(Z)-2-(3,4-Dichlorophenyl)-3-(1H-Pyrrol-2-yl)Acrylonitrile Exhibits Selective Antitumor Activity in Breast Cancer Cell Lines via the Aryl Hydrocarbon Receptor Pathway^[S]

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ABSTRACT

We have previously reported the synthesis and breast cancer selectivity of (Z)-2-(3,4-dichlorophenyl)-3-(1H-pyrrol-2-yl)-acrylonitrile (ANI-7) in cancer cell lines. To further evaluate the selectivity of ANI-7, we have expanded upon the initial cell line panel to now include the breast cancer cell lines (MCF7, MCF7/VP16, BT474, T47D, ZR-75-1, SKBR3, MDA-MB-468, BT20, MDA-MB-231); normal breast cells (MCF-10A); and cell lines derived from colon (HT29), ovarian (A2780), lung (H460), skin (A431), neuronal (BE2C), glial (U87, SJG2), and pancreatic (MIA) cancers. We now show that ANI-7 is up to 263-fold more potent at inhibiting the growth of breast cancer cell lines (MCF7, MCF7/VP16, BT474, T47D, ZR-75-1, SKBR3, MDA-MB-468) than normal breast cells (MCF-10A) or cell lines derived from other tumor types. Measures of growth inhibition, cell cycle analysis,

morphologic assessment, Western blotting, receptor binding, gene expression, small interfering RNA technology, reporter activity, and enzyme inhibition assays were exploited to define the mechanism of action of ANI-7. In this work, we report that ANI-7 mediates its effects via the activation of the aryl hydrocarbon receptor (AhR) pathway and the subsequent induction of CYP1-metabolizing mono-oxygenases. The metabolic conversion of ANI-7 induces DNA damage, checkpoint activation, S-phase cell cycle arrest, and cell death in sensitive breast cancer cell lines. Basal expression of AhR, the AhR nuclear translocator, and the CYP1 family members do not predict for sensitivity; however, inherent expression of the phase II-metabolizing enzyme sulfur transferase 1A1 does. For the first time, we identify (Z)-2-(3,4-dichlorophenyl)-3-(1H-pyrrol-2-yl)-acrylonitrile as a new AhR ligand.

Introduction

Breast cancer is the most common cancer in women both in the developed and less developed world, and the incidence is on the rise. Early-stage breast cancer treatments include surgery and radiotherapy, whereas chemotherapy, hormonal, and targeted therapies are considered for more aggressive tumors. Tamoxifen and anastrozole are standard treatment of hormone-sensitive tumors; however, drug resistance is often induced and tumor selectivity is poor (Ma et al., 2015). Herceptin selectively targets the human epidermal growth factor receptor (HER2); however, 70% of HER2-positive patients fail to respond to treatment, with resistance developing rapidly. Herceptin also induces significant cardiac dysfunction in 2%–7% of patients. Even fewer options are available for triple-negative tumors that

are receptor negative for estrogen (ER), progesterone, and HER2. Triple-negative breast cancers are vastly heterogeneous, hindering targeted therapy development (Sharma, 2016). Critically and irrespective of the hormonal status, 33% of patients with initial breast cancer experience recurrence or metastasis, whereas 5% of new breast cancer patients present with metastases at diagnosis. The 5-year survival for these advanced breast cancer patients is only 25%, and, despite all efforts, metastatic breast cancer remains incurable (Steeg, 2016). The need to identify better therapies and translate these discoveries into the clinic has never been greater.

We have previously reported the synthesis of (Z)-2-(3,4-dichlorophenyl)-3-(1H-pyrrol-2-yl)acrylonitrile (ANI-7, Fig. 1A) and identified it as a potent and selective inhibitor of cell growth in MCF-7 breast cancer cells (Tarleton et al., 2011), while having minimal to no effect on the growth of normal nontumor-derived breast cells or cells derived from other tumor types, including colon, ovarian, lung, skin, neuronal, glial, and pancreatic. Spurred on by this discovery, we set out to investigate the breast cancer selectivity of ANI-7 and to identify its mode of action using standard cell-based technologies. In

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; ANI-7, (Z)-2-(3,4-dichlorophenyl)-3-(1H-pyrrol-2-yl)acrylonitrile; ARNT, AhR nuclear translocator; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P450, cytochrome p450; PB, phosphate buffer; siRNA, small interfering RNA; SULT, sulfur transferase; XRE, xenobiotic response element.

Fig. 1. Structure of (A) ANI-7; (B) 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (aminoflavone, AhR agonist); (C) tamoxifen (ER antagonist); (D) 4-hydroxytamoxifen (ER antagonist); (E) raloxifene (ER antagonist/AhR agonist); (F) anastrozole (aromatase inhibitor); (G) (Z)-3-(3,5-dichlorophenyl)-2-pyridin-3-ylprop-2-enenitrile (tyrphostin, RG14620, EGFR inhibitor).

this study, we compare the growth inhibition qualities of ANI-7 with other structurally comparable analogs and standard breast cancer treatments (Fig. 1) in a broader cell line panel and report that ANI-7 mediates its breast cancer-selective effects via the aryl hydrocarbon receptor (AhR) pathway.

The AhR is a member of the basic-helix-loop-helix transcription factor family and is commonly known for its ability to mediate the effects of polycyclic and polyhalogenated aromatic hydrocarbon ligands, including environmental toxins (Okey, 2007; Androutsopoulos et al., 2009; Walsh et al., 2013; Kolluri et al., 2017). However, many endogenous ligands also exist, including bilirubin, prostaglandins, tryptophan, and even plant-derived ligands, such as resveratrol and flavones (Murray et al., 2014; Tian et al., 2015). As more evidence becomes available, the complexity of the AhR pathway is ever increasing; indeed, the AhR can influence gene transcription and various cellular events even in the absence of ligand binding (Barhoover et al., 2010; Murray et al., 2014).

The AhR is constitutively localized within the cell cytosol, where it is part of a complex of two heat-shock proteins: heat-shock protein 90, prostaglandin E synthase 3, and a single molecule of the immunophilin-like protein hepatitis B virus X-associated protein 2. This chaperone complex protects the AhR from degradation, constrains the AhR in a conformation receptive to ligand binding, and prevents the inappropriate binding of the AhR nuclear translocator (ARNT) (Denison and Nagy, 2003; Okey, 2007; Androutsopoulos et al., 2009). Ligand binding to the AhR triggers a conformational change that exposes a nuclear localization sequence that facilitates ARNT binding and translocation to the nucleus. Within the nucleus, the AhR:ARNT heterodimer complex binds to specific DNA recognition sites known as xenobiotic response elements (XREs), leading to the transcriptional activation of genes that possess XRE in their promoter sequences. AhR-activated genes encode phase I metabolic enzymes such as cytochrome p450 (P450), CYP-1A1, -1A2, and -1B1. The unliganded AhR is then exported back to the cytosol (Okey, 2007; Androutsopoulos et al., 2009).

Materials and Methods

Growth Inhibition. All test agents were prepared as stock solutions (20 mM) in dimethylsulfoxide (DMSO) and stored at -20°C . Tamoxifen, 4-hydroxytamoxifen, anastrozole, CH223191, raloxifene, tyrphostin, and α -naphthoflavone were purchased from Sigma-Aldrich (Sydney, Australia). Aminoflavone was obtained from the National Cancer Institute. Cell lines used in the study included MCF-7, MDA-MB-468, T47D, ZR-75-1, SKBR3, BT474, BT20, MDA-MB-231, and MCF7/VP16 (breast carcinoma); HT29 (colorectal carcinoma); U87, SJ-G2, and SMA (glioblastoma); A2780 (ovarian carcinoma); H460 (lung carcinoma); A431 (skin carcinoma); Du145 (prostate carcinoma); BE2-C (neuroblastoma); and MiaPaCa-2 (pancreatic carcinoma) together with the one nontumor-derived normal breast cell line (MCF10A). All cell lines were incubated in a humidified atmosphere, 5% CO_2 at 37°C . The cancer cell lines MCF7, MCF7/VP16, MDA-MB-231, HT29, U87, SJ-G2, SMA, A2780, H460, A431, DU145, BE2-C, and MIAPaCa2 were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with fetal bovine serum (10%), sodium pyruvate (10 mM), penicillin (100 IU/ml $^{-1}$), streptomycin (100 $\mu\text{g}/\text{ml}^{-1}$), and L-glutamine (2 mM). The cancer cell lines MDA-MB-468, T47D, ZR-75-1, SKBR3, and BT474 were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with fetal bovine serum (10%), sodium pyruvate (10 mM), penicillin (100 IU/ml $^{-1}$), streptomycin (100 $\mu\text{g}/\text{ml}^{-1}$), L-glutamine (2 mM), and HEPES (10 mM). The noncancer MCF10A cell line was maintained in DMEM:F12 (1:1) cell culture media and 5% heat-inactivated horse serum, supplemented with penicillin (50 IU/ml $^{-1}$), streptomycin (50 $\mu\text{g}/\text{ml}^{-1}$), HEPES (20 mM), L-glutamine (2 mM), epidermal growth factor (20 ng/ml $^{-1}$), hydrocortisone (500 ng/ml $^{-1}$), cholera toxin (100 ng/ml $^{-1}$), and insulin (10 mg/ml $^{-1}$). Cytotoxicity was determined by plating cells in duplicate in medium (100 μl) at a density of 2500–4000 cells/well in 96-well plates. On day 0 (24 hours after plating), when the cells were in logarithmic growth, medium (100 μl) with or without the test agent was added to each well. After 72-hour drug exposure, growth-inhibitory effects were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and absorbance was read at 540 nm. An eight-point dose-response curve was produced, as shown in Fig. 2 using MS Excel software. Each data point is the mean $\pm$ S.E.M. calculated from four to five replicates, which were performed on separate occasions and separate cell line passages. From these dose-response curves, the GI_{50} value was

Fig. 2. Growth inhibition response (MTT assay) of ANI-7 in various breast (red) and nonbreast (blue)-derived cell lines after 72-hour continuous exposure showing sensitive (solid line) and non-sensitive (dashed line) cell populations. Each data point is the mean $\pm$ S.E.M. from four to five replicates.

calculated, representing the drug concentration at which cell growth was inhibited by 50% based on the difference between the optical density values on day 0 and those at the end of drug exposure (Tarleton et al., 2011).

Cell Cycle Analysis. Tumor cells in logarithmic growth were transferred to six-well plates at a density of 2×10^5 – 2.5×10^5 cells/well. On day 0 (24 hours after plating), the cells were treated with or without the test agent. The cells were harvested 24 hours after drug treatment and washed twice in phosphate-buffered saline, fixed in 70% ethanol, and stored overnight at -20°C . The cell pellet was incubated in 600 μl phosphate-buffered saline containing propidium iodide ($40 \mu\text{g}/\text{ml}^{-1}$) and RNase ($200 \mu\text{g}/\text{ml}^{-1}$) for at least 30 minutes at room temperature. The samples (1.5×10^4 events) were analyzed for fluorescence (FL2 detector, filter 575/30-nm band pass) using a FACScan (BD Biosciences, Franklin Lakes, NJ). Cell cycle distribution was assessed using CellQuest software. Experiments were each performed on three separate occasions. Values are the percentage distribution for each phase of the cell cycle.

Morphologic Assessment. Live cells were examined for morphologic alterations after 24-hour exposure with and without ANI-7, using phase-contrast microscopy (Olympus CKX41 inverted microscope 100 $\times$ magnification) (Supplemental Fig. 1).

Western Blotting. Cells (3×10^5) were plated in six-well plates in DMEM containing test agent. At the indicated times, the cells were harvested and protein content was determined (Lowry Modified/Bio-Rad Protein Assay). Equal aliquots (20 μg) of total protein from whole-cell lysates were fractionated on a 10% denaturing SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Nonspecific interactions were blocked with 5% nonfat milk/0.05% Tween 20. Proteins were identified using rabbit monoclonal antibodies against γH2AX and pCHK2 (Cell Signaling Technology, Danvers, MA) and mouse monoclonal antibody CHK2. Membrane-bound antibodies were detected using goat anti-rabbit and anti-mouse secondary antibodies (Abcam, Cambridge, UK) and Clarity Western ECL (Bio-Rad, Hercules, CA).

ER Binding. Competition-binding assays were performed by using an enzyme fragment complementation method described in the HitHunter (Freemont, CA) enzyme fragment complementation estrogen chemiluminescence assay kit, according to the manufacturer's instructions. Briefly, competing ligands at final concentrations ranging from 25 pM to 2 μM were incubated with 5 nM recombinant ER α (Invitrogen, Carlsbad, CA) and 17 β -estradiol-conjugated enzyme donor for 1.5 hours. The enzyme acceptor was then added, followed by the chemiluminescence substrate, and incubated for 1 hour. Relative luminescence was determined by using

a GloMax Explorer plate reader (Promega, Madison, WI). Sigmoidal standard curves were created by Excel.

Aromatase Assay. Aromatase reactions were carried out, as previously described (Matsui et al., 2005). Test chemicals were dissolved in DMSO and diluted 1:10 in diluent 1 [0.1% bovine serum albumin (BSA), 50 mM phosphate buffer (PB), pH 7.2]. Sample (10 μl) was added to a 96-well plate (on ice), followed by 50 μl ice-cold R1 solution (0.1% BSA, 50 mM PB, pH 7.2, 3.3 mM NADP-2Na; BD Biosciences), 0.8 μM glucose-6-phosphate, and 62.5 nM testosterone (Sigma-Aldrich). R2 solution (50 μl , 0.1% BSA, 50 mM PB, pH 7.2, 8.3 mM magnesium chloride, and 1 U/ ml^{-1} glucose-6-phosphate dehydrogenase) was added to each test sample. Ten microliters diluted P450arom (1 pg/ ml , 0.1% BSA, 50 mM PB, pH 7.2; BD Biosciences) was added to a second 96-well plate on ice. Ninety microliters of sample reaction was transferred to the P450arom and incubated for 20 minutes at 37°C . The reaction was terminated with 10 μl 500 μM α -naphthoflavone. After completion of the P450aromase reaction, 50 μl sample was transferred to an enzyme-linked immunosorbent assay plate. The amount of estradiol in each sample was determined using the Estradiol EIA kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. Absorbance of each sample was proportional to the amount of bound estradiol tracer that was inversely proportional to the amount of estradiol.

Kinase Inhibition. A dry sample of ANI-7 was sent to Reaction Biology (PA) and International Centre for Kinase Profiling (University of Dundee, Dundee, UK) for kinase inhibition assays. Both organizations use the ^{33}P ATP radioactive filter-binding assay (Hastie et al., 2006). A stock solution of ANI-7 was prepared in DMSO, and kinase inhibition assays were conducted in duplicate in the presence of a single concentration of ANI-7 (10 μM). Data represent percentage kinase enzyme activity; the lower the value, the greater the enzyme inhibition.

Knockdown of AhR Expression. Transient knockdown of AhR in MDA-MB-468 cells was performed through transfection of small interfering RNAs (siRNA) targeting AhR (Qiagen) and the AllStars Negative Control nonsilencing siRNA (Qiagen). The AhR siRNA contained four siRNAs for the AhR target (FlexiTube GeneSolution GS196). Cells were transfected with Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. Briefly, 8×10^3 MDA-MB-468 cells were plated into each well of a 96-well plate and allowed to adhere for 24 hours. Opti-MEM media (Invitrogen) containing 0.3 ml Lipofectamine 3000 transfection reagent and 0.3 pmol siRNA were added to each well. After 6 hours of incubation, transfection media were replaced with growth media containing 1.0 μM ANI-7. Cells were incubated for an additional 72 hours prior to MTT analysis.

TABLE 1

Growth inhibition response (MTT assay, 72 hours) (GI_{50} values micromolars, concentration that inhibits growth by 50%) of ANI-7 and various breast cancer-targeting agents in a broad panel of cancer cell lines

Data represent the mean $\pm$ S.E.M. of four to five replicates.

Cell Line	ANI-7	Tamoxifen	Hydroxy Tamoxifen	Raloxifene	Anastrozole	Tyrphostin RG14620	Amino Flavone
MCF-7 ^a	0.38 $\pm$ 0.03	7.7 $\pm$ 0.6	4.2 $\pm$ 1.2	8.7 $\pm$ 3.3	35 $\pm$ 16	12 $\pm$ 1	0.006 $\pm$ 0.001
A431 ^b	0.51 $\pm$ 0.05	6.5 $\pm$ 1.0	8.8 $\pm$ 0.4	14 $\pm$ 2	>50	12 $\pm$ 0.75	7.4 $\pm$ 0.7
H460 ^c	3.0 $\pm$ 0.4	9.5 $\pm$ 0.3	2.5 $\pm$ 0.4	12 $\pm$ 0.8	>50	16 $\pm$ 0.4	0.16 $\pm$ 0.05
HT29 ^d	6.0 $\pm$ 0.2	8.2 $\pm$ 1.9	2.0 $\pm$ 0.1	11 $\pm$ 2	>50	5.8 $\pm$ 1.5	21 $\pm$ 5
A2780 ^e	13 $\pm$ 2	13 $\pm$ 1.2	7.1 $\pm$ 0.7	9.5 $\pm$ 0.4	>50	10 $\pm$ 1.0	0.32 $\pm$ 0.09
BE2-C ^f	18 $\pm$ 2	16 $\pm$ 0.3	2.8 $\pm$ 0.3	15 $\pm$ 0	>50	10 $\pm$ 0.67	21 $\pm$ 2
SMA ^g	20 $\pm$ 2	12 $\pm$ 0.3	7.9 $\pm$ 0.2	16 $\pm$ 2	>50	15 $\pm$ 2.0	14 $\pm$ 1
SJ-G2 ^h	23 $\pm$ 2	12 $\pm$ 2	8.6 $\pm$ 0.5	17 $\pm$ 0	>50	9.5 $\pm$ 1.3	12 $\pm$ 1
Du145 ⁱ	27 $\pm$ 1	10 $\pm$ 3	8.9 $\pm$ 0.6	12 $\pm$ 0	>50	11 $\pm$ 0.43	12 $\pm$ 1
U87 ^h	36 $\pm$ 3	18 $\pm$ 2	9.5 $\pm$ 0.6	21 $\pm$ 1	>50	13 $\pm$ 0.82	14 $\pm$ 1
SW480 ^d	39 $\pm$ 3	16 $\pm$ 0.3	2.0 $\pm$ 0.1	nd	>50	18 $\pm$ 0.86	nd
MIA ^j	42 $\pm$ 3	10 $\pm$ 0.3	6.0 $\pm$ 0.5	14 $\pm$ 2	>50	12 $\pm$ 0.0	13 $\pm$ 2

nd, Not determined.

^aBreast carcinoma.

^bSkin carcinoma.

^cLung carcinoma.

^dColon carcinoma.

^eOvary carcinoma.

^fNeuroblastoma.

^gSpontaneous murine astrocytoma.

^hGlioblastoma.

ⁱProstate carcinoma.

^jPancreatic carcinoma.

AhR Reporter Luciferase Assay. The activity of the AhR signaling pathway was measured using the Cignal Xenobiotic Response (XRE) Reporter Assay Kit from Qiagen, according to the manufacturer's instructions. Briefly, MDA-MB-468 cells were reverse transfected with the Cignal XRE Reporter (containing an AhR-responsive luciferase construct and a constitutively expressing *Renilla* luciferase) as well as positive and negative controls. After 20 hours of transfection, medium was changed to assay medium (DMEM + 0.5% fetal bovine serum + 0.1 mM NEAA). After 24 hours of transfection, cells were treated with ANI-7 (0.2 and 2.0 μ M) for 6 hours. The Dual-Glo Luciferase Assay System (Promega) was performed after 30 hours of transfection using the GloMax Explorer Luminescence plate reader. The promoter activity was replicated twice, and values are expressed as arbitrary units using *Renilla* reporter for internal normalization.

Gene Expression Analysis. For each cell population, total RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Rotor-Gene SYBR Green polymerase chain reaction kit (Qiagen) was used to perform quantitative polymerase chain reaction for AhR, CYP1A1, CYP1A2, CYP1B1, sulftransferase (SULT)1A1, and ARNT on a Rotor-Gene 3000 Thermo-Cycler Instrument using β_2 -microglobulin as a housekeeping gene (Qiagen). The primer sequences were purchased from Qiagen as follows: AhR (QT02422938), CYP1A1 (QT00012341), CYP1A2 (QT00000917), CYP1B1 (QT00209496), SULT1A1 (QT01665489), ARNT (QT00023177), and β_2 M (QT00088935). HotStar Taq activation took place at 95°C for 5 minutes, 40 cycles of denaturation (95°C for 5 seconds), and annealing/extension (60°C for 10 seconds). The comparative C_t value method was used for data analysis. Endogenous gene expression was examined in cell harvests from each of our cell lines, whereas ANI-7-induced gene expression was examined in MDA-MB-468 cells following treatment with 2 μ M ANI-7 for 1, 2, 4, 8, 12, and 24 hours.

Results

ANI-7 Selectively Targets Breast Cancer Cell Populations. We have previously shown that ANI-7 induces potent selective growth inhibition in MCF-7 breast cancer cells (GI_{50} = 0.5 μ M) when compared with cell lines derived

from other tumor types (GI_{50} = 3.2–46 μ M) (Tarleton et al., 2011). At the time of this discovery, the mechanism of action of ANI-7 was unknown, and the selectivity was only examined in a limited number of breast cancer cell lines (MCF-7 and MDA-MB-231). In the present study, we set out to compare the action of ANI-7 with other well-known breast cancer-targeting drugs (Fig. 1) and to expand the panel of breast cancer cell lines to now include cell lines of ER⁺ luminal A (MCF-7, T47-D, and ZR-75-1 cells), ER⁺ luminal B (BT-474 cells), HER2⁺ (SKBR-3), basal (triple negative for ER, PR, and HER2; MDA-MB-468, BT20, and MDA-MB-231 cells), and MCF-7/VP16 cells, which overexpresses the drug resistance ABCC1 gene. The results of our extended growth inhibition analysis clearly show that ANI-7 potently inhibits the growth of not only MCF-7 breast cancer cells but also other breast cancer-derived cell lines (Fig. 2, solid lines), while showing negligible activity in a broad range of nonbreast-derived cell lines (dashed lines). Indeed, comparisons of the GI_{50} values (Table 1) show that ANI-7 produces a GI_{50} value of 0.38 $\pm$ 0.03 μ M in MCF-7 cells, whereas values of 3.0–42 μ M were observed in cell lines from lung, colon, ovary, neuronal, glial, prostate, and pancreas. The only other tumor type that showed appreciable growth inhibition by ANI-7 was the A431 vulva cell line (GI_{50} = 0.51 $\pm$ 0.05 μ M).

Comparison with other well-known breast cancer-targeting drugs shows that the ER antagonists tamoxifen, its metabolite hydroxy tamoxifen, and raloxifene did not selectively inhibit the growth of MCF-7 (GI_{50} = 7.7 $\pm$ 0.6, 4.2 $\pm$ 1.2, and 8.7 $\pm$ 3.3 μ M, respectively) cells when compared with cell lines derived from other tumor types (GI_{50} range, 6.5–18, 2–9.5, and 11–21 μ M, respectively). Although the aromatase inhibitor, anastrozole, showed a slight preference for MCF-7 cells (GI_{50} = 35 $\pm$ 16 μ M versus >50 μ M), it was essentially ineffective at inhibiting cell growth in this model system. Similarly, the epidermal growth factor receptor (EGFR) inhibitor tyrphostin RG14620 also failed to show any selective preference toward the growth inhibition of MCF-7 cells (GI_{50} = 12 $\pm$ 1 μ M versus a range of

TABLE 2
Growth inhibition response (MTT assay, 72 hours) (GI₅₀ values micromolars, concentration that inhibits growth by 50%) of ANI-7 and various breast cancer–targeting agents in a panel of breast cancer cell lines
Data represent the mean ± S.E.M. of four to five replicates.

Cell Line	ANI-7	Tamoxifen	Hydroxy Tamoxifen	Raloxifene	Anastrozole	Tyrphostin RG14620	Amino Flavone
T47D ^a	0.16 ± 0.02	4.7 ± 0.3	3.1 ± 0.4	7.2 ± 0.5	26 ± 0	9.6 ± 0.6	0.04 ± 0.02
ZR-75-1 ^a	0.18 ± 0.02	5.8 ± 0.3	4.8 ± 1.2	7.2 ± 1.2	22 ± 14	14 ± 2	0.002 ± 0.02
MCF-7/VP16 ^a	0.21 ± 0.4	nd	nd	nd	33 ± 17	11 ± 0.0	nd
MCF-7 ^a	0.38 ± 0.03	7.7 ± 0.6	4.2 ± 1.2	8.7 ± 3.3	35 ± 16	13 ± 1.3	0.02 ± 0.0
SKBR3 ^b	0.21 ± 0.03	7.1 ± 0.2	7.2 ± 0.1	7.3 ± 0.9	nd	8.5 ± 0.5	0.005 ± 0.03
MDA-MB-468 ^c	0.23 ± 0.01	8.0 ± 0.0	8.2 ± 0.3	14 ± 0.7	nd	8.8 ± 0.2	0.001 ± 0.01
BT474 ^a	1 ± 0.3	7.4 ± 0.4	7.4 ± 1.0	9.0 ± 2.1	nd	10 ± 1	0.8 ± 0.3
BT20 ^c	2 ± 0.4	8.5 ± 0.0	9.0 ± 0.0	16 ± 0.8	nd	13 ± 0.7	7.0 ± 1.5
MDA-MB-231 ^c	17 ± 4	9.0 ± 0.03	12 ± 0.3	20 ± 0.6	33 ± 18	17 ± 1	20 ± 0.7
MCF10A ^d	26 ± 3	9.7 ± 0.6	11 ± 0.3	16 ± 0.3	>50	11 ± 1	16 ± 0.3

nd, Not determined.
^aER positive.
^bER negative, HER2 positive.
^cTriple negative for ER, progesterone receptor, and HER2.
^dNormal breast cell line.

5.8–18 μM). However, in contrast with these standard breast cancer treatments, the aryl-hydrocarbon agonist, aminoflavone, mimicked the breast cancer selectivity of ANI-7 with a GI₅₀ value of 0.006 ± 0.001 μM in MCF-7 cells and a GI₅₀ range of 0.16–21 μM in all other cell types.

In our expanded breast cancer panel (Table 2), ANI-7 potently inhibited the growth of T47D, ZR-75-1, MCF-7, SKBR3, and MDA-MB-468 breast cancer cells (GI₅₀ range of 0.16–0.38 μM), moderately inhibited the growth of BT20 and BT474 cells (GI₅₀ range of 1–2 μM), and essentially failed to inhibit the growth of MDA-MB-231 and MCF10A cells (GI₅₀ range of 17–26 μM). Moreover, ANI-7 maintained its ability to inhibit the growth of drug-resistant cells (MCF-7/VP16: GI₅₀ = 0.21 ± 0.4 μM). Tamoxifen, hydroxytamoxifen, and raloxifene produced GI₅₀ values of 4.7–9.7, 3.1–12, and 7.2–20 μM, respectively, with T47D cells the most sensitive and MDA-MB-231 the least sensitive of the breast cancer cell lines. Anastrozole again induced minimal effects on growth inhibition across all cell lines (GI₅₀ range 22 to >50 μM), whereas tyrphostin RG14620 induced moderate inhibition (GI₅₀ 8.5–17 μM). Although aminoflavone was more potent than ANI-7, it again mimicked the response of ANI-7—producing GI₅₀ range of 0.001–0.04 μM in T47D, ZR-75-1, MCF-7, SKBR3, and MDA-MB-468 cells; moderate inhibition in BT474 and BT20 (GI₅₀ range of 0.8–7.0 μM); and minimal effects in MDA-MB-231 and MCF10A (GI₅₀ range of 16–20 μM) cells.

ANI-7 Induces Cell Cycle Arrest Checkpoint Activation and DNA Damage. To further investigate the mode of action of ANI 7, we chose to focus on the cell cycle events induced in MDA-MB-468 cells in response to ANI-7. Cell cycle analysis (Fig. 3) and morphologic assessment (Supplemental Fig. 1)

of ANI-7 confirmed the negligible effect of ANI-7 (2.5 μM) on the growth of normal breast MCF10A cells within 24 hours (Fig. 3, A and B), whereas ANI-7 induced significant S-phase and G₂ + M-phase cell cycle arrest within 24 hours of treatment in MDA-MB-468 cells (Fig. 3, C and D). Western blot analysis confirmed the induction of cell cycle checkpoint activation within 12 hours of treatment with ANI-7 (2 μM) in MDA-MB-468 cells, via a significant increase in the content and phosphorylation of CHK2 (25-fold increase) (Fig. 4A). Concomitantly, ANI-7 (2 μM) induced a significant increase in H2AXγ (3.5-fold increase) in MDA-MB-468 cells within the same timeframe, indicative of DNA double-strand damage (Fig. 4B). The ability of ANI-7 to selectively target breast cancer cells and to induce S-phase cell cycle arrest was the initial clue that led us to examine the role of ANI-7 in the aryl hydrocarbon receptor pathway, as similar events have been described for aminoflavone (Meng et al., 2006).

Inhibition of the AhR Pathway Ameliorates the Effects of ANI-7. Using the MTT growth inhibition assay, we observed that treatment of MDA-MB-468 cells with CH223191 (5 μM), a known antagonist of the AhR (Choi et al., 2012), significantly reduced the growth-inhibitory effects of both ANI-7 (0.1 μM) (from 60% to 17% relative growth inhibition) and aminoflavone (3 pM) (from 100% to 28% relative growth inhibition) (Fig. 5A). As the AhR pathway is also known to induce the expression of phase 1–metabolizing enzymes, including cytochrome P450 (CYP1) enzymes, we also observed that the specific CYP1 inhibitor α-naphthoflavone (10 μM) ameliorated the growth-inhibitory effects of both ANI-7 (0.5 μM) and aminoflavone (3 pM) from near-total growth inhibition to negligible growth inhibition (Fig. 5B). Further to

Fig. 3. Cell cycle analysis (percentage distribution) of MCF10A (A and B) and MDA-MB-468 (C and D) cells treated with (B and D) or without (A and C) ANI-7 (2.5 μM) for 24 hours. Analysis was replicated on three occasions with one representative set shown.

Fig. 4. MDA-MB-468 cells were treated with ANI-7 (2.0 μ M) for 0, 12, and 24 hours, and examined for (A) checkpoint activation (CHK2, pCHK2) and (B) DNA damage (H2AX γ) by Western blotting. The relative optical density normalized to actin content is also shown. Data were replicated on two separate occasions, with one representative set shown. Data for (A) and (B) were from the same experiment and blots were performed on the same gel, therefore the same loading control was used for both panels.

this, siRNA knockdown of AhR expression (by 60%, Fig. 5C) in MDA-MB-468 cells enhanced the survival of cells from 26% to 57% following treatment with ANI-7 (1 μ M) (Fig. 5D).

Collectively, these data confirm the role of the AhR and P450s in mediating the effects of ANI-7 pathway.

ANI-7 Activates XRE Activity and Expression of the AhR and CYP1 Members. The ability of ANI-7 to induce binding of the AhR with the XRE promotor was determined using a XRE reporter assay. The ANI-7-sensitive cell line MDA-MB468 was transfected with a XRE reporter plasmid, as well as control reporter plasmids. Treatment with ANI-7 at concentrations of 0.2 and 2.0 μ M significantly induced promotor activity by up to twofold within 6 hours (Fig. 6A), confirming XRE activation. Treatment of these cells with ANI-7 also induced a modest increase in the expression of AhR by up to 3.6-fold within 24 hours (Fig. 6B). Analysis of CYP1A1 expression over the same 24-hour exposure showed a 28-fold increase in CYP1A1 expression within 1 hour, reaching a maximum fold increase of 252 by 8 hours post-treatment (Fig. 6C). CYP1A2 and CYP1B1 also showed an increase in expression within 1 hour of treatment, reaching a maximum fold increase of 21 and 13 by 12 hours post-treatment, respectively (Fig. 6, D and E). Interestingly, the expression of SULT1A1 did not alter following treatment with ANI-7 (Fig. 6F). To further define the role of the AhR pathway in mediating the effects of ANI-7, we examined the basal expression of AhR, ARNT, CYP1 family, and SULT1A1 in our large panel of cell lines (Fig. 7) and compared the expression with ANI-7 sensitivity (Fig. 2). Interestingly, the cell lines displayed a varying profile of gene expression of the key members of the AhR pathway. Of note, the inherent expression of AhR, ARNT,

Fig. 5. Growth inhibition response (MTT assay) in MDA-MB-468 cells after 72 hours of ANI-7 (0.1 μ M) and aminoflavone (3 pM) in the presence and absence of (A) the AhR antagonist CH223191 (5 μ M) and (B) the CYP1 inhibitor α -naphthoflavone (10 μ M). Each data point is the mean $\pm$ S.E.M. of three replicates. (C) Fold change in expression of AhR in MDA-MB-468 cells in the presence (AhR siRNA) or absence (scrambled siRNA) of AhR knockdown. (D) Growth inhibition response in MDA-MB-468 cells after 72 hours of ANI-7 (1.0 μ M) in the presence (AhR siRNA) or absence (scrambled siRNA) of AhR siRNA. Each data point is the mean $\pm$ S.E.M. of two replicate experiments.

Fig. 6. (A) Induction of XRE activity using a reporter assay in MDA-MB-468 cells after 6 hours of ANI-7 (0.2 and 2 μ M) treatment. Each data point is the mean $\pm$ S.E.M. of two replicate experiments. (B–F) Change in gene expression (quantitative polymerase chain reaction) in MDA-MB-468 cells of (B) AhR, (C) CYP1A1, (D) CYP1A2, (E) CYP1B1, and (F) SULT1A1 after 1- to 24-hour treatment of ANI-7 (2 μ M) compared with untreated control cells (Unt).

and CYP1 members did not predict for ANI-7 activity; however, SULT1A1 expression did. Collectively, the data show that ANI-7 is mediating its effects via the AhR pathway with a particular enhancement of CYP1A1 expression.

ANI-7 Does Not Interact with Other Standard Breast Cancer Targets. During our initial investigations into the mechanism of action, we also examined the ability of ANI-7 to interact with the ER pathway. Thus, we examined the ability of ANI-7 to bind to the ER and to inhibit aromatase activity. The data in Table 3 clearly show that although tamoxifen (IC_{50} 0.012 μ M) and hydroxytamoxifen (IC_{50} 0.0017 μ M) are potent inhibitors of ER and anastrozole (IC_{50} 0.12 μ M) is a potent inhibitor of aromatase activity, ANI-7 failed to inhibit either target at concentrations 1000 times greater than the IC_{50} values for these targeted therapies. To further characterize the activity of ANI-7, we also screened its ability to inhibit the activity of a panel of protein kinases at a concentration of 10 μ M (Table 4). The data clearly show that ANI-7 does not significantly alter the kinase activity of a very broad panel of kinase enzymes, including tyrosine kinase receptors, lipid kinases, or those specific to the phosphatidylinositol 3-kinase/mammalian target of rapamycin or mitogen-activated protein kinase pathway.

Discussion

In this study, we report that ANI-7 is a potent (micromolar) and selective (up to 263-fold) inhibitor of cell growth in breast

cancer cell lines (Fig. 2; Tables 1 and 2). The sensitive lines represent cancers from the main molecular subtypes of luminal A (MCF-7, T47D, ZR-75-1), luminal B (BT474), basal (MDA-MB-468, BT20), and HER2 (SKBR3), with varying receptor status (ER, PR, HER2) (Table 2). Also included is one line with a drug-resistant phenotype (MCF-7/VP16), overexpressing the p-glycoprotein drug transporter ABCB1. Interestingly, all ER-positive lines were sensitive to growth inhibition by ANI-7. Of the ER-negative cell lines, the MDA-MB-468 line was the most sensitive. Sensitivity of this cell line and other ER-negative cells to AhR ligands has previously been described (Bradshaw et al., 2008; Zhang et al., 2009; Brinkman et al., 2014). The only nonsensitive breast cancer cell line was the MDA-MB-231 line with a basal subtype and triple negative for receptor status, with amplifying mutations in KRas and BRAf activity (Eckert et al., 2004). Although these mutations are relatively common in colon cancer, less than 5% of breast cancer tumors carry this genotype (Bos, 1989). The resistance of MDA-MB-231 to AhR activation has been observed with other AhR ligands, including aminoflavone (Callero and Loaiza-Pérez, 2011; Fukasawa et al., 2015). The only other tumor type that showed appreciable sensitivity to ANI-7 was the A431 vulva cell line, which is ER positive and overexpresses the EGFR growth receptor (Rexer et al., 2009). Initially, our investigations were focused on the possibility that ANI-7 was a selective inhibitor of ER⁺ cell populations (Tarleton et al., 2011); however, it became clear that ANI-7

Fig. 7. Inherent gene expression (quantitative polymerase chain reaction) of (A) AhR, (B) ARNT, (C) SULT1A1 (D) CYP1A1, (E) CYP1A2, and (F) CYP1B1 in our panel of cell lines.

was not a ligand for the ER or a substrate for aromatase function (Table 3). ANI-7 also failed to inhibit a broad range of kinase activity (Table 4), further excluding ANI-7 from mediating its effects by traditional breast cancer-related biochemical pathways, including the EGFR/HER2, phosphatidylinositol 3-kinase/mammalian target of rapamycin, and mitogen-activated protein kinase pathways. We also show that the growth inhibition profile of ANI-7 differs considerably from that induced by ER (tamoxifen, hydroxytamoxifen, raloxifene), aromatase (anastrozole), and EGFR (tyrphostin) antagonism.

Our desire to determine the mode of action of ANI-7 led us to examine the effect of ANI-7 on the cell cycle (Fig. 3), whereby ANI-7 induced S-phase cell cycle arrest. This single observation led us to examine the possibility that ANI-7 was mediating its effects, as described for the halogenated aryl hydrocarbon, aminoflavone (Meng et al., 2005), i.e., via activation of the AhR pathway. Aminoflavone was first described

by Akama et al. (1996) as a selective inhibitor of the growth of breast cancer cells. Subsequent studies have shown that the selectivity of aminoflavone relies on the localization of the AhR in the cytoplasm rather than the nucleus of cells (Callero and Loaiza-Pérez, 2011). Structurally aminoflavone can be metabolized by CYP1A1 at two amino groups to form N-hydroxyl metabolites that are substrates for bioactivation by sulfur transferase (SULT1A1) (Meng et al., 2006). N-sulfoxy groups are further converted to active nitrenium ions, which form DNA adducts and induce cell death (Meng et al., 2006). The ability of aminoflavone to be metabolized at two amino groups compared with ANI-7's single amino group may account for their differing potency. Although aminoflavone presents with greater potency (Table 2), this does not preclude the development of ANI-7 as a new clinical lead. Indeed, highly potent molecules are often difficult to detect in blood and present with a narrow therapeutic index with unpredictable toxicity, off-targets, and poor pharmacokinetics; i.e., high potency does not necessarily equate with greater clinical efficacy (Waldman, 2002).

Using standard cell biology methods, we show that ANI-7 binds to the AhR, induces translocation to the nucleus, activates the XRE (Fig. 6A), and induces CYP1 activity (Fig. 6, C–E), culminating in cell cycle arrest (Fig. 3), checkpoint activation (Fig. 4A), DNA damage (Fig. 4B), and cell death (Fig. 2; Supplemental Fig. 1). Of note is the significant induction of CYP1 expression within 1 hour following treatment, with CYP1A1 dominating the effect (Fig. 6C). Although ANI-7 clearly mediates its effects via the AhR pathway, the inherent expression of each pathway member (AhR, ARNT, and CYP1) (Fig. 7) does not predict for sensitivity, highlighting

TABLE 3

In vitro ER and aromatase inhibition (IC_{50} μ M) assay in the presence of ANI-7, tamoxifen, hydroxy tamoxifen, and anastrozole. Data represent the mean $\pm$ S.E.M. of three replicate experiments.

	ER Inhibition	Aromatase Inhibition
	(IC_{50} μ M)	(IC_{50} μ M)
ANI-7	>2	>100
Tamoxifen	0.012 $\pm$ 0.009	nd
4-Hydroxy tamoxifen	0.0017 $\pm$ 0.0007	nd
Anastrozole	nd	0.12 $\pm$ 0.07

nd, Not determined.

TABLE 4

Kinase inhibition data showing percentage kinase activity in the presence of 10 μ M ANI-7
Data represent the mean $\pm$ S.E.M. of duplicate experiments.

Kinase Classification			
Receptors		Phosphatidylinositol 3-kinase (PI3K)/mTOR pathway	
EGFR	101 $\pm$ 1	^a	PDK1/PDPK1 95 $\pm$ 1.4 ^a
ERBB2/HER2	95 $\pm$ 3	^a	SGK1 95 $\pm$ 0.8 ^a
ERBB4/HER4	102 $\pm$ 6	^a	SGK2 93 $\pm$ 1.1 ^a
IGF1R	90 $\pm$ 7.2	^b	SGK3/SGKL 99 $\pm$ 3.1 ^a
PDGFR α	87 $\pm$ 8.5	^a	AKT1 94 $\pm$ 2.3 ^a
PDGFR β	102 $\pm$ 2.3	^a	AKT2 91 $\pm$ 1.9 ^a
Lipid kinases			AKT3 104 $\pm$ 2.0 ^a
Choline kinase α	97 $\pm$ 2.4	^b	COT1/MAP3K8 95 $\pm$ 0.8 ^a
Choline kinase β	97 $\pm$ 4.1	^b	GSK3a 80 $\pm$ 0.3 ^a
DGK β	93 $\pm$ 30	^b	GSK3b 89 $\pm$ 1.5 ^a
DGK γ	83 $\pm$ 20	^b	mTOR/FRAP1 98 $\pm$ 0.7 ^a
DGK ζ	88 $\pm$ 14	^b	ROCK1 95 $\pm$ 2.3 ^a
PI3K α	84 $\pm$ 1.9	^a	ROCK2 98 $\pm$ 0.3 ^a
PI3K β	95 $\pm$ 6.9	^a	P70S6K 103 $\pm$ 3.0 ^b
PI3K δ	77 $\pm$ 8.3	^a	Mitogen-activated protein kinase pathway
PI3K γ	66 $\pm$ 12	^a	
PI4K2A	94 $\pm$ 8.4	^b	RAF1 106 $\pm$ 1.3 ^a
PIP5K2A	83 $\pm$ 0.6	^b	ARAF 100 $\pm$ 2.3 ^a
Sphingosine kinase 1	65 $\pm$ 23	^b	BRAF 101 $\pm$ 1.2 ^a
Sphingosine kinase 2	80 $\pm$ 8.9	^b	MEK1 101 $\pm$ 1.5 ^a
Others			MEK2 92 $\pm$ 0.3 ^a
			ERK1 100 $\pm$ 1.6 ^a
			ERK2/MAPK1 98 $\pm$ 0.7 ^a
			c-Src 87 $\pm$ 4.6 ^a
BRK	91 $\pm$ 4.6	^a	
PKBa	101 $\pm$ 0.2	^b	
PKD1	119 $\pm$ 0.1	^b	
PKBb	99 $\pm$ 9.0	^b	

^aReaction Biology Corporation, Malvern, PA.

^bThe International Centre for Kinase Profiling, University of Dundee, UK.

the well-known inducible nature of this pathway rather than constitutive activity. In contrast, the inherent expression of SULT1A1 did predict for ANI-7 sensitivity, and its expression was not altered following treatment, indicating that it functions independently of the AhR pathway. Notwithstanding this, the role of SULT1A1 is clearly an important determinant for the breast cancer selectivity of ANI-7. Studies of amino-flavone activity and gene expression in NCI-60 cell line panel (National Cancer Institute Developmental Therapeutics Program) showed that selectivity was highly correlated with the expression of SULT (Meng et al., 2006). Moreover, the transfection of SULT1A1 into MDA-MB-231 aminoflavone-resistant cells restored sensitivity (Meng et al., 2006).

In the present study, we also show that the growth-inhibitory effect of ANI-7 is ameliorated in the presence of the AhR antagonist CH223191 (Choi et al., 2012), AhR siRNA (Fig. 5, A, C, and D), and the pan CYP1 inhibitor α NF (Fig. 5B), confirming the need for each step of the AhR pathway and the metabolism of ANI-7 to an active intermediary. The presence of the DNA damage marker γ H2AX and the cell cycle checkpoint activation suggests that ANI-7 is metabolized to a DNA-interacting compound. Although we have not identified the precise ANI-7 metabolites, it is likely that the amino group of ANI-7 is metabolized to an active nitrenium ion, as observed for aminoflavone. Furthermore, the sensitivity of the cell lines to ANI-7 and the induction of DNA damage are independent of their p53 status, i.e., MCF-7 (sensitive) and MCF10A (insensitive) cells are both p53 wild type, whereas MDA-MB-468 (sensitive) and MDA-MB-231 (insensitive) are p53 mutant.

Other halogenated aryl-hydrocarbon molecules have been shown to selectively target breast cancer cells via activation of the AhR pathway (Loaiza-Pérez et al., 2002; Callero and Loaiza-Pérez, 2011; Fukasawa et al., 2015), including

2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F-203) and (5S,7S)-7-methyl-3-(3-(trifluoromethyl)phenyl)-5,6,7,8-tetrahydrocinnolin-5-ol (NK150460). Of note, the amino-containing compound 5F-203 has been shown to induce S-phase accumulation and DNA-adduct formation (Trapani et al., 2003). In contrast, the nonamino-containing compound, NK150460, failed to induce γ H2AX expression, indicating that DNA-adduct formation was not induced (Fukasawa et al., 2015). Not surprisingly, the initial chemical scaffold clearly dictates the structure and function of active metabolites produced via this pathway.

Other acrylonitrile compounds [(Z)-2,3-bis(4-nitrophenyl)acrylonitrile] have been shown to activate the AhR, including its translocation to the nucleus; however, (Z)-2,3-bis(4-nitrophenyl)acrylonitrile does not activate CYP1 expression in cell models (Guyot et al., 2012). Thus, whereas halogenated aryl-hydrocarbons and acrylonitrile compounds have been shown to activate the AhR pathway, the specific steps and mechanisms do differ, underscoring the importance of characterizing the mode of action. Exploring the differential effects of AhR agonists open the way to exploit their subtle differences for the clinical treatment of disease, including management of potential clinical toxicities, which have been described for prodrugs of aminoflavone and 5F-203 (Behrsing et al., 2013; <https://dctd.cancer.gov/featured-agents/pdfs/710464aminoflavonotoxabstract.pdf>).

The AhR has been previously described in the initiation and progression of breast cancer (Nebert et al., 2004; Schlezinger et al., 2006; Vinotini and Nagini, 2010; Powell et al., 2013; Go et al., 2015). The metabolism of environmental toxins by the AhR leads credence to the proposal that the initial insult that caused the breast cancer was a fat-soluble xenobiotic element that was metabolized to a DNA-damaging compound. The ongoing hyperactivation of the AhR in breast cancers and its

ability to control many oncogenic pathways further suggest a role of AhR in progression of this disease. Of note, the AhR is known to control the transcription of the ER gene. The cross-talk between these two pathways is complex (Safe and Wormke, 2003; Callero and Loaiza-Pérez, 2011; Go et al., 2015). Indeed, it has been proposed that a complex of AhR-ARNT and AhR agonist may dimerize with an ER α -ER agonist complex, leading to the elevated expression of CYP1A1 and CYP1B1 (Go et al., 2015). Such a proposal may explain our observation that all ER-positive cell lines ($n = 5$) tested in our study were sensitive to ANI-7; however, we clearly show that inherent CYP1 expression is not related to ANI-7 sensitivity. Furthermore, various AhR ligands have been shown to induce the proteasome-dependent degradation of ER α protein (Wormke et al., 2000, 2003) and also directly target E2 (estradiol)-responsive gene promoters (Krishnan et al., 1995).

This study for the first time has identified (Z)-2-(3,4-dichlorophenyl)-3-(1H-pyrrol-2-yl)acrylonitrile as a new AhR ligand and a substrate for CYP1 metabolism, culminating in DNA damage and growth inhibition in breast cancer cells. The unique structure of ANI-7 provides a new platform for the design and development of novel breast cancer-selective molecules exploiting the activation of the AhR pathway and the induction of CYP1s. This pharmacophore substantially adds to the ever-increasing development of novel AhR-targeting molecules for the treatment of cancer (Fukasawa et al., 2015; Yurttaş et al., 2015; Callero et al., 2017; Kolluri et al., 2017; Luzzani et al., 2017).

Authorship Contributions

Participated in research design: McCluskey, Sakoff.

Conducted experiments: Gilbert, De Iuliis, Tarleton.

Contributed new reagents or analytic tools: Tarleton.

Performed data analysis: Gilbert, McCluskey, Sakoff.

Wrote or contributed to the writing of the manuscript: Gilbert, McCluskey, Sakoff.

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