeting Chimeric moleculeS). PROTACS is a novel approach of tagging small recognition sequences of a specific E3 ubiquitin ligase complex to a known ligand for the receptor of interest (AHR) for targeting its degradation. Here, we present the design and initial characterization of AHR targeting PROTACS (Apigenin-Protac) designed to degrade and inhibit the AHR in epithelial cells. Our results demonstrate the "proof of concept" of this approach in effectively blocking AHR activity in cultured cells.

Exposures to environmental factors are believed to play a substantial role in the development of many human cancers (Boffetta and Nyberg, 2003; Boffetta, 2004; Poirier, 2004; Luch, 2005). For example, in a Swedish study that encompassed 9.6 million individuals, it was reported that environmental factors account for 69% of colon cancers, 79% of lung cancers, and 78% of kidney cancers (Czene et al., 2002). Although the specific causative agents are difficult to identify, one class of human carcinogens that is believed to be important because of its high prevalence in the environment and relatively well-characterized mode of action in animal models is that of the aromatic hydrocarbons (Luch, 2005). Members of this group include benzo[*a*]pyrene, polychlorinated biphenyls, and 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), which are contaminants present in cigarette smoke and other environmental sources. The carcinogenic actions of many polycyclic aromatic hydrocarbons typically occur after their binding to and activation of the aryl hydrocarbon receptor (AHR). Although the AHR is typically believed to be involved largely in the genotoxic actions of environmental carcinogens, emerging evidence indicates a role of the AHR in tumor promotion and progression (Luch, 2005).

At the cellular level, the AHR is present in the cytoplasm as a component of an AHR chaperone complex in association with heat shock protein-90, XAP2/ARA9, and p23 (Kewley et al., 2004). Upon activation by its agonists, the AHR translocates into the nucleus, where it dissociates from its chaperone complex and binds with its dimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT). The AHR-ARNT dimer then interacts with its DNA recognition sites, dioxin response elements (DREs), and subsequently regulates a battery of AHR target genes such as CYP1A1 and CYP1B1. Although CYP1A1 and CYP1B1 are perhaps the best-characterized AHR target genes and are typically considered to be biomarkers of the AHR pathway, it has not yet been established whether up-regulation of CYP1A1 and CYP1B1 is required for all of the carcinogenic effects elicited by AHR agonists.

The promise associated with using the AHR as a target for effective chemopreventive approaches has been demonstrated by the use of AHR antagonists like 3′-methoxy-4′-nitroflavone

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; MNF, 3′- methoxy-4′-nitroflavone; NHK, normal primary human keratinocyte; PROTACS, Proteolysis-targeting chimeric molecules; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DRE, dioxin response element; DMSO, dimethyl sulfoxide; RT, reverse transcriptase; PCR, polymerase chain reaction; ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; EMSA, electrophoretic mobility shift assay.

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(MNF) (Dertinger et al., 2001) and resveratrol (Revel et al., 2003). For example, in vivo treatment of mice with MNF has been shown to inhibit the genotoxicity induced by benzo[*a*]pyrene and completely block that induced by cigarette smoke condensate (Dertinger et al., 2001). Likewise, use of resveratrol that naturally occurs in red wine has also been shown to inhibit benzo[*a*]pyrene-induced genotoxicity in vivo (Revel et al., 2003). The problems currently associated with further development of these AHR antagonists as chemopreventive agents lie primarily in either their lack of specificity (resveratrol) (Signorelli and Ghidoni, 2005) or their actions as partial AHR agonists (MNF) (Zhou and Gasiewicz, 2003).

In considering additional approaches that may be used to generate a small molecule capable of blocking the actions of the AHR, we turned to the use of PROTACS. The PROTACS (PROteolysis-TArgeting Chimeric moleculeS) approach is a novel technology developed by us (Zhang et al., 2004a,b; Bargagna-Mohan et al., 2005) and others (Sakamoto et al., 2001, 2003; Schneekloth et al., 2004) that target proteins of interest for degradation via the ubiquitin-proteasome pathway. PROTACS are chimeric molecules composed of a specific E3 ubiquitin ligase recognition motif and a ligand that binds to a target protein that then recruit the targeted protein to the specific E3 ligase complex for ubiquitination and initiates its degradation by the proteasome. Because PROTACS can be used to control intracellular levels of specific proteins post-translationally, these novel molecules provide a direct means to probe protein functions and in this manner may be used for therapeutic intervention by down-regulating disease-promoting proteins. Thus far, PROTACS that target androgen receptor (Schneekloth et al., 2004), estrogen receptor (Sakamoto et al., 2003; Zhang et al., 2004a,b; Bargagna-Mohan et al., 2005), MetAP-2 (Sakamoto et al., 2001), and FKBP (Schneekloth et al., 2004) have been successfully developed. These studies demonstrate the feasibility of the PROTACS approach for use in inhibiting the actions of a number of disease targets. We envision that PROTACS developed to target the AHR (Fig. 1A) may prove useful as a molecular probe to delineate the role of the AHR in environmentally related disease processes as well as serving as a future chemoprevention agent.

Materials and Methods

Materials. Apigenin-Protac and Apigenin-Protac[Ala] were synthesized as described previously (Lee et al., 2007). TCDD was a generous gift from Dr. Stephen H. Safe (Texas A&M University, College Station, TX). [^{3}H]TCDD was obtained from ChemSyn Laboratories (Lenexa, KS). Unless otherwise mentioned, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture. Neonatal primary human keratinocytes (NHKs) were purchased from Cascade Biologics (Portland, OR). The cells were grown in Epilife medium with EDGS (Cascade Biologics) at 37°C and 5% CO_2. Murine (Hepa1c1c7) and human hepatoma (HepG2) cells were maintained in Dulbecco's modified Eagle's media with glucose and glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2.

Electrophoretic Mobility Shift Assays. The impact of Apigenin-Protac on the AHR/ARNT DNA binding complex formed in cultured cells was determined by pretreating HepG2 cells with Apigenin-Protac for 7 h before the administration of either DMSO (0.1%) or TCDD (1 nM). After a 1-h incubation, the cells were harvested, and nuclear extracts were prepared using the Nucbuster protein extraction kit (Novagen, Madison, WI). Aliquots of the extracts (12 μg) were incubated with salmon sperm DNA (1 μg) and KCl (0.1 M final concentration) at room temperature for 15 min. The samples were then incubated for an additional 15 min at room temperature with the radiolabeled (^{32}P) consensus DRE sequences, (forward) TCGAGCTGGGGGCATTGCGTGACATTAC and (reverse) TCGAGGTATGTCACGC AATGCCCCCAGC, as described previously (Hoagland et al., 2005; Puppala et al., 2006). After a 15-min incubation, the samples were separated using 4% polyacrylamide nondenaturing electrophoretic gel and 0.5× Tris borate-EDTA (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA, pH 8.0) as the running buffer.

Ligand Binding Assays. Competitive ligand binding assays were performed as described previously (Hoagland et al., 2005; Puppala et al., 2006) using varying concentration of either apigenin or Apigenin-Protac. The IC_{50} values were determined using Prism 3.0 (GraphPad Software, San Diego, CA).

Western Blot Analyses. NHKs were seeded into six-well plates. Once they reached approximately 70% confluence, they were treated with the indicated chemicals for varying time periods as described in the figure legends. The cells were harvested, and the total cellular extracts were prepared using radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, and 0.5% sodium deoxycholate). The protein concentrations were estimated using BCA analysis (Pierce, Rockford, IL). Aliquots of the cellular extracts (approximately 100 μg) were separated using SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes. After a brief incubation in blocking buffer, the blots were probed using antibodies that recognized AHR (Abcam, Cambridge, MA), CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma-Aldrich).

Proliferation/Viability Analyses. NHKs were seeded into 96-well plates. Once they reached approximately 70% confluence, treatments of DMSO, TCDD, or Apigenin-Protac were added, and the cells were incubated for the time periods described in the figure legends. The cells were then subjected to MTT analyses (American Type Culture Collection, Manassas, VA) according to the manufacturer's protocol.

RT Real-Time PCR. The cells were harvested at the time points described in the figure legends, and the RNA was extracted using TRIzol reagent (Invitrogen). For RT real-time PCR, the cDNA was prepared using the manufacturer's protocol for Omniscript RT kit (Qiagen, Valencia, CA) and random primers (Invitrogen). The cDNA was then analyzed using Brilliant SYBR Green QPCR Master Mix and Human QPCR Reference RNA (Stratagene). Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and were specifically designed using Vector NTI 9.0.0 (InforMax; Invitrogen Life Science Software, Frederick, MD) to amplify regions spanning exon junctions. For CYP1A1, the primer sequences were the following: forward, 5′-CAAAACCTTTGAGAAGGGCCACATC-3′; and reverse, 5′-GACAGCTGGACATTGGCGTTCTC-3′. For CYP1B1, the primer sequences were the following: forward, 5′-GCTGCTCCTCCTCTTCACCAGGTA-3′; and reverse, 5′-GCTGGTCACCCATACAAGGCAGAC-3′.

Proteasome Inhibition Kinetics Assays. Apigenin, Apigenin-Protac, or epoxomicin was mixed with a fluorogenic peptide substrate and assay buffer (20 mM Tris, pH 8.0, 0.5 mM EDTA, and 0.035% SDS) in a 96-well plate. The chymotrypsin-like activity was assayed using the fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (Sigma-Aldrich). Hydrolysis was initiated by the addition of human erythrocyte 20S proteasome (Biomol International, Plymouth Meeting, PA), and the reaction was monitored by fluorescence (360 nm excitation/460 nm detection) using a Microplate Fluorescence Reader (FL600; Bio-Tek Instruments, Inc., Winooski, VT) using the software KC4 v.2.5 (Bio-Tek Instruments). The reactions were allowed to proceed for 90 min, and fluorescence was detected every 1 min. Fluorescence was quantified as arbitrary units, and progression curves were plotted for each reaction as a function of

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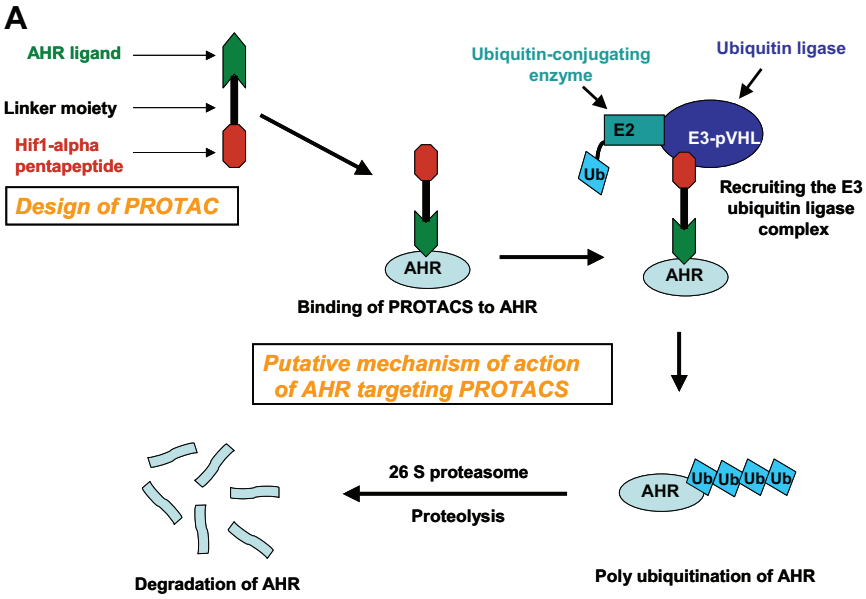
time. The range of concentrations tested was chosen such that several half-lives could be observed during the course of the analyses.

Statistical Analysis. The data were analyzed using analysis of variance (ANOVA) with Bonferroni's multiple comparison test as mentioned in the figure legends.

Results

Apigenin-Protac Specifically Interacts with the AHR. The apigenin-based AHR-targeting PROTACS (Apigenin-Protac) was synthesized as described previously (Lee et al., 2007) (Fig. 1B). Apigenin-Protac[Ala] was synthesized using similar strategies. Apigenin-Protac[Ala] lacks the hydroxyproline moiety in the E3 ubiquitin recognition sequence, which is required for recruiting the specific ubiquitination machinery (Hon et al., 2002) and thereby serves as a negative control. Given that we had modified the existing natural compound (apigenin), we first verified that Apigenin-Protac, like the parent compound, could still specifically interact with the ligand binding site of the AHR. Toward this end, we performed competitive ligand binding assays wherein we used protein extracts from the Hepa1c1c7 cell line (a murine hepatoma cell line) that expresses high levels of a very stable form of the AHR (Holmes and Pollenz, 1997). As shown in Fig. 2, increasing concentrations of either apigenin (the parent compound) or the Apigenin-Protac compound were effective in competing with tritiated TCDD for binding to the AHR. IC_{50} values obtained from these analyses revealed that the AHR binding affinity of Apigenin-Protac is less than that of apigenin (3.8×10^{-6} M and 2.9×10^{-7} M, respectively).

Impact of Apigenin-Protac on Cell Viability. Our next goal was to determine the effect of Apigenin-Protac in cultured cells. For the majority of our experiments, we have



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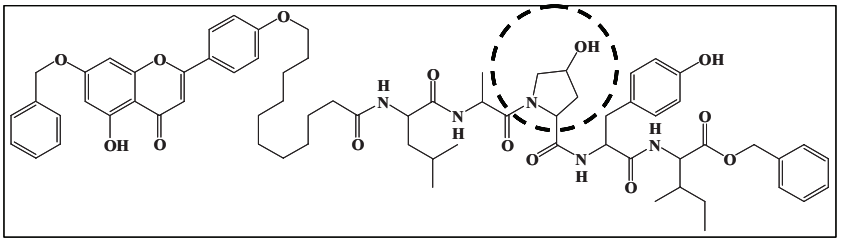
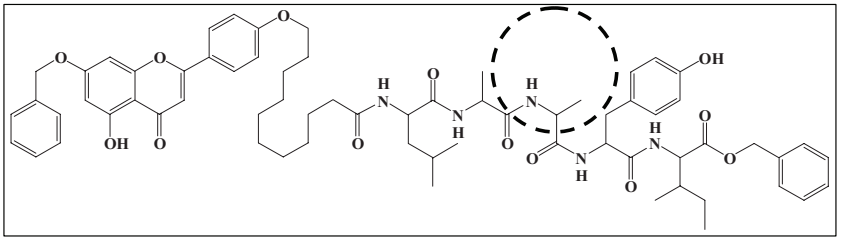


Chemical structure of Apigenin-Protac



Chemical structure of Apigenin-Protac[Ala]

Fig. 1. A, schematic representation of the design and putative mechanisms of action of AHR-based PROTACS, Apigenin-Protac. The design of PROTACS consists of three different moieties: an AHR ligand (i.e., apigenin) (a), a linker moiety composed of a chain of 11 linear hydrocarbons (b), and a penta peptide sequence composed of the specific E3-pVHL ubiquitin ligase recognition motif (c). The mechanism by which Apigenin-Protac degrades the AHR would involve recognition of the ligand apigenin by the AHR, recruitment of the E3 ubiquitination machinery, polyubiquitination of AHR, and finally AHR degradation by the 26S proteasome. B, chemical structures of Apigenin-Protac and Apigenin-Protac[Ala]. Apigenin-Protac and Apigenin-Protac[Ala] were synthesized as described previously (Lee et al., 2007). To generate the negative control of Apigenin-Protac, hydroxy proline, the key amino acid in the pentapeptide pVHL E3 ubiquitin ligase recognition site (Hon et al., 2002) was replaced by alanine, resulting in Apigenin-Protac [Ala].

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chosen to use NHKs. The rationale for use of NHKs is based on the evidence that the AHR plays a major role in maintaining appropriate cellular homeostasis of the skin (Fernandez-Salguero et al., 1997; Panteleyev and Bickers, 2006). Thus, these cells are a good model for examining the AHR signaling pathway in normal epithelial cells. To verify that Apigenin-Protac was not overtly toxic to the cells, we performed MTT assays (Fig. 3). These analyses revealed that the viability of the cells treated with Apigenin-Protac was not significantly different from that of the DMSO control at the time points tested.

Apigenin-Protac, Unlike Apigenin, Does Not Inhibit Proteasome Activity. Earlier reports have indicated that the parent compound, apigenin, inhibits the proteasomal pathway (Chen et al., 2005, 2007). To determine whether the modified apigenin compound (i.e., Apigenin-Protac) similarly altered the proteasomal pathway, we performed enzyme kinetic assays using increasing concentrations of Apigenin-Protac. As shown in Fig. 4, the inhibitory effect of Apigenin-Protac (10 μM) on the proteolytic activity of the proteasome was significantly less than that of the positive controls, epoxomicin and apigenin. Comparison the IC_{50} values of the proteosome inhibitor, epoxomicin and Apigenin-Protac reveals that the inhibitor effect of Apigenin-Protac is negligible.

Apigenin-Protac Decreases AHR Protein Levels and Inhibits Agonist Induction of CYP1A1 Expression. Our next goal was to determine whether Apigenin-Protac is capable of degrading the AHR receptor protein and antagonizing the actions of the prototypical AHR agonist, TCDD. Toward this end, we treated NHK with TCDD in the presence or absence of Apigenin-Protac. As shown in Fig. 5 (lanes 1 and 2), TCDD treatment decreased the protein levels of the AHR as observed previously in the NHKs using similar conditions (Ray and Swanson, 2004). Also as expected, TCDD treatment resulted in an increase in the expression levels of the AHR target gene, CYP1A1 (Fig. 5, lanes 1 and 2). Although treatment with Apigenin-Protac also decreased the protein levels of the AHR, it did not induce protein levels of CYP1A1, indicating that Apigenin-Protac lacks the ability to act as an AHR agonist (Fig. 5, lanes 1 and 3). The antagonistic properties of Apigenin-Protac, however, are evident by the ability of Apigenin-Protac to inhibit TCDD-induced CYP1A1 protein levels (Fig. 5, lanes 2 and 4). To confirm whether the actions of Apigenin-Protac on AHR protein levels are mediated via the 26S proteasomal pathway, we treated the cells with the proteasomal inhibitor epoxomicin both in the presence and absence of Apigenin-Protac (Fig. 5, lanes 5 and 6). Treatment with epoxomicin alone increased the protein levels of the AHR consistent with earlier reports (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Pollenz, 2007), indicating a role of the 26S proteasome in regulating endogenous AHR levels.

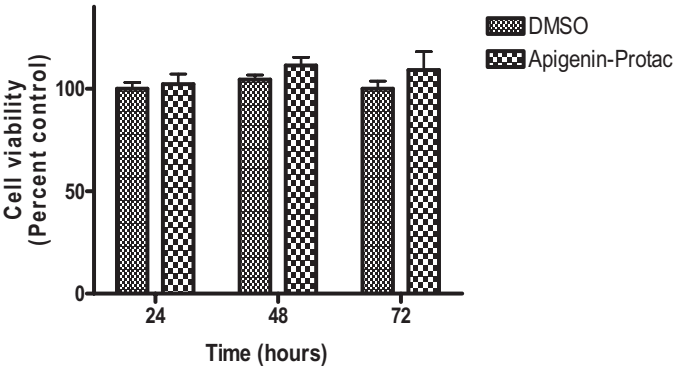


Fig. 3. Impact of Apigenin-Protac on cell viability. NHKs were cultured in 96-well plates for 48 h before the treatments. When 50 to 70% confluent, the cells were incubated with either DMSO (0.1%), or Apigenin-Protac (10^{-5} M). At the indicated time points, the cells were analyzed using the MTT assay. The values are expressed relative to the DMSO control and are representative of two independent experiments. The data were analyzed using GraphPad Prism 3.0 with ANOVA analysis followed by Bonferroni's multiple comparison test ($p < 0.01$ as indicated). The Apigenin-Protac-treated samples were not statistically different from the DMSO control at any time point examined.

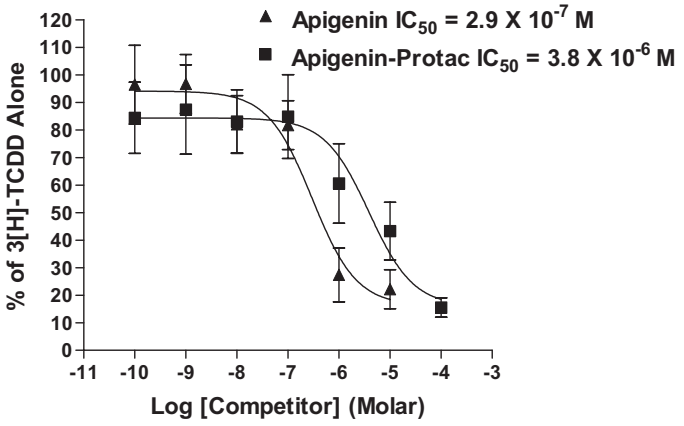
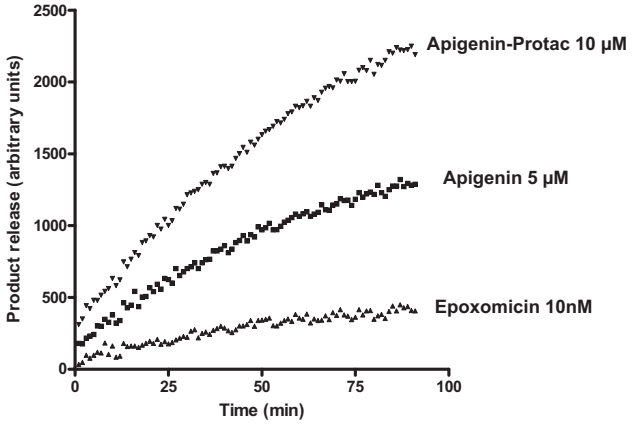


Fig. 2. Relative affinity of Apigenin and Apigenin-Protac for the AHR as determined by competitive ligand binding analyses. Tritiated TCDD, in the absence or presence of varying concentrations of apigenin or Apigenin-Protac (10^{-10} to 10^{-5} M) was incubated with protein extracts prepared from Hepa1c1c7 cells. After incubation at room temperature for 2 h, the nonspecific binding was removed using hydroxyapatite. A 200-fold molar excess of 2,3,7,8-tetrachlorodibenzofuran, an analog of TCDD, was used to estimate the nonspecific binding of TCDD. All of the values are expressed as percentages of the value obtained using TCDD alone. The data are averages ± S.D. of three independent experiments. The IC_{50} values were determined using Graph Pad Prism.



Compound	Apigenin-Protac	Apigenin	Epoxomicin
IC_{50}	125.36 µ M ± 12.67	4.61 µ M ± 0.15	5.80 nM ± 0.23

Fig. 4. Effect of apigenin and Apigenin-Protac on proteasomal activity. Apigenin-Protac (10 μM), apigenin (5 μM), and epoxomicin (10 nM) were combined with the fluorogenic peptide substrate and assay buffer in a 96-well plate and assayed as described under *Materials and Methods*. The reactions were allowed to proceed for 90 min, and fluorescence data were evaluated in 1-min intervals. Fluorescence was quantified as arbitrary units, and progression curves were plotted for each reaction as a function of time. The corresponding IC_{50} values for each treatment are indicated in the table.

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More importantly, cotreatment of epoxomicin with Apigenin-Protac blocked the ability of Apigenin-Protac to alter AHR protein levels, supporting the idea that Apigenin-Protac decreases AHR protein expression via a mechanism that includes degradation by the 26S proteasomal pathway.

The Ability of Apigenin-Protac to Decrease AHR Protein Levels and Inhibit TCDD-Induced CYP1A1 and CYP1B1 mRNA Levels Occurs in a Time- and Dose-Dependent Manner. We then determined the optimal time and dose required for the actions of the Apigenin-Protac on the AHR pathway by performing time- and dose-response experiments. These experiments were performed similarly to those described in Fig. 5 except that the protein levels of the AHR were examined using Western blotting (Fig. 6), and the mRNA levels of the AHR target genes CYP1A1 and CYP1B1 were examined using RT real-time PCR (Fig. 7A). As shown in Fig. 6, degradation of the AHR by Apigenin-Protac was evident after 16 and 24 h of treatment. Furthermore, the observation that similar treatment with Apigenin-Protac[Ala] failed to reduce AHR protein levels again indicates that recognition of E3 ligase is required for the actions of Apigenin-Protac on the AHR.

Examination of the mRNA levels of the AHR target genes CYP1A1 and CYP1B1 (Fig. 7A) after treatments similar to those described in Fig. 6 revealed that although the ability of Apigenin-Protac to significantly decrease AHR protein levels requires at least 16 h, its ability to block TCDD-induction of CYP1A1 and CYP1B1 mRNA levels occurs after only 8 h of treatment. Apigenin-Protac significantly inhibited TCDD-induced mRNA levels of both CYP1A1 and CYP1B1 by at least 80% at all time points examined (8, 16, and 24 h). Apigenin-Protac seems to exert minimal agonistic activities. At the 8-h time point, a small but statistically insignificant increase (compared with the DMSO control) was observed. This change in CYP1A1 and CYP1B1 mRNA levels induced by Apigenin-Protac seemed to diminish after 16 and 24 h of treatment.

To determine the optimal dose required to block the AHR pathway, we performed dose-response studies at the 16-h time point (Fig. 7B). Analyses of the mRNA levels of CYP1A1 and CYP1B1 using RT real-time PCR revealed that Apigenin-Protac failed to exhibit agonistic activity at any concentration tested. More importantly, Apigenin-Protac inhibited the ability of TCDD to up-regulate either CYP1A1 or CYP1B1 mRNA levels in a dose-dependent manner.

Apigenin-Protac Inhibits the Ability of TCDD to Induce Formation of the AHR/ARNT/DNA Complex. Induction of CYP1A1 and CYP1B1 mRNA expression by TCDD is preceded by binding of the AHR/ARNT heterodimer to its DNA recognition sequences. To determine whether Apigenin-Protac could inhibit this crucial step in the AHR signaling pathway, we performed EMSA analysis of nuclear extracts obtained from human hepatoma (HepG2) cells that were incubated with TCDD in the absence or presence of Apigenin-Protac. HepG2 cells were chosen for these experiments because the amount of AHR expressed in these cells is higher than that expressed in the primary NHKs and hence are more amenable for EMSA analyses (S. Ray and H. Swanson, unpublished observations). As shown in Fig. 8 (lanes 1 and 2), treatment with TCDD resulted in formation of the AHR/ARNT/DNA binding complex similar to that reported previously (Hoagland et al., 2005; Puppala et al., 2006). Although AHR/ARNT/DNA binding was not detected after incubation with Apigenin-Protac alone, treatment with Apigenin-Protac before the addition of TCDD significantly ($p < 0.001$) inhibited formation of the AHR/ARNT/DNA binding complex (Fig. 8, lanes 3 and 4).

Discussion

In this report, we performed an initial characterization of an AHR targeting PROTACS (Apigenin-Protac) and demonstrated its effectiveness in decreasing the protein levels of the AHR and inhibiting the ability of the prototypical AHR agonist, TCDD, to activate the AHR signaling pathway in cultured cells. These results indicate that Apigenin-Protac may be a useful tool for delineating the role of the AHR in human diseases and perhaps to inhibit the progression of at least some of these disease states, such as environmentally induced cancers. The idea that Apigenin-Protac may be effective in treating human cancers at several different stages is based on observations that the AHR plays roles in not only

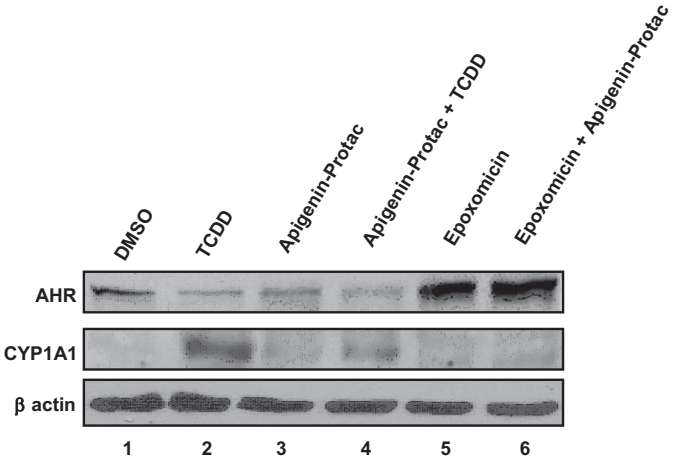

Fig. 5. Treatment with Apigenin-Protac decreases AHR protein levels and inhibits TCDD-induced CYP1A1 protein levels. NHKs were incubated with DMSO (0.1%), TCDD (1 nM), Apigenin-Protac (10^{-5} M), or epoxomicin (10^{-7} M) as indicated. Incubations with DMSO, Apigenin-Protac, or epoxomicin were for 12 h. When coincubated with TCDD, Apigenin-Protac was added 4 h before the 8-h incubations with TCDD. The cells were then harvested and subjected to Western blot analyses as described under *Materials and Methods*. The results are representative of three independent experiments.

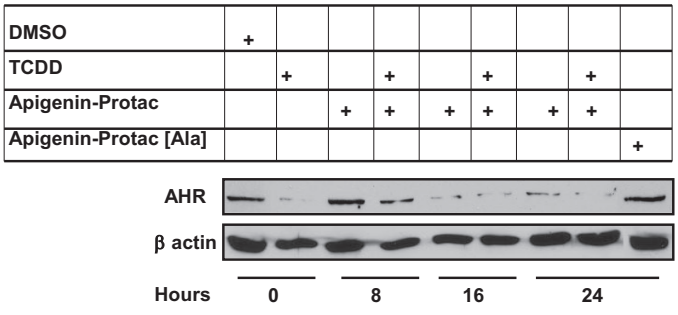

Fig. 6. Treatment with Apigenin-Protac but not Apigenin-Protac[Ala] decreases AHR protein levels. NHKs were incubated with either DMSO (0.01%), Apigenin-Protac (10^{-5} M), or Apigenin-Protac [Ala] (10^{-5} M) for 0 to 24 h. The time points indicated in the figure refer to only Apigenin-Protac or Apigenin-Protac [Ala] treatments. TCDD (1 nM) was administered at 4 h before harvesting. The cells were then harvested, and lysates were prepared and subjected to Western blot analyses. The data are representative of three independent experiments.

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the initiation step of cancer but also in many of the subsequent steps associated with cancer progression (Luch, 2005).

Although a number of research tools have been developed to probe the biology of the AHR pathway, their use has been somewhat limited. For example, in our laboratory, adenoviruses that were successfully engineered to contain either a dominant-negative form of the AHR or antisense AHR and were effective in blocking the actions of the AHR also exhibited high viral-specific effects (Swanson et al., 2005). Likewise, the use of short interfering RNA was found to be effective in blocking the AHR during short (Ray and Swanson, 2004) but not during extended time periods (S. Ray and H. Swanson, unpublished results). These problems would probably be insurmountable if attempted in in vivo studies. In addition, use of the AHR-null mouse, a highly effective research tool is limited by the possibility that some of the disease endpoints may be significantly altered by events that have occurred during development in the absence of endogenous AHR signaling. Thus, these animals may be limited in the study of disease processes that occur in the adult. Thus, the advantages of the PROTACS approach, by virtue of its small-molecule attributes, lie in its ability to overcome these disadvantages and to advance the possible use of the AHR as a therapeutic target. Finally, use of a small-molecule approach bears the distinct advantage of accelerating the bench-to-bedside transition.

In designing Apigenin-Protac, we chose to use an AHR antagonist as the moiety that facilitates recognition of the AHR protein because of the possibility that use of an AHR agonist (i.e., TCDD) in this role might allow liberation of the agonist during metabolic degradation of the AHR-targeting PROTAC and thus activate AHR-mediated adverse effects. Given the requirements dictated by the chemical synthesis of PROTACS, we also focused on compounds with the appropriate functional groups (i.e., –OH or $–NH_2$ moieties) that will allow for further modifications. A final consideration was that the chosen AHR ligand must interact with the AHR with relatively high affinity and must exhibit little or no toxicity. For these reasons, we turned to the flavonoids. A number of laboratories have shown that several naturally occurring flavonoids exhibit AHR antagonistic activities (Surh, 2003; Zhang et al., 2003). To identify candidate AHR antagonists to be used for the development of an AHR-targeting PROTACS, we performed a screen of 15 naturally occurring flavonoids (Puppala et al., 2006). The results from this study identified apigenin as an appropriate candidate and led to the idea that modifying apigenin using the PROTACS approach would allow for inhibition of the AHR pathway and degradation of the receptor protein. An area of concern with the use of apigenin is that although apigenin blocked the AHR pathway at high concentrations (i.e., 10 μM), at lower concentrations (i.e., 10 nM), apigenin activated the AHR pathway (Puppala et al.,

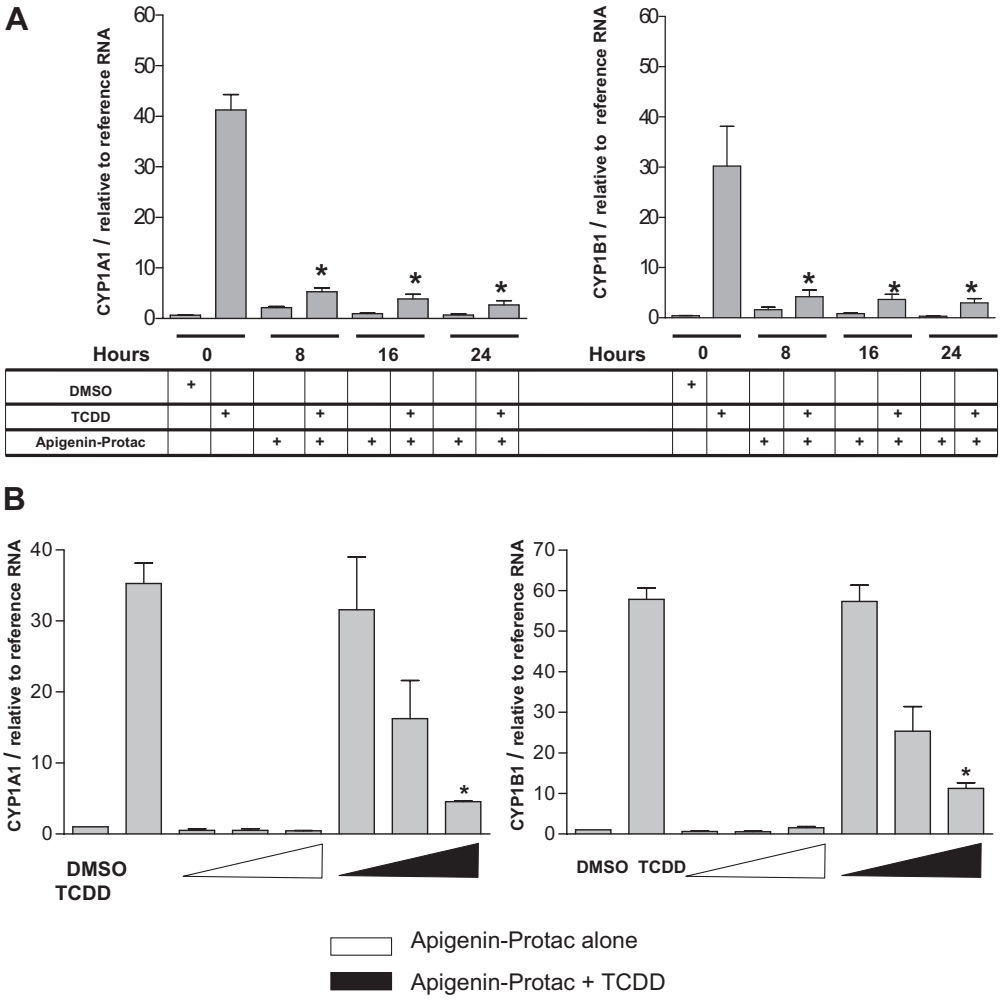

Fig. 7. Apigenin-Protac inhibits the ability of TCDD to induce CYP1A1 and CYP1B1 levels in a time-dependent (A) and dose-dependent (B) manner. For the time dependent experiments, NHKs were incubated with either DMSO (0.1%) or Apigenin-Protac (10^{-5} M) for 0 to 24 h as described in Fig. 6. The time points indicated in the figure refer only to Apigenin-Protac. TCDD (1 nM) was administered at 4 h before harvesting. The cells were then harvested; lysates were prepared and subjected to RT real-time PCR analysis. For the dose-response experiments, the NHKs were treated with increasing concentrations of Apigenin-Protac (10^{-7} to 10^{-5} M), harvested at the 16-h time point, and then subjected to RT real-time PCR analysis (Fig. 7B). The data are representative of three independent experiments. The data were analyzed using GraphPad Prism 3.0 ± S.E. with ANOVA analysis followed by Bonferroni's multiple comparison test. *, $p \leq 0.001$ compared with TCDD.

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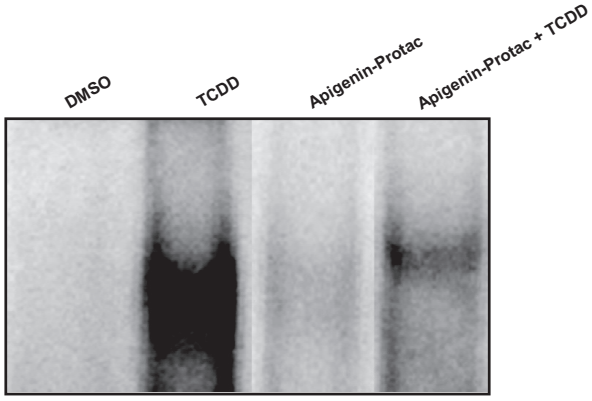
2006). Our results shown in the current study indicate that Apigenin-Protac lacks significant agonistic activity at any concentrations tested here (Fig. 7B). Furthermore, Apigenin-Protac seems to antagonize the AHR signaling pathway and degrades the AHR protein at a concentration of 10 μM. Finally, we have demonstrated that like the parent compound, apigenin, Apigenin-Protac competitively displaces TCDD from the AHR ligand binding site with moderate affinity (Fig. 2) that seems to be sufficient for eliciting AHR degradation.

Our studies indicate that the biological properties of Apigenin-Protac are distinct from those of the parent compound, apigenin. For example, apigenin has been shown previously to induce cell cycle arrest in cultured cells (Reiners et al., 1999). In addition, apigenin has been reported to induce apoptosis by activating protein kinase Cδ and caspases in different cell lines (Khan and Sultana, 2006; Vargo et al., 2006). The data shown in the current study indicate that the modified form of apigenin, Apigenin-Protac, lacks the ability to both significantly induce CYP1A1 and CYP1B1 expression (Fig. 6B) and alter cell viability (Fig. 3). An additional activity attributed to apigenin is its inhibition of the proteasomal pathway (Chen et al., 2007). Our analyses indicate that Apigenin-Protac compound seems to lose this property of the parent compound as indicated by our proteosomal activity analyses (Fig. 4).

In developing our experimental approach to be used for testing the effectiveness of Apigenin-Protac, we considered several of the properties of the prototypical AHR agonist, TCDD. For example, TCDD is highly lipophilic and has a half-life of approximately 7 to 9 years in the human body (Pirkle et al., 1989). This would suggest that even a minute amount of TCDD that remains bound to the AHR may be sufficient for maintaining activation of the AHR pathway. With this in mind, we used a pretreatment incubation that would be sufficient for degrading the maximal amount of AHR that is present in the cell before its exposure to TCDD, thereby ensuring that the AHR was unavailable for binding to TCDD and activation of the AHR pathway. However, it should be noted that a number of compounds that are present in the environment and act as potent AHR agonists vary significantly in their chemical properties (Denison and Nagy, 2003). These ligands are classified into "classic" (i.e., TCDD or β naphthoflavone) and "nonclassic ligands" of AHR (i.e., like omeprazole or thiabendazole). Thus, effectiveness of Apigenin-Protac may be dependent on which AHR agonist is used. This approach will help us better understand the mechanisms involved in agonist-induced activation of the AHR pathway and could also validate the idea regarding AHR as a target for chemoprevention strategies.

Although we envision the use of Apigenin-Protac or a similar AHR-targeting PROTACS as chemopreventive agents, a number of possible limitations must first be addressed. With respect to efficacy, pharmacokinetics and disposition studies need to be performed to ensure that sufficient concentrations reach the appropriate target tissue. Given the chemical structure and high molecular weight of Apigenin-Protac, issues pertaining to metabolism and tissue permeability may be challenging. Use of the AHR$^{-/-}$ mice would facilitate the determination of off-target effects and guide decisions pertaining to the choice of biomarkers, disease end points, and putative adverse effects.

In conclusion, this work is a "proof of concept" that demonstrates the feasibility of the PROTACTS approach to be used for blocking the AHR pathway. Our future efforts will be focused on determining the specificity of Apigenin-Protacs for the AHR and in elucidating AHR-dependent mechanisms involved in human disease states such as tumor promotion.



Lane	1	2	3	4
Values (Range)	16.14(+/-0.7)	100 (+/-7.03)	23.9 (+/-2.03)	46.7(+/-4.15)

Fig. 8. Apigenin-Protac inhibits the ability of TCDD to induce formation of the AHR/ARNT/DNA binding complex in cultured HepG2 cells. HepG2 cells were cultured with either DMSO (0.1%) or TCDD (1 nM) for 1 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of a 7-h pretreatment with Apigenin-Protac (10^{-5} M). The cells were harvested, nuclear extracts were prepared, and EMSAs were performed using the ^{32}P-labeled DRE as the radiolabeled probe. These experiments were quantitated by PhosphorImager analyses (Image Quant software) and are expressed relative to the TCDD-treated samples. The data depict the averages of two independent experiments, and the original values are expressed in terms of range.

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