Endocrine Disruptors Fludioxonil and Fenhexamid Stimulate miR-21 Expression in Breast Cancer Cells

Yun Teng,* Tissa T. Manavalan,* Chuan Hu,* Svetlana Medjakovic,† Alois Junghauer,† and Carolyn M. Klinge*,2

*Department of Biochemistry & Molecular Biology and Center for Genetics and Molecular Medicine, University of Louisville School of Medicine, Louisville, Kentucky 40292; and †Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Muthgasse 18, Vienna, Austria

†These authors contributed equally to this study.

2To whom correspondence should be addressed at Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Delia Baxter Building, 581 South Preston Street, Louisville, KY 40202. Fax: (502) 852-3659. E-mail: carolyn.klinge@louisville.edu.

Received June 25, 2012; accepted September 26, 2012

Fenhexamid and fludioxonil are antifungal agents used in agricultural applications, which are present at measurable amounts in fruits and vegetables. Fenhexamid and fludioxonil showed endocrine disruptor activity as antiandrogens in an androgen receptor reporter assay in engineered human breast cancer cells. Little is known about how environmental chemicals regulate microRNA (miRNA) expression. This study examined the effect of fenhexamid and fludioxonil on the expression of the oncomiR miR-21 in MCF-7, T47D, and MDA-MB-231 human breast cancer cells and downstream targets of miR-21 in MCF-7 cells. Fenhexamid and fludioxonil stimulated miR-21 expression in a concentration-dependent manner and reduced the expression of miR-21 target Pdcd4 protein. Antisense to miR-21 blocked the increase in Pdcd4 protein by fenhexamid and fludioxonil. Fenhexamid and fludioxonil reduced miR-125b and miR-181a, demonstrating specificity of miRNA regulation. Induction of miR-21 was inhibited by the estrogen receptor antagonist fulvestrant, by androgen receptor antagonist bicalutamide, by actinomycin D and cycloheximide, and by inhibitors of the mitogen-activated protein kinases and phosphoinositide 3-kinase pathways. Fenhexamid inhibited C3-ketoreductase, which is involved in ergosterol biosynthesis (Fillinger et al., 2008). Fludioxonil is the ISO-approved name for 4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile. Fludioxonil was not carcinogenic in rats or mice (www.federalregister.gov). Both fenhexamid and fludioxonil are considered to have high exposure scores based on usage and residual concentrations on fruits and vegetables (Orton et al., 2011). Fenhexamid and fludioxonil are sprayed on tomatoes and appear to be present on the harvested fruits at concentrations below the legal maximum residue levels (Angioni et al., 2012; Cabizza et al., 2012). Human tissue or serum levels of fenhexamid and fludioxonil have not been examined.

Endocrine disrupting chemicals (EDC) are environmental chemicals that mimic or block transcriptional activation elicited by naturally circulating steroid hormones by binding to steroid hormone receptors and acting as either agonists or antagonists of that receptor (Diamanti-Kandarakis et al., 2009). In addition, EDC may also act at the level of enzymes involved in steroid hormone synthesis or metabolism and may alter the expression or activities of transcriptional coregulators (Diamanti-Kandarakis et al., 2009). The role of EDC in breast cancer is suspected but not proven (Weyandt et al., 2008). Pesticides and fungicides have been reported to be tumorigenic in rodent models of breast cancer (e.g., Ross and Leavitt, 2010). Based on their widespread use, environmental persistence, and high incidence of breast cancer, the possible role of EDC in stimulating breast tumorigenesis is of clear importance (Casals-Casas and Desvergne, 2011; Diamanti-Kandarakis et al., 2009; Weyandt et al., 2008).

Although lifetime estrogen exposure clearly increases breast cancer risk (Wiseman, 2004), the role of androgens is still undefined (Kotsopoulos and Narod, 2012). Androgen receptor (AR) is expressed in most breast tumors and is associated with endocrine sensitivity in estrogen receptor α (ERα)+ tumors (Lundin...
et al., 2011); however, AR is overexpressed in ERα/HER2+ and triple negative breast cancers, indicating that androgens may play a role in the pathogenesis of these tumors (Ni et al., 2011). Fenhexamid (IC50 ~8µM) and fludioxonil (IC50 ~2µM) had antiandrogenic activity in an engineered MDA-MB-453 breast cancer cell line stably expressing an mouse mammary tumor virus (MMTV) promoter luciferase reporter (called MDA-kb2) (Orton et al., 2011). Fludioxonil and fenhexamid were ERα agonists (EC50 values of 3.7 and 9.0µM, respectively) in a yeast-based reporter assay (Jungbauer, unpublished data). The effect of fenhexamid and fludioxonil on endogenous microRNA (miRNA) gene expression in breast cancer cells is unknown.

miRNA silence gene expression by basepairing to the 3’-UTR of their target mRNAs resulting in translational repression and mRNA degradation (Klinge, 2012). The current miRBase release (19.0) contains 1527 human miRNAs (August 2012, http://www.mirbase.org/). Each miRNA may repress hundreds of mRNAs. Changes in miRNA expression are implicated in human cancer (Iorio and Croce, 2012). Roles for miRNAs in environmental toxicology and as possible biomarkers of exposure have been reviewed (Koturbash et al., 2012).

Recent reports have identified miR-21 as an oncogenic overexpressed in different types of tumors, including breast cancer (reviewed in Fu et al. [2011]). Inhibition of miR-21 by chemically modified antisense oligonucleotides reduced proliferation and tumor growth of MCF-7 cells (Li et al., 2009). miR-21 regulates invasion and metastasis by inhibiting tumor suppressors, e.g., programmed cell death 4 (PDCD4), PTEN, and Bcl-2, an antiapoptotic protein (Wickramasinghe et al., 2009). miR-21 potentiates Ras signaling through inhibition of multiple negative regulators of the Ras/MEK/ERK pathway, e.g., SPRY1, SPRY2, BTG2, and PDCD4 (Hatley et al., 2010). There is only one report examining how EDC affect miRNA expression (Tilghman et al., 2012). That study showed that, like E2 (Wickramasinghe et al., 2009), o,p'-dichlorodiphenyltrichloroethane and bisphenol A (BPA) activate ERα in MCF-7 cells and downregulate miR-21 (Tilghman et al., 2012). There are no published studies on how fungicides regulate miRNAs in normal breast or in breast carcinoma and/or other neoplasias. Here, we examined how fenhexamid and fludioxonil affect the expression of the oncogenic miR-21 in MCF-7, T47D, and MDA-MB-231 breast cancer cells.

MATERIALS AND METHODS

Chemicals and antibodies. Fludioxonil (no. 46102), fenhexamid (no. 31713), 17 α-estradiol (E2, no. E8875), wortmannin (phosphoinositide 3-kinase [PI3K] inhibitor, no. W1628), and α-naphthoflavone (α-NF, an arylhydrocarbon receptor [AhR] antagonist, no. N5755) were purchased from Sigma-Aldrich (St Louis, MO). ICI 182, 780 (fulvestrant, no. 1047) was purchased from Tocris (Ellisville, MO). PD98059 (MEK1/2 inhibitor, no. 1213) was purchased from Cell Signaling (Beverly, MA). Dihydrotestosterone (DHT, no. 521-18-6) was purchased from Steraloids (Wilton, NH). The antiandrogen bicalutamide (Casodex) was generously provided by AstraZeneca (Macclesfield, UK). The antibodies against Pdcd4 (sc-130545), Bcl-2 (sc-509), PARP (Poly(ADP-ribose) polymerase-1, (sc-7150), and ERα (HC20, sc-545) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Pten (138G6), ERK1/2 (9102), and phosphor-ERK1/2 (9101S) were from Cell Signaling Technology (Beverly, MA); α-tubulin (MS-S81-P1) was from Thermo Scientific (Kalamazoo, MI); β-actin was from Sigma-Aldrich.

Cell lines and maintenance. The human breast cancer cell lines MCF-7, T47D, and MDA-MB-231 and the normal MCF-10A breast epithelial cell line were purchased from the American Type Culture Collection (Rockville, MD). MCF-7 and MDA-MB-231 cells were cultured in IMEM (Invitrogen, Carlsbad, CA). T47D cells were maintained in RPMI-1640 media containing insulin (5 µg/ml, Invitrogen), and MCF-10A was maintained in MEGM (Lonza, Walkersville, MD; cat no. CC-3150) containing 100 ng/ml cholera toxin. Cells were cultured in their respective growth medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen) at 37°C under an atmosphere of 5% CO2.

Treatments. Prior to treatment, the cells were “serum starved” in phenol red–free IMEM supplemented with 5% dextran charcoal–stripped fetal bovine serum (DCC-FBS) and 1% penicillin/streptomycin for 72 h to remove endogenous hormones from the serum and allow hormone-dependent responses to be detected in the cells (Bissas and Vonderhaar, 1987; Gill et al., 1991). Cells were then treated with dimethylsulfoxide (DMSO, the vehicle control), 10 nM E2, fludioxonil, or fenhexamid (at concentrations indicated in Figures) for 6 or 24h, as indicated in Figures. Stock solutions for each compound were prepared in DMSO and frozen in aliquots of 100 µl. The final concentration of DMSO in the culture medium was <0.1%.

RNA extraction and quantitative real-time reverse transcription-PCR. Total RNA was isolated with the miRCURY RNA Isolation Kit (Exiqon, Copenhagen, DEN) according to the manufacturer’s instructions. For analysis of miRNA expression, cDNA was prepared with the mercury LNA Universal RT microRNA PCR Kit (Exiqon).

Quantitative real-time reverse transcription-PCR (qPCR) was performed using the miRCURY LNA SYBR Green Master Mix (Exiqon) and miRNA primer sets for miR-21, miR-22, miR-125b, miR-181a, and miR-200a (Exiqon). RN48 and RN38 were used for normalization of miRNA expression. For analysis of primary miR-21 and gene expression, cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions, and individual mRNAs were monitored with the following inventoried TaqMan assays (Applied Biosystems): human primary-miR-21, THEM49 (VMP1), PDCD4, PTEN, and BCL2. 18S RNA was used for normalization of mRNA expression. The ABI 7900HT Fast Real-Time PCR System was used for all PCR (Applied Biosystems), done in triplicate. Thermal cycling parameters comprised stage 1: a 50°C Uracil-DNA glycosylase activation step for 2 min; stage 2: 95°C for 10 min; stage 3: 40 cycles of 95°C for 15 s and 60°C for 1 min; stage 4: 95°C for 15 s; 60°C for 15 s. Changes in expression were calculated by the change in threshold cycle (Ct) method with endogenous control and were normalized to results obtained with untreated cells.

Transfection of anti-miR miRNA inhibitor and Pre-miR miRNA. For transfection of pre-miRNA and anti-miRNA, 2 × 105 MCF-7 cells were transfected with 90 pmol of pre-miR miR-21 precursors and anti-miR miR-21 inhibitors (Ambion) with 7 µl of Lipofectamine RNAiMAX (Invitrogen) in antibiotics-free medium. Cells were harvested for RNA isolation 48 h after transfection.

Western blot. Cells were treated as indicated in individual Figures. Whole cell extracts were prepared in modified RIPA buffer (10 mM sodium phosphate, pH 7.2; 1% NP-40; 1% Na-deoxycholate; 150 mM NaCl; 2 mM ethylendiaminetetraacetic acid; 0.2 mM NaVO4; 50 mM NaF; and 1 mg/ml each of aprotinin, leupeptin, and pepstatin; complete protease inhibitor (Roche); and 1 mM PMSF). Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Lysates were immunoblotted according to standard methods (Klinge et al., 2010). Ten micrograms of protein (Figs. 3B and 4B) and 30 µg of protein (Fig. 5) were separated by 10% SDS-PAGE, transferred to polyvinylene difluoride membranes (Millipore, Thermo Scientific).
Fludioxonil and Fenhexamid Increase miR-21 Expression in MCF-7 Cells

Because the role of EDC in regulating oncomiR miR-21 expression in breast cancer is largely undefined, we determined the effect of fludioxonil and fenhexamid on mature miR-21 expression in MCF-7 breast cancer cells. Both fludioxonil and fenhexamid increased miR-21 expression in MCF-7 cells (Fig. 1A). Fludioxonil showed a concentration-dependent increase with an apparent saturation of the response at 1–10µM and then miR-21 expression returned to basal at 50µM (Fig. 1A). The maximum activity detected was similar to the EC₅₀ values of 3.7 and 9.0µM, respectively, for fludioxonil and fenhexamid for ERα in a yeast reporter assay (Jungbauer, unpublished data). Similarly to other EDC (Vandenberg et al., 2012), fenhexamid did not show a linear or saturation response although, like fludioxonil, the peak induction of miR-21 was detected at 10µM and was reduced at 50µM (Fig. 1A). The fenhexamid response appears biphasic with an initial response peak at 10–100nM and a second peak at 10 µM. Importantly, neither fludioxonil nor fenhexamid altered the expression of RNU38 or RNU48 that was used as controls for qPCR for miRNA expression (Supplementary fig. 2 and data not shown).

DHT Increased miR-21 Expression in MCF-7 Cells

DHT increased miR-21 in both LNCaP and VCaP prostate cancer cells (Waltering et al., 2011), but the effect of DHT on miR-21 expression in MCF-7 cells is unknown. MCF-7 cells express AR (Horwitz et al., 1975) although not all investigators find AR protein in MCF-7 cells (Robinson et al., 2011). Because fludioxonil and fenhexamid act as antiandrogens in a genetically engineered breast cancer cell line and inhibited MMTV luciferase reporter activity, we examined the effect of DHT alone or with fludioxonil or fenhexamid cotreatment. DHT increased miR-21 expression in MCF-7 cells and neither fludioxonil nor fenhexamid blocked this response (Fig. 1B). The antiandrogen bicalutamide inhibited the increase in miR-21 stimulated by DHT, fludioxonil, and fenhexamid, suggesting that AR is involved in induction of miR-21 expression (Fig. 1B). These results indicate that fludioxonil and fenhexamid are acting as AR agonists, not antagonists.
Actinomycin D and Cycloheximide Block Fludioxonil, Fenhexamid, and DHT-Mediated miR-21 Expression

To determine whether the increase in miR-21 expression detected with fludioxonil, fenhexamid, and DHT is a direct effect at the genomic level or requires synthesis of a secondary protein, MCF-7 cells were pretreated with the transcriptional inhibitor actinomycin D (ActD) or the protein synthesis inhibitor cycloheximide (CHX) and then treated with fludioxonil, fenhexamid, and DHT for 6 h (Fig. 1C). CHX alone increased miR-21 expression to a level similar to that induced by fludioxonil, fenhexamid, and DHT (Fig. 1C). CHX superinduction of gene expression is a well-established phenomenon mediated by a reduction in turnover of mRNA, an increase in p38MAPK kinase, and NFκB activation although the mechanisms responsible for this cell-specific and gene-specific effect are not understood (Hershko et al., 2004). Pretreatment with ActD and CHX blocked fludioxonil-, fenhexamid-, and DHT-mediated miR-21 expression, suggesting that the mechanism by which they increase miR-21 involves both transcriptional (primary genomic) and secondary mechanisms.

Fludioxonil and Fenhexamid Affect the Expression of Other miRNAs

To determine whether the increase in miR-21 expression by fludioxonil and fenhexamid was attributable to a general increase in all miRNAs, we examined the effect of these fungicides on the expression of miR-125b, which has tumor suppressor (O’Day and Lal, 2010) and oncomiR (Rajabi et al., 2010; Wang et al., 2012) activities, and three tumor suppressor miRNAs: miR-181a, miR-22, and miR-200a. As reported (Manavalan et al., 2011), E2 does not affect the expression of these miRNAs (Fig. 1D). Fludioxonil and fenhexamid inhibited miR-125b and miR-181 expression in MCF-7 cells (Fig. 1D). Fludioxonil reduced whereas fenhexamid increased miR-22. Both fludioxonil and fenhexamid increased miR-200a. These data indicate that these fungicides are not stimulating the expression of all
miRNAs and that they are likely to regulate miRNAs in a gene-specific manner. Because there are no published studies on DHT regulation of miRNAs in breast cancer cells and fludioxonil and fenhexamid were reported to have antiandrogenic activity (Orton et al., 2011), we also examined the effect of 10nM DHT on the expression of these four miRNAs. Interestingly, the effect of DHT was similar to that of fenhexamid: inhibition of miR-125b and miR-181a expression and stimulation of miR-22 and miR-200a expression (Fig. 1D). Because of its role as an oncomiR in breast cancer, we focused on miR-21 regulation by fludioxonil and fenhexamid in further studies.

Fludioxonil and Fenhexamid Increase miR-21 Expression in T47D but not in ERα-Negative MCF-10A and MDA-MB-231 Cells

To examine whether the induction of miR-21 expression with fludioxonil and fenhexamid is unique to MCF-7 or can be detected in another luminal breast cancer cell line, we examined miR-21 expression in T47D cells (Fig. 2A). Fludioxonil and fenhexamid increased miR-21 in T47D cells in a concentration-dependent manner. E2 repressed miR-21 expression in T47D cells. In contrast, E2, fludioxonil, and fenhexamid did not affect miR-21 expression in normal, ERα-MCF-10A breast epithelial cells or in basal-like, triple negative MDA-MB-231 breast cancer cells.

miR-21 is located in the 10th intron of the TMEM49/VMP1 gene (Fujita et al., 2008). Previous results indicate that miR-21 and TMEM49 are independently regulated in HL-60 cells treated with phorbol 12-myristate 13-acetate (PMA) to activate AP-1 (Fujita et al., 2008), in XG-1 cells treated with IL-6 to activate Stat3 (Löffler et al., 2007), and in MCF-7 cells treated with E2 (Wickramasinghe et al., 2009). Recently a read-through transcript from VMP1 including miR-21 was identified as a source for miR-21 expression in MCF-7 breast, DU-145 prostate, and HL-60 promyelocytic leukemia cells (Ribas et al., 2012). Fludioxonil and fenhexamid increased pri-miR-21, but not TMEM49 in T47D cells (Figs. 2B and 2C). Fludioxonil and fenhexamid increased TMEM49 in MDA-MB-231 cells, and this increase was reflected in increased pri-miR-21 in fludioxonil-treated cells but not in fenhexamid-treated cells. These data indicate that the increase in pri-miR-21 may result from a read through of TMEM49 transcription in fludioxonil-treated cells. Overall, these data indicate that increase in miR-21 in response to fludioxonil and fenhexamid detected in MCF-7 cells (Fig. 1) is not cell-line specific but may be related to ERα expression.

Fludioxonil and Fenhexamid Increase pri-miR-21 Expression in MCF-7 Cells

Pri-miRNAs are the primary transcripts of miRNAs that are sequentially processed first to pre-miRNAs by Drosha in the nucleus and then by Dicer in the cytoplasm to miRNA (Gregory et al., 2004; Klinge, 2012). Fludioxonil and fenhexamid increased pri-miR-21 transcript levels in MCF-7 cells (Fig. 3A). No significant change was seen with E2, but DHT increased pri-miR-21 (Fig. 3A). The increase in pri-miR-21 in cells treated with fludioxonil and fenhexamid was blocked by ActD, suggesting a direct transcriptional response. ActD only partially inhibited DHT-induced pri-miR-21 expression. These data indicate that the increase in mature miR-21 in response to fludioxonil and fenhexamid, but not DHT, appears to be a primary transcriptional event.

Fenhexamid Increases TMEM49 Transcription

In MCF-7 cells, Fenhexamid and DHT increased TMEM49 but fludioxonil and E2 did not (Fig. 3B). ActD reduced basal
TMEM49 expression and ablated the induction by fludioxonil and fenhexamid but did not affect DHT-induced TMEM49 expression. These data indicate that the increase in TMEM49 expression by fenhexamid results from increased transcription, whereas the mechanism by which DHT increases TMEM49 expression is independent of new transcription. Testosterone increased the expression of Tmem49 in the meibomian glands of orchidectomized BALB/c mice treated for 14 days with testosterone, but the mechanism was not examined (Sullivan et al., 2009). Because the focus of this study was the effects of fludioxonil and fenhexamid on miR-21 expression, the DHT observations were not further dissected.

Effects of Fludioxonil and Fenhexamid on mRNA and Protein Targets of miR-21

miR-21 regulates the translation of PDCD4, PTEN, and BCL2 (Wickramasinghe et al., 2009). To determine whether the increase in miR-21 in response to fludioxonil and fenhexamid correspondingly reduced the expression of these miR-21 target mRNAs, qPCR was performed for PDCD4, PTEN, and BCL2. mitoR was treated with E2 in parallel as a positive control (Wickramasinghe et al., 2009). Fludioxonil and fenhexamid decreased the mRNA levels of PDCD4 and PTEN, in agreement with the increase in miR-21 expression (Fig. 4A). However, like E2, fludioxonil and fenhexamid increased anti-apoptotic BCL2 expression. This effect agrees with the decrease in miR-21 by E2 but is the opposite of the increase in miR-21 by fludioxonil and fenhexamid. The E2-mediated increase in mRNA levels PDCD4 and BCL2, but not PTEN, agrees with our previous report (Wickramasinghe et al., 2009). Fludioxonil and fenhexamid reduced and E2 increased the levels of PDCD4 mRNA in T47D cells (Fig. 4B) in inverse correlation with miR-21 (Fig. 2A). E2 had no effect on PDCD4 expression in MCF-10A cells (Fig. 4B), reflecting the lack of impact of E2 on miR-21 (Fig. 2A). Fludioxonil and fenhexamid, 10nM, but not 100nM, reduced PDCD4 mRNA expression ~15% in MCF-10A, an effect that appears to be independent of miR-21 regulation because neither of these fungicides affected miR-21 expression (Fig. 2A). E2 increases BCL2 transcription in MCF-7 cells via ERα-Sp1 and AP1 interactions (Dong et al., 1999); thus, the increase in BCL2 mRNA with E2 is not solely mediated by a decreased miR-21 (Wickramasinghe et al., 2009). The increase in BCL2 mRNA with fludioxonil and fenhexamid appears to be independent of their stimulation of miR-21 expression.

In agreement with their upregulation of miR-21, both fludioxonil and fenhexamid reduced Pdcd4 (Fig. 4B) and Bcl-2 (Fig. 5B) protein expression. These data are consistent with the inhibition of PDCD4 mRNA; however, fludioxonil and fenhexamid increased BCL2 mRNA. Thus, the reduced Bcl-2 protein is consistent with increased miR-21 and inhibition at the translational level. Because miRNAs can reduce translation while not modifying mRNA levels (Iorio and Croce, 2012), this finding is consistent with an increase in miR-21 reducing Bcl-2 at the mRNA level, but not at the protein level. Pdcd4 and Bcl-2 protein expression was increased in MCF-7 cells treated with E2, in agreement with previous findings (Wickramasinghe et al., 2009) (Figs. 4B and 5B).

AS-miR-21 Inhibits Fludioxonil and Fenhexamid-Mediated Inhibition of Pdcd4 Protein Expression

To determine whether the inhibition of Pdcd4 and Bcl-2 protein expression in MCF-7 cells by fludioxonil and fenhexamid was mediated directly by miR-21, cells were transfected with antisense-miR-21 oligonucleotide (AS-miR-21) or an AS control for 48 h and then treated with E2, fludioxonil, or fenhexamid for 6 or 24 h for RNA and protein studies, respectively (Fig. 5). AS-miR-21 completely ablated miR-21 expression...
As expected, E₂ increased Pdcd4 and Bcl-2 protein and AS-miR-21 further increased Pdcd4 and Bcl-2 protein (Fig. 5B). AS-miR-21 increased basal Pdcd4 protein expression in MCF-7 cells but had no effect on basal Bcl-2 protein (Fig. 5B). AS-miR-21 blocked the repression of Pdcd4 and Bcl-2 protein by fludioxonil and fenhexamid (Fig. 5B). These data are commensurate with a direct stimulation of miR-21 by fludioxonil and fenhexamid that in turn reduces Pdcd4 and Bcl-2 protein levels.

### Fludioxonil and Fenhexamid Directly Regulate miR-21 Repression of Pdcd4 Through the 3' UTR

To determine whether fludioxonil and fenhexamid directly regulate miR-21 interaction with the 3' UTR of Pdcd4, MCF-7 cells were transiently transfected with a PDCD4-3'UTR construct cloned downstream of the Renilla luciferase reporter. Cells were also transfected with AS-miR-21 or an AS control and then treated with E₂, fludioxonil, or fenhexamid (Fig. 5B). These data are commensurate with a direct stimulation of miR-21 by fludioxonil and fenhexamid that in turn reduces Pdcd4 and Bcl-2 protein levels.

### Fludioxonil and Fenhexamid Inhibit MCF-7 Cell Viability

To determine the functional consequence of fludioxonil and fenhexamid’s induction of miR-21 in MCF-7 cells, MCF-7 cells were treated with a range of concentrations of fludioxonil and fenhexamid, and cell viability was measured by an MTT assay after 48-h treatment (Fig. 6A). E₂ was included as a positive control and increased MCF-7 viability. Fludioxonil had a biphasic effect, first increasing proliferation at 10nM and then inhibiting cell viability at higher concentrations. Fenhexamid had no effect on cell viability at concentrations from 1nM to 10µM.

Fludioxonil and fenhexamid inhibited E₂-induced cell viability, a finding in agreement with their ability to block E₂ inhibition of miR-21 expression (Fig. 1A). There was no evidence that fludioxonil and fenhexamid induced apoptosis in MCF-7 cells (Fig. 6B), even after 96-h treatment (Supplementary fig. 4). With 48-h treatment, fludioxonil increased intact poly(ADP-ribose) polymerase (PARP), a finding that reflects the lack of effect of fludioxonil on cell viability.

### Fludioxonil and Fenhexamid Inhibit E₂-Regulated Gene Transcription in MCF-7 Cells

To determine whether fludioxonil and fenhexamid exhibit antiestrogenic activity on ERα-regulated gene targets, the expression of E₂-regulated genes cyclin D1 (CCND1) and progesterone receptor (PGR) was measured by quantitative real-time PCR along with the expression of ERα (ESR1) and ERα (ESR2). As a positive control, E₂ increased the expression of
Fludioxonil and fenhexamid inhibited expression of CCND1, PGR, and ESR1. Fludioxonil inhibited ESR2 and fenhexamid had no effect on ESR2. 18S rRNA control gene expression was not altered by any treatment (Supplementary fig. 6).

Fludioxonil and Fenhexamid Regulate BCL2 Gene Expression in ERα-Negative MDA-MB-231 Cells

In addition, we tested the effect of fludioxonil and fenhexamid on three well-established E2-ERα-induced genes: PGR, CCND1, and BCL2 in ERα-negative, ERβ-positive (Dotzlaw et al., 1999), MDA-MB-231 cells. PGR was not expressed in MDA-MB-231 cells (CT > 38, data not shown). Neither fludioxonil nor fenhexamid increased the expression of cyclin D1 (CCND1) (Supplementary fig. 7). Fludioxonil inhibited the expression of BCL2, whereas fenhexamid increased BCL2 (Supplementary fig. 7). Fludioxonil did not inhibit BCL2 expression in MCF-7 cells, whereas fenhexamid increased BCL2 in MCF-7 cells (Fig. 4), indicating cell-specific regulation of BCL2 expression by these fungicides.

Fludioxonil and Fenhexamid Inhibit MCF-7 Cell Mobility in a Wound-Healing Assay

To address the dichotomy of the increase in miR-21 expression by fludioxonil and fenhexamid with the inhibition of MCF-7 cell viability by fludioxonil, we examined the effect
of these fungicides on MCF-7 cell motility in a wound-healing assay (Figs. 7A and B). E2 increased MCF-7 cell motility. Fludioxonil and fenhexamid slowed cell migration compared with DMSO at the 24- and 48-h time points.

**DISCUSSION**

A role for EDCs in breast cancer is suspected but unproven in humans (Weyandt et al., 2008). The mechanisms by which EDC might stimulate breast tumorigenesis and disease progression involve potential effects on a wide range of processes involved in hormone action. Here we report for the first time that the commonly used fungicides fludioxonil and fenhexamid increase the expression of miR-21 in MCF-7 breast cancer cells at the gene transcription level. Similar results were observed in another luminal A breast cancer cell line, T7D, but not in two ERα-negative cell lines: MCF-10A and MDA-MB-231 cells.

The rationale for examining miR-21 is that it is the most overexpressed miRNA in breast tumors and correlates with poor prognosis (Yan et al., 2008). The increase in oncomiR miR-21 expression induced by fludioxonil and fenhexamid was blocked by fulvestrant and bicalutamide, suggesting that these fungicides act through ERα and AR to increase miR-21. Overexpression of miR-21 in breast tumors has been associated with reduced survival time, and knockdown of miR-21 in breast cancer cell lines has been associated with increased responses to taxol in vitro (Corcoran et al., 2011). Hence, it is possible that women exposed, perhaps repeatedly through ingestion of fruits with residual fludioxonil and fenhexamid pesticides, might have an increase in miR-21 in breast tumor cells, which would be expected to increase cell proliferation and reduce responses to anticancer agents.

In contrast to results from a reporter assay in stably transformed MDA-MB-231 cells (Orton et al., 2011), fludioxonil and fenhexamid did not act as antiandrogens in MCF-7 cells, suggesting cell-specific responses to these fungicides. We note that fludioxonil and fenhexamid reduced the expression of tumor suppressors miR-125b and miR-181a but stimulated the expression of tumor suppressor miR-200a. Increased miR-200a would be predicted to inhibit cell motility, commensurate with our observations in the wound-healing assay, but further detailed studies are necessary to elucidate the mechanisms involved. miR-22 is a tumor suppressor and downregulates ESR1 (ERalpha) (Klinge, 2012). Interestingly, the small increase and decrease in ERα protein in MCF-7 cells treated with fludioxonil and fenhexamid, respectively (Supplementary fig. 3), reflect the decrease and increase, respectively, in miR-22 seen with these fungicides. Overall, the fungicides fludioxonil and fenhexamid regulate miRNA transcription in a gene-specific manner.

Our study also shows for the first time that 1 and 10nM DHT increase miR-21, miR-22, and miR-200a and reduce miR-125b and miR-181a in MCF-7 cells. Although full dissection of the mechanism and meaning of these results will require further investigation, it appears that DHT, like fludioxonil and fenhexamid, regulates miRNA expression in a gene-specific manner. The increase in tumor suppressor miR-22 and miR-200a is in agreement with the ability of DHT to inhibit MCF-7 cell proliferation (Macedo et al., 2006). There is currently much interest in the role of androgens in breast cancer because epidemiological studies suggest an association between higher circulating

---

**FIG. 7.** Fludioxonil and fenhexamid inhibit MCF-7 cell migration in a wound-healing assay. MCF-7 cells were seeded in six-well plates and incubated overnight in phenol red–free IMEM + 5% DCC-FBS before wounding with a plastic pipette tip. The cells were treated with DMSO (vehicle control), 10nM E2, 100nM fludioxonil, or 100nM fenhexamid, as indicated, for 48 h total. (A) Cells were photographed at the indicated times, the bar is 1000 µm. (B) The wound closure was analyzed at the indicated times using NIH Image J software. Values are the mean ± SD of duplicate measurements.
levels of androgens, as well as estrogens, in postmenopausal breast cancer (Kotsopoulos and Narod, 2012).

OncomiR miR-21 downregulates PDCD4, BCL2, and PTEN as well as other targets in breast cancer cells (O’Day and Lal, 2010). As expected, commensurate with their ability to increase miR-21, fludioxonil and fenhexamid reduced PTEN and BCL2 protein levels. Fludioxonil and fenhexamid also reduced PDCD4 and PTEN mRNA but not BCL2 mRNA in MCF-7 cells. We also detected reduced PDCD4 in T47D cells but not in ERα-negative MCF-10A cells. These results indicate, as modeled in Fig. 8, that fludioxonil and fenhexamid activate ERα and AR to increase miR-21 expression, which, in turn, directly downregulated PDCD4, PTEN, and BCL2. In agreement with this model, we demonstrated that AS-miR-21 blocked fludioxonil and fenhexamid upregulation of miR-21 and thus derepressed PDCD4 and BCL2 protein expression.

Although previous studies reported that miR-21 inhibits apoptosis by reducing Bcl-2 in a breast cancer mouse model (Si et al., 2007), here fenhexamid inhibited MCF-7 cell proliferation despite increasing miR-21 expression. This observation is similar to other paradoxical data showing that all-trans-retinoic acid (ATRA) increased miR-21 in MCF-7 and other ERα-expressing cells and inhibited cell proliferation and decreased cell motility (Terao et al., 2011). In contrast, fludioxonil had no effect on cell viability and increased the amount of intact PARP seen at 48 h, an effect that was ablated at 96 h, although no increase in cleaved PARP was detected. These data indicate different mechanisms for fludioxonil and fenhexamid in MCF-7 cells and suggest a potentially beneficial role for the observed increase in miR-21 with fludioxonil and fenhexamid, i.e., reducing cell motility, an event adversely affecting breast cancer prognosis.

Fenhexamid and DHT, but not fludioxonil or E2, increased expression of the TMEM49 in which miR-21 is encoded in MCF-7 cells, and fludioxonil and fenhexamid increased TMEM49 in MDA-MB-231 cells. Although exploring the significance of increased TMEM49 expression is beyond the scope of this study, TMEM49/VMP1 has been suggested to be a 7-transmembrane protein (Sauermann et al., 2007). When overexpressed in HEK-293 cells, VMP1 is located in the endoplasmic reticulum and vacuoles and plays a role in the plasma membrane by initiating maturation of tight junctions and adherens junctions (Sauermann et al., 2007). Knockdown of VMP1 in HEK-293 cells revealed that VMP1 plays a role in cell-cell adhesion and that cells with low VMP1 showed increased invasiveness. VMP1 expression was reported to be reduced with increasing tumor grade in invasive ductal breast carcinomas. The authors concluded that VMP1 plays a role in maintaining cell-cell interaction similar to that of E-cadherin, and when VMP1 is reduced, cells become more migratory and metastatic (Sauermann et al., 2007). Because fenhexamid increased TMEM49/VMP1 and reduced cell migration in MCF-7 cells, these results are consistent with the role of overexpressed VMP1 in HEK-293 cells (Sauermann et al., 2007).

The AHR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) reduced Tmem49 expression in fetal mouse hearts in a dose-dependent manner (Thackaberry et al., 2005). We observed that treating MCF-7 cells with AHR antagonist α-NF increased Tmem49 expression ~sevenfold (data not shown). The finding that α-NF had a small, but statistically significant, effect inhibiting fenhexamid-stimulated miR-21 expression is consistent with some activity of fludioxonil via AHR but is inconsistent with the reported repressive effect of AHR on E2-ERα regulated genes (Madak-Erdogan and Katzenellenbogen, 2012). Knockdown of AHR increases the expression of some ERα-regulated genes in response to E2 treatment by dismissing the corepressor RIP140 from the gene promoters (Madak-Erdogan and Katzenellenbogen, 2012). Given the Geoprofiles data GDS2744 and GDS2745 (GSE7765) showing that a 16-h treatment of MCF-7 cells with 100nM TCDD increased miR-21 and Tmem49 expression, it is possible that the increase in miR-21 is a consequence of a read through of AHR regulation of Tmem49 transcription. This hypothesis is indicated as a possibility in Figure 8 and requires further exploration. Overall, we...
FUNGICIDES INCREASE miR-21

It is likely that fludioxonil and fenhexamid target multiple receptors and pathways, albeit with relatively lower affinity than the natural ligands for these pathways.

We also report that inhibitors of MAPK and PI3K pathways inhibit fludioxonil- and fenhexamid-induced miR-21 expression. However, because TRBP is regulated by MAPK phosphorylation which increases Dicer activity, the ablation of miR-21 induction by the MEK1 inhibitor PD98059 may reflect inhibition of Dicer activity and thus not be informative of the mechanism by which these fungicides increase miR-21 (Klinge, 2012). Likewise, AKT was identified as a positive upstream regulator of miR-21 in rat cardiomyocytes because wortmannin upregulated Fasl and downregulated miR-21 (Sayed et al., 2010). However, that study used 5µM wortmannin for 24 h compared with 100nM wortmannin for 7 h total in our experiments. Nonetheless, we cannot conclude a role for the PI3K/AKT pathway in mediating the increase in miR-21 by fludioxonil and fenhexamid in MCF-7 cells.

In summary, the fungicides fludioxonil and fenhexamid increase the expression of miR-21 in an ER- and AR-dependent manner in MCF-7 breast cancer cells. The requirement for ERα was reflected in similar findings in T47D luminal A breast cancer cells, but not in ERα-negative MCF-10A normal breast epithelial cells or MDA-MB-231 triple negative breast cancer cells. Fenhexamid also appears to increase miR-21 expression through AHR activation. The fludioxonil- and fenhexamid-induced decrease in Pdcd4 and Bcl-2 proteins was mediated by an increase in miR-21. Fludioxonil inhibited MCF-7 cell viability, whereas fenhexamid had no impact on cell viability at concentrations from 1nM to 10µM. This is in agreement with results of Jungbauer (unpublished data) although in that report 100µM fenhexamid inhibited MCF-7 cell viability. Both fungicides reduced cell motility. Although the relationship of the concentrations of fludioxonil and fenhexamid as residuals in fruits and vegetables and the 100nM concentration used in most of these experiments is unknown, we suggest that depending on the time of harvest after spraying and subsequent washing procedures, a 100nM concentration might be found. Rats gavaged with fludioxonil at 5mg/kg bw had serum levels of 0.065 and 0.027 mg/l for males and females, respectively, 15 min after treatment (World Health Organization, 2006). These serum levels are ~250–100nM, similar to the concentrations used in this study. Likewise, rats gavaged with fenhexamid at 1mg/kg bw showed serum levels of 0.065 and 0.027 µg/ml for males and females, respectively (UN FAO, 2005), which is ~200-1000nM, again similar to concentrations used here. Although studies of fludioxonil sprayed on tomatoes showed no fludioxonil 24 days after field spraying (Cabizza et al., 2012), further testing of human exposure levels may be warranted. The ability of fenhexamid and fludioxonil to act as endocrine disruptors by activating endogenous ERα and AR and increasing expression of the oncomiR miR-21 in human breast cancer cells requires further study for its significance for fetal development and human health.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Institutes of Health (CA138410 to C.M.K.).

ACKNOWLEDGMENT

We thank Brandie J. Radde for performing some Western blots.

REFERENCES


FUNGICIDES INCREASE miR-21


