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Omeprazole Inhibits Pancreatic Cancer Cell Invasion Through a Non-genomic Aryl Hydrocarbon Receptor Pathway

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Abstract

Omeprazole and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are aryl hydrocarbon receptor (AhR) agonist that inhibit invasion of breast cancer cell through inhibition of CXCR4 transcription. Treatment of highly invasive Panc1 pancreatic cancer cells with TCDD, omeprazole and seven other AhR-active pharmaceuticals showed that only omeprazole and tranilast but not TCDD inhibited invasion in a Boyden chamber assay. Similar results were observed in MiaPaCa2 cells, another quasimensenchymal pancreatic ductal adenocarcinoma (QM-PDA) pancreatic cancer cell line, whereas invasion was not observed in BxPC3 or L3.6pL cells which are classified as “classical” (less invasive) pancreatic cancer cells. It was also observed that in the QM-PDA cells that TCDD, omeprazole and tranilast did not induce CYP1A1 or CXCR4 and treatment with these compounds did not result in nuclear uptake of the AhR. In contrast, treatment of BxPC3 and L3.6pL cells with these AhR ligands resulted in induction of CYP1A1 (by TCDD) and nuclear uptake of the AhR which was similar to that observed for Ah-responsive MDA-MB-468 breast and HepG2 liver cancer cell lines. Results of AhR and AhR nuclear translocator (Arnt) knockdown experiments in Panc1 and MiaPaCa2 cells demonstrate that omeprazole- and tranilast-mediated inhibition of invasion was AhR-dependent but Arnt-independent. These results demonstrate that in the most highly invasive sub-type of pancreatic cancer cells (QM-PDA), the selective AhR modulators omeprazole and tranilast inhibit invasion through a non-genomic AhR pathway.

Graphical abstract

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Cytosolic; omeprazole; tranilast; QM-PDA

Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated receptor that binds the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) with high affinity to induce a set of well-characterized biochemical and toxic responses.^{1, 2} The classical molecular mechanism of action of the AhR involves ligand (TCDD) binding to the cytosolic AhR and nuclear uptake of the liganded AhR which forms a transcriptionally active complex with the AhR nuclear translocator (Arnt) and binds *cis*-xenobiotic response elements (XREs) to modulate gene expression.^{1, 2} This pathway is consistent with the induction of CYP1A1 by TCDD and other AhR ligands in many organs/tissues and cell lines. However, since the discovery of the AhR as the intracellular target for TCDD and related halogenated aromatics,³ it has been shown that the AhR and various ligands play an important endogenous role in organ/tissue development, inflammation, autoimmune and immune responses, and carcinogenesis.⁴⁻¹⁹ Moreover, the AhR not only binds and mediates the effects of TCDD and other toxicants but also structurally diverse flavonoids and other

phytochemicals with health promoting activity, a large number of pharmaceuticals,²⁰⁻²³ and several endogenous biochemicals including kynurenine and 6-formylindolo(3,2-b)carbazole (FICZ) which have been proposed as endogenous ligands for the AhR.²⁴⁻²⁶

The toxicities of TCDD and related compounds have been associated with persistent occupation of the nuclear AhR. Structurally diverse AhR antagonists have also been identified²⁷⁻³⁰ and these compounds inhibit TCDD-induced genes and pathways. It is clear that the effects of AhR ligands depend not only on their structure, but also on the target organ and downstream responses and genes. In addition to the classical nuclear AhR:Arnt-mediated response, non-genomic AhR pathways have also been reported,³¹ and recent studies show that a nuclear AhR-Krüppel-like factor 4 (KLF4) complex mediates activation of p21 and plasminogen activator inhibitor-1 through a non-consensus-XRE.^{32, 33}

Research in this laboratory has identified selective AhR modulators (SAhRMs) that act as AhR antagonists for some TCDD-induced responses but also function as AhR agonists and inhibit growth of estrogen receptor (ER)-positive and ER-negative breast cancer cells and tumors.⁷ We have also investigated AhR-active pharmaceuticals²⁰ for their inhibition of breast cancer cell invasion *in vitro* and have identified the proton pump inhibitor omeprazole as an effective inhibitor of invasion (*in vitro*) and metastasis *in vivo*.^{34, 35} In this study, we initially screened eight AhR-active pharmaceuticals previously investigated in breast cancer cells and these include 4-hydroxytamoxifen, flutamide, leflunomide, mexiletine hydrochloride, nimodipine, omeprazole, sulindac and tranilast as inhibitors of Panc1 pancreatic cancer cell invasion using a Boyden chamber assay. The results showed that only omeprazole and tranilast, but not the other AhR-active pharmaceuticals or TCDD, inhibited cell invasion in Panc1 and other pancreatic cancer cell lines. Mechanistic studies showed cell context- and ligand-dependent differences in the induction of CYP1A1 by TCDD, omeprazole and tranilast. However, using the highly invasive Panc1 cells as a model, we showed that omeprazole and tranilast inhibited invasion through a novel non-genomic pathway that does not involve ligand-induced nuclear translocation.

Experimental Procedures

Cell lines, antibodies, and reagents

Human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.37% sodium bicarbonate, 0.011% sodium pyruvate, 0.058% L-glutamine, 10% fetal bovine serum (FBS) or RPMI medium supplemented with 0.2% sodium bicarbonate, 0.03% L-glutamine, 10% FBS, purchased from GenDEPOT (Barker, TX). CYP1A1, AHR, and β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Flag, Arnt, and HIF-1 α antibodies were purchased from Cell Signaling Technology and p84, GAPDH, and RNA polymerase II antibody were purchased from GeneTex (Irvine, CA). All compounds and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Cell proliferation (MTT) assay

Cells (5×10^3 per well) were plated in 96-well plates and allowed to attach for 16 hr. The medium was then changed to DMEM medium containing 2.5% FBS, and either vehicle [dimethyl sulfoxide (DMSO)] or different concentrations of the compounds were added. After 24 hr, treatment medium was replaced with fresh medium containing 0.05 mg of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) per 100 μ L and incubated for 4 hr. Medium was then removed, and 100 μ L of dimethyl sulfoxide was added to wells. The optical density of each sample was read on a microplate reader (FLUOstar OPTIMA) at 570 nm against a blank prepared from cell-free wells. Cell proliferation was expressed as a % of relative absorbance of untreated controls.

Survival analysis of microarray data

Pancreatic cancer patient gene profiling data (GSE2501) were obtained from Gene Expression Omnibus (GEO). The patient samples were classified into two groups according to the AhR mRNA expression level (high, top 50% vs. low, bottom 50%). Kaplan-Meier plot and the log-rank test were performed to estimate patient prognosis. Overall survival was defined as the time interval between the date of histological diagnosis and the date of death from any cause. Statistical analysis was tested with the survival package in R (<http://www.r-project.org>). The level of AhR mRNA in several normal and tumor tissues (Fig. 1) was measured using the following data set: Breast (GSE9574 and GSE5764); Lung (GSE2514, GSE10072 and GSE19804), Pancreas (GSE16515), Prostate (GSE6919), Stomach (GSE2685), Colon (GSE4107), Thyroid (GSE3678), and Cervical (GSE7803).

Invasion assay

For the invasion assay, the BD-Matrigel Invasion Chamber (24-transwell with 8 μ m pore size polycarbonate membrane) was used in a process of modified Boyden chamber assay. The medium in the lower chamber contained 10% FBS. Cells (5×10^4 cells/insert) in serum-free medium were plated into the upper chamber with or without various concentrations of compounds and incubated for 18 hr at 37°C, 5% CO₂. After incubation, the non-invading cells were removed from the upper surface of the membrane with a wet Q-tip/cotton swab. The invading cells on the lower surface of the membrane were fixed with 10% formalin for 10 min, stained in hematoxylin, and eosin Y solution (H&E). After washing and drying, the numbers of cells in five adjacent fields of view were counted.

Transfection of siRNA and quantitative real-time PCR

Cells were transfected with 100 nM of each siRNA duplex for 6 hr using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer's protocol. The sequence of AhR siRNA oligonucleotide was 5'-UAA GGU GUC UGC UGG AUA AUU (UU)-3' and Arnt siRNA was purchased from Santa Cruz Biotechnology. As a negative control, a nonspecific scrambled small inhibitory RNA (siCT) oligonucleotide was used (Qiagen). Total RNA was isolated from harvested cells with an RNeasy Mini Kit (Qiagen) using the manufacturer's protocol. For RT-PCR assay, cDNA was prepared from the total RNA of cells with amfiRivert cDNA Master Mix Platinum (GenDEPOT, Barker, TX). The real-time PCR was performed using SYBR Green Mastermix (Applied Biosystems) as previously described.³⁵ The

sequences of the primers used for real-time PCR were as follows: AhR sense, 5'-AGT TAT CCT GGC CTC CGT TT-3'; antisense, 5'-TCA GTT CTT AGG CTC AGC GTC-3'; CYP1A1 sense, 5'-GAC CAC AAC CAC CAA GAA C-3'; antisense, 5'-AGC GAA GAA TAG GGA TGA AG-3'; CYP1B1 sense, 5'-ACC TGA TCC AAT TCT GCC TG-3'; antisense, 5'-TAT CAC TGA CAT CTT CGG CG-3'; CXCR4 sense, 5'-TTT TCT TCA CGG AAA CAG GG-3'; antisense, 5'-GTT ACC ATG GAG GGG ATC AG-3'; and TBP sense, 5'-TGC ACA GGA GCC AAG AGT GAA-3'; antisense, 5'-CAC ATC ACA GCT CCC CAC CA-3'.

Subcellular fractionation and Western blot analysis

The cytosolic and nuclear protein fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following the manufacturer's protocol. Western blot was performed as previously described.³⁵

Subcellular localization assays

Cells on a cover slip were fixed in 10% formalin in PBS (pH 7.4), then washed with PBST and permeabilized by immersing the cells in 0.3% Triton X-100 solution in PBST for 10 min. After blocked with 5% BSA in 1x PBST, cells were then incubated with anti-rabbit Arnt antibody (Santa Cruz) in 5% BSA in 1x PBST for 6 hr, followed by anti-rabbit IgG conjugated with FITC (Santa Cruz). Cells were mounted in mounting medium containing DAPI (Vector Lab., CA). Fluorescent images were collected and analyzed using EVOS® FL fluorescence microscope (Life Technologies, Grand Island, USA).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using ChIP-IT Express Magnetic Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA) in according to the manufacturer's protocol as previously described.³⁵ Cells (5×10^6 cells) were treated with TCDD, omeprazole, or tranilast for 2 hr. The CYP1A1 primers were 5'-TCA GGG CTG GGG TCG CAG CGC TTC T-3' (sense), and 5'-GCT ACA GCC TAC CAG GAC TCG GCA G-3' (antisense), amplifying 122-bp region of human CYP1A1 promoter which contained the AhR binding sequences.

Construction of recombinant adenoviruses expressing wild type (WT) and constitutively active (CA) AhR

The CA-AhR construct was cloned by recombination-mediated PCR as previously described.^{36, 37} Two sites adjacent to the PAS B domain of *AhR* were amplified to generate Flag-tagged CA-AhR using the following primer sets: hAhR-1-F, ACG CGG CCG CGA TGA ACA GCA GCA GCG; hAhR-293-R, AGT CCT TAG TGG TAG TTT GTG TTT GGT TCT AAA G (containing 12 bp of right adjacent of PAS B domain); hAhR-428-F, CCA AAC ACA AAC TAC CAC TAA GGA CTA AAA ATG G (containing 14 bp of right adjacent of PAS B domain); hAhR-848-R, ACG GTA CCT TAC AGG AAT CCA CTG G. Two amplified PCR fragments were ligated and re-amplified using primer hAHR-1-F and hAhR-848-R, containing *Not*I and *Kpn*I restriction enzyme sequences, respectively. The PCR products were digested with *Not*I and *Kpn*I and then ligated into the corresponding

sites of p3X-Flag-CMV 10 (Sigma-Aldrich), thus the PAS B domain was removed without any linker. *WT-AhR* was also prepared using primer hAhR-1-F and hAhR-848-R and with same processes. *WT*- and *CA-AhR* containing Flag tag sequences were transferred into *Sal*I and *Eco*R I sites of pENTRA 1A dual selection vector (Invitrogen, Grand Island, NY) using the primer set, hAhR-pENTRA-F, ACG TCG ACT AGT GAA CCG TCA GAA TTA and hAhR-pENTRA-R, ACG ATA TCT TAC AGG AAT CCA CTG G. *AhR* gene constructs were transferred again into pAD/CMV/V5-DEST using pAD/CMV/V5-DEST Gateway® Vector Kits (Invitrogen). Adenoviruses containing Flag-tagged *WT*- and *CA-AhR* were generated using the ViraPower™ Adenoviral Expression System (Invitrogen) following by manufacturer's instructions.

Statistics

All of the experiments were repeated a minimum of three times. Statistical significance was analyzed using Student's t-test. The results are expressed as means with error bars representing 95% confidence intervals for three experiments for each group unless otherwise indicated, and a *P* value of less than 0.05 was considered statistically significant.

Results

The AhR is expressed in pancreatic tumors and there is now increasing evidence that the AhR is also a prognostic factor and a potential drug target for multiple tumors.⁷ We have expanded on the previous study on pancreatic tumors and show the expression and prognostic significance of the AhR using data from publically available databases containing over 100 patients. Figure 1A compares AhR mRNA levels in eight different cancers compared to non-cancer tissues, and pancreatic cancer was among four tumor types in which AhR expression was high compared to normal tissue. Kaplan-Meier analysis of the prognostic significance of AhR expression in a pancreatic tumor array (GSE16515) showed that high expression predicts a longer disease-free survival than low expression of the receptor (Fig. 1B). These results demonstrate that the AhR is overexpressed in pancreatic tumors and a prognostic factor and based on results of our recent study in breast cancer cells showing that AhR ligands inhibited invasion,³⁵ we investigated the activity of eight AhR-active pharmaceuticals as inhibitors of pancreatic cancer cell invasion.

Panc1 cells are highly invasive quasimesenchymal pancreatic ductal adenocarcinoma (QM-PDA) cells³⁸ and were used to screen AhR-active pharmaceuticals and TCDD for their inhibition of cell invasion in a Boyden chamber assay (Fig. 1C) using concentrations that were not cytotoxic (< 20% growth inhibition as determined in an MTT assay, Suppl. Fig. S1). These pharmaceuticals have potential anticancer activity and were previously investigated for their inhibition of breast cancer cell invasion.^{34, 35} 4-Hydroxytamoxifen (2 and 4 μM), flutamide (10 and 20 μM), leflunomide (20 and 40 μM), mexiletine-hydrochloride (200 and 400 μM), nimodipine (6 and 10 μM), sulindac (50 and 100 μM), and TCDD (10 nM) did not significantly inhibit invasion. In contrast, tranilast and omeprazole (100 and 200 μM) significantly inhibited Panc1 cell invasion.

Omeprazole, tranilast and TCDD were further used to investigate the Ah-responsiveness of prototypical QM-PDA (Panc1 and MiaPaCa2) and classical (BxPC3 and L3.6pL) pancreatic

cancer cell lines with the classical cells representing a less-invasive phenotype compared to QM-PDA cells.³⁸ In Panc1 cells, neither omeprazole, tranilast nor TCDD induced *CYP1A1* gene expression and minimal effects were observed after transfection with siAhR (Fig. 2A). Supplemental Figures S2 and S3 show that the eight AhR-active pharmaceuticals did not induce CYP1A1 or CYP1B1 in Panc1 cells, and a time course study showed that tranilast and omeprazole did not induce CYP1A1 in Panc1 cells (Suppl. Fig. S4). Omeprazole and tranilast but not TCDD decreased *CYP1B1* gene expression, and the former two responses were not reversed by siAhR, whereas in the absence of AhR, CYP1A1 was increased by TCDD. CXCR4 downregulation in breast cancer cells by omeprazole was AhR-dependent³⁵ and in Panc1 cells, CXCR4 was also decreased by omeprazole and tranilast (but not TCDD). Basal expression of CXCR4 was decreased after AhR knockdown and in Panc1 cells treated with omeprazole or tranilast, transfection with siAhR further decreased CXCR4 mRNA levels, suggesting that the AhR may play a role in ligand-induced CXCR4 downregulation. In MiaPaCa2 cells (Fig. 2B), omeprazole, tranilast and TCDD did not induce CYP1A1 and after AhR knockdown, there was a decrease in CYP1A1 indicating that basal CYP1A1 expression was AhR-regulated. Only tranilast induced levels of CYP1B1 in MiaPaCa2 cells and after transfection with siAhR, CYP1B1 was unchanged in all treatment groups. Omeprazole and tranilast but not TCDD decreased CXCR4 mRNA levels and these responses were unchanged in MiaPaCa2 cells after AhR knockdown. The pattern of ligand-induced CYP1A1 in BxPC3 cells (Fig. 2C) demonstrated that omeprazole and tranilast were weak agonists compared to TCDD for induction of CYP1A1; however, results of AhR knockdown confirmed that these responses were AhR-dependent. Interestingly, CYP1B1 was induced only by TCDD in BxPC3 cells and the induction response was increased after AhR knockdown and this was similar to that observed in Panc1 cells. In L3.6pL cells (Fig. 2D), omeprazole did not induce CYP1A1; however, induction by tranilast and TCDD was AhR-dependent. The magnitude of the decrease in TCDD-induced CYP1A1 after transfection with siAhR (C and D) was less than expected due, in part, to incomplete AhR knockdown. CYP1B1 was induced by tranilast and TCDD but levels of CYP1B1 mRNA were unchanged after AhR knockdown, indicating that this response was AhR-independent. CXCR4 mRNA expression was decreased only by omeprazole in L3.6pL cells and the magnitude of this response was unaffected by AhR knockdown. The QM-PDA and classical pancreatic cancer cell lines all expressed AhR mRNA levels which were decreased after AhR knockdown by RNAi; however, it was evident that CYP1A1 was induced in the classical but not QM-PDA pancreatic cancer cells and therefore, the differences between their Ah-responsiveness (i.e. CYP1A1 induction) were further investigated.

Western blot analysis of cytosolic and nuclear fractions from Panc1 and MiaPaCa2 cells treated with DMSO (control), 10 nM TCDD, and 200 μ M omeprazole and tranilast for 90 min showed that AhR and Arnt were primarily cytosolic and, not surprisingly, CYP1A1 protein expression was not induced after treatment for only 90 min (Fig. 3A). Moreover, immunostaining of Arnt in Panc1 cells also shows that Arnt is primarily cytosolic (Suppl. Fig. S5). In contrast, nuclear expression of AhR and Arnt proteins were observed in L3.6pL and BxPC3 cells in all treatment groups (Fig. 3B) and ligand-induced increases were primarily observed in BxPC3 cells. MDA-MB-468 and HepG2 cells are Ah-responsive cell lines^{34, 35, 39} and were used as “controls” for this study. TCDD induced nuclear uptake of

the AhR in both cell lines; in contrast, omeprazole but not tranilast also increased levels of the nuclear AhR after treatment of these cells for 90 min. GAPDH and p84 were used as cytosolic and nuclear markers, respectively, to confirm the integrity of the isolation of the cytosolic and nuclear extracts. Thus, the rapid (within 90 min) ligand (TCDD)-induced nuclear uptake of the AhR was not detected in Panc1 or MiaPaCa2 cells, whereas increased levels were observed in BxPC3 and L3.6pL cells and also in MDA-MB-468 and HepG2 cells (Figs. 3B and 3C) and increased nuclear AhR levels were prominent in the latter two cell lines. We also observed that Arnt expression was primarily cytosolic in the pancreatic cancer cell lines, whereas in MDA-MB-468 and HepG2 cells, there were increased levels of nuclear Arnt which was further increased after treatment with TCDD. Nuclear expression of Arnt has been characterized extensively in HepG2 and other liver cancer cell lines; however, the cytosolic location of this protein has also previously been reported.⁴⁰

The effects of the same AhR ligands on AhR location after treatment for 24 hr were also investigated. TCDD and omeprazole (Panc1) and TCDD, omeprazole and tranilast (MiaPaCa2) induced cytosolic AhR degradation; however, this was not accompanied by nuclear uptake of the receptor or induction of CYP1A1 protein (Fig. 4A). In L3.6pL and BxPC3 cells, only TCDD induced degradation of the cytosolic AhR and this was accompanied by induction of CYP1A1 protein (Fig. 4C), and similar effects were observed for TCDD in MDA-MB-468 and HepG2 cells (Fig. 4C). Previous studies showed that TCDD either did not induce⁴¹ or significantly induced (minimal)⁴² CYP1A1 protein levels in Panc1 cells; this study detects endogenous expression of this protein but no induction. Omeprazole and tranilast had minimal effects on AhR protein levels (cytosolic or nuclear) in L3.6pL, BxPC3, MDA-MB-468 or HepG2 cells; omeprazole induced CYP1A1 protein in only one (MDA-MB-468) of the four cell lines, and tranilast did not induce CYP1A1 protein. TCDD-induced downregulation of the AhR by activation of proteasomes is well known; however, the effects of other AhR ligands on the AhR are ligand- and cell context-dependent.^{34, 35} The cell context-dependent differences in the induction of CYP1A1 and other Ah-responsive genes/proteins by the AhR-active pharmaceuticals has previously been observed in other cancer cell lines^{34, 35} and is consistent with their SAhRM-like activity. We used a ChIP assay to examine ligand-induced recruitment of the AhR to the XRE sequence on the CYP1A1 promoter (Fig. 4D). The results showed that TCDD, tranilast and omeprazole did not induce recruitment of the AhR to the CYP1A1 promoter in Panc1 cells, whereas TCDD and to a lesser extent omeprazole and tranilast increased AhR complex binding to the CYP1A1 promoter in BxPC3, L3.6pL and HepG2 cells. The classical BxPC3 and L3.6pL pancreatic cancer cells are Ah-responsive with respect to induction of CYP1A1 via ligand-induced activation of the nuclear AhR which is a well-recognized characteristic of Ah-responsiveness. In contrast, inhibition of Panc1 cell invasion by omeprazole and tranilast in Panc1 cells (Fig. 1C) is either AhR-independent or due to a non-genomic pathway and this was further investigated.

Transfection of Panc1 cells with a non-specific oligonucleotide or siAhR followed by treatment with omeprazole, tranilast and TCDD showed that only the former two compounds decreased invasion which was attenuated after AhR knockdown (Fig. 5A). Similar results were observed in MiaPaCa2 cells, another QM-PDA cell line (Fig. 5B), suggesting that AhR-dependent inhibition of invasion of Panc1 and MiaPaCa2 cells by

omeprazole and tranilast was non-genomic. The partial reversal of omeprazole-induced invasion in Panc1 and MiaPaCa2 cells transfected with siAhR is due, in part, to incomplete AhR knockdown. Previous studies on other AhR-dependent non-genomic pathways also did not require Arnt,^{43, 44} and results in Figures 5C and 5D demonstrate that omeprazole- and tranilast-mediated inhibition of Panc1 and MiaPaCa2 cell invasion was unaffected by Arnt knockdown. We also examined the potential effects of omeprazole, tranilast and TCDD in classical BxPC3 and L3.6pL cells; however, these cells did not invade in a Boyden chamber assay and were not further investigated.

The failure of the endogenous AhR in Panc1 and MiaPaCa2 cells to undergo ligand-induced nuclear translocation was further investigated using Panc1 cells as a model. These cells were transfected with FLAG-tagged wild-type (WT-AhR) and constitutively-active (CA-AhR) AhR (Fig. 6A). CA-AhR induces CYP1A1 in cell culture and animal models in the absence of ligand;⁴⁵ however, in Panc1 cells transfected with WT-AhR and CA-AhR, these receptors were detected only in the cytosolic fraction (Fig. 6B), and immunostaining with FLAG antibodies and merging with DAPI-stained cells confirmed that the AhR was extranuclear (Fig. 6C). Figure 6D confirms the overexpression of both WT-AhR and CA-AhR in Panc1 cells and the failure of CA-AhR to induce CYP1A1 as a functional marker of nuclear AhR. In contrast, transfection of WT-AhR and CA-AhR in BxPC3, MDA-MB-468 and HepG2 cells results in nuclear accumulation of CA-AhR and induction of CYP1A1 mRNA in all three cell lines (Fig. 6E).

Thus, the results with endogenous and transfected AhR and CA-AhR in Panc1 cells show that this receptor accumulates in the cytosol and does not undergo nuclear translocation. It is possible that this may be due, in part, to the failure of Arnt to accumulate in the nucleus; however, treatment of Panc1 cells with cobaltous chloride (CoCl₂) to induce hypoxia resulted in increased formation of Arnt and HIF-1 α but not AhR (transfected or endogenous) in the nucleus (Fig. 7A). Treatment of Panc1 cells transfected with WT-AhR and CA-AhR with 200 μ M omeprazole and tranilast and 10 nM TCDD also did not induce nuclear translocation of the AhR (Fig. 7B). These studies further confirm that in contrast to HepG2, MDA-MB-468, BxPC3 and L3.6pL cells (Figs. 3 and 4), AhR ligands including TCDD do not induce nuclear uptake of the AhR and induction of CYP1A1 in Panc1 and MiaPaCa2 cells, suggesting that Arnt-independent but AhR-dependent inhibition of invasion (Figs. 1 and 2) is due to a novel non-genomic pathway. Moreover, we also did not observe Src activation (data not shown) which has previously been reported as a non-genomic mechanism of action^{43, 44} (Fig. 7C).

Discussion

Several reports demonstrate multiple functions of the AhR in normal physiology and its importance in immunity and autoimmunity, inflammation, and carcinogenesis.⁴⁻¹⁹ The role of the endogenous AhR in mediating these responses is highly variable and tissue-specific and this receptor is emerging as an important drug target for application of selective AhR agonists or antagonists. For example, the AhR plays a role in expression of the pro-survival and interleukin-6 genes in head and neck cancer cells and TCDD enhances cell migration, whereas AhR antagonists repress head and neck cancer cell migration.⁴⁶ In this study, we

show that high expression of the AhR in pancreatic tumors predicts a longer disease-free survival and the prognostic significance varies with different tumor types.⁷ It is possible that patients with tumors expressing high AhR may be more responsive to AhR-active therapeutics; however, this has not been clinically determined. In order to develop AhR agonists or antagonists for therapeutic applications, it is necessary to understand the mechanisms of AhR action for specific diseases. Recent studies in this laboratory have focused on investigating the potential anticarcinogenic activities of AhR-active compounds in treating ER-negative breast cancer,^{34, 35} a disease for which current treatments with cytotoxic drugs have limited effectiveness and are accompanied by highly toxic side-effects. AhR ligands inhibited invasion in these cell lines and based on the results of an extensive screening of pharmaceuticals²⁰, we selected eight of the more active compounds for subsequent studies on their activities as inhibitors of breast^{34, 35} and pancreatic cancer cell invasion (Fig. 1C). This initial screening approach presupposes that identification of a drug approved for treating other diseases can be more readily repositioned for cancer chemotherapy.

Omeprazole is an AhR ligand that has been extensively used for treating acid reflux and our recent studies show that omeprazole inhibits ER-negative breast cancer cell invasion (*in vitro*) and metastasis in a mouse model.^{34, 35} In ER-negative breast cancer cells, omeprazole (and TCDD) downregulate the pro-invasion factor CXCR4 through interaction of the nuclear AhR with a *cis*-acting XREs in the CXCR4 promoter.³⁵ The AhR mRNA and protein are highly expressed in pancreatic tumors compared to non-tumor tissue (Fig. 1A), and previous studies show that some SAHRMs inhibit anchorage-independent growth of pancreatic cancer cells⁴¹. Initial screening of eight AhR-active pharmaceuticals as inhibitors of Panc1 cells invasion in a Boyden chamber assay identified omeprazole and tranilast but not TCDD as inhibitors of invasion (Fig. 1C), and we used these ligands as models for investigating the Ah-responsiveness of pancreatic cancer cells. However, we now show that the AhR is a potential drug target for pancreatic cancer and if doses of compounds such as omeprazole are too high, there are several more clinically-approved and experimental benzimidazoles that may be more effective and will be investigated in the future.

Panc1 and MiaPaCa2 cells are classified as QM-PDA cells based on gene expression patterns³⁸. They are models for the most aggressive type of pancreatic tumors and are associated with the lowest rates of patient survival.³⁸ Both cell lines express AhR mRNA (Figs. 2A and 2B) and protein (Figs. 3A and 4A), and treatment with omeprazole and TCDD but not tranilast for 24 hr decreased expression of the AhR protein in Panc1 cells, whereas in MiaPaCa2 cells levels of the AhR protein were decreased by all three ligands. In contrast, only TCDD decreased AhR protein expression in the less-invasive classical pancreatic cancer cell lines (BxPC3 and L3.6pL); all four pancreatic cancer cell lines as well as the Ah-responsive MDA-MB-468 breast cancer cells expressed high levels of cytosolic AhR in the absence or presence of ligands. The major exception was the high levels of nuclear (vs. cytosolic) AhR in HepG2 cells treated with TCDD (Fig. 4C). Using induction of *CYP1A1* gene expression as a marker of Ah-responsiveness, we observed a striking difference between the QM-PDA and the classical pancreatic cancer cells. TCDD, omeprazole and tranilast did not induce CYP1A1 mRNA or protein (Figs. 2A, 2B and 4A), and this was consistent with the failure of these ligands to induce formation of the nuclear AhR (Fig. 4A)

or AhR binding to the CYP1A1 promoter (Fig. 4D). In contrast, TCDD induced *CYP1A1* gene expression and nuclear uptake of the AhR in BxPC3, L3.6pL, HepG2 and MDA-MB-468 cells. Similar cell context-dependent effects were also observed for omeprazole and tranilast and this was consistent with results of previous studies with these AhR-active pharmaceuticals.^{34, 35} We also compared nuclear uptake of transfected AhR and CA-AhR in Panc1 vs. Ah-responsive BxPC3, MDA-MB-468 and HepG2 cells and it was evident that in the former cell line, there was a block in nuclear uptake of the receptor in absence or presence of ligand (Figs. 6 and 7). Moreover, inhibition of nuclear translocation was specific for the AhR and not Arnt (Fig. 7A) and this represents a novel Arnt-independent AhR-mediated non-genomic pathway, and we are currently using the QM-PDA cells as models to understand the cellular and functional bases of this nuclear translocation deficit.

It has been previously reported in breast cancer cells that omeprazole and TCDD but not tranilast inhibit invasion by AhR-dependent downregulation of CXCR4.³⁵ In this study, the effects of TCDD, omeprazole and tranilast on CXCR4 were highly variable and cell context-dependent; moreover, we observed that only the QM-PDA Panc1 and MiaPaCa2 but not the classical BxPC3 and L3.6pL cells were invasive in a Boyden chamber assay (Fig. 5). Results of RNA interference assays showed that AhR but not Arnt knockdown partially reversed the inhibition of Panc1 and MiaPaCa2 cell invasion by omeprazole and tranilast (Fig. 5). Previous studies have observed non-genomic AhR-mediated responses in both transformed and non-transformed cell lines;^{31, 43, 44, 47, 48} however, in many of these studies, the effects of TCDD were also accompanied by the classical AhR-dependent activation of CYP1A1. For example, a recent report shows that TCDD rapidly induces focal adhesion kinases in HepG2 cells through a non-genomic pathway and this contributes to activation of a cell migration program.⁴⁸ However, TCDD also activates the nuclear AhR in HepG2 cells (e.g. Fig. 4C), and invariably studies on non-genomic AhR-mediated responses are accompanied by classical (genomic) induction of CYP1A1. Thus, our results in QM-PDA cells define a novel non-genomic pathway in cells where there appears to be a block on ligand-induced AhR nuclear translocation and we are currently using these cell lines to further understand the molecular mechanisms associated with this pathway. Although omeprazole inhibits invasion of pancreatic and breast cancer cells through different AhR-mediated pathways, this does not exclude a role for AhR-independent effects and these are currently being investigated for omeprazole and related benzimidazoles and tranilast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AhR	aryl hydrocarbon receptor
AhRc	cytosolic AhR
AhRn	nuclear AhR

Arnt	aryl hydrocarbon receptor nuclear translocator
ChIP	chromatin immunoprecipitation
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
ER	estrogen receptor
FBS	fetal bovine serum
GEO	Gene Expression Omnibus (GEO)
KLF-4	Krüppel-like factor-4
QM-PDA	quasimensenchymal pancreatic ductal adenocarcinoma
SAhRMs	selective aryl hydrocarbon receptor modulators
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
XRE	xenobiotic response element

Figure 1.

AhR expression and inhibition of Panc1 cell invasion by AhR pharmaceuticals. Analysis of AhR expression (A) in GEO data sets of pancreatic ductal adenocarcinomas and Kaplan Meier analysis (B) of patient survival based on high or low (50:50) AhR expression in the data set GSE16515. Kaplan-Meier analysis of high and low Arnt gave expression mixed results based on two measurements of Arnt on the array (data not shown). (C) Panc1 cells were treated with 8 AhR-active pharmaceuticals and their effects on cell invasion were determined in a Boyden chamber assay as outlined in the Materials and Methods. Results are

expressed as means $\pm$ SE for 3 separate determinations, and significant ($p < 0.05$) inhibition of invasion is indicated (*).

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Figure 2.

Role of the AhR in ligand-induced changes in gene expression. Panc1 (A), MiaPaCa2 (B), BxPC3 (C), and L3.6pL (D) cells were transfected with a non-specific control oligonucleotide or siAhR (AhR knockdown) and treated with DMSO, 200 μ M omeprazole, 250 μ M tranilast, or 10 nM TCDD for 18 hr. Expression of CYP1A1, CYP1B1, CXCR4 and AhR mRNA levels were determined by real time PCR as outlined in the Materials and Methods. Results are expressed as means $\pm$ SE for 3 determinations and significant ($p < 0.05$)

loss of activity after AhR knockdown are indicated (*) and differences between control and treatment groups are also indicated (#).

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Figure 3.

Effects of AhR ligands after treatment of cancer cells for 90 min. Panc1 and MiaPaCa2 (A), L3.6pL and BxPC3 (B), MDA-MB-468 and HepG2 (C) cells were treated with DMSO, 200 μ M omeprazole (OME), 200 μ M tranilast or 10 nM TCDD and, after 90 min, cell lysates were obtained and cytosolic and nuclear fractions were analyzed by western blots as outlined in the Materials and Methods. GAPDH and p84 antibodies were used as markers for cytosolic and nuclear proteins, respectively, in the two fractions. The results were similar in replicate (3) experiments.

Figure 4.

Effects of AhR ligands after treatment of cancer cells for 24 hr and ChIP analysis. Panc1 and MiaPaCa2 (A), L3.6pL and BxPC3 (B), and MDA-MB-468 and HepG2 (C) cells were treated with DMSO, 200 μ M omeprazole (OME), 200 μ M tranilast and 10 nM TCDD or 24 hr. Cell (nuclear and cytosolic) lysates were analyzed by western blots using GAPDH (cytosolic) and p84 (nuclear) to demonstrate the purity of the subcellular fractions. (D) Cells were treated as described in (A)-(C) for 2 hr and recruitment of the AhR to the CYP1A1

promoter was determined by ChIP assays as outlined in the Materials and Methods. The results were similar in replicate (3) experiments.

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Figure 5.

Role of AhR and Arnt in ligand-dependent inhibition of QM-PDA cell invasion. Panc1 (A) and MiaPaCa2 (B) cells were transfected with siCtl or siAhR, and treated with DMSO, 200 μ M omeprazole, 200 μ M tranilast or 10 nM TCDD for 18 hr. Cell invasion was determined using a Boyden chamber assay as outlined in the Materials and Methods. Panc1 (C) and MiaPaCa2 (D) cells were transfected siCtl or siArnt and treated as described in (A)/(B), and effects on invasion were determined in a Boyden chamber assay. Results are expressed as means $\pm$ SE for 3 replicate determinations, and significant ($p < 0.05$) attenuation of ligand-

induced responses by siAhR or siArnt are indicated (*). Results of AhR and Arnt knockdown by RNAi are also shown in each panel (A – D).

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Figure 6.

Nuclear uptake and CYP1A1 induction in cells transfected with wild-type (WT) or constitutively active (CA) AhR. (A) Human WT- and CA-AhR containing N-terminal FLAG-tags. (B) Panc1 cells were transfected with empty vector (V), WT-AhR (WT) or CA-AhR (CA) and after 48 hr, cytosolic and nuclear fractions were analyzed by western blots as outlined in the Materials and Methods. Panc1 cells were also transfected as outlined in (B) and after 48 hr, cells were co-stained with DAPI and AhR antibodies (C) or analyzed by real time PCR (D). Significant ($p < 0.05$) induction (*) is indicated (means $\pm$ SE of 3 replicate

determinations). (E) BxPC3, MDA-MB-468 and HepG2 cells were transfected as described in (A). Nuclear and cytosolic extracts were analyzed by western blots and induction of mRNA levels were determined by real time PCR. Significant ($p < 0.05$) induction is indicated (*) (means $\pm$ SE for 3 replicate determinations).

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Figure 7.

Ligand-dependent effects on nuclear AhR uptake in Panc1 cells transfected with WT-AhR (WT) and CA-AhR (CA). Panc1 cells were transfected with empty vector, WT or CA expression plasmids and treated with 100 μ M CoCl_2 for 24 hr (A) and then treated with DMSO, 200 μ M omeprazole (OME), 200 μ M tranilast or 10 nM TCDD for 24 hr (B). Cytosolic and nuclear fractions were then analyzed by western blots with GAPDH and p84

serving as cytosolic and nuclear marker proteins, respectively. (C) Model for ligand- and AhR-dependent inhibition of QM-PDA cell invasion.

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