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Evidence for ligand-mediated selective modulation of aryl hydrocarbon receptor activity.

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Abstract.

The concept of selective receptor modulators has been established for the nuclear steroid hormone receptors. Such selective modulators have been utilized therapeutically with great success in the treatment of cancer. However, this concept has not been examined with regard to the aryl hydrocarbon receptor (AHR) because of the latent toxicity commonly associated with AHR activation. AHR-mediated toxicity is primarily derived from AHR binding to its dioxin response element (DRE) and driving expression of *CYP1* family members, which have the capacity to metabolize procarcinogens to genotoxic carcinogens. Recent evidence using a non-DRE binding AHR mutant has established the DRE-independent suppression of inflammatory markers by the AHR. We wished to determine if such DRE-independent repression with wild-type AHR could be dissociated from canonical DRE-dependent transactivation in a ligand-dependent manner and, in doing so, prove the concept of a selective AHR modulator (SAhRM). Here, we identify the SERM Way-169916 as a dually selective modulator, binding both ER and AHR. Inflammatory gene expression associated with the cytokine-inducible acute phase response (APR) *e.g.* *SAA1* and *CRP* is diminished by Way-169916 in an AHR-dependent manner. Furthermore, activation of AHR by Way-169916 fails to stimulate canonical DRE-driven AHR-mediated *CYP1A1* expression, thus eliminating the potential for AHR-mediated genotoxic stress. Such anti-inflammatory activity in the absence of DRE-mediated expression fulfills the major criteria of a SAhRM, which suggests that selective modulation of AHR is possible and renders the AHR a therapeutically viable drug target for the amelioration of inflammatory disease.

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Introduction.

For many decades the ascribed function of the aryl hydrocarbon receptor (AHR) has been that of a xenobiotic sensor, modulating gene expression, principally P450 detoxifying enzymes, *e.g. CYP1A1*, in response to environmental ligands, *e.g.* dioxin. Such contaminants are products of the industrial age and fail to account for the evolutionary persistence of the AHR. Recently, a paradigm shift has occurred regarding AHR function. Whilst the detoxification role of AHR is not in doubt, the development of *Ahr* null and transgenic rodent models indicate physiological roles for AHR beyond that of xenobiotic metabolism. Reports provide evidence for the involvement of AHR in immune function from both toxicological and physiological perspectives. AHR ligands alter embryonic immune development and programming (Hogaboam et al., 2008); induce thymic atrophy in rodents through enhanced FasL-mediated apoptosis (Camacho et al., 2005; Kamath et al., 1997); diminish B-lymphopoiesis (Schneider et al., 2008) and the B-cell IgM response (North et al., 2009), and promote the polarization of $T_H 1/2$ cells, generating a $T_H 1$ bias (Negishi et al., 2005). Recently, focus has turned to the role of the AHR in facilitating the differentiation of CD4+ lymphocytes into $T_H 17$ and T_{Reg} cells. The $T_H 17$ population is sub-optimal in the presence of an AHR antagonist (Veldhoen et al., 2009) with the implication that endogenous AHR ligands stimulate $T_H 17$ commitment. Furthermore, *Ahr* knockout models exhibit attenuated $T_H 17$ differentiation, substantiating AHR involvement (Kimura et al., 2008). Despite the diversity of immunological effects prompted by the AHR, little is known regarding a mode of action, but it is likely to involve cross-talk mechanisms. Notwithstanding, the immune-suppressive activity exhibited by the AHR raises the question- can the AHR represent a novel drug target for the treatment of inflammatory or autoimmune conditions?

Recently, an established *in vivo* model of multiple sclerosis, experimental acute encephalitis (EAE) has been shown to be ameliorated by the prototypical AHR agonist 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) and yet, be enhanced by FICZ, also an AHR ligand (Quintana et al., 2008; Veldhoen et al., 2008). Additionally, non-obese diabetic (NOD) mice predisposed to autoimmune diabetes fail to develop

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diabetes whilst exposed to TCDD (Kerkvliet, 2009). Both are suspected to involve reprogramming of T cells. Whilst illustrating the involvement of AHR, the use of polycyclic hydrocarbon agonists is not therapeutically viable due to the perceived inherent toxicity associated with AHR activation. Indeed, the AHR has a dubious reputation as a mediator of various modes of toxicity, including the conversion of procarcinogens into genotoxic intermediates through enhanced phase I bioactivity (Sagredo et al., 2006). Deleterious effects result from ligand-activated AHR in combination with its dimerization partner ARNT binding to DREs within AHR target genes, thus facilitating the expression of downstream effectors. We hypothesize that a selective AHR ligand with the capacity to promote dissociation between the beneficial cross-talk modes of AHR action away from its DRE-dependent toxic activity could render the AHR a viable therapeutic target.

This hypothesis of ligand-dependent but DRE-independent AHR activity has been tested utilizing a DNA-binding AHR mutant, identifying numerous genes which were suppressed in response to ligand (Patel et al., 2009). Predominant among these were components of the inflammatory acute phase response (APR), *e.g.* serum amyloid-associated (*Saa1*) and C-reactive protein (*Crp*). These targets emphasized the involvement of AHR in the modulation of inflammatory signaling and established a subset of targets to screen and validate potential selective AHR modulators (SAhRMs) *i.e.* AHR ligands with the capacity to promote DNA-independent AHR activity at the expense of DRE-mediated expression. The concept of selective receptor modulators is well established for nuclear hormone receptors, especially within the context of the estrogen receptor, and has been utilized clinically in the treatment of hormone-sensitive cancer. The selective modulator concept has expanded to include other nuclear receptors, however, the idea and therapeutic viability of SAhRMs has not been expounded.

In line with its function as a xenobiotic sensor, the AHR is remarkably promiscuous, binding diverse ligands, including some ER ligands *e.g.* resveratrol, which antagonize canonical AHR signaling (Casper et al., 1999). Like TCDD, resveratrol ameliorates EAE by impacting T cell function (Singh et al., 2007).

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Intriguingly, the selective ER modulator (SERM) Way-169916 demonstrates anti-inflammatory properties in models of rheumatoid arthritis, suppressing the expression of the same APR targets identified with our DNA-binding AHR mutant (Chadwick et al., 2005; Harnish et al., 2006), thus presenting a potential for Way-169916 to influence the DNA-independent repressive action of AHR. We investigated this hypothesis and present evidence that Way-169916 is an AHR ligand and represents the first report of a SAhRM with anti-inflammatory properties.

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Materials and Methods.

Materials. TCDD was a generous gift provided by Dr. Stephen Safe (Texas A. & M., TX). Way-169916 was initially a kind gift from Dr. Robert Steffan (Wyeth Research, PA) and subsequently synthesized in-house. Human and murine recombinant cytokines, IL1beta and IL6 were obtained commercially (PeproTech, Rocky Hill, NJ).

Chemical synthesis. 4-[1-allyl-7-(trifluoromethyl)-1*H*-indazol-3-yl]benzene-1, 3-diol (Way-169916) was synthesized as previously described with minor modifications (Steffan et al., 2004). Comprehensive methods and synthesis scheme are detailed in supplemental information. The structure and purity of Way-169916 were verified by ESI-MS and N.M.R.

Cell culture. The human hepatoma Huh7 cell line was maintained in α-minimal essential medium (α-MEM) (Sigma, St. Louis, MO) supplemented with 8% fetal bovine serum (Hyclone Labs, Logan, UT), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma). Cells were cultured at 37°C in a humidified atmosphere comprised of 95% air and 5% CO_2.

Electromobility shift assay. DRE-specific electrophoretic mobility shift assays (EMSA) were performed using *in vitro* translated human AHR and ARNT proteins. Expression vectors for each protein were translated separately using the T7 TNT kit (Promega Corp., Madison, WI), reactions were modified to include 1.25 mM sodium molybdate to enhance AHR stability. Aliquots (4µl) of AHR and ARNT were combined at a 1:1 molar ratio in HEDG (25 mM HEPES, 1 mM EDTA, 10 mM sodium molybdate and 10% glycerol, pH 7.5) buffer, followed by the addition of 0.25 µl ligand or vehicle, for a total reaction volume equaling 10 µl. Transformation reactions were incubated for 90 min at room temperature, followed by the addition of 15 µl of oligonucleotide binding buffer (42 mM Hepes, 0.33 M KCl, 50%

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glycerol, 16.7 mM DTT, 8.3 mM EDTA, 0.125 mg/ml CHAPS, 42 ng/µl poly dI:dC). After 15 min of

incubation in binding buffer, ~2 x 10^5 cpm of ^{32}P-labeled DRE was added to each reaction. The samples

were mixed with an appropriate amount of 5X loading dye and a half of each sample resolved on a 6%

non-denaturing polyacrylamide gel (Novex, Invitrogen). DRE oligonucleotides (5'-

GATCTGGCTCTTCTCACGCAACTCCG-3' and 3'-ACCGAGAAGAGTGCGTTGAGGCCTAG-5')

were a gift from Dr. M.S. Denison (University of California, Davis, CA.).

Competitive AHR ligand binding assay. Competitive ligand binding assays were performed as

previously described (Flaveny et al., 2009). Briefly, The AHR photoaffinity ligand; 2-azido-3-[^{125}I]iodo-

7, 8-dibromodibenzo-*p*-dioxin (PAL) was synthesized as described previously (Poland et al., 1986).

Hepatic cytosol extracts were isolated from B6.Cg-*Ahr*$^{tm3.1 \ Bra}$ Tg (Alb-cre, Ttr-AHR)1GHP "*humanized*"

AHR mice (Flaveny et al., 2009) by homogenization in MENG (25 mM MOPS, 2 mM EDTA, 0.02%

NaN$_3$, 10% glycerol pH 7.4) containing 20 mM sodium molybdate and protease inhibitor cocktail

(Sigma), followed by centrifugation at 100,000 x g for 1 h. All binding experiments were conducted in

the dark until photo-crosslinking of the PAL had occurred. A saturating amount of PAL (0.21 pmol *i.e.* 8

$\times 10^5$ cpm / tube) were added to 150 µg of cytosolic protein. Samples were then co-incubated with Way-

169916 for 20 min at room temperature. Samples were photolyzed (402 nm, 8 cm, 4 min), and 1 %

dextran-coated charcoal added, followed by centrifugation at 3,000 x g for 10 min to remove unbound

PAL. Labeled samples were resolved on 8 % Tricine polyacrylamide gels, transferred to PVDF

membrane and visualized by autoradiography. Radioactive AHR bands were excised and quantified by γ-

counting.

Competitive ER ligand binding polarization assay. Competitive ER ligand binding assays were

performed using the PanVera Estrogen Receptor-α Competitor Assay (Invitrogen) following

manufacturer's directions. Briefly, vehicle (DMSO) or test compounds were diluted to twice their final

assay concentrations in PanVera supplied ES2 screening buffer and added to 60 x 150 mm borosilicate glass cell culture tubes. A master mix was made of screening buffer with human recombinant ERα added for a final concentration of 6 pmol/µl, and ES2 fluoromone added for a final concentration of 400 nM. The master mix was added to diluted test compounds in a 1:1 volume, mixed gently, and incubated in the dark at room temperature for 2 h. Samples were then measured for fluorescence polarization using the PanVera Beacon 2000 polarization reader with 485 nm excitation and 530 nm emission filters at 25° C.

Short-interfering RNA-mediated knockdown. Huh7 cells were seeded into 6-well plates and cultured overnight in serum and antibiotic-free media. Cells were transfected using the Geneporter 3000 reagent (Thermo Scientific) following the manufacturer's directions. siRNA oligonucleotides (Dharmacon,Lafayette, CO) were transfected at final concentration of 50 nM. Cells were cultured for a further 24 h prior to treatment, as indicated. RNA and protein were harvested as detailed.

Protein isolation and expression analysis. Total protein was isolated from Huh7 cells by lysis with MENG (25 mM MOPS, 2 mM EDTA, 0.02% NaN$_3$, 10% glycerol pH 7.4)/1% NP-40/1x protease inhibitor cocktail. Protein concentrations were assayed using the BCA kit (Thermo Fisher Scientific Inc, Rockford, IL). Protein samples were resolved on 8% Tricine-SDS-PAGE gels and subsequently transferred to PVDF membrane (Millipore, Billerica, MA Membranes were probed as indicated with the following antibodies: rabbit anti-AHR Rpt1 (Affinity Bioreagents, Golden, CO), mouse anti-ARNT MA1-515 (Affinity Bioreagents), rabbit anti-ERα sc-543 (Santa Cruz, Santa Cruz, CA.), rabbit anti-ERβ sc-8974 (Santa Cruz) and mouse anti-β-actin sc-47778 (Santa Cruz).

RNA isolation and reverse transcription. Total RNA was isolated from Huh7 cells cultured in six-well plates using Trizol (Invitrogen). RNA concentration was determined via spectrophotometry at λ 260 nm

and 280 nm. Total RNA was reverse transcribed to cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR. Quantitative PCR was performed on a DNA Engine Opticon® system using DyNAmo™ SYBR® Green reagent (New England Biolabs, Ipswich, MA) following manufacturers instructions. Nucleotide sequences of primers (Integrated DNA Technologies, Coralville, IA) used in this study are as previously published (Patel et al., 2009). In all cases, melting point analysis revealed amplification of a single amplicon. Data acquisition and analyses were achieved using MyIQ software (Biorad, Hercules, CA).

Statistical Analysis. Data were analyzed using the Graphpad Prism 5.0 software package. One-way ANOVA with Tukey-Kramer multiple comparison post-test and Student's t-test were applied to determine statistical significance, $P < 0.05$ were deemed significant and are indicated with an asterisk.

Results.

Way-169916 induces AHR/ARNT dimerization & weakly stimulates DRE binding through direct interaction with AHR. Whilst investigating the mutual cross-talk between AHR and ER, we obtained data which suggested that the non-steroidal SERM Way-169916 (Fig. 1B) may also bind to the human AHR. To confirm this hypothesis we examined the potential of Way-169916 to mimic the characteristics of an AHR agonist *i.e.* to promote the dimerization and subsequent binding of AHR:ARNT to the DRE containing *CYP1A1* enhancer. Electromobility shift analyses using *in vitro* translated AHR identified the formation of an AHR/ARNT/DRE complex following exposure to increasing (1, 2, 5 and 10 μM) concentrations of Way-169916. However, the level of complex formation elicited by Way-169916, even at the highest dose examined, was significantly less than that observed with 20 nM TCDD, the prototypical AHR agonist (Fig. 2A). To further substantiate the status of Way-169916 as a putative AHR ligand we performed competitive *in vitro* ligand binding assays using a high-affinity photo-reactive AHR ligand and human AHR expressing Huh7 hepatoma cytosol. Competition for photoaffinity ligand binding was not detected with vehicle alone but was observed with increasing concentrations of α-naphthoflavone (αNF), an established AHR ligand with a maximal displacement of ~80% achieved at 0.1 μM. Similarly, increasing concentrations of Way-169916 were able to effectively compete with the photoaffinity ligand for AHR binding with ~15% displacement at 0.1 μM, ~50% at 1 μM and ~65% at 100 μM (Fig. 2B). Such data confirm the status of Way-169916 as a ligand for the human AHR.

The AHR ligand Way-169916 fails to stimulate xenobiotic gene expression. Having established Way-169916 as a ligand for AHR, we wished to examine the potential of Way-169916 to drive AHR-dependent gene expression. Exposure of Huh7 cells to 10 nM TCDD for 5 h resulted in an expected robust (>100-fold) increase in *CYP1A1* mRNA level over vehicle treated controls, as assessed by quantitative PCR (Fig. 3A). In contrast, Way-169916 failed to elicit a significant induction of *CYP1A1*

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mRNA above that of vehicle-treated control over the same time frame. Similarly, the AHR-responsive target genes *CYP1A1* and *CYP1A2*, whilst expressed in response to TCDD, were refractory to induction by Way-169916 in MCF-7 cells, revealing that failure to induce AHR-responsive genes is neither gene nor cell-type specific (Supp. Fig. 2.). Further examination using HepG2 cells stably integrated with a *CYP1A1* enhancer-luciferase reporter construct revealed that the failure of Way-169916 to induce AHR dependent gene expression was not associated with dose or temporal issues (Fig. 3B). Although a modest increase in reporter activity was observed with Way-169916 at 3 h, this proved not to be statistically significant, suggesting very weak agonist activity. We also examined the capacity of Way-169916 to antagonize TCDD-mediated expression of an AHR-dependent reporter construct. HepG2 (40/6) cells harboring the DRE-driven pGudluc 6.1 reporter were exposed to vehicle, TCDD, or in combination with increasing concentrations of Way-169916, and reporter expression was assayed. Exposure to TCDD prompted a robust induction of reporter activity, which was significantly repressed upon co-exposure to Way-169916 (Fig. 4.). These data in conjunction with our ligand-binding data indicate that Way-169916 is an extremely weak agonist with the capacity to be an effective competitive antagonist with regard to canonical DRE-dependent AHR transactivation.

Way-169916 stimulates DRE-independent AHR activity to repress APR gene expression. Evidence exists indicating the capacity of AHR to influence gene expression through the phenomenon of receptor cross-talk. Recent data obtained using a non-DRE binding AHR mutant has identified cytokine regulated components of the inflammatory acute phase response (APR) as being negatively regulated by AHR in the absence of cognate response element interaction (Patel et al., 2009). We thus assessed the potential of Way-169916 to modulate non-DRE dependent gene expression using APR targets as a model. Exposure of Huh7 cells to interleukin-1 beta (IL1β) elicited the expression of the APR genes s*erum amyloid-associated-1* (*SAA1*), *C-reactive protein* (*CRP*) and *haptoglobin* (*HP*), as determined by quantitative PCR (Figs. 5A). Pre-treatment with AHR ligands, αNF, βNF, B[*a*]P, M50354 or TCDD,

prior to IL1β exposure has been shown to result in a significant attenuation of acute phase gene expression (Patel et al., 2009). Treatment with 10 µM Way-169916 also resulted in significant repression of *SAA1*, *CRP* and *HP* mRNA following IL1β induction (Fig. 5.). Furthermore, exposure of Huh7 cells to increasing concentrations of Way-169916 identified a dose-dependent suppression of cytokine-mediated APR expression (Fig. 5B.).

Attenuation of APR gene expression by Way-169916 is independent of ER. Way-169916 was originally developed as a SERM and has been shown to repress gene expression in rodent models of inflammation by a mechanism involving the estrogen receptor (Chadwick et al., 2005). Using a fluorescent polarization competition assay we were able to confirm the status of Way-169916 as a ligand for ER (Fig. 6A). Thus, despite being a ligand for AHR, our data illustrating attenuation of APR gene expression could be attributed to the SERM nature of Way-169916. We examined this scenario by co-treating Huh7 cells with Way-169916 and the ER antagonist ICI-182780 (ICI) at a dose previously shown to effectively inhibit ER-dependent signaling, prior to IL1β induction of APR gene expression. Analysis of human *SAA1* mRNA revealed the same level of *SAA1* repression with Way-169916 regardless of co-exposure to ICI (Fig. 6B). Further confirmation of the ER-independent action of Way-169916 in the context of Huh7 cells was obtained from protein expression studies which demonstrated a lack of detectable ERα or ERβ expression by this cell line (Fig. 6C). Such data indicate that ER expression is not necessary to facilitate repression of *SAA1* by Way-169916 at least in the context of the Huh7 cell line.

Suppression of APR expression by Way-169916 is AHR-dependent. Having established that Way-169916 is an AHR ligand with the capacity to repress the cytokine-mediated expression of acute phase reaction components independently of ER, we wished to investigate if the AHR/ARNT complex is a requirement for repression. Previous data indicated APR repression following exposure to AHR antagonists thus rendering them unsuitable for AHR inhibition in this context (Patel et al., 2009). We

therefore utilized siRNA to ablate AHR activity. Preliminary studies demonstrated that siRNA targeted against AHR alone was inefficient with regards to lowering AHR protein levels to a point that influenced repression of APR expression. We therefore used a double knockdown approach; using siRNA targeted against AHR and its obligatory dimerization partner ARNT. Huh7 cells transfected with a non-targeting scrambled siRNA oligonucleotide were sensitive to Way-169916-mediated repression of *SAA1* (Fig.7.). By contrast, cells with diminished protein expression of AHR and ARNT arising from transfection with siRNA oligonucleotides targeted against AHR and ARNT were refractory to Way-169916 and demonstrated no significant repression of *SAA1* (Fig. 7B.). These data indicate that the AHR/ARNT complex is necessary and sufficient to mediate Way-169916-mediated APR repression.

Way-169916 attenuates cytokine-mediated gene expression in a context-specific manner. To determine if the failure of DRE-dependent transcription together with the repression of APR gene expression is due to a globally repressive nature of Way-169916, we examined its effect upon additional inflammatory targets. Exposure of Huh7 cells to IL1β can elicit induction of the pro-inflammatory cytokine *IL8* (Fig. 8). In contrast to the observed Way-169916-mediated repression of the APR targets *SAA1*, *CRP* and *HP*, *IL8* exhibited no significant repression following co-treatment with IL1β and Way-169916 (Fig. 8). Such data indicates that repression of inflammatory gene expression by the AHR ligand Way-169916 is context-specific, eliminating a role for Way-169916 as a global inflammatory transcription inhibitor.

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Discussion.

The clinical application of SERMs has been a reality for many years, even pre-dating the evolution of the SERM concept. It was the seminal observation that nuclear hormone receptors, particularly the steroid receptors, *e.g.* estrogen and glucocorticoid receptors, exhibit the phenomenon of DNA-independent transrepression and are not restricted solely to direct gene activation events through their cognate response elements and thus identified the physiological essence for selectivity (Reichardt et al., 1998). Whilst evolutionarily and structurally dissimilar to the steroid nuclear hormone receptor family, the aryl hydrocarbon receptor (AHR), the only known ligand-activated member of the basic helix-loop-helix/PAS family of transcription factors behaves in an analogous fashion to elicit gene induction (Beischlag et al., 2008). Binding of an agonist *e.g.* TCDD, increases the affinity of AHR for its cognate response element and facilitates the expression of AHR-responsive genes, principally members of the CYP1 P450 family, often leading to genotoxic stress or endocrine disruption (Safe, 1995; Sagredo et al., 2006). Despite the functional similarity between AHR and the nuclear receptor family, the notion of therapeutic modulation of AHR has, with the notable exception of its ability to antagonize ER function and mitigate hormone-sensitive tumor growth, largely been overlooked (Safe and McDougal, 2002). However, numerous recent reports have demonstrated the integration of the AHR into signaling pathways not directly connected with xenobiotic metabolism. Activation of AHR with putative endogenous ligands has been shown to exert profound effects upon the immune system, suggesting a physiological role for AHR (Stevens et al., 2009).

The association between AHR and the immune system, whilst known for a number of years from a toxicological immunosuppressive perspective (Kerkvliet, 2002), is only now being considered for its potential therapeutic applications in the treatment of autoimmune or inflammatory disease (Kerkvliet, 2009; Quintana et al., 2008; Veldhoen et al., 2008). Currently, the highly characterized role of AHR in mediating various aspects of toxicity has been a major obstacle in realizing the latent therapeutic modulation of immune signaling by the AHR. Recently, we have demonstrated that a number of genes associated with the inflammatory cytokine-mediated acute phase response, *e.g. Saa1* and *Crp* are

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negatively regulated by exposure to AHR agonists (Patel et al., 2009). The promoters of both genes contain putative but degenerate DRE sequences and could therefore be influenced through AHR binding to these elements. However, *in vivo* studies utilizing an AHR mutant (AHR[A78D]) which confers non-DRE binding status upon the AHR, whilst retaining ligand binding and heterodimerization functions, revealed these APR genes as being repressed by AHR in a DRE-independent fashion, possibly involving a cross-talk mode of action and the inhibition of NF-κB function in a promoter-specific manner. Intriguingly, the weak AHR partial agonist alpha-naphthoflavone (αNF) retained the capacity to repress APR gene induction with minimal CYP1A1 induction and thus supports the SAhRM hypothesis.

The concerted identification of AHR-mediated APR repression and establishment of the SAhRM hypothesis prompted us to screen for compounds with SAhRM activity. Preliminary evidence suggested that Way-169916, a non-steroidal SERM, may be an AHR ligand. Competitive binding assays confirmed Way-169916 as an AHR ligand and corroborated its status as an ER ligand, although with an apparent affinity three orders of magnitude less relative to ER. Thus, like resveratrol, Way-169916 represents a dual specificity ER/AHR ligand (Bhat and Pezzuto, 2001; Casper et al., 1999). Interestingly, resveratrol exhibits antagonistic activity for both ER and AHR. We therefore investigated the ability of Way-169916 to promote AHR transformation, DRE binding and stimulate AHR-dependent gene expression. Way-169916 proved to be a very weak partial AHR agonist in the context of both endogenous *CYP1A1* mRNA expression and heterologous DRE-reporter activity. This weak activity can be attributed to greatly diminished association between AHR and its response element relative to typical AHR agonists. At this time, it is unclear whether Way-169916 is inefficient at inducing AHR transformation from the chaperone-bound complex into its heterodimeric 6S conformation and/or influences AHR structurally to directly diminish DRE affinity. In its capacity as a SERM, Way-169916 could influence DRE-dependent gene expression through the documented cross-talk of ER and AHR (Matthews and Gustafsson, 2006). This scenario was eliminated in the context of our Huh7-based expression analyses due to the absence of

ERα and ERβ expression in this cell line, but emphasizes a requirement to fully characterize putative SAhRMs due to the frequent overlap between AHR and ER with regard to ligands, modes of activation, repression and mutual antagonism.

The revelation that Way-169916 binds AHR yet invokes minimal canonical DRE-driven AHR signaling and is thus unlikely to promote subsequent AHR toxicities fulfills one of the major criteria of a putative SAhRM. Indeed, previous *in vivo* gene profiling analyses in multiple tissues did not highlight the induction of any characteristic DRE-driven AHR targets in response to Way-169916 (Keith et al., 2005). The second defining facet of SAhRM activity is demonstrated with the repression of inflammatory APR gene targets by Way-169916. Anti-inflammatory action by Way-169916 is reported in various rodent models of inflammation, including rheumatoid arthritis, chronic intestinal inflammation, and ischemia-reperfusion injury, which highlights an ER-dependent suppression of NF-κB activity to account for diminished APR expression (Booth et al., 2007; Chadwick et al., 2005; Harnish et al., 2006). Whilst this mechanism of action is valid, it fails to explain the observed repression in Huh7 cells which lack ER expression. Previous data using the DNA-binding AHR mutant, various structurally diverse AHR ligands, and the observation that Way-169916 is an AHR ligand, all support our contention that repression of APR targets can occur through AHR independently of ER. Although different experimental approaches were employed, it is interesting to note that despite having a much lower affinity for AHR, the Way-169916-mediated reduction in APR expression reported here is similar to that obtained through the ER-dependent mechanism (Keith et al., 2005).

As proof of the SAhRM concept, an examination of the mechanism behind the AHR-dependent APR repression is beyond the scope of this manuscript. It is plausible that inhibition through AHR cross-talk is the underlying cause of repression. Such cross-talk between AHR and inflammatory mediators is not without precedent. Indeed, many of the toxic immunosuppressive outcomes attributed to sustained AHR

activation by environmental contaminants are thought to involve cross-talk signaling (Tian et al., 2002). Studies showing APR repression with TCDD indicate a requirement for AHR translocation into the nucleus, as an AHR mutant deficient in translocation failed to mediate repression, thus eliminating a mechanism involving the AHR-mediated nuclear blockade of a necessary regulator (Patel et al., 2009). Furthermore, heterodimerization is also a prerequisite for AHR-mediated APR repression, as evidenced by AHR mutants lacking affinity for ARNT. We are currently investigating the molecular basis for SAhRM activity with the aim of identifying novel SAhRMs that exhibit enhanced efficacy and selectivity.

In conclusion, it is our contention that the anti-inflammatory non-steroidal SERM Way-169916 also exhibits affinity for the AHR and in doing so retains anti-inflammatory characteristics with regard to APR expression. Furthermore, the AHR-mediated anti-inflammatory activity occurs in the absence of potentially toxic canonical DRE-dependent gene expression. As such, Way-169916 represents the first example of a selective AHR modulator. Importantly, these data lend credence to the SAhRM concept and support the notion that the AHR represents a viable therapeutic target for the treatment of inflammatory disorders.

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Footnotes

Figure legends.

Figure 1. Chemical structures of AHR ligands. The chemical structures of the prototypical AHR agonist TCDD (**A**) is illustrated together with the putative selective AHR modulator (SAhRM) Way-169916 (**B**).

Figure 2. Way-169916 induces AHR/ARNT dimerization and weakly promotes DNA binding through direct interaction with AHR. (**A**) Gel shift analysis of AHR/ARNT DNA binding in the presence of Way-169916. *In vitro* translated AHR and ARNT were incubated with a ^{32}P-labeled DNA probe encompassing the *Cyp1a1* enhancer element. Binding reactions were co-incubated with vehicle, TCDD, or increasing concentrations of Way-169916, as indicated. Specific binding of transformed AHR/ARNT to the probe is indicated with *. (**B**) AHR ligand binding analysis. Competitive *in vitro* ligand binding assays were performed using human AHR containing Huh7 cytosol. Cytosolic extracts were incubated with vehicle, increasing concentrations (0.1, 1, 10 and 100 μM) of Way-169916 or α-napthoflavone (αNF) in the presence of the photo-reactive AHR ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin , as indicated.

Figure 3. Way-169916 is a weak partial agonist for AHR. (**A**) Huh7 cells were treated with either vehicle (DMSO), 10 nM TCDD or 10 μM Way-169916, as indicated for 4 h. Total RNA was isolated, reverse transcribed to cDNA and used as a template for quantitative RT-PCR analysis of human *CYP1A1* expression. Data represent mean *CYP1A1* mRNA level normalized against human r*L13A* mRNA. (**B**) Hepa1.1 cells were treated with either vehicle (DMSO), 10 nM TCDD, or 10 μM Way-169916. At the indicated time-points, cells were lysed and luciferase activity determined. Data represent mean luciferase activity ± SEM. Significant induction is indicated by asterisks, n.s. (not significant).

Figure 4. Way-169916 competes with AHR agonists to antagonize DRE-mediated expression.
HepG2 (40/6) cells harboring the stably integrated AHR-dependent pGudluc 6.1 reporter construct were treated with either vehicle (DMSO), 2 nM TCDD, or TCDD, together with increasing concentrations of Way-169916, as indicated for 5 h. Cells were lysed and luciferase activity determined. Data represent mean luciferase activity ± SEM.

Figure 5. **Way-169916 suppresses cytokine-mediated expression of acute phase reaction components.** (**A**) Huh7 cells were pre-treated for 1 h with vehicle (DMSO) or 10 μM Way-169916, then were incubated for a further 4 h while exposed to 2 ng/ml IL1β. Total RNA was isolated, reverse transcribed to cDNA and used as a template for quantitative RT-PCR analysis of human *SAA1*, *CRP* and *HP* expression. Data represent mean *SAA1*, *CRP* and *HP* mRNA level normalized against human r*L13A* mRNA. (**B**) dose-response analysis of Way-169916-mediated *SAA1* repression. Huh7 cells were pre-treated for 1 h with vehicle (DMSO) or increasing concentrations of Way-169916, then were incubated for a further 4 h while exposed to 2 ng/ml IL1β. Total RNA was isolated, reverse transcribed to cDNA, and used as a template for quantitative RT-PCR analysis of human *SAA1* expression. Data represent mean *SAA1* mRNA level normalized against human r*L13A* mRNA.

Figure 6. Way-169916 mediated repression of inflammatory gene expression is independent of ERα/β. (**A**) ER competition binding assays were performed using the PanVera Estrogen Receptor-α Competitor Assay. Fluoromone polarization was determined at 485 nm and 530 nm. Data represent mean polarization ± SEM. (**B**) Huh7 cells were pre-treated for 1 h with 1 μM ICI 182780 or 10 μM Way-169916 in isolation or in combination prior to 4 h exposure to 2 ng/ml IL1β. Total RNA was isolated, reverse transcribed to cDNA and used as a template for quantitative RT-PCR analysis of human *SAA1* expression. Data represent mean *SAA1* mRNA level normalized against human r*L13A* mRNA. (**C**)

Protein expression of ERα/β in Huh7 cells was analyzed by western immunoblot. Protein from MCF-7 cells was used as a positive control for ER expression. β-actin was used as a loading control.

Figure 7. Way-169916-mediated SAA1 repression is AHR-dependent. (**A**) siRNA-mediated knockdown of AHR/ARNT expression in Huh7 cells transfected with scrambled control or AHR and ARNT-specific siRNA was assessed by western immunoblotting. 48 h post-transfection, cells were harvested and protein isolated. Protein blots were probed for AHR, ARNT and β-actin was used as a loading control. (**B**) 48 h post-transfection with scrambled control or AHR and ARNT-specific siRNA Huh7 cells were pre-treated for 1 h with vehicle (DMSO) or 10 μM Way-169916. Cells were then incubated with 2 ng/ml IL1β for a further 4 h. Total RNA was isolated, reverse transcribed to cDNA and used as a template for quantitative RT-PCR analysis of human *SAA1* expression. Data represent mean *SAA1* mRNA level normalized against human r*L13A* mRNA.

Figure 8. Way-169916 mediated repression of cytokine mediated gene expression is context specific. Huh7 cells were pre-treated for 1 h with 10 nM TCDD or 10 μM Way-169916 prior to 4 h exposure to 2 ng/ml IL1β. Total RNA was isolated, reverse transcribed to cDNA, and used as a template for quantitative RT-PCR analysis of human *IL8* expression. Data represent mean *IL8* mRNA level normalized against human r*L13A* mRNA.
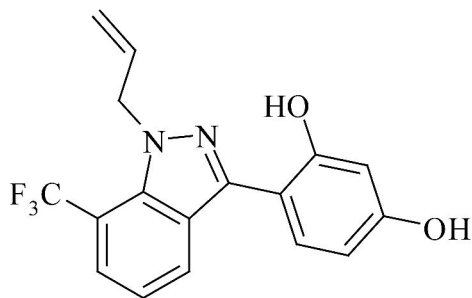
Figure 1

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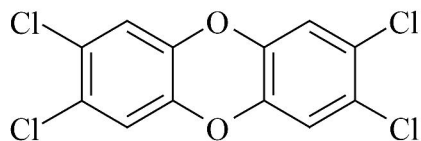
TCDD
2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin

B

Way-169916
4-[1-allyl-7-(trifluoromethyl)-1H-indazol-3-yl]benzene-1, 3-diol

Figure 2

A

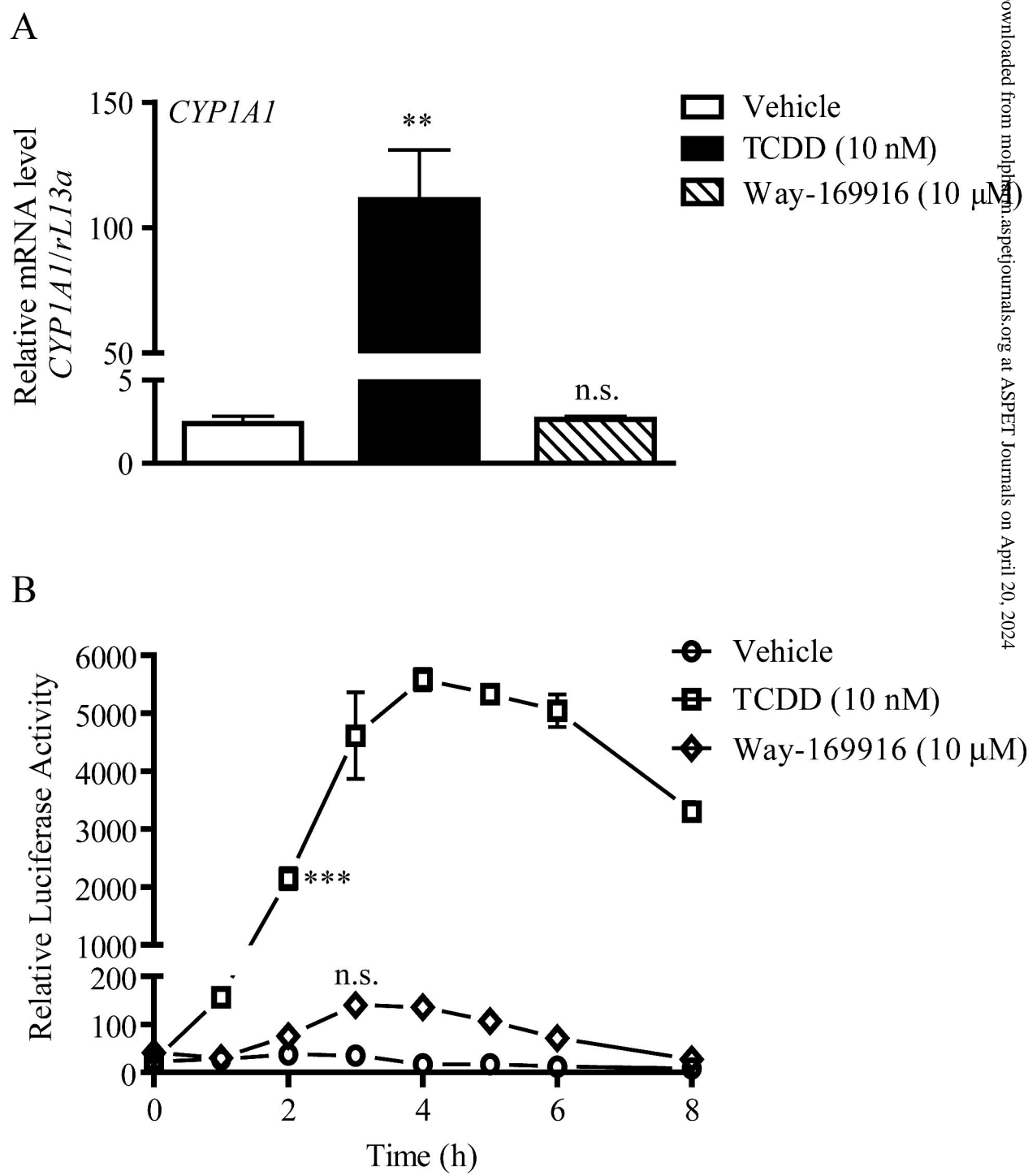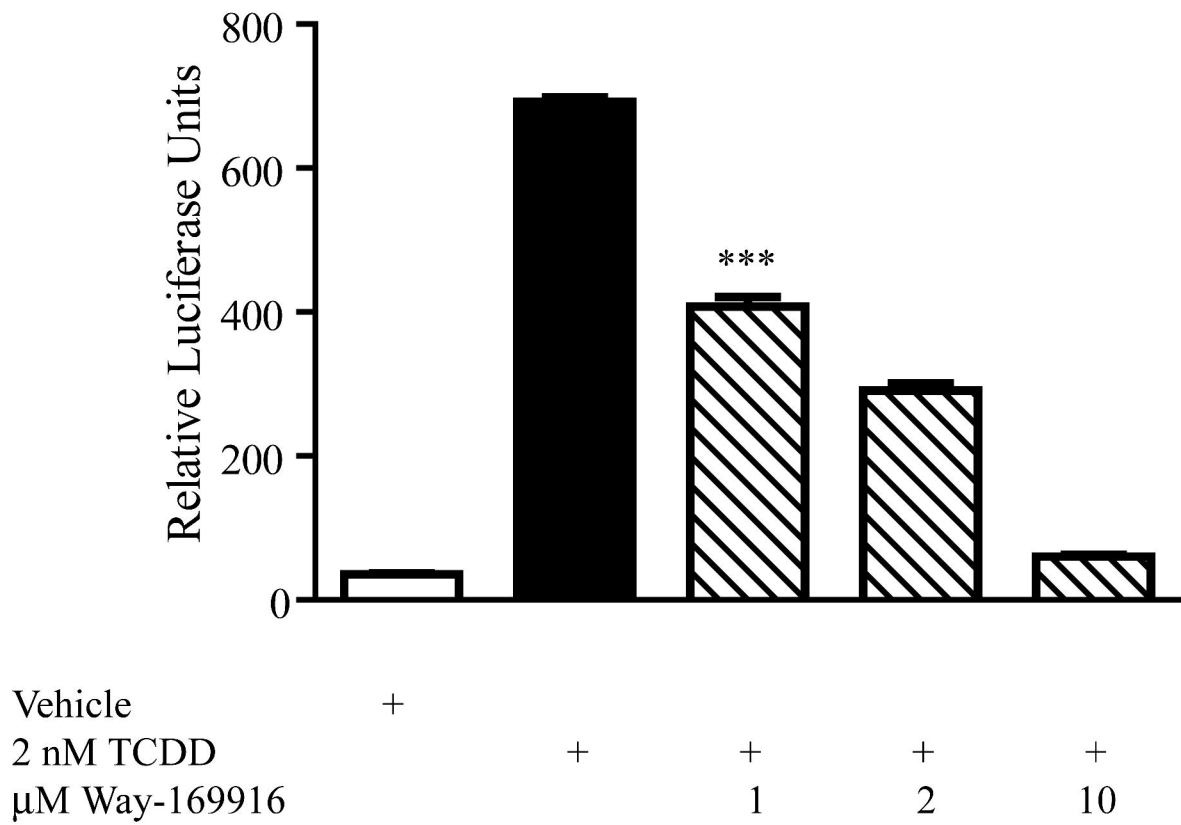
* ◀AHR/ARNT

◀non-specific

AHR	+	+	+	+	+	+	+	+
ARNT		+	+	+	+	+	+	+
Vehicle			+					
20 nM TCDD				+				
Way-169916					1	2	5	10 μM

B

* ◀AHR

	0.1	1	10	100		0.1	1	10	100	(μM)
Vehicle	+									
Way-169916		+	+	+	+					
α-NF							+	+	+	+

Figure 3

Figure 5

A

SAA1

Relative mRNA level *SAA1*/rL13a

***

CRP

Relative mRNA level *CRP*/rL13a

***

HP

Relative mRNA level *HP*/rL13a

***

Vehicle	+		
2 ng/ml IL1β		+	
10 µM Way-169916			+	+

B

SAA1

Relative mRNA level *SAA1*/rL13a

Vehicle	+				
2 ng/ml IL1β		+	+	+	+	+
µM Way-169916			1	2	5	10

Figure 6

A

- competitor
vehicle
1 nM E$_2$
10 nM E$_2$
50 nM E$_2$
100 nM E$_2$
1 µM E$_2$
10 µM E$_2$
10 µM Way 169916

***

Polarization (mP)

0   50   100   150   200

B

Relative mRNA level hSaa-1/hrL13a

2.5
2.0
1.5
1.0
0.5
0.0

$P<0.05$

* *

Vehicle	+					
2 ng/ml IL-1β/IL-6		+		+	+	+
1 µM ICI 182780			+	+	+	+
10 µM Way 169916				+	+	+

C

66 kDa-   ERα   ERβ
           MCF-7 Huh-7   MCF-7 Huh-7

42 kDa-   β-actin   β-actin
           MCF-7 Huh-7   MCF-7 Huh-7

Figure 7

Figure 8