

Ah Receptor Antagonism Represses Head and Neck Tumor Cell Aggressive Phenotype

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Abstract

The aryl hydrocarbon receptor (AhR) has been shown to play a role in an increasing number of cellular processes. Recent reports have linked the AhR to cell proliferation, cytoskeletal arrangement, and tumor invasiveness in various tumor cell types. The AhR plays a role in the de-repression of the interleukin (*IL*)6 promoter in certain tumor cell lines, allowing for increased transcriptional activation by cytokines. Here, we show that there is a significant level of constitutive activation of the AhR in cells isolated from patients with head and neck squamous cell carcinoma (HNSCC). Constitutive activation of the AhR in HNSCCs was blocked by antagonist treatment, leading to a reduction in *IL6* expression. In addition, the AhR exhibits a high level of expression in HNSCCs than in normal keratinocytes. These findings led to the hypothesis that the basal AhR activity in HNSCCs plays a role in the aggressive phenotype of these tumors and that antagonist treatment could mitigate this phenotype. This study provides evidence that antagonism of the AhR in HNSCC tumor cells, in the absence of exogenous receptor ligands, has a significant effect on tumor cell phenotype. Treatment of these cell lines with the AhR antagonists 6, 2', 4'-trimethoxyflavone, or the more potent GNF351, decreased migration and invasion of HNSCC cells and prevented benzo[*a*]pyrene-mediated induction of the chemotherapy efflux protein ABCG2. Thus, an AhR antagonist treatment has been shown to have therapeutic potential in HNSCCs through a reduction in aggressive cell phenotype. *Mol Cancer Res*; 10(10); 1369–79. ©2012 AACR.

Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that has been largely regarded as a mediator of xenobiotic metabolism for decades (1). Unliganded AhR is typically found in a cytoplasmic complex with the 90-kDa hsp90 and the X-associated protein 2 (XAP2). Agonist binding leads to nuclear translocation of the receptor, where it releases the chaperone complex and heterodimerizes with the AhR nuclear translocator (ARNT). Prototypical AhR agonists include a variety of xenobiotics, including polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]pyrene (B[*a*]P) and 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). PAHs are common environmental pollutants resulting from car-

exhaust, manufacturing, and cigarette smoke, in addition to other sources. The xenobiotic response initiated by the AhR centers on its ligand-mediated binding to dioxin response elements (DRE) in the promoters of *CYP1A1*, *CYP1B1*, and *CYP1A2*, which express enzymes that act in phase I xenobiotic metabolism, as well as other target genes.

Recent research has provided evidence that there are a myriad of endogenous roles for the AhR, both in the presence and absence of exogenous ligands. Examples of physiologic activities in which the AhR plays a part include attenuation of the acute phase response, cytokine signaling, T helper (T_H)17 immune cell differentiation, modulation of NF- κ B activity, and regulation of hormonal signaling (2–7). This multifaceted aspect of endogenous AhR activity arises not only through the AhR binding to its cognate response element but also through protein–protein interactions. This latter mechanism can mediate transcription factor sequestration away from a gene promoter or tethering of the AhR to a transcription factor on a promoter. In support of the concept of endogenous ligands as a source of receptor activation, a growing list of endogenous ligands have been identified, such as the uremic toxin, 3-indoxyl sulfate, transient metabolites of the arachidonic acid pathway, and byproducts of the tryptophan oxidation pathway (8–10). This implies that there is potential for constitutive or transient *in vivo* activation of the receptor in certain tissue types, resulting in a wide range of effects.

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We have previously shown in the MCF-7 breast cancer cell line that activation of the AhR by TCDD treatment induces binding of the receptor to DREs, approximately 3 kb upstream from the transcription start site of the interleukin (*IL*)6 promoter. This has the effect of priming the DNA for IL1B-mediated NF- κ B binding and a subsequent increase in transcription. In this context, the binding of the AhR coincides with de-repression of the gene by dismissal of histone deacetylases (HDAC) from the proximal promoter (11, 12). In the absence of AhR expression, IL1B only poorly induces *IL6* expression. Our research has focused on head and neck squamous cell carcinoma (HNSCC), which often shows constitutively high cytokine expression regardless of the tissue of origin (13–15). Analysis of the *IL6* promoter in multiple HNSCC cell lines revealed a high level of AhR presence in the absence of exogenous ligand, apparently maintaining the promoter in the de-repressed state. For this reason, basal *IL6* production was higher than in MCF-7 cells, and IL1B readily induced *IL6* transcription on its own. Treatment of HNSCCs with the AhR antagonist 6, 2', 4'-trimethoxyflavone (TMF) for 12 hours or longer resulted in a significant reduction in the level of AhR found at the *IL6* promoter and a corresponding increase in the amount of HDAC1 present (12). This reversal of constitutive de-repression through removal of the AhR from the *IL6* promoter led to decreases in both basal and IL1B-induced *IL6* transcription and subsequent IL6 secretion. Thus, AhR antagonist treatment has proven to be a viable method to decrease progrowth IL6 in HNSCC cell culture models.

Having shown that AhR antagonism effectively limits the secretion of *IL6* in HNSCC cell lines, we then focused on the phenotypic effects of AhR antagonism on HNSCC. HNSCC is regarded as an aggressive form of carcinoma, with a 5-year overall survival rate below 50% and high levels of metastasis in patients (16). Current treatment for HNSCCs centers on radical neck dissection with or without adjuvant radiation therapy and/or chemotherapy. While high IL6 levels in HNSCCs correlate with disease aggressiveness and poorer patient prognosis (17), it has not been proven to be a cause-and-effect relationship. The possibility remains that the higher IL6 levels are, due in part to, higher AhR activity, and this activated AhR itself has numerous other effects on cellular phenotype. In this context, we assessed the ability of AhR antagonist treatment to abrogate multiple aspects of the aggressive phenotype of HNSCCs. Results presented here reveal that blocking AhR activity can, in a relatively short time frame, lead to decreased HNSCC migration, invasion, and proliferation.

Materials and Methods

Cell culture

HN13, HN30, HN2095 HNSCC cell lines were maintained at 37°C, 5% CO₂ in a high glucose 1:1 DMEM:F12 (Sigma), supplemented with 10% FBS (Hyclone Labs.), 1,000 units/mL penicillin, and 0.1 mg/mL streptomycin (Sigma). Human epidermal keratinocytes (HEK) were purchased from Cell Applications, Inc. These cells were

maintained in adult keratinocyte growth medium and passaged using the Subculture Reagent Kit (Cell Applications, Inc.).

Chemicals

TMF was purchased from Indofine Chemical Company. TCDD was kindly provided by Dr. Steve Safe, Texas A&M University (College Station, TX). The synthesis of GNF351 [*N*-(2-(1*H*-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9*H*-purin-6-amine] is described in the Supplementary Methods.

Gene expression

Treatment of cells was conducted by diluting compounds to the desired working concentration in serum-free media supplemented with 5 mg/mL bovine serum albumin (BSA) or low serum (2%) media. Total RNA was extracted from the cells using TRI reagent (Sigma) as specified by the manufacturer. The ABI High-Capacity cDNA Archive Kit (Applied Biosystems) was used to prepare cDNA from isolated RNA. mRNA expression was measured by quantitative real-time PCR (qRT-PCR) using the Quanta SYBR Green Kit on an iCycler DNA engine equipped with the MyiQ single color real-time PCR detection system (Bio-Rad). Expressed quantities of mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels and plotted using GraphPad Prism 4.0 (GraphPad Software). Histograms are plotted as mean values of biologic replicates; error bars represent the SD of replicates. qRT-PCR primers used are presented in Supplementary Materials.

Immunoblotting

Whole-cell extracts were prepared by lysing cells in radioimmunoprecipitation assay (RIPA) buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 0.5 mmol/L EGTA, 140 mmol/L NaCl, 1% Triton X-100, 0.1% Na deoxycholate, 0.1% SDS] supplemented with 1% IGEPAL, 300 mmol/L NaCl, and protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 21,000 × *g* for 30 minutes at 4°C, and the soluble fraction was collected as whole-cell extract. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad). Protein samples were resolved by tricine SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Primary antibodies used to detect specific proteins are shown in Supplementary Materials and were visualized using biotin-conjugated secondary antibodies (Jackson ImmunoResearch) in conjunction with ¹²⁵I-streptavidin (Amersham). In the case of ABCG2 protein, protein was detected by enhanced chemiluminescence (Pierce).

HN30 and human epidermal keratinocyte comparative analysis

AhR protein levels were assessed after lyses of cells in MENG (25 mmol/L MOPS, 2 mmol/L EDTA, 0.02% NaN₃, 10% glycerol, pH 7.5) plus 1% IGEPAL CA-630, 300 mmol/L NaCl, 20 mmol/L molybdate, and protease

inhibitors. Cell lysates were centrifuged at $13,000 \times g$ for 10 minutes at 4°C , and soluble extracts containing both nuclear and cytosolic proteins were collected. The extracts were resolved by 8% tricine SDS-PAGE and transferred to a PVDF membrane. Relative AhR and β -actin protein levels were determined. For the isolation of cytosolic and nuclear extracts, cells were collected and suspended in MENG plus 20 mmol/L molybdate, pH 7.4 (molybdate buffer), with the addition of protease inhibitors. The cells were homogenized and centrifuged at $1,000 \times g$ for 20 minutes at 4°C . The soluble phase was saved as the cytosolic fraction. The pellet was washed with molybdate buffer and centrifuged at $1,000 \times g$ for 5 minutes at 4°C for a total of 3 wash cycles. Molybdate buffer + 50 mmol/L NaCl was added to the pellet, and the extracts were centrifuged at $1,000 \times g$ for 20 minutes at 4°C . The pellet was incubated on ice for 1 hour with high salt buffer (molybdate buffer, 500 mmol/L NaCl, and protease inhibitors). The samples were then centrifuged for 1 hour at $100,000 \times g$ at 4°C , and the nuclear fraction was collected. Samples were resolved using tricine SDS-PAGE and protein transferred to a PVDF membrane. The blot was probed for AhR, lamin A/C, and β -actin using the appropriate primary antibodies, secondary biotinylated antibodies, and ^{125}I -streptavidin.

Chromatin immunoprecipitation assays

HN13 cells were grown to approximately 90% confluency in 150-cm^2 dishes and serum-starved 18 hours before treatment. Cells were treated in serum-free media supplemented with 5 mg/mL BSA by diluting compounds to the desired working concentrations. Chromatin immunoprecipitation assays were conducted as previously described (11). The primers used for PCR analysis and antibodies used are given in the Supplementary Materials.

Gene silencing

Specific protein levels were decreased using the Dharmacon siRNA (control oligo D-001810-0X, AhR oligo J-004990-07). Electroporation/nucleofection was conducted using the Amaxa Nucleofection System essentially as described in manufacturer's protocols. Briefly, cells were washed and suspended at a concentration of 2.0×10^6 cells per 100 μL of nucleofection solution. Control or targeted siRNA was added to the cells for a final concentration of 1.5 $\mu\text{mol/L}$. Cells were electroporated using manufacturer's MCF-7 high-efficiency program.

Migration assay

All HNSCC cell lines were plated and treated in serum-free media for 24 hours, after which they were trypsinized, counted, and normalized for cell number between treatments. Cells were then subjected to migration assay through an 8- μm pore polycarbonate filter (Neuro Probe #PFB8) using the Neuro Probe Multiwell Chemotaxis Chamber according to manufacturer's specifications. Briefly, lower well chambers were filled with either serum-free or 10% FBS-containing media, along with vehicle or treatment compound. Polycarbonate membrane was then placed over

the lower wells, with the upper well plate was secured on top. Upper wells were then filled with cell suspensions in serum-free media, containing vehicle or treatment compound. Cells were incubated for 48 hours, after which nonmigratory cells were removed and membranes fixed and stained (Dade Behring Diff-Quik stain set). Membranes were analyzed under $\times 3.2$ and $\times 10$ objectives and imaged. Quantification is the result of cell counts on biologic triplicates of imaged membranes.

Agarose spot migration assay

HNSCC cell lines were treated in low serum media for 24 hours, after which they were trypsinized, counted, and normalized for cell number between treatments. Cells were then subjected to agarose spot assay as described in the study by Wiggins and Rappoport (18). Briefly, 0.5% agarose solution was heated to dissolution, cooled, mixed with PBS or EGF, and spotted onto glass cover slides at concentration of 300 ng EGF/spot. Cover slides were placed in 6-well culture plates, placed at 4°C to solidify, and cell suspensions were then transferred into wells. Forty-eight hours later, spots were imaged at $\times 10$ objective, and migratory cells that had passed under the horizon of the agarose spot were counted.

Invasion assays

HN30 cells were plated and treated in serum-free media for 24 hours, after which they were trypsinized, counted, and normalized for cell number between treatments. Cells were then subjected to 1 of 2 invasion assays. Cells were either plated in serum-free media into the Cell Biolabs CBA-110 CytoSelect Basement Membrane Boyden chamber or plated in serum-free media into Corning Costar Transwell plates (#3422) containing 8- μm polycarbonate filter coated in $0.5\times$ basement membrane extract (BME; Trevigen #3455-096-02, 3455-096-03). In both cases, samples were plated with and without 10% FBS in the lower chamber as a chemoattractant, and all samples and treatments were plated in triplicate. Treatment compounds were contained in the media of both the upper and lower chambers. Forty-eight hours following upper well seeding, nonmigratory cells and basement membrane coating were removed, inserts were stained, rinsed, imaged, and cells were dissociated from the membrane. Colorimetric absorbance of stained cell containing solution was read at 570 nm.

Lactate dehydrogenase assay

Cytotoxicity of AhR ligands was measured by treating HNSCC cell lines for 24 or 48 hours and subjecting culture media and cell lysates to Sigma-Aldrich Tox-7 lactate dehydrogenase (LDH) assay, as per manufacturer's specifications.

Proliferation

HNSCCs were counted and normalized and plated at 5% confluency in 10% serum-containing media in 6-well culture plates. Treatments were added 18 hours after seeding, once cells were adherent. Cells were grown in a reduced serum medium (2% FBS) from time of initial treatment up

to 48 hours. Cells were treated every 12 hours with either 10 $\mu\text{mol/L}$ TMF or 100 nmol/L GNF351, and at 24 hours, the media were changed. Cells were trypsinized and counted using a hemocytometer. HN30 cells were also grown in 16-well plates and growth monitored using an xCELLigence RTCA DP instrument (Roche) with the same media conditions.

Zymography

HN13 cells were plated in 100-mm culture plates for 24 hours, changed to serum-free media and treated for 48 hours, after which media were collected and cells were lysed. Amicon Centricon centrifugal filtration columns were used to concentrate media. Gelatin zymography was conducted using a modified version of the protocol described by Troeberg and Nagase (19). Briefly, samples were denatured using only SDS and run on an 8% PAGE containing 1 mg/mL gelatin. The gel was then washed in 2.5% Triton X-100 to remove SDS, and enzymes were reactivated and allowed to digest gelatin overnight. Gel was then stained with Coomassie blue and destained over 36 hours.

Statistical analysis

All experiment treatments were carried out in triplicate, and the experiments repeated at least twice. Statistical analyses were conducted using Prism 5 graphing and statistical analysis software (GraphPad Software Inc.). Data were analyzed by 1-way ANOVA, Tukey multiple comparison, or Student's *t* tests. $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

The AhR antagonist GNF351 effectively represses constitutive AhR activity in HNSCC cells

We have previously shown that multiple HNSCC cell lines exhibit a constitutively active AhR that is able to maintain the *IL6* promoter in a state of de-repression by preventing HDAC occupancy (17). Treatment of these cells with 10 $\mu\text{mol/L}$ of the AhR antagonist TMF reduced basal *IL6* and *CYP1A1* expression. Chemical screens conducted by the Genomics Institute of the Novartis Research Foundation (San Diego, CA) led to the development of a number of high-affinity AhR antagonists, one of which is the compound GNF351 (20). Prior studies have shown that GNF351 is a more potent AhR antagonist than previously available AhR antagonists. Treatment of HN30 cells with 100 nmol/L GNF351 for 24 hours led to a significant decrease in *IL6* transcription (Fig. 1A), similar to results seen after treatment with 10 $\mu\text{mol/L}$ TMF (11). Thus, the higher affinity AhR antagonist exhibited a similar effect on constitutive *IL6* expression at 100-fold lower dose. In addition, basal *CYP1A1* activity is almost completely ablated following GNF351 treatment (Fig. 1B). Treatment of HN2095 cells with 100 nmol/L GNF351 for 12 hours revealed identical effects on protein occupancy of the *IL6* promoter as shown in our previous studies with TMF (Fig.

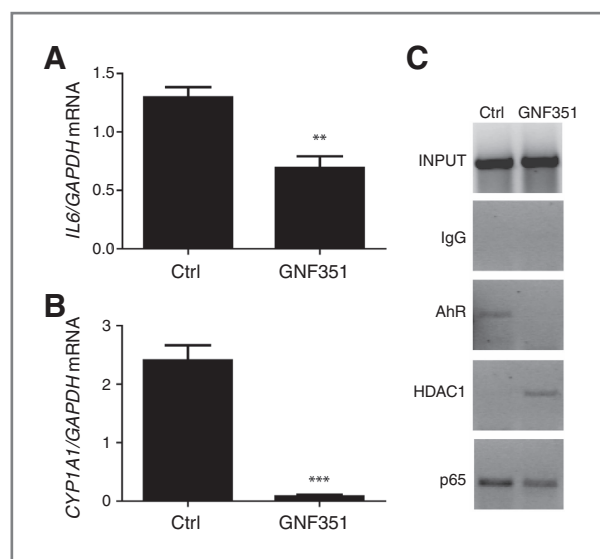


Figure 1. GNF351 is a potent AhR antagonist that reduces *IL6* expression via transcriptional repression. Serum-starved HN2095 cells were treated with vehicle or 100 nmol/L GNF351 for 12 hours. The relative levels of *IL6* (A) and *CYP1A1* (B) mRNA were determined by qRT-PCR. C, chromatin immunoprecipitation (ChIP) analysis of the *IL6* promoter in HN2095 cells following 12-hour treatment with 100 nmol/L GNF351. Ctrl, control.

1C; ref. 12). The constitutive presence of the AhR upstream from the *IL6* transcription start site acts to dismiss the presence of transcriptionally repressive HDAC1 and thus allows for robust NF- κ B-driven transcription, as shown by the enhanced presence of p65 on the *IL6* promoter in the absence of GNF351 treatment. Antagonist treatment of HNSCCs prevents the nuclear localization of AhR and subsequent heterodimerization with ARNT, leading to AhR dismissal from and return of HDAC1 to the *IL6* promoter with a subsequent loss of transcriptional potential. HEKs were also examined for constitutive level of *IL6* expression and the effect of GNF. Results reveal that relative to HN30 cells, HEK cells exhibit extremely low levels of *IL6* expression with no significant effect of GNF351 on expression, in contrast to AhR antagonist repression of *IL6* expression (Supplementary Fig. S1).

HNSCC30 cells exhibit elevated constitutive nuclear levels of AhR in comparison to HEK

The ability of HN30 cells to constitutively express *IL6* is, in part, due to the presence of AhR at the *IL6* promoter. We wanted to assess whole-cell extracts, cytosolic, and nuclear levels of the AhR in HN30 cells in reference to HEKs (Fig. 2). The results revealed that HN30 cells have 7-fold more AhR and exhibited significant levels of AhR in the nucleus. In contrast, nuclear AhR was essentially undetectable in nuclear extracts of HEKs under the experimental conditions used. This would suggest that AhR antagonist treatment would have a greater effect on HNSCCs than normal keratinocytes. This underscores the potential of AhR antagonists as a targeted treatment of tumor cells relative to the surrounding tissue.

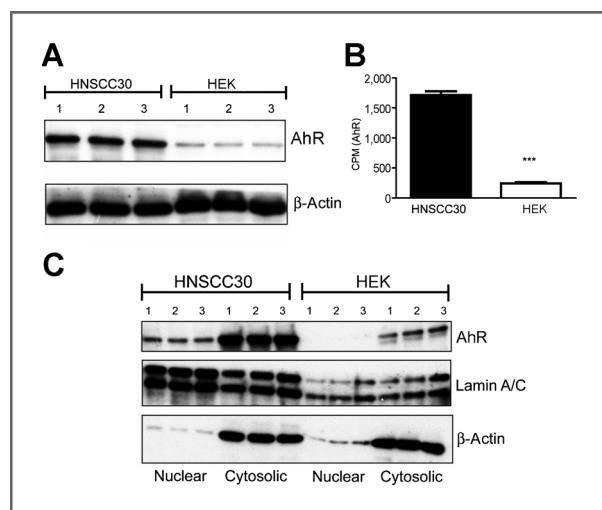


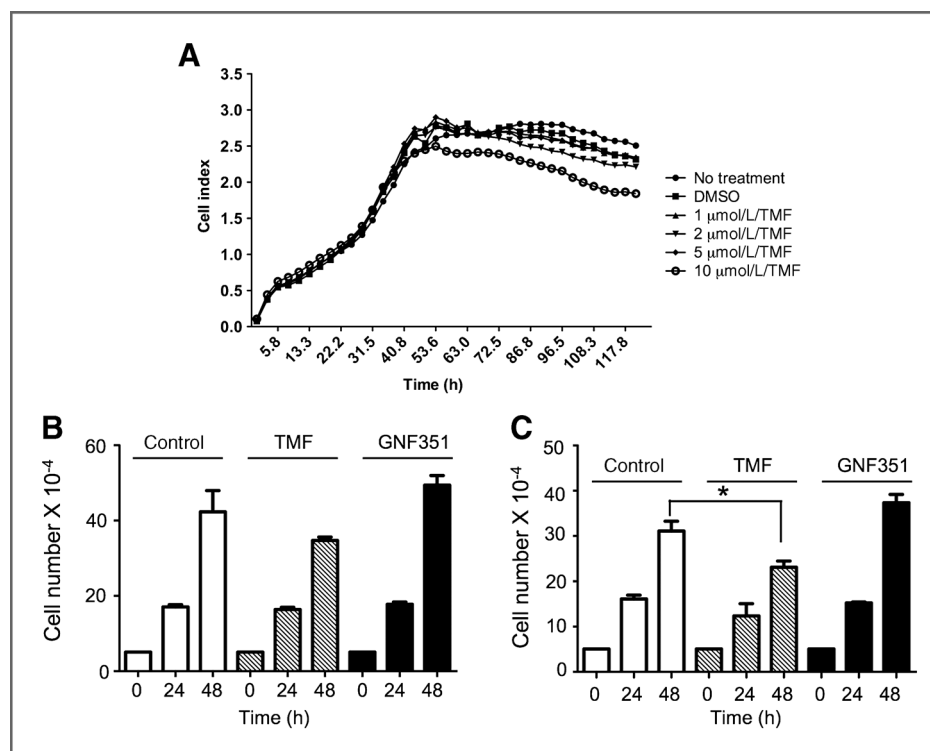
Figure 2. HN30 cells express high overall levels of AhR, with significant levels detected in nuclei, when compared with HEKs. A, whole-cell extracts were prepared from HNSCC30 cells and HEKs in triplicate. Levels of AhR and β -actin were assessed by Western blotting. B, quantification of AhR protein levels shown in A. C, nuclear and cytosolic fractions were isolated from HNSCC30 cells and HEKs in triplicate. Levels of AhR, lamin A/C, and β -actin were assessed by Western blotting.

AhR antagonism has modest effects on HNSCC cellular proliferation and viability

Evidence suggests that the AhR can play a role in cellular proliferation through numerous mechanisms, including affecting cell-cycle progression, progrowth signaling, and anti-apoptotic pathways, although species- and tissue-specific

differences have been noted (21). The use of potent antagonists will allow for investigations into the manner in which constitutively active AhR augments the typically aggressive phenotype of human HNSCCs. Our initial experiments used a real-time cell analyzer to measure proliferation of HN30 cells in a dose-response study with the AhR antagonist TMF (Fig. 3A). The data revealed that TMF had no significant repressive effect on the proliferative ability through the rapid growth phase. However, treatment with 10 μ mol/L TMF did have an effect on cells after they reached the stationary phase. Because we were addressing the question as to whether AhR antagonist had an effect on rapid proliferation, additional studies were conducted at 24 and 48 hours. Further assessments of AhR antagonism on the phenotype of HNSCC cell lines used the HN13 and HN30 cell lines. Cells were treated with either 10 μ mol/L TMF or 100 nmol/L GNF351, an inhibition of proliferation was only seen with TMF at 48 hours in HN13 cells (Fig. 3B and C). To rule out the possibility that AhR antagonism exerts a negative effect on proliferation simply by being cytotoxic, the culture supernatants from the HN13 and HN30 cells were subjected to LDH assay, a marker of cell damage (22). Neither TMF nor GNF351 showed any change in LDH levels in the culture media of HN30 over 24 or 48 hours at the doses used in the proliferation study (Fig. 4). In contrast, TMF induced a significant increase in LDH levels in the media after 24 hours, whereas at 48 hours, there was no additional increase in activity. No change in LDH activity was observed in HN13 cells after GNF treatment. These results would suggest that AhR antagonism does not affect cellular proliferation to a significant extent.

Figure 3. AhR antagonist treatment fail to inhibit HNSCC proliferation. A, HN30 cells were plated a low density into E-plates 16 and cell growth monitored using an xCELLigence system over time in the presence of various concentrations of TMF. HN30 (B) and HN30 (C) cells were plated at low confluency for 18 hours and then placed in low serum media and treated with either carrier solvent (control) or 10 μ mol/L TMF or 100 nmol/L GNF351 for 24 or 48 hours. After multiple doubling times, cells were trypsinized and counted. DMSO, dimethyl sulfoxide.



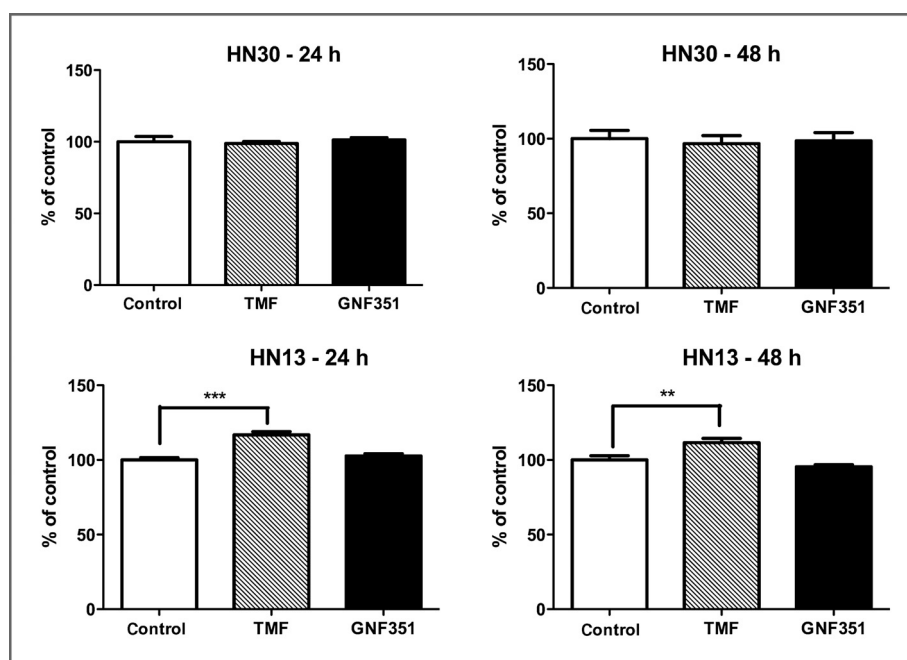


Figure 4. AhR antagonists are not cytotoxic. HN30 and HN13 cells were treated with vehicle or 10 $\mu\text{mol/L}$ TMF or 100 nmol/L GNF for 24 or 48 hours, the media were collected from the experiment in Fig. 3 and LDH activity levels were determined.

Optimal HNSCC cellular migration requires AhR expression

There has been a link suggested between the AhR and cellular motility or migration (23). In light of this information, AhR antagonists were analyzed for their ability to inhibit the highly migratory HN30 cell line in Transwell assays. Using FBS as a chemoattractant, vehicle-treated HN30 cells were found to undergo a significant increase in migration toward the lower chamber. Treatment with the potent agonist TCDD had no effect on basal or chemoattractant-induced migration. In contrast, both TMF and GNF351 were able to significantly inhibit migration in the absence and the presence of FBS as a chemoattractant (Fig. 5A). To assess the role of the AhR in cellular migration, HN30 cells were transfected with AhR siRNA and subsequently plated for Transwell migration assays. Nearly complete AhR protein ablation (Fig. 5B) resulted in a significant loss of migratory ability for HN30 cells and one that is comparable with GNF351 treatment of control siRNA-transfected cells (Fig. 5C). As expected, AhR siRNA transfection also resulted in a loss of GNF351-mediated repressive effects in FBS-exposed samples.

A cell culture-based migratory assay that is more representative of a tumor microenvironment has been developed (18). Liquid agarose containing the chemoattractant EGF is spotted in cell culture plates and allowed to solidify, after which cells are plated around the spots. Given the reliance on EGF signaling for many characteristics of HNSCC cell line phenotype (24), the cells migrate toward and then under the agarose spots in an effort to reach the point of highest EGF density. HN30 cells show a significant reduction in their ability to migrate under EGF-containing agarose spots when treated with the AhR antagonists TMF or GNF351 (Fig. 6A). Note that the cells also appear more rounded after

GNF351 treatment. HN13 and HN2095 also exhibited reduced migration in the agarose spot assay (Supplementary Fig. S2). Interestingly, TCDD also repressed migration in HN13 cells but not in HN2095 cells.

Treatment of HNSCC cells with AhR antagonists inhibits invasive potential

With the finding that AhR antagonist treatment of HNSCCs prevented cellular migration, the focus then moved to determining the effect of GNF351 on invasive potential. Because of their lack of ability to migrate through a polycarbonate membrane containing 8- μm pores, HN13 cells were insufficient for use in Transwell invasion assays. Therefore, HN30 cells were subjected to Transwell assays in which a membrane was coated with BME. These cells showed a significant impairment in their ability to move through the BME layer and migrate through a polycarbonate filter in the presence of TMF (Fig. 6B) or GNF351 (Fig. 6C). This finding suggests that an AhR antagonist may be preventing expression of proteinases required to degrade BME components in addition to affecting migratory ability.

Matrix metalloproteinases (MMP) are a family of proteinases commonly studied in the context of their secretion by tumor cells for the purpose of degrading extracellular matrix proteins to allow for invasion into neighboring tissue (25, 26). MMPs have been shown to play a role in HNSCC disease progression, and MMP9 in particular has even been suggested as an indicator of relapse-free survival, where higher MMP9 expression correlates with a poorer disease prognosis (27). MMP9 is a gelatinase that has also been shown to be upregulated in certain tumor types by AhR activation (28–30). While qRT-PCR for MMP9 expression showed a relatively low, unchanging level of mRNA in HNSCC cell lines (data not shown), gelatin zymography

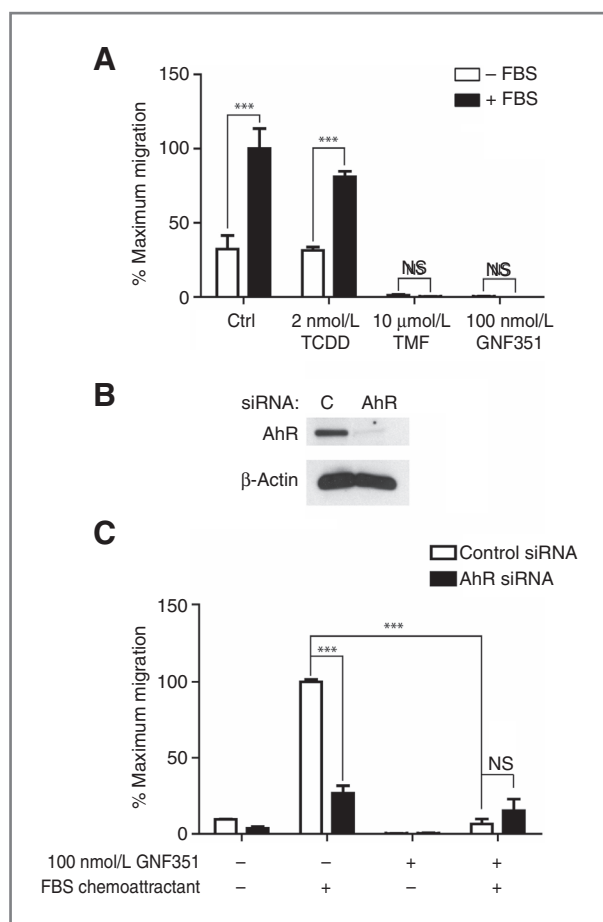


Figure 5. Antagonism of AhR inhibits cellular migration. A, HN30 cells were serum-starved and treated with control, TCDD, TMF, or GNF351 for 24 hours, after which they were normalized and plated into a Transwell migration assay using FBS as a chemoattractant. Cells were then allowed to migrate over a 48-hour time period after which polycarbonate membranes were fixed, stained, and migratory cells counted. B, HN30 cells were subjected to electroporation with AhR siRNA oligonucleotides, after which cells were plated in full serum media for 24 hours, then changed to serum-free media for 48 hours. Cells were then lysed, and whole-cell extract was subjected to Western blotting for AhR and β -actin protein levels. C, HN30 cells were electroporated with nontargeting or AhR-targeting siRNA and plated into a Transwell migration assay with control or GNF351 treatment using FBS as a chemoattractant. Cells were then allowed to migrate over a 48-hour time period after which polycarbonate membranes were fixed, stained, and migratory cells counted. Ctrl, control; NS, not statistically significant.

was used to illustrate the role of the AhR in the secretion of active MMP9. HN13 cells treated with an AhR antagonist had lower levels of MMP9 secreted into the media over 48 hours (Fig. 6D).

Treatment of HNSCC cells with an AhR antagonist inhibits ABCG2 induction

The sensitivity of tumor cells to chemotherapy treatment has been shown to decrease with increased expression of membrane pumps that work in drug efflux. One component of this protein network is ATP-binding cassette sub family G member 2 (ABCG2/BCRP). The AhR has been shown to be

a ligand-activated transcription factor that can mediate induction of human ABCG2 transcription (31, 32). Pretreatment of HN30 cells for 6 hours with GNF351 prevented B[a]P-mediated ABCG2 transcription after an 18-hour exposure (Fig. 7A). As a component of a membrane-bound drug efflux pump, ABCG2 protein levels take longer to increase in response to stimuli. Treatment of HN30 cells for 48 hours with GNF351 followed by 48 hours with 5 μ mol/L B[a]P revealed that GNF351 pretreatment was also able to mitigate protein increase after 96 hours (Fig. 7B).

Discussion

Recent reports have shown that enhanced IL6 expression in head and neck tumor cells stimulates cell growth and epithelial-mesenchymal transition (33, 34). Our previous studies have investigated the role of the AhR in the expression of the pro-survival cytokine IL6 in tumor cells (12). We have shown in MCF-7 breast cancer cells that activation of the AhR with an exogenous ligand such as TCDD can mediate synergistic induction of *IL6* upon stimulation with IL1B (3). Several HNSCC cell lines were subsequently shown to exhibit constitutive AhR occupancy at the *IL6* promoter, leading to higher basal and readily inducible *IL6* expression patterns. Treatment of these cells with an AhR antagonist successfully reduced the expression of *IL6* within 12 hours of the initial dose (12). The potential for constitutively active AhR in HNSCCs, in combination with the understanding that known direct AhR target genes represent only a small segment of receptor-affected genes, points to the AhR as a possible mediator of numerous pathways that enhance the aggressive nature of HNSCCs beyond anti-apoptotic IL6 expression.

The ability of AhR antagonists to block endogenous and exogenous ligand-mediated (e.g., PAHs) receptor activity makes their use a promising method for dissecting the role of the receptor in the HNSCC phenotype. In addition, the lack of cellular toxicity at higher doses coupled with long exposure times would suggest that TMF or GNF351 can be used in long-term experiments *in vivo*. Another layer of complexity is the role of the AhR in cell-cycle progression and in intracellular progrowth signaling. It is quite possible that both an AhR agonist and antagonist can alter their own unique subset of genes, which then leads to the same phenotype. The AhR has been shown to modulate the activity of the retinoblastoma protein and cyclin-dependent kinase 2, both positively and negatively affecting cell-cycle progression, respectively (35–37). Another example is the ability of liganded AhR to block p300 recruitment to cell-cycle genes (38). Introduction of a constitutively active AhR leads to spontaneous stomach and liver tumors in mice, suggesting that sustained AhR activation leads to outgrowth of tumor cells (39, 40). Activation of the receptor through TCDD treatment has shown results similar to those seen with EGF ligand treatment, pointing to the AhR as a mediator of downstream kinase activity that mimics EGF receptor (EGFR) signaling (41, 42). The fact that EGFR signaling is amplified in numerous HNSCC cell lines points

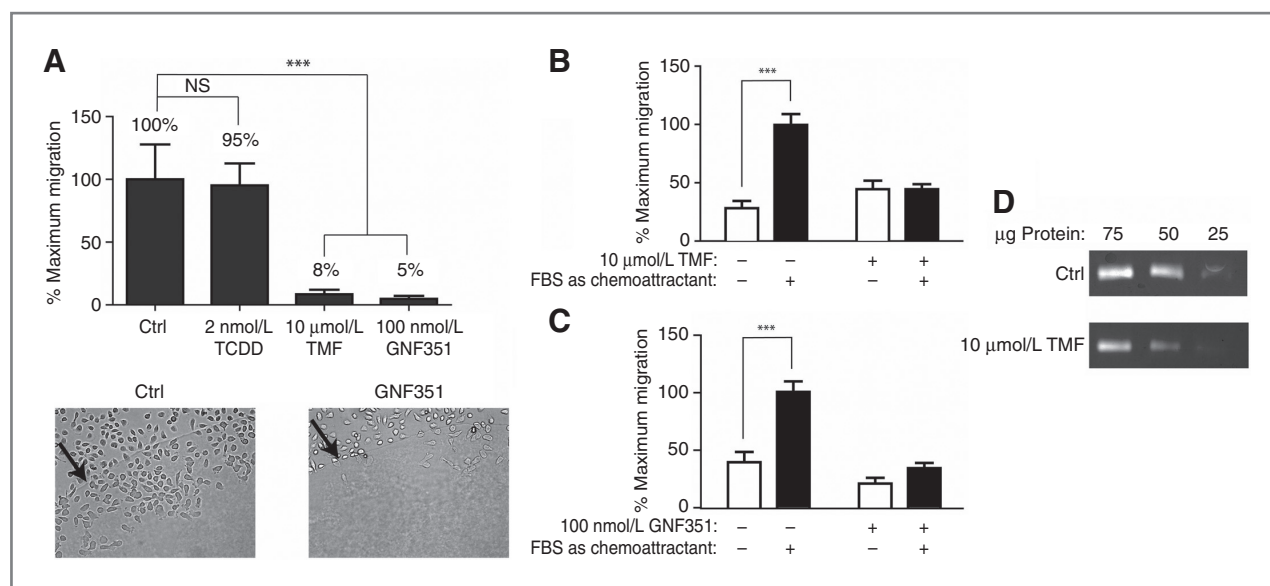


Figure 6. AhR antagonists inhibit HNSCC migration in agarose spot assay and in a Transwell invasion assay. **A**, HN30 cells were treated in low serum media with control, TCDD, TMF, or GNF351 for 24 hours, after which they were trypsinized and plated in treatment-containing, serum-free media into culture plates containing dried agarose spots with or without 300 ng EGF. Cells were then allowed to migrate toward and under agarose spots over 48 hours, at the end of which the spots were imaged and migratory cells were counted. Data represent the mean of cell counts in 4 fields of view, with 2 biologic replicates. **B** and **C**, HN30 cells in serum-free media were treated with either TMF or GNF351 for 24 hours, respectively. The cells were subsequently trypsinized and plated into Transwell invasion assays. After 48 hours, the number of cells on each side of the membrane was determined. **D**, HN13 cells were treated for 48 hours in serum-free media with vehicle or 10 μmol/L TMF. After 48 hours, the media were collected, concentrated, and protein content was determined. Samples were subjected to zymolytic analysis. Ctrl, control; NS, not statistically significant.

to the possibility that EGFR and AhR ligands can act cooperatively, mediating accelerated cellular growth.

Patients with HNSCCs tend to present with later stage disease and are at a high risk of recurrence. Because of the proximal location of lymph nodes and the ability of primary tumors to migrate and metastasize, formation of secondary tumors is a common occurrence. Adjuvant treatment that can mitigate cellular migration and invasion to neighboring tissue would therefore have an impact on preventing disease progression. In 2 different migration assays, AhR antagonists

were able to almost completely ablate migration of several HNSCC cell lines. While TCDD treatment had little effect on the movement of most HNSCC cell lines, both TMF and GNF351 prevented the ability of cell passage through an 8-μm pore-containing polycarbonate membrane, as well as cellular migration toward and under EGF-containing agarose spots. Knockdown of AhR protein in HN30 cells led to a similar outcome in the Transwell migration assay, highlighting the effect of receptor expression on HNSCC cell movement. The role that the AhR plays in cytoskeletal

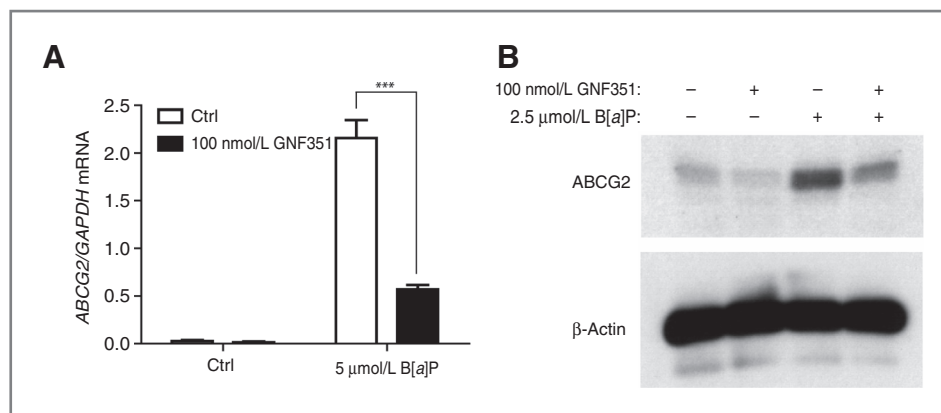


Figure 7. Antagonism of AhR can inhibit the upregulation of ABCG2. **A**, HN30 cells were plated and serum-starved with 6-hour control or 100 nmol/L GNF351 pretreatment, after which control or 5 μmol/L B[a]P was added for further 18 hours. Total RNA was isolated, cDNA prepared, and relative ABCG2 mRNA measured by qRT-PCR. **B**, HN30 cells were plated in low serum media with pretreatment of control or 100 nmol/L GNF351. After 48 hours, cells were further treated for 48 hours with control or 500 nmol/L B[a]P, at the end of which cells were lysed and whole-cell extract was subjected to Western blotting for ABCG2 and β-actin protein levels. Ctrl, control.

organization and migratory ability remains unclear, but evidence has shown that AhR-null fibroblasts have lower migration due to deregulated cytoskeletons, and treatment of cells with AhR ligands results in a decrease in cell–cell contact and an increase in cell–extracellular matrix contact (43, 44). One potential pathway through which these changes could occur is the AhR-mediated regulation of VAV3, which is itself a mediator of Rho GTPases and downstream cytoskeletal organization (45, 46). Clearly, the targets of AhR antagonism in HNSCCs will need to be explored.

The AhR has been shown to affect the invasiveness of numerous cancer cells, such as the increase in estrogen receptor–negative breast cancer cell migration following B[a]P treatment (47). One mechanism by which this may occur centers on the effect of the AhR on MMP expression. Antagonist treatment of HN30 cells has a significant effect on their invasiveness as measured by extracellular matrix–coated Transwell assays. While TCDD treatment has been shown to have an effect on the expression of numerous MMPs, the most widely studied interaction involves the gelatinase MMP9 (28–30). Gelatin zymography highlights the decrease in secreted MMP9 following AhR antagonist treatment in HN13 cells. Considering the lack of a difference in mRNA levels of MMP9 upon GNF351 treatment, the mechanism of repressed MMP9 secretion would appear to be posttranscriptional.

Little progress has been made over the past decades with regard to treatment of HNSCCs. The 5-year overall survival rate remains below 50%, and the heterogeneity of tumors under the HNSCC classification precludes a single breakthrough treatment that would target one pathway and affect all primary malignancies. HNSCC is classified as only being moderately radiosensitive, and the efficacy of chemotherapy is questionable. Recent research has shown that higher EGFR expression in HNSCCs correlates with poorer efficacy of radiation treatment and assessments are ongoing using concomitant EGFR inhibitors (48). This treatment would, by definition, only be effective through one molecular mechanism and only in certain patients. However, the

ability of AhR antagonist pretreatment to sensitize HNSCCs to a more aggressive adjuvant therapy could have a large impact on treatment outcomes. For example, treatment of HNSCC cell lines with GNF351 dramatically attenuates increases in the drug efflux pump ABCG2 in the presence of B[a]P, which is known to actively remove the chemotherapy drugs doxorubicin and mitoxantrone from the cell (49). In this way, enhanced expression of ABCG2 in cancer cells, such as in cigarette smokers, could be reduced before treatment, allowing for longer half-life of chemotherapeutics within the cell and/or lower chemotherapy dosages to achieve similar efficacy endpoints. Radiosensitization is another method by which nontoxic therapies can influence the outcome of HNSCC survival in patients. For example, EGFR inhibitors have been shown to increase the number of cells in G₂–M phase, with S-phase growth arrest following radiation treatment (50). Thus, other therapies will likely need to be coupled with AhR antagonist treatments to test whether synergistic tumor cell toxicity can be achieved.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: B.C. DiNatale, S.G. Amin, G.H. Perdev
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.C. DiNatale, K. John, G.H. Perdev
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.C. DiNatale, K. Smith, K. John, G.H. Perdev
Writing, review, and/or revision of the manuscript: B.C. DiNatale, K. Smith, S.G. Amin, G.H. Perdev
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.C. DiNatale, G.H. Perdev
Study supervision: G.H. Perdev

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