Discovery and mechanistic characterization of a novel AhR receptor ligand that induces apoptosis in cancer cells

Edmond O'Donnell

School of Medicine, University of California, Davis

Hyo Sang Jang

Oregon State University

Daniel Liefwalker

Oregon Health & Science University University Hospital: Oregon Health & Science University Hospital

Nancy Kerkvliet

Oregon State University

Siva Kumar Kolluri (✉ siva.kolluri@oregonstate.edu)

Oregon State University

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Abstract

Background: The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor and a member of the bHLH/PAS (basic Helix-Loop-Helix/Per-Arnt-Sim) family of proteins. The AhR was cloned and characterized for its role in mediating the toxicity of dioxins. Subsequent research has identified AhR's role in the suppression of cancer cell growth. We hypothesized that the AhR is a molecular target for therapeutic interventions, and that activation of the AhR by select AhR modulators in cancer cells could have anti-cancer properties including cell death. This study describes the discovery and characterization of a new class of anti-cancer agents targeting the AhR.

Methods: We employed two independent small molecule screening approaches to identify novel modulators of the AhR with unique anti-cancer properties. We established the AhR selective effects of a highly selective modulator in cancer cells with or without the AhR expression and identified the mechanism of action of this anti-cancer compound.

Results: We report the identification of CGS-15943, uniquely selective AhR modulator, that activates AhR signaling and induces apoptosis in an AhR-dependent manner in liver and breast cancer cell models. Investigation of the downstream signaling pathway of this newly identified modulator revealed novel upregulation of Fas-ligand, which is required for AhR-mediated apoptosis.

Conclusions: We identified CGS-15943 as a novel AhR-selective modulator with anti-cancer properties using two parallel and distinct screening strategies. This compound induced AhR-dependent apoptosis in multiple mouse and human liver cancer cells. Our results provide a basis for the development of a new class of anti-cancer therapeutics targeting an underappreciated molecular target, the AhR.

Background

The aryl hydrocarbon receptor is a ligand-activated transcription factor belonging to the bHLH-PAS (basic-helix-loop helix-Per/ARNT/Sim) protein family [1]. The AhR is localized in the cytosol [2] where it is complexed with the chaperone molecules HSP90 [3], p23 [4, 5] and XAP2 (reviewed in [1]). Upon ligand binding, the AhR translocates to the nucleus where it complexes with its obligate heterodimer partner, Aryl Hydrocarbon Receptor Nuclear Translocation (ARNT) [6], to regulate the transcription of gene targets. The AhR has been extensively studied for its role in mediating the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [7]. The AhR activates a diverse range of genetic programs in the cell. The most well-known gene targets of AhR-activation are those involved in xenobiotic metabolism. Many ligands of the AhR upregulate phase I and phase II metabolism genes, including CYP1A1, CYP1B1, and UGT1A1 (Reviewed in [1]). In addition to metabolism-associated genes, the AhR has been shown to regulate transcription of genes involved in proliferation, development, and immunomodulation [1, 8, 9]. In particular, the AhR functions associated with cellular proliferation have been studied both in cell culture models [10, 11] and in AhR-positive and AhR-null mice, revealing a potential role of the AhR as a tumor

suppressor gene [12–14]. TCDD inhibits proliferation of hepatocytes by increasing expression of the CDK inhibitor p27^{kip1} [10], and loss of AhR expression in hepatocytes results in decreased proliferation [11].

Just as the range of gene targets of the AhR is broad, so too are the ligands capable of initiating these responses. These include TCDD as well as dioxin-like compounds (DLCs), halogenated polyaromatic hydrocarbons, and putative endogenous AhR activators including tryptophan metabolites 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) [15]. In recent years there has been a steady increase in the identification and understanding of the diverse array of AhR ligands. For example, there is significant interest in the AhR-mediated immunomodulatory effects of the tryptophan metabolite kynurenine [16]. An aryl-hydrocarbon receptor antagonist purine derivative StemRegenin1 was shown to promote expansion of human hematopoietic stem cells (HSCs) [17], which has recently progressed to clinical trials for expanding CD34 + HSCs umbilical cord blood [18]. Excitingly, the landscape of AhR activators and their phenotypic effects continues to expand.

In addition to the ligand-mediated actions, *in vivo* studies have also suggested a role for the AhR in cancer outside of exogenous ligand activation. AhR-null transgenic adenocarcinoma of the mouse prostate (TRAMP) mice develop prostate tumors faster and more aggressively than AhR-positive TRAMP mice [13]. Similarly, AhR null mice injected with diethylnitrosamine (DEN), which induces liver tumors after a single dose, exhibit increased expression of markers of proliferation and decreased expression of tumor suppressor genes compared with AhR-positive counterparts [12]. Taken together, there is a role for the AhR in regulating cancer growth [24], and activation of the anti-cancer effects of the AhR with appropriate ligands is an exciting opportunity.

Given the therapeutic potential of AhR modulation, it is necessary to tease apart AhR activation from the adverse effects of dioxins in order to realize a new class of AhR-based therapeutics. Thus, we identified and characterized small molecules already in use in the clinic that activate the AhR. We and others previously showed that the immunomodulatory agent leflunomide, used in the treatment of rheumatoid arthritis, is an AhR ligand [19, 20]. In addition, we showed that the AhR mediates some of the effects of leflunomide in inhibiting melanoma cells [21]. With respect to cellular proliferation, we have shown that the tyrosine kinase inhibitor and AhR-ligand SU5416 (semaxanib) induces a p21-dependent growth inhibition in liver cancer cells. Likewise, our group has shown that the anti-androgen flutamide is an AhR ligand that suppresses hepatocellular carcinoma cell proliferation through AhR-dependent induction of TGF- β 1 [22]. Beyond suppression of cellular proliferation, we have also shown that the selective-estrogen receptor modulator raloxifene activates the AhR and induces apoptosis in liver and MDA-MB-231 breast cancer cells in an AhR-dependent manner [23].

In the present study, we utilized two independent small molecule screening approaches to identify AhR ligands with ability to activate the AhR without necessarily requiring canonical xenobiotic response element driven transcription. Excitingly, we identified the triazoloquinazoline non-xanthine adenosine A₁ and A_{2A} antagonist CGS-15934 as a novel AhR ligand [25]. We showed the ability of CGS-15943 to induce

AhR-dependent apoptosis in mouse hepatoma and human hepatocellular carcinoma cells, as well as in triple negative breast cancer cells. The selective AhR-dependent nature of CGS-15943 in these model systems is unique. In characterizing the downstream effects of this novel AhR-ligand, we showed that CGS-15943 induces apoptosis thorough induction of FasL and subsequent activation of the extrinsic and intrinsic caspase cascades.

Methods

Reagents

CGS-15943 and MRS1220 were purchased from Tocris. The purity of CGS-15943 was 100% as determined by HPLC analysis. The purity of MRS 1220 was 99.1% as determined by HPLC analysis. Cycloheximide was purchased from Sigma. All other reagents were purchased from Sigma unless otherwise indicated.

Cell culture models of differential AhR expression

Hepa1, TAO, C4, and vT{2} cells were used as described previously [5]. HEK293T, HepG2, and MDA-MB-468 cells were purchased from ATCC. All cells were cultured in DMEM with antibiotics (streptomycin and penicillin) from Mediatech, Inc. supplemented with 10% FBS (Tissue Culture Biologicals). Cells were grown in a humidified 5% CO₂ atmosphere.

AhR ligand binding assays

Competitive ligand binding assays were performed with [³H]-3MC as described previously [44]. Differential proteolysis assays were performed by incubating whole cell Hepa1 extracts prepared as described previously [21] with subtilisin at pre-determined time intervals, quenching reactions with SDS-containing buffer, and analyzing differential AhR proteolysis (band intensity) by Western blot.

Immunofluorescence and flow cytometry

Immunofluorescence experiments for nuclear translocation were performed as described previously [5, 16]. DAPI staining was used to assess nuclear fragmentation as described previously [45]. Briefly, cells undergoing apoptosis were collected by centrifugation, fixed, and stained overnight with DAPI (Molecular Probes, Invitrogen). Quantitation of apoptosis was determined by dividing the number of apoptotic cells (condensed/fragmented nuclear morphology) by the total number of cells counted. At least 300 cells were counted per slide, and experiments were performed in triplicate.

Flow cytometry was performed with an FC500 as described previously [20, 21]. Annexin V detection was performed with the Biovision Annexin V kit, using a FITC tagged Annexin V antibody for single stain experiments, and Annexin V-PE-Cy5 for dual stain (GFP) experiments. The JO2 and CD95 antibodies for detection of Fas and FasL, respectively, were a kind gift from the laboratory of Dr. Nancy Kerkvliet.

Reporter gene analysis

XRE-based reporter gene assays were performed as described previously [20]. Briefly, Hepa1.1 cells were plated in 96 well plates at a density of 1×10^4 cells/well, grown overnight, and treated with compounds the following day for 24 hours [46, 47]. For the GAL4/AhR screening assay, HEK293T cells were seeded in 96 well plates at a density of 1×10^4 cells/well in 100 μ L of 10% DMEM and grown overnight. The following day, each well was transfected with 70 ng of Gal-Luciferase and 10 ng each of GAL-AhR, GAD-ARNT, and B-GAL, a plasmid expressing beta-galactosidase under a constitutive promoter that was used to normalize for transfection efficiency. Transfections were performed by combining (per well) DNA with 200 nL of PLUS reagent (Invitrogen, Carlsbad USA) in a total of 10 μ L of OPTI-MEM (Invitrogen), incubating this mixture for 15 minutes, and combining it with 200 nL Lipofectamine (Invitrogen) diluted in 10 μ L of OPTI-MEM. The resulting solution (20 μ L/well) was incubated for an additional 15 minutes and then added directly to cells. Transfection solutions were prepared as a master mix to minimize well-to-well transfection variations. Transfected cells were incubated overnight and treated with compounds as described above for the XRE-based screen. Analysis of luciferase activity from the GAL/AhR screening system was performed essentially as described above, with minor modifications. Cells were lysed in 150 μ L of cell lysis reagent, for which 100 μ L of the lysate was used for measurement of luciferase activity and 10 μ L was used for analysis of beta-galactosidase activity.

Gene expression analysis

Semi-quantitative gene expression analysis was performed as described previously [5, 17]. qPCR analysis was performed as described previously [21]. The relevant primers for qPCR analysis are described in the aforementioned references. Additional semi-quantitative PCR primer sequences were as follows: BAX FP: 5'-CAA GAA GCT GAG CGA GTG TC-3', RP: 5'-TCA GCC CAT CTT CTT CCA G-3'. Additional qPCR primer sequences were as follows: mouse CYP1A1: FP 5'-GAC CCT TAC AAG TAT TTG GTC GT-3', RP 5'-GGT ATC CAG AGC CAG TAA CCT-3'; mouse FasL FP: 5'-GCC CAT GAA TTA CCC ATG TCC-3', RP: 5'-ATT TGT GTT GTG GTC CTT CTT CT-3'; mouse GAPDH: FP 5'-AGG TCG GTG TGA ACG GAT TTG-3', RP 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'; and human FasL FP: 5'-GCT GAA AGA GGC CAA TGC CCC A-3', RP: 5'-CTG TTA GTT TCA CCG ATG GCT CAG G-3'.

For targeted gene expression analysis of apoptosis associated genes, we used the PAMM-012A Apoptosis PCR Array from SA Biosciences (Qiagen) according to the manufactures recommended protocol. Data were analyzed using the online SA Biosciences analysis tools available at: <http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>.

AhR knockdown

Generation of human HepG2 cells stably expressing shRNAs to knockdown AhR was performed by lentiviral-mediated transduction as described previously [16]. For doxycycline-inducible AhR knockdown, we used the same vectors described previously [16]. Generation of HepG2 cells stably expressing shRNAs (non-inducible), we used the pLKO.1 lentivirus system. All vectors were purchased from Open Biosystems (Thermo Scientific) and Addgene. Transient knockdown of AhR in Hepa1 cells was achieved with morpholinos targeting the AhR transcription start site. Morpholinos were linked to a polyarginine-tag to facilitate uptake after addition to cell culture media, and a FITC label to confirm uptake. Morpholinos

were a kind gift from the laboratory of Dr. Nancy Kerkvliet. Transient knockdown of AhR in human HepG2 cells was performed using siRNAs from Dharmacon (Thermo Scientific) for luciferase (control) and AhR (proprietary sequence). Transfection of siRNAs was performed with DharmaFECT-1 (Thermo Scientific) according to the manufacturer's recommended protocol. MDA-MB-468 inducible knockdowns were prepared as described previously [21].

FasL knockdown

Knockdown of FasL in mouse Hepatoma (Hepa1 cells) was performed with pGIPZ vectors (shScramble or shFasL) purchased from Open Biosystems. Stable-expressing cells were generated by lentiviral-mediated transduction as described previously [21]. Cell sorting (Mo-Flow XP) was used to obtain a population of stably infected cells using the co-expressed GFP reporter contained on the GIPZ vector. Knockdowns were confirmed by qPCR.

Viability and proliferation assays

Viability and proliferation assays were performed as described previously [21]. Briefly, cell viability was determined using the Promega GLO-titer reagent and analyzed with a luminometer. Plates were allowed to equilibrate to room temperature prior to addition of the GLO-Titer reagent, and incubated for a minimum of 30 minutes before analysis with a luminometer. Real time analysis of cellular proliferation was performed using a DP-RTCA assay as described previously [21]. Colony forming assays were performed as described previously [45].

Caspase Activity Assays

Analysis of caspase 3/7, caspase 8, and caspase 9 activation was performed using kits from Promega (Caspase-GLO assays) according to the manufacturer's recommended protocol.

Western blotting

Western blotting was performed as described previously [21]. Antibodies used were as follows: AhR (1:1000, Enzo Life Sciences), PARP (1:200, Santa Cruz 7150), Caspase 3 (1:500, Biosource/Invitrogen CPP32), and GAPDH (1:100, Santa Cruz 137179).

Data analysis

Data were analyzed by ANOVA as appropriate, and multiple comparisons were performed with Tukey's post-hoc analysis. All Caspase, Annexin V, Real-time proliferation, and viability assays are representative of at least three similar experiments. Flow cytometry data was analyzed with FlowJo (Tree Star Inc). Values of $p < 0.05$ were considered statistically significant.

Results

A dual screening approach with XRE-reporter and Heterologous AhR reporter genes identifies CGS-15943 as a novel AhR activator.

Our study began with an attempt to identify novel ligands of the AhR and study their functional effects in cells, with the goal of isolating molecules with anti-cancer potential. XRE reporter genes are based on the canonical XRE binding sequence such as those found in the promoter of CYP1A1, and are efficiently activated by AhR ligands [26]. However, because it is these same ligands that are often associated with dioxin-like toxicity, we considered that it would be advantageous to employ a second screening approach that would allow us to identify ligands capable of bringing the AhR into a transcriptionally activate state without the strict requirement of canonical XRE binding *per se*. Thus, we employed a heterologous yeast GAL4/AhR fusion reporter in which the AhR DNA-binding domain (DBD) was replaced with the GAL4 yeast DBD coupled with a GAL-activating domain fused with ARNT. Together, these constructs were capable of activating a luciferase reporter gene driven by a GAL4-response element. A schematic of the screening constructs used in this study are shown in Fig. 1A. We used two independent screenings in parallel, Hepa1 cells expressing the pGudLuc1.1 reporter gene for the XRE-based screen, and heterologous GAL4-reporter based AhR activation screen. Using these two systems, we screened the library of pharmacologically active compounds (LOPAC1280) and 50,000 Chembridge pharmacophore library. The screening results with LOPAC library are shown in Fig. 1B. There were unique hits in each reporter system, but among the top 10 hits for each screen, only 4 compounds were shared in common (Fig. 1D). We focused on those compounds highly active in the non-canonical XRE system but minimal activity in the XRE screen.

Confirmatory screening of CGS-15943 showed that it activated the GAL4/AhR reporter system in 239T cells in a dose-dependent manner, and was also capable of activating the reporter system to a much greater extent than the relative maximal concentration of TCDD (1 nM) (Fig. 1D). To our surprise, confirmatory screening in the XRE-system performed at a shorter timepoint (4 hrs vs ~ 24 for the screen) showed that CGS-15943 was also capable of dose-dependently activating the XRE-reporter system (Fig. 1E). We did note a high degree of cell death in the Hepa1 cells treated with CGS-15943 for prolonged treatment times. Thus, CGS-15943 does activate AhR transcription, but on our initial screen was falsely negative in the XRE-based screen due to a high proportion of cell death.

CGS-15943 is a ligand of the AhR

Having identified CGS-15943 as an AhR activator, we next performed in vitro ligand-displacement assays to confirm specific AhR binding. CGS-15943 exhibited an EC₅₀ for binding of 96 nM, while TCDD had an EC₅₀ of 3.2 nM (Fig. 1G), consistent with previous observations for TCDD [15]. To provide a separate line of evidence for direct binding of CGS-15943 to the AhR, we next showed that CGS-15943 alters the proteolysis of AhR in limited proteolysis experiments, which has been demonstrated for TCDD [27] (Fig. 1H). We also confirmed that CGS-15943 induces nuclear translocation of the AhR using immunohistochemical studies (Fig. 1I). Because we observed XRE-activation in a shorter-term reporter assay, we next tested whether CGS-15943 could activate several known classic AhR-target genes. Consistent with this possibility, CGS-15943 activated the AhR target genes CYP1A1 and NADPH: quinone oxidoreductase (NQO1) in mouse Hep1c1c7 cells (Fig. 1K), and CYP1A1 in the HepG2 human hepatoma

cell line (Fig. 1J), in both cases to a similar degree as 1 nM TCDD. Taken together, these results showed that our screening strategy was effective in identifying novel AhR ligands, including CGS-15943.

CGS-15943 induces apoptosis

In confirmatory screening, we observed that CGS-15943 potently induces cell death in cultures of Hepa1 cells. Given its ability to activate the AhR, we were intrigued by the possibility the apoptotic effects of CGS-15943's may be mediated by the AhR. To address this possibility, we evaluated the effects of CGS-15943 on Hepa1 cells with time lapse video microscope (Video S1). We noted significant rounding of cells and membrane blebbing in cells treated with CGS-15943 (Fig. 2A), consistent with apoptosis. We quantitatively evaluated the effect of CGS-15943 on Hepa1 cells using an xCelligence assay, which we have used previously to study the phenotypic effects of AhR-ligands [23]. Addition of CGS-15943 to proliferating Hepa1 cells decreased the normalized cell index. Concentrations of CGS-15943 from 20 μ M to 2 μ M led to a dramatic decline in cell abundance, approaching that of the initial cell index at plating by 48 hours (Fig. 2B). Quantitative analysis of the normalized cell index values at 24 hours showed a statistically significant decrease in normalized cell index at concentrations of CGS-15943 down to 500 nM, and by 48 hours a significant effect of 100 nM CGS-15943 was also observed (Fig. 2B). Using DNA staining and microscopy, we visually confirmed that CGS-15943 induces nuclear fragmentation in Hepa1 cells, consistent with apoptosis (Fig. 2C). We also confirmed that CGS-15943 induces apoptosis using Annexin V binding (Fig. 2D), and activation of Caspases 3/7 (Fig. 2E). In all of our studies, equivalent AhR-activating concentrations of TCDD (1 nM) had no effect on Hepa1 cell with respect to induction of apoptosis.

AhR-dependent induction of apoptosis by CGS-15943

Given that the induction of apoptosis by CGS-15943 occurred at concentrations similar to those required to activate the AhR, we next asked if the effects were AhR-dependent. To address this possibility we first utilized transient knockdown of the AhR using morpholino oligonucleotides targeting the AhR and conjugated with a cell-penetrating peptide [28]. We confirmed successful knockdown of AhR using a morpholino targeting the AhR AUG start codon (Fig. 3A). Using these knockdown and control cells, we quantitatively assessed apoptosis by counting the number of cells with nuclear fragmentation. We found that AhR knockdown significantly decreased the percentage of cells with nuclear fragmentation in response to treatment with CGS-15943 compared to control cells (Fig. 3B). Visually, we also saw a noticeable difference in morphology between AhR knockdown cells treated with CGS-15943 compared to treated control cells, with decreased rounding of cells (Fig. 3C). These results indicated that the induction of apoptosis by CGS-15943 are mediated through AhR activation.

We have previously used Hepa1 cell culture models of high and low expression of AhR to investigate the AhR-dependent phenotypic effects of various ligands [28]. In the present study, we first turned to C12 and C12 + AhR cells, which are derivative cell lines of the Hepa1 cell line that have low and stably-restored expression of AhR, respectively (Fig. 3D). Using these cells, we found that CGS-15943 increased Annexin V staining only in the presence of AhR (Fig. 3E). Consistent with the results of the transient knockdown

experiments, there was a significant decrease in the degree of nuclear fragmentation in the absence of AhR compared to AhR-expressing cells (Fig. 3F). Absence of AhR (C12 cells) was associated with a significant increase in the number of viable cells compared to C12 + AhR cells (Fig. 3G).

To provide a third independent line of evidence for the requirement for AhR in mediating the apoptotic effects of CGS-15943, we utilized Hepa1 and TAO cells, which we and others have also used to characterize the effects of AhR ligands [28], as TAO cells express very low levels of AhR (Fig. 3H). Using these cells, we first performed real-time analysis of cellular proliferation as described above. TAO cells were significantly resistant to apoptosis induced by CGS-15943 compared to WT Hepa1 cells, but did exhibit some changes presumably due to low-baseline AhR levels (Fig. 3I). We also performed time-lapse microscopy in Hepa1 and TAO cells cultured side-by-side but physically separated in a small culture flask to observe the effects of the compound on the two cell types simultaneously. Consistent with the results of the xCelligence assay (Fig. 3I), TAO cells remained viable in the presence of CGS-15943 while Hepa1 cells underwent rapid and efficient apoptosis (Figure S2 and Video S2). We confirmed these results with cellular viability assays, in which there was a clear demarcation in the number of viable cells according to AhR expression (Fig. 3J). Interestingly, while CGS-15943 influenced TAO cell proliferation, there was absolutely no activation of caspase 3/7 in these cells at concentrations of CGS-15943 up to 20 μ M (Fig. 3K). In contrast, Hepa1 cells exhibited an approximately 20-fold increase in caspase 3/7 activity that appeared to saturate at a concentration of 5 μ M. In colony forming assays, pulsing Hepa1 or TAO cells with CGS-15943 for 24 hours with CGS-15943 at 10 μ M or 5 μ M (Fig. 3L) led to a complete loss of Hepa1 cell colonies, whereas TAO cells were virtually unaffected. Further, to provide additional evidence for a lack of activation of apoptosis in TAO cells, we performed Western blot analysis for PARP and caspase 3. As shown in Fig. 3M, TAO cells were completely resistant to the formation of cleaved PARP and caspase 3 in response to treatment with CGS-15943. In addition, we found that CGS-15943 decreased levels of AhR in treated Hepa1 cells, consistent with its ability to bind the AhR and induce the phenomenon of receptor recycling [28].

ARNT is required for CGS-15943 mediated apoptosis

ARNT is the obligate heterodimer of the AhR, and is required for the transcriptional activity of the AhR [28]. Thus, it was logical to ask whether ARNT is also required for CGS-15943 mediated induction of apoptosis. As we have done previously to study the ARNT-dependent effects of SU5416, Leflunomide, and Raloxifene [28], we used a cell culture model consisting of C4 cells, which express a mutant and transcriptionally inactive ARNT, and vT{2} cells, which stably re-express a WT ARNT protein. We first confirmed that ARNT is required for transcriptional activation of the AhR gene target CYP1A1 by CGS-15943 using semi-quantitative PCR (Fig. 4A). Next, we performed real-time growth profiling of C4 and vT{2} cells using the xCelligence assay. C4 cells were completely resistant to the effects of CGS-15943, appearing no different than cells treated with vehicle or 1 nM TCDD (Fig. 4B). Conversely, the normalized cell index values of vT{2} cells treated with CGS-15943 declined precipitously. Time lapse video microscopy using C4 and vT{2} cells in the parallel culture assay confirmed these findings (Figure S3 and Video S3). C4 cells were largely resistant to the effects of CGS-15943 in colony forming assays during

continuous exposure to CGS-15943 (Fig. 4C). Nuclear fragmentation was also almost completely abrogated in C4 cells treated with CGS-15943 compared to vT{2} cells (data not shown). Likewise, only vT{2} cells treated with CGS-15943 exhibited increased staining with Annexin V (Fig. 4D) or activation of caspase 3/7 (Fig. 4E). Together, these results clearly showed that ARNT is required for the induction of apoptosis by CGS15943. Furthermore, the absence of any significant effect of CGS-15943 on C4 cells argue for an AhR-dependent growth-inhibitory pathway activated by CGS-15943.

Identification of Fas ligand as a putative target of CGS-15943 mediated AhR activation

Upregulation of the pro-apoptotic molecule Bax has been described in literature as an AhR-target gene responsible for oocyte destruction mediated by the PAH 7,12-dimethylbenz[a]anthracene (DMBA) [29].

Thus, we asked if the potent induction of apoptosis by CGS-15943 led us to investigate the potential involvement of BAX. CGS-15943 did not increase the expression of BAX at the transcriptional level (Figure S4) or at the level of protein (data not shown). However, stable knockdown of BAX in Hepa1 cells partially blocked the induction of apoptosis by CGS-15943 (Figure S4). Based on these data, we hypothesized that the CGS-15943 mediated apoptosis may involve the intrinsic cell death pathway.

We next used a focused RT-qPCR-gene array to identify mediators of CGS-15943 induced apoptosis downstream of AhR activation in Hepa1 cells. To narrow the potential list of gene targets, we performed the screen in parallel with cells treated with 1 nM TCDD, reasoning that any genes also upregulated by TCDD would be unlikely candidates for mediators of CGS-15943 induced apoptosis (Fig. 5A). Several putative gene targets were potently upregulated by CGS-15943 but not TCDD, the most notable of which was Fas ligand (FasL, CD95L). FasL is involved in immune cell regulation, inducing death of target cells expressing the Fas receptor (CD95). To ensure that induction of FasL could be a regulator of apoptosis in Hepa1 cells, presumably in an autocrine fashion, we confirmed that Hepa1 cells express Fas receptor (Fig. 5B). We next determined if induction of FasL by CGS-15943 correlated with AhR expression. FasL was significantly upregulated by CGS15943 in Hepa1 cells and vT{2} cells, but not the reciprocal AhR and ARNT null cell lines, respectively (Figs. 5C-D). Similarly, surface expression of FasL as determined by flow cytometry was increased to a greater extent in Hepa1 cells than Tao cells in response to CGS-15943 (Fig. 5E). To investigate whether FasL is a direct or indirect target of the AhR, we performed experiments in which Hepa1 cells were co-treated with CGS-15943 and the protein synthesis inhibitor cycloheximide (CHX). Addition of cycloheximide to Hepa1 cells treated with TCDD results in a super-induction of CYP1A1 [28], while blockade of protein synthesis would be expected to inhibit the transcription of any secondary gene targets. We found that CHX co-treatment with CGS-15943 led to a superinduction of CYP1A1, but blocked the increase in FasL mRNA as determined by qPCR (Figure S5).

FasL is required for CGS-15943 induced apoptosis

Having shown that CGS-15943 induces apoptosis in Hepa1 cells in a strongly AhR and ARNT dependent manner, and identified FasL and BAX as putative downstream regulators, we next asked if FasL is required for CGS-15943 mediated apoptosis in Hepa1 cells. To address this possibility, we generated

stable Hepa1 cells lines expressing shRNAs directed against FasL. We confirmed basal knockdown of FasL in these cells by qPCR, and also showed that the knockdown blocked induction of FasL in response to treatment with CGS-15943 (Fig. 5F). Knockdown of FasL significantly decreased caspase 3/7 activation compared with WT Hepa1 cells, indicating that FasL is required for CGS-15943 mediated cell death (Fig. 5H).

FasL-mediated apoptosis can occur through type I signaling, involving only the extrinsic cell death pathway, or type II signaling, which involves cross-talk with the intrinsic mitochondrial-dependent cell death pathway [30, 31]. Our results with Bax knockdown (Figure S4) suggested mitochondrial involvement. Thus, to determine if CGS-15943 induces cell death occurs through the intrinsic cell death pathway, or involves the mitochondria, we asked if CGS-15943 could increase the activity of caspases 8 and 9. Specifically, activation of both caspases would suggest a mechanism by which FasL signaling activates caspase 8 to cleave the pro-apoptotic protein BID to induce mitochondrial-mediated apoptosis and subsequent activation of caspase 9 [30, 31]. We observed increased activity of both caspases 8 and 9 in an AhR-dependent manner (Fig. 5H), suggesting that apoptosis induced by CGS-15943 involves both the intrinsic and extrinsic cell death pathways through a Type II signaling mechanism.

MRS1220 is a CGS-15943 derivative with unique properties

Given the unique ability of CGS-15943 to induce AhR-dependent apoptosis in such a selective manner, we were next interested in identifying similar related molecules. We investigated a series of derivatives of CGS-15493, including those with additions of various moieties at the free amine group, for their ability to induce apoptosis in Hepa1 cells. From this effort, we identified one compound, MRS1220, henceforth named CGS_B, with properties similar to CGS-15943 (Fig. 6A). Interestingly, CGS_B was largely inactive in short-duration XRE-reporter assays (Fig. 6B), and did not appear to significantly increase expression of CYP1A1 in semi-quantitative RT-PCR experiments (Fig. 6C). On the other hand, while not as potent as the parent molecule, CGS_B retained the ability to induce apoptosis in Hepa1 cells according to xCelligence (Fig. 6D) and caspase 3/7 activation (Fig. 6E). Thus, while MRS1220 does not appear to exhibit increased potency with respect to the induction of apoptosis compared with CGS-15943, it may selectively regulate AhR transcription and is thus an attractive target for future studies.

CGS-15943 induces AhR-dependent apoptosis in human liver and breast cancer cells

Having characterized the effects of CGS-15943 in a mouse liver cancer cell line, we were next interested in expanding the repertoire of cell types in which CGS-15943 may have efficacy as an anti-cancer agent. We first investigated whether CGS-15943 has activity against human HepG2 liver cancer cells. We found that CGS-15943 decreased HepG2 cell viability, and that transient knockdown of AhR with an siRNA rescued HepG2 cells from CGS-15943 induced cell death (Figs. 7A; Figure S6 and Video 4). CGS-15943 increased caspase 3/7 activation in HepG2 cells (Fig. 7B), and increased the expression of FasL mRNA (Fig. 7C). Thus, CGS-15943 induces apoptosis in a human liver cancer cell line, also consistent with our previous observations for the AhR-ligand raloxifene [28]. In subsequent evaluation of additional cell lines

susceptible to CGS-15943, we identified the triple-negative (ER-/PR-/HER2-) MDA-MB-468 cell line. To demonstrate that apoptosis induced by CGS-15943 in these cells required the AhR, we generated a stable cell line expressing a doxycycline inducible shRNA against the AhR and co-expressed with an RFP-reporter (Fig. 7D). Partial knockdown of AhR in these cells (Fig. 7D) led to significantly decreased activation of Caspase 3/7 in response to treatment with CGS-15943 (Fig. 7E). In addition, the AhR knockdown in MDA-MB-468 cells significantly rescued the decreased cell viability seen with CGS-15943 (Fig. 7F).

Discussion

In the present study, we described the pro-apoptotic functions of a novel AhR ligand CGS-15943 in mouse (Figs. 2–4) and human liver cancer cells (Fig. 7), and MDA-MB-468 triple negative breast cancer cells (Fig. 7). In each of these cell lines, we found that the effects of CGS-15943 required expression of the AhR. We also identified a derivative of CGS-15943, MRS1220 (CGS_B), that induces apoptosis in an AhR-dependent manner, but does not significantly induce the AhR target gene CYP1A1 or an XRE-reporter gene (Fig. 6).

Mutants of the AhR expressed in AhR-null cells support the ability of the AhR in inducing apoptosis. A constitutively active AhR mutant, termed as CA-AhR, lacking part of the ligand binding domain, induced apoptosis in Jurkat T lymphoma cells [36]. We previously reported that Raloxifene induces apoptosis in Hepa1 cells and MDA-MB-231 breast cancer cells [23]. The results of the current study indicate that CGS-15943 anti-cancer effects can be attributed completely to AhR and ARNT activation (Figs. 3–4). Indeed, C4 cells containing an inactive ARNT protein are completely resistant to the effects of CGS-15943. Thus, CGS-15943 represents a novel mediator of AhR-dependent apoptosis. AhR-selective ligands such as CGS-15943 may exert their anti-cancer effects by activating AhR tumor suppressive signaling. We observed no such effects with well-studied AhR ligand TCDD (Figs. 2D, 3G, and 4B) strongly supporting ligand-selective effects of the AhR.

Pro-apoptotic protein Bax is not induced, but is required for AhR-mediated apoptosis upon treatment with CGS-15943. We identified for the first time induction of *FasL as a pathway that mediates AhR-regulated apoptosis in cancer* cells. Interestingly, involvement of Fas-signaling and the AhR has been reported previously in various contexts [39, 40]. We observed that induction of FasL by CGS-15943 was completely blocked by CHX co-treatment, as was cell death (Figure S5). These results may suggest that fasL is an indirect gene target of the AhR, and that activation of an intermediate product is required for CGS-15943 mediated apoptosis upstream of fasL. Consistent with this possibility, only Fas, but not FasL, contain XRE sequences in their promoter [41]. Alternatively, these results may be explained by a fast-decaying factor induced by the CGS-15943 through the AhR that is required to stabilize increased abundance of FasL mRNA. Both of these possibilities will be investigated in the future.

CGS 15943 is a triazoloquinazoline non-xanthine adenosine antagonist with nanomolar affinity for adenosine receptors A1 and A2A [25]. Interestingly, both adenosine and CGS-21680 have been reported to

induce apoptosis in Human HepG2 cells via mitochondrial mediated cytochrome c release and subsequent activation of caspases 3 and 9, an effect that could be abrogated by knockdown of the A2A adenosine receptor [42]. The results reported by Tamura et al. were obtained with adenosine receptor agonists, whereas CGS-15943 is an A2A antagonist, and because an A2A agonist blocked the effects of CGS-21860 [42], these results appear to be separate from those observed in our study. Conversely, another study showed that adenosine induces apoptosis in HepG2 cells, but that the effect is independent of mitochondrial-mediated cell death and is not blocked by A2A antagonists [43]. With respect to CGS-15943, the differences in concentrations associated with its adenosine receptor antagonist functions [25] vs its AhR-dependent effects (nanomolar and low-micromolar, respectively), as well as the significant degree to which the effects of CGS-15943 require both expression of AhR and Arnt (Figs. 2–4) as well as FasL (Fig. 5), argue against involvement of adenosine receptor activity in its biological effects.

Conclusions

In conclusion, we have identified CGS-15943 as an novel AhR-selective ligand with anti-cancer properties using two parallel and distinct screening strategies. CGS-15943 induced AhR-dependent apoptosis in mouse and human liver cancer cells. Discovery and development of CGS-15943 as an AhR-selective anti-cancer ligand to investigate AhR mediated anti-cancer effects has been an important area of research in our laboratory for the past several years. Both CGS-15943 and its analog MRS1220 represent AhR-targeted therapeutics for the treatment of liver and breast cancer. Our results argue for the use of phenotypic AhR-dependent screening strategies for identifying novel AhR ligands with anti-cancer actions and we have successfully implemented this approach to discover a novel AhR signaling with utility for cancer treatment.

List Of Abbreviations

Aryl-hydrocarbon Receptor (AhR); Aryl Hydrocarbon Receptor Nuclear Translocation (ARNT); bHLH-PAS (basic-helix-loop helix-Per/ARNT/Sim); diethylnitrosamine (DEN); DNA-binding domain (DBD); hematopoietic stem cells (HSCs); NADPH: quinone oxidoreductase (NQO1); Transgenic adenocarcinoma of the mouse prostate (TRAMP); 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE); 6-formylindolo[3,2-b]carbazole (FICZ); 7,12-dimethylbenz[a]anthracene (DMBA).

Declarations

Author Contributions

EFO contributed to study design, execution of experiments, data analysis, and wrote the manuscript. HSJ performed experiments and contributed to data analysis. DCK performed experiments, contributed to data analysis, and provided input on study design. NIK provided input on study design and provided material support. SKK designed and supervised the study, and helped prepare and revise the manuscript.

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Competing interests

The authors have no conflict of interests to disclose.

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Not Applicable

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Figures

Figure 1

A small molecule screen identified CGS-15943 as a novel activator of the Aryl Hydrocarbon Receptor. (A) Schematic of the reporter constructs used in the screen. (B) LOPAC1280 screen reports for the XRE reporter (top) or GAL-AhR reporter (bottom) sorted according to the results of the opposing screen. (C) Diagram showing the relationship between the top 10 hits of each screen. The structure of CGS-15943, which was part of the GAL4-only screen results, is shown. (D) Confirmation of the GAL/AhR screen results. CGS-15943 dose-dependently activates the reported. (E) Hepa1.1 XRE is also dose-dependently upregulated by CGS-15943. (F) CGS-15943 activates the heterologous GAL4/AhR screening system in Hepa1 cells in a dose-dependent manner. (D-F, data is plotted as the mean $\pm$ SEM ($n \geq 3$ for all experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ for all panels). (G) Competitive [3H]-3MC ligand binding assays with TCDD and CGS-15943 with mouse hepatoma whole cell extracts. (H) In vitro subtilisin protease assay shows delayed proteolysis by CGS-15943. (I) CGS-15943 induces AhR nuclear localization similar to that of TCDD. Cells were treated for 4 hours. (K-J) Activation of endogenous AhR target genes in Hepa1 and HepG2 mouse and human hepatoma cells, results are representative of at least three similar experiments.

Figure 2

CGS-15943 induces apoptosis in hepatoma cells. (A) Microscopy still images of video-time lapse analysis of CGS-15943 (20 μ M) induced cell death in Hepa1 cells (Video 1, under supplementary data). (B) Real-time analysis of Hepa1 cellular proliferation with xCelligence assay. Plots labeled “24 Hrs” and “48 Hrs” correspond to values taken at that timepoint after adding the indicated concentrations of CGS-15943. Data are analyzed as the mean $\pm$ SEM, n=2. Results are representative of two similar experiments. (C) Representative images DAPI-stained Hepa1 cells treated for 18 hours with CGS-15943 shows significant nuclear fragmentation and condensation. (D-E) CGS-15943 increases caspase 3/7 activation and Annexin V+ positive cells in a dose-dependent manner measured with a kit and by flow cytometry, respectively (For all panels: * p<0.05, ** p<0.01, *** p<0.001, and **** p<0.0001).

Figure 3

Apoptosis induced by CGS-15943 requires the aryl hydrocarbon receptor. (A-C) Morpholino mediated AhR knockdown in Hepa1 cells. (A) Western blot showing knockdown of AhR by the AUG_PMO morpholino. (B) Analysis of nuclear fragmentation of Hepa1 cells treated with control or AUG_PMO followed by CGS-15943 at the indicated concentration. (C) Images of Hepa1 cells treated with the AUG_PMO for AhR knockdown followed by CGS-15943. (D-G) Analysis of the effects of CGS-15943 in C12 and C12+AhR cells. (D) Western Blot showing the relative levels of AhR expression. (E) Annexin V assay in C12 and C12+AhR cells. (F) Analysis of nuclear fragmentation in C12 and C12+AhR cells treated with 20 μ M or 5 μ M CGS-15943. (G) MTT viability assay for C12 and C12+AhR cells treated with the indicated concentrations of CGS-15943 overnight. (H-M) Analysis of the effects of CGS-15943 in Hepa1 and TAO cells (Video 2 under supplementary data). (H) Western blot showing the relative levels of AhR expression. (I) Real-time analysis of cellular proliferation shows an AhR-dependent decrease in cellular impedance measurements in Hepa1 cells compared with TAO cells. (J) Dose-response analysis of viability (ATP-content based luciferase assay) in Hepa1 and TAO cells treated with CGS-15943. (K) Measurement of Caspase 3/7 activity under conditions identical to those in B. (L) Colony forming assays performed with a 24 hours pulse of the indicated treatments (TCDD 1 nM) followed by washing the cells with PBS and refeeding with normal medium until visible colonies were present. Colonies were stained with a methylene blue solution, and two representative plates from a total of six are shown. Data are representative of three similar experiments. (M) Western blot analysis of apoptotic-markers caspase 3 and cleaved PARP in WT Hepa1 and TAO cells treated with 20 μ M CGS-15943 for 18 hours. The lysates are triplicate biological replicates. (For all panels * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

Figure 4

Apoptosis induced by CGS-15943 requires ARNT. (A) Semi-quantitative RT-PCR results for vT{2} and C4 cells treated with vehicle (0.1% DMSO), TCDD (1 nM), and CGS-15943 (20 μM). The initial data are from 20 cycles, and +3 refers to three additional rounds of PCR. (B) ARNT deficient Hepa1 cells (C4) are completely resistant to the effects of CGS-15943 in an xCelligence assay, while vT{2} cells are highly susceptible (Video 3 under supplementary data). (C) Colony forming assay with C4 and vT{2} cells. A total of 300 cells per 60 mm dish were plated, grown overnight, and then treated with the indicated compounds for approximately 10 days until cell colonies were visible. Colonies were stained with a methylene blue solution for visualization. (D) Annexin V and (E) Caspase 3/7 activation assays for C4 and vT{2} cells treated with CGS-15943.

Figure 5

CGS-15943 increases FasL expression in hepatoma cells in an AhR-dependent manner and induces Type II extrinsic cell death involving mitochondria. (A) Focused qPCR array in Hepa1 cells shows upregulation of apoptosis-associated genes by CGS-15943 but not TCDD. (B) Confirmation of Fas receptor on Hepa1c1c7 cells with the JO2 antibody. (C-D) CGS-15943 increases mRNA abundance of FasL in an AhR- and Arnt-dependent manner, respectively. (E) Flow cytometry analysis shows a greater increase in expression of surface FasL on Hepa1 cells treated with CGS-15943 compared to TAO cells. (F) FasL mRNA expression in purified shScramble and shFasL cell lines co-expressing GFP with the pGIPZ shRNA hairpin. (G) Caspase 3/7 activation assay in shScramble or shFasL expression Hepa1 cells treated for 18

hours with the indicated concentrations of CGS-15943. (H) Caspases 3/7, 8, and 9 are activated by CGS-15943 in Hepa1 cells in an AhR-dependent manner, experiments were performed in parallel. Data are representative of three independent experiments.

Figure 6

MRS1220, a structural analog of CGS-15943, induces AhR-dependent apoptosis in Hepa1 cells but does not activate the AhR target gene CYP1A1. (A) Structures MRS1220 (CGSB). The pink highlighted area shows the difference with the parent molecule (see Figure 1). (B) XRE-based reporter gene assays with CGS-15943 and CGSB in Hepa1 cells, performed at 4 hours. (C) Semi-quantitative RT-PCR analysis of CYP1A1 induction by CGS-15943 and CGSB in Hepa1 cells. (D-E) CGSB induces apoptosis in Hepa1 cells similar to CGS-15943 according to real-time proliferation analysis (xCelligence) and caspase 3/7 activation assays.

Figure 7

Induction of apoptosis in human HepG2 and MDA-MB-468 cells by CGS-15943 requires expression of the AhR. (A) % Viability of HepG2 cells transfected with either siLuciferase or siAhR (Video 4 under supplementary data). (B) Caspase 3/7 activation after 48 hr treatment with 20 μ M CGS-15943 in HepG2 cells. (C) qPCR analysis of FasL mRNA levels after treatment with 40 and 20 μ M CGS-15943 for 24 hours. (D-G) MDA-MB-468 cells expressing an inducible AhR shRNA. (D) Western blot and flow cytometry profiling of AhR knockdown and pTRIPZ RFP reporter expression, respectively. (E) Caspase 3/7 assay of MDA-MB-468 cells treated with CGS-15943 for 24 hours at the indicated concentrations in the presence or absence of established AhR knockdown (doxycycline >3 days). (F) Viability analysis of MDA-MB-468

cells treated with CGS-15943 for 24 hours at the indicated concentrations in the presence or absence of established AhR knockdown. (G) Flow cytometry results showing RFP and Annexin V staining of the indicated cell and treatment groups. Data are representative of three independent experiments.

Supplementary Files

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