Nuclear receptor 4A1 as a drug target for breast cancer chemotherapy

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Abstract

The orphan nuclear receptor 4A1 (NR4A1) is overexpressed in mammary tumors and breast cancer cell lines. The functional activity of this receptor was investigated by RNA interference with oligonucleotides targeted to NR4A1 (siNR4A1) and by treatment with NR4A1 antagonists. Breast cancer cells were treated with NR4A1 antagonists or transfected with siNR4A. Effects on cell proliferation and apoptosis as well as specific genes associated with these responses were investigated in MCF-7, SKBR3, and MDA-MB-231 cells, and in athymic nude mice bearing MDA-MB-231 cells as xenografts. Transfection of MCF-7, MDA-MB-231, and SKBR3 breast cancer cells with siNR4A1 decreased cell proliferation and induced apoptosis in these cell lines. Transfection of breast cancer cells with siNR4A1 also decreased expression of Sp-regulated genes including survivin, bcl-2, and epidermal growth factor receptor, inhibited mTOR signaling in MCF-7 cells that express WT p53, and activated oxidative and endoplasmic reticulum stress through downregulation of thioredoxin domain-containing 5 and isocitrate dehydrogenase 1. 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes (C-DIMs) are NR4A1 ligands that act as NR4A1 antagonists. Treatment with selected analogs also inhibited breast cancer cell and tumor growth and induced apoptosis. The effects of C-DIM/NR4A1 antagonists were comparable to those observed after NR4A1 knockdown. Results with siNR4A1 or C-DIMs/NR4A1 antagonists in breast cancer cells and tumors were similar to those previously reported in pancreatic, lung, and colon cancer cells. They demonstrate the potential clinical applications of NR4A1 antagonists in patients with tumors that overexpress this receptor.

Key Words

NR4A1
indole derivative
antagonists

Introduction

Nuclear receptor 4A1 (NR4A1, Nur77, TR3) is a member of the NR4A orphan receptor sub-family of nuclear receptors. NR4A receptors (NR4A1, NR4A2, and NR4A3) play essential roles in metabolic processes, inflammation, vascular function, steroidogenesis, and the CNS (Maxwell & Muscat 2006, Pearen & Muscat 2010, Lee et al. 2011). NR4A1 is over-expressed in multiple tumors and cancer cell lines. Results of receptor knockdown by RNA interference (RNAi) demonstrate that in solid tumors the receptor is pro-oncogenic and regulates cell growth and survival (Uemura & Chang 1998,
NR4A1, indole derivative, antagonists

Endocrine-Related Cancer

Several pro-apoptotic agents including phorbol esters and adamantly-derived retinoids induce expression and nuclear export of NR4A1 which subsequently binds mitochondrial bcl-2 to form a pro-apoptotic complex that decreases mitochondrial membrane potential (Li et al. 2000, Lin et al. 2004, Zhang 2007). This has led to the development of peptide mimics that convert bcl-2 into an apoptotic complex; similar results have been reported for the taxane-derived anticancer agent paclitaxel (Kolluri et al. 2000, Lee et al. 2003, Kolluri et al. 2004, Zeng et al. 2006, Lee et al. 2009, 2010, 2012, 2014a, Wu et al. 2011). Since NR4A1 exhibits pro-oncogenic activity, we have been investigating NR4A1 in cancer cell lines but this was not accompanied by nuclear export of NR4A1 (Lee et al. 2010, 2012, 2014a). Ethyl 2-(2,3,4-trimethoxy-6-(1-octanoyl)phenyl)acetate inactivates nuclear NR4A1, whereas 1-(3,4,5-trihydroxyphenyl)no-nan-1-one and cytosporone B induce nuclear export of NR4A1.

Studies in this laboratory have been investigating a series of 1,1-bis(3’-indoly)-1-(p-substituted phenyl) methane (C-DIM) analogs and their effects on NR4A1 and NR4A1-dependent transactivation (Chinthalapalli et al. 2005, Lee et al. 2009, 2010, 2012, 2014a, Cho et al. 2010). Since NR4A1 exhibits pro-oncogenic activity, we have been focused on the identification of C-DIMs that inactivate NR4A1. The p-hydroxyphenyl analog (DIM-C-pPhOH) was characterized as a compound that inactivated nuclear NR4A1 in cancer cell lines but this was not accompanied by nuclear export of NR4A1 (Lee et al. 2010, 2012, 2014a). Subsequent studies comparing the effects of DIM-C-pPhOH and knockdown of NR4A1 (siNR4A1) by RNAi identified three major pro-oncogenic pathways and associated genes regulated by NR4A1 that were inhibited by DIM-C-pPhOH: i) NR4A1 regulates expression of genes such as survivin through interactions with specificity protein 1 (Sp1) bound to their proximal GC-rich promoters (Lee et al. 2010); ii) NR4A1 inactivates p53 to enhance mTOR signaling in lung and colon cancer cells expressing WT p53 (Lee et al. 2012, 2014b); and iii) NR4A1 regulates expression of thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) to maintain low levels of oxidative stress (Lee et al. 2014a; Fig. 1A).

Recent studies show that NR4A1 is overexpressed in ER-positive and ER-negative breast tumors (Muscat et al. 2013). NR4A1 expression in breast tumors is correlated with decreased relapse-free survival (Zhou et al. 2014). Results of NR4A1 overexpression in breast cancer cells suggest that NR4A1 may be anti-migratory (Alexopoulos et al. 2010). However, a recent study reported pro-migratory activity for this receptor (Zhou et al. 2014). Research in this laboratory has demonstrated pro-oncogenic functions of NR4A1 in pancreatic, colon, and lung cancer cells. This...
study investigates the functions of this receptor in breast cancer cells and the effects of C-DIM/NR4A1 antagonists. The results clearly demonstrate the pro-oncogenic functions of NR4A1 in breast cancer and also demonstrate that C-DIM/NR4A1 antagonists represent a potential novel approach for treating breast cancer patients that over-express this orphan receptor.

**Materials and methods**

**Cell lines and antibodies**

MCF-7, MDA-MB-231, and SKBR3 human breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and were kept frozen until initiation of these studies. The cells were received at low passage (<15) and new frozen stocks were used every 6–8 weeks. The three cell lines were authenticated by Biosynthesis (Lewisville, TX, USA) on 3rd February 2015. Cells were maintained at 37 °C in the presence of 5% CO2 in DMEM/Ham’s F-12 medium with 10% fetal bovine serum with antibiotic. β-actin antibody and DMEM were purchased from Sigma–Aldrich. Sp1 antibody was purchased from Millipore (Temecula, CA, USA). Caspases 7 and 8, sestrin 2 (SESN2), bcl2, CHOP, ATF4, IDH1, IRE, ATF6, GRP78, and epidermal growth factor receptor (EGFR) antibodies were purchased from Santa Cruz Biotechnology. Caspase 3, cleaved poly ADP ribose polymerase (c-PARP; 9541), phospho mTOR, mTOR, phospho AMPKβ, AMPKα, phospho p70S6K, p70S6K, phospho S6RP, S6RP, phospho 4EBP1, 4EBP1, and survivin antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). TXNDC5 antibody was purchased from Genetex (Irvine, CA, USA). XBP-1s and phospho PERK were obtained from Santa Cruz Biotechnologies. Caspase 3, cleaved poly ADP ribose polymerase (c-PARP; 9541), phospho mTOR, mTOR, phospho AMPKβ, AMPKα, phospho p70S6K, p70S6K, phospho S6RP, S6RP, phospho 4EBP1, 4EBP1, and survivin antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). TXNDC5 antibody was purchased from Genetex (Irvine, CA, USA). XBP-1s and phospho PERK were obtained from Genetex (San Diego, CA, USA). The Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Immobilon western chemiluminescence substrates (Millipore) were used to develop images captured on a Kodak 4000 MM Pro image station, from Advanced Microscopy. The proportion of apoptotic cells was determined by the amount of green fluorescence observed in the treatment groups relative and normalized to the control group.

**Cell proliferation assay**

MCF-7, MDA-MB-231, and SKBR3 breast cancer cells (1.0×10⁵/well) were plated in 12-well plates and allowed to attach for 24 h. Cells were treated with 1,1-bis(3’-indolyl)-1-(p-carboxymethylphenyl)methane (DIM-C-pPhCO2Me) in DMSO for 24 or 48 h or transfected with siNR4A1 or iGL2 (control siRNA) in lipofectamine for 72 h. Cells were then trypsinized and counted using a Coulter Z1 cell counter and growth inhibition was determined. Each experiment was carried out in triplicate and results were expressed as the mean ± S.E.M. for each set of experiments. Cells were also treated with C-DIMs after NR4A1 knockdown.

**Annexin V staining**

MCF-7, MDA-MB-231, and SKBR3 cells (1.0×10⁵/well) were seeded in two-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific (Waltham, MA, USA) and were allowed to attach for 24 h. The medium was then changed to DMEM/Ham’s F-12 medium containing 2.5% charcoal-stripped fetal bovine serum, and either DMSO or DIM-C-pPhCO2Me (15 μM) was added for 24 h. For siRNA treatment, cells were transfected with iGL2 or 100 nm siNR4A1 (1 or 2) for 72 h. Apoptosis was analyzed by Apoptotic and Necrotic Assay Kit (Biotium), which contained FITC–Annexin V, ethidium homodimer III, and Hoechst 3342. Apoptosis, Necrotic, and Healthy Cell Detection Kit was used according to the manufacturer’s protocol and cells were visualized under an EVOS fl, fluorescence microscope, from Advanced Microscopy. The proportion of apoptotic cells was determined by the amount of green fluorescence observed in the treatment groups relative and normalized to the control group.

**Western blot analysis**

Breast cancer cells (3.0×10⁵/well) were seeded in DMEM/Ham’s F-12 medium in six-well plates. Cells were allowed to attach for 24 h and treated with varying concentrations of DIM-C-pPhCO2Me for 24 h or with 100 nm of siNR4A1 for 72 h. Cells were lysed with high salt lysis buffer (with protease inhibitor cocktail) and quantitated with a Bradford reagent. Lysates were then analyzed by SDS–PAGE and transferred onto a PVDF membrane by wet electroblotting. Membranes were then incubated with a primary antibody, followed by a secondary antibody. Western blot analysis was determined as described and Immobilon western chemiluminescence substrates (Millipore) were used to develop images captured on a Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR, USA).

**siRNAi assay**

Breast cancer cells were seeded (1.2×10⁵/well) in six-well plates in DMEM/Ham’s F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and left to

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attain for 24 h. Knockdown of NR4A1 was carried out using Lipofectamine 2000 reagent according to the manufacturer’s protocol. Small inhibitory RNAs and GL2 (non-specific oligonucleotide) were prepared and purchased from Sigma–Aldrich. The siRNA complexes used in the study are as follows: siGL2-5′-CGU ACG CGG AAU ACU UCG A-3′; siNR4A1 (1)-SASI_Hs02_00333289; siNR4A1 (2)-SASI_Hs01_00182072.

Generation and measurement of reactive oxygen species (ROS)

Cellular ROS levels were ascertained using the cell permeable probe CM-H2DCFDA (5-(and-6)-chloromethyl-2/7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen. Following treatment of the cells for 12 or 24 h with DIM-C-pPhCO2Me or siNR4A1 for 72 h, cells that were plated on a six-well culture plate were trypsinized, neutralized, then loaded with 10 μM of probe for 20 min, and washed once with serum free medium. ROS was then measured by flow cytometry using Accuri’s C6 Flow Cytometer (Accuri, Ann Arbor, MI, USA).

Triple negative breast cancer (TBNC) orthotopic xenograft model

Female BALB/c nude mice (6–8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA, USA) and maintained under specific pathogen-free conditions, housed in isolated vented cages and allowed to acclimate for 1 week with a standard chow diet. The animals were housed at Florida A&M University in accordance with the standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The protocol of the animal study was approved by the Institutional Animal Care and Use Committee (IACUC), Florida A&M University, FL, USA. MDA-MB-231 cells (1×10⁶ cells) were detached, resuspended in 100 μl of PBS with matrigel (BD Bioscience, Bedford, MA, USA), and implanted subcutaneously in the mammary fat pad of mice. When tumors reached about 40–50 mm³ in size, the animals were randomized into control and treatment groups (six animals per group) and mice were treated with placebo or DIM-C-pPhCO2Me or 1,1-bis(3′-indolyl)-1-(p-cyanophenyl)methane (DIM-C-pPhCN) (50 mg/kg per day) in nano liquid carrier (administered in sodium carboxymethyl cellulose) by oral gavage every 2nd day for 4 weeks. Tumor volumes and weights, and body weight were determined. The tumor size was measured using Vernier calipers, and the tumor volume was estimated by the formula: tumor volume (mm³) = (L × W²) × ½, where L is the length and W is the width of the tumor. Tumor lysates were obtained and analyzed for protein expression by western blots.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by Student’s t-test. The results are expressed as means with error bars representing 95% CIs for three experiments for each group unless otherwise indicated. A P value < 0.05 was considered statistically significant. All statistical tests were two-sided.
Results

Inhibition of cell proliferation by siNR4A1 and DIM-C-pPhCO₂Me

The orphan nuclear receptor NR4A1 is overexpressed in ER-positive and ER-negative breast cancer cells (Muscat et al. 2013). The role of this receptor in regulating breast cancer cell growth and survival was investigated by RNAi in ER-positive (MCF-7), ER-negative (MDA-MB-231), and erbB2 (SKBR3) overexpressing breast cancer cell lines. Cells were transfected with two different oligonucleotides against NR4A1 (siNR4A1-1/siNR4A1-2). This resulted in ≥ 50% growth inhibition in MCF-7 and SKBR3 cells, and 35% inhibition of MDA-MB-231 cell proliferation (Fig. 1B). The C-DIM compounds with a p-carboxymethylphenyl group (DIM-C-pPhCO₂Me) and cyano substituent (DIM-C-pPhCN) have been identified as an NR4A1 antagonists (Lee et al. 2014b). Figure 1C and Supplemental Figure S1A, see section on supplementary data given at the end of this article demonstrate that the former compound significantly inhibits growth of MCF-7, MDA-MB-231 cells after treatment for 24 and 48 h. IC₅₀ values were 20, 19, and 19 μM after treatment of MCF-7, MDA-MB-231, and SKBR3 cells, respectively, for 24 h and 13, 19, and 12 μM after treatment for 48 h. In contrast, treatment of non-transformed MCF-10A mammary cells with the high affinity NR4A1 ligand DIM-C-pPhOH (30 μM) for 24 or 48 h did not affect cell proliferation (Supplemental Figure S1B). Moreover, in an orthotopic model for breast cancer in athymic nude mice using MDA-MB-231 cells treatment with 50 mg/kg per day and weight compared to the corn oil controls (Fig. 1D). In contrast, after knockdown of NR4A1 in these cells, treatment with DIM-C-pPhCO₂Me resulted in only minimal growth inhibition, which confirms a role for NR4A1 in mediating the growth inhibitory effects of DIM-C-pPhCO₂Me (Fig. 1E).

siNR4A1 and DIM-C-pPhCO₂Me induce apoptosis and activate growth inhibitory genes/pathways in breast cancer cells

NR4A1 also regulates pro-survival genes and pathways in pancreatic and lung cancer cells. Figure 2A results show transfection of breast cancer cells with siNR4A1 induced cleavage of caspases 8 and 7 as well as PARP cleavage. Moreover, siNR4A1 also induced Annexin V staining MCF-7 (Fig. 2B), MDA-MB-231 (Fig. 2C), and SKBR3 (Fig. 2D) cells confirming that NR4A1 regulated anti-apoptotic pathways in these cell lines. Treatment of the cells with DIM-C-pPhCO₂Me for 24 h also induced cleavage (activation) of caspases 7 and 8 and PARP (Fig. 3A). Similar results were observed for the p-cyanophenyl compound (DIM-C-pPhCN) which is an NR4A1 ligand and antagonist in colon cancer cells (Lee et al. 2014b; Supplemental Figure S1C). DIM-C-pPhCO₂Me also enhanced Annexin V staining in MCF-7, MDA-MB-231, and SKBR3 cell lines (Fig. 3B). Moreover, DIM-C-pPhCO₂Me-induced apoptosis (PARP cleavage) was reversed after co-administration with the pancaspase inhibitor ZVAD–FMK (Fig. 3C). Thus, both NR4A1 knockdown and NR4A1 antagonists decreased

Figure 3

DIM-C-pPhCO₂Me induces apoptosis and siNR4A1 and DIM-C-pPhCO₂Me decrease expression of selected genes with GC-rich promoters in breast cancer cells. (A) Cells were treated with DIM-C-pPhCO₂Me for 24 h and whole cell lysates were analyzed by western blots. (B) MCF-7, MDA-MB-231, and SKBR3 cells were treated with DIM-C-pPhCO₂Me for 24 h and Annexin V staining was determined and quantitated. Quantitative results are means ± S.E.M. for three separate determinations and significant (P < 0.05) induction of Annexin V is indicated (*). (C) Cells were treated with DMSO or DIM-C-pPhCO₂Me alone or in combination with the pan-caspase inhibitor ZVAD–FMK, and PARP cleavage was analyzed by western blots. Cells were transfected with siNR4A1 (D) or treated with DIM-C-pPhCO₂Me (E), and whole cell lysates were analyzed by western blots.
breast cancer cell growth and induced apoptosis as well as decreased NR4A1/Sp1-regulated genes as previously described (Lee et al. 2014b). Figure 3D shows that after knockdown of NR4A1 (siNR4A1) in MCF-7, MDA-MB-231, and SKBR3 cells, there was a significant decrease in expression of several Sp1-regulated genes including EGFR, survivin, and bcl-2. However, Sp1 protein levels were unchanged. Similar results were observed in the same cell lines after treatment with the NR4A1 antagonists DIM-C-pPhCO2Me (Fig. 3D) and DIM-C-pPhCN (Supplemental Figure S1D).

siNR4A1 and DIM-C-pPhCO2Me induce ROS and endoplasmic reticulum stress

It was recently reported that NR4A1 regulates expression of genes such as IDH1 and TXNDC5 that maintain high levels of reducing equivalents and minimize ROS-mediated cellular stress (Lee et al. 2014a,b). Knockdown of NR4A1 by RNAi induced ROS by two- to fourfold in MCF-7, MDA-MB-231, and SKBR3 cells (Fig. 4A). This was accompanied by decreased expression of both TXNDC5 and IDH1 in these cell lines (Fig. 4B). Moreover, after transfection with siNR4A1, we also observed enhanced markers of endoplasmic reticulum (ER) stress including increased phosphorylation of PERK and increased expression of ATF-4, CHOP, GRP78, IRE1, ATF6, and spliced XBP-1 (XBP-1s) in the breast cancer cell lines (Fig. 4C). Treatment of MCF-7, MDA-MB-231, and SKBR3 cells with the NR4A1 antagonist DIM-C-pPhCO2Me also increased ROS after 12 and 24 h (Fig. 5A, B and C). This was also accompanied by decreased expression of TXNDC5 and IDH1 (Fig. 5D) as well as induction of markers of ER stress (p-PERK, ATF4, CHOP, ATF6, IRE1, GRP78, and XBP-1s; Fig. 5E), as previously observed in pancreatic cancer cells (Lee et al. 2014a). The role of ROS in mediating the effects of DIM-C-pPhCO2Me on activation of stress gene products was investigated in MCF-7, SKBR3, and MDA-MB-231 cells by cotreatment with the antioxidant GSH which reversed induction of the stress genes by the C-DIM/NR4A1 antagonist (Fig. 5F). We also observed that the NR4A1 antagonist DIM-C-pPhCN decreased expression of IDH1 and TXNDC5 in breast cancer cells (Supplemental Figure S1E). In addition, western blot analysis of tumor lysate from control and DIM-C-pPhCO2Me-treated mice confirmed that DIM-C-pPhCO2Me significantly induced PARP cleavage, decreased expression of Sp-regulated survivin, EGFR and bcl2 gene products and also decreased levels of TXNDC5 and IDH1 (Supplemental Figure S2A and B, see section on supplementary data given at the end of this article).

siNR4A1 and DIM-C-pPhCO2Me inhibit mTOR signaling

Transfection of p53-positive MCF-7 cells with siNR4A1 (Fig. 6A) or treatment with DIM-C-pPhCO2Me (Fig. 6B) induced SESN2, activated AMPKα (phosphorylation), and decreased phosphorylation of mTOR and mTOR downstream genes. Similar results were observed for DIM-C-pPhCN (Supplemental Figure S1F). These results are consistent with previous studies in lung cancer cell lines where inactivation of NR4A1 activated p53-dependent activation of SESN2 which in turn activated AMPKα (Lee et al. 2012). Surprisingly, we also observed that transfection of p53-mutant MDA-MB-231 and SKBR3 cells with siNR4A1 induced SESN2, activated AMPKα, and inhibited mTOR signaling (Fig. 6C). Similar results were observed...
after treatment with DlM-C-pPhCO₂Me. We also recently observed similar results in a p53-mutant renal adenocarcinoma cell line (Hedrick et al. 2015) which showed that induction of SESN2 was ROS-dependent. This was consistent with previous reports showing that SESN2 was an oxygen-sensing gene (Budanov et al. 2002). Figure 6D shows that induction of SESN2 and activation of AMPKα in MDA-MB-231 and SKBR3 cells after treatment with DlM-C-pPhCO₂Me was inhibited after cotreatment with GSH. These data are consistent with the induction of ROS in these cell lines after transfection with siNR4A1 (Fig. 4A) or treatment with DlM-C-pPhCO₂Me (Fig. 5A, B and C). Thus, results of this study demonstrate that NR4A1 regulates multiple pro-oncogenic pathways in breast cancer cells. Immunostaining for NR4A1 demonstrates that the receptor is nuclear and remains in the nucleus after treatment with an antagonist (Supplemental Figure S3, see section on supplementary data given at the end of this article). Thus, C-DIM/NR4A1 antagonists inhibit multiple nuclear NR4A1-mediated genes/pathways (Fig. 1A) and represent a new class of mechanism-based drugs for breast cancer chemotherapy.

Discussion
NR4A1 exhibits pro-oncogenic activity in cancer cell lines derived from solid tumors and is overexpressed in tumors from lung, pancreatic, and colon cancer patients (Chintharlapalli et al. 2005, Cho et al. 2010, Lee et al. 2010, 2012, 2014a, Wu et al. 2011). Moreover, in lung cancer patients, high expression of NR4A1 is a prognostic indicator for decreased survival (Lee et al. 2012). Similar results were recently reported for the expression and prognostic activity of NR4A1 in breast cancer patients. It was also observed that NR4A1 was one of only a few nuclear receptors overexpressed in patients with both ER-positive and ER-negative breast tumors (Muscat et al. 2013, Zhou et al. 2014). Since an early report indicated that NR4A1 is more highly expressed in early- vs late-stage aggressive breast tumors and exhibited some tumor suppressor-like activity (Alexopoulou et al. 2010), we investigated the function of NR4A1 in three different breast cancer cell lines by RNAi and treatment with NR4A1 antagonists. We have recently demonstrated that several C-DIMs including the ρ-hydroxy, carbomethoxy, cyano,
and bromophenyl analogs directly bind the ligand binding domain of NR4A1 and exhibit NR4A1 antagonist activity in colon cancer cells (Lee et al. 2014b). The p-carboxmethoxyphenyl analog (DIM-C-pPhCO₂Me) and to a lesser extent DIM-C-pPhCN were used as prototypical C-DIMs/NR4A1 antagonists for investigating the anticancer activities of NR4A1 antagonists in breast cancer cells with a focus on inhibition of three previously identified pro-oncogenic NR4A1-regulated pathways (Fig. 1A).

Initial studies investigated the role of NR4A1 in the growth of three prototypical breast cancer cell lines (ER-positive MCF-7, erbB2 overexpressing SKBR3, and triple negative MDA-MB-231 cells). Knockdown of NR4A1 using two different oligonucleotides significantly decreased proliferation of all three breast cancer cell lines and similar growth inhibitory effects were observed for DIM-C-pPhCO₂Me (Fig. 1B and C). Moreover, after knockdown of NR4A1 in MCF-7, MDA-MB-231, and SKBR3 cells, treatment with DIM-C-pPhCO₂Me had minimal effects (Fig. 1D), suggesting that the growth inhibitory effects of DIM-C-pPhCO₂Me were primarily NR4A1-dependent. The effects of NR4A1 knockdown or treatment with DIM-C-pPhCO₂Me on several markers of apoptosis, including cleavage of caspases 7 and 8 and PARP as well as induction of Annexin V staining, were also determined in the breast cancer cell lines (Figs 2 and 3). These results demonstrate that NR4A1 regulates pathways that contribute to the growth and survival of breast cancer cell. This parallels the functions previously observed for this receptor in pancreatic, lung, and colon cancer cells (Lee et al. 2010, 2012, 2014a,b).

NR4A1 binds and inactivates p53 (Zhao et al. 2006), whereas knockdown of NR4A1 or treatment of p53 WT lung cancer cells with an NR4A1 antagonist or transfection with siNR4A1 results in activation of p53 and induction of SESN2 which activates AMPKα and inhibits the mTOR pathway (Lee et al. 2012). mTOR pathway inhibitors have been extensively developed for cancer chemotherapy (Baselga et al. 2012, Ciruelos Gil 2014). C-DIM/NR4A1 antagonists represent a new class of mTOR inhibitors which block NR4A1-regulated mTOR activation in cancer cells expressing WT p53 (Lee et al. 2012). Results illustrated in Fig. 6A and B show that both siNR4A1 and DIM-C-pPhCO₂Me inhibited mTOR pathway in MCF-7 breast cancer cells that express WT p53. In p53 WT lung cancer cells, siNR4A1 and NR4A1 antagonists also induced SESN2 which activates AMPKα and inhibits mTOR, whereas this is not observed in lung cancer cells expressing mutant p53 (Lee et al. 2012). Interestingly, DIM-C-pPhCO₂Me and siNR4A1 also induced SESN2 and inhibited mTOR in p53 mutant SKBR3 and MDA-MB-231 cells (Fig. 6C). The DIM-C-pPhCO₂Me-mediated induction of SESN2 and activation of AMPKα were reversed after cotreatment with GSH (Fig. 6D). These results are consistent with induction of ROS by DIM-C-pPhCO₂Me (Fig. 5A, B and C) and the fact that SESN2 is an oxygen-sensing gene that is induced by ROS (Budanov et al. 2002).

Like other nuclear receptors, NR4A1 interacts with the Sp1 transcription factor bound to GC-rich sites to activate survivin and other anti-apoptotic/growth promoting genes. siNR4A1 or treatment with a NR4A1 antagonist decreases expression of these genes (Liu & Simpson 1999, Pipaon et al. 1999, Suzuki et al. 1999, Lu et al. 2000, Shimada et al. 2001, Sugawara et al. 2002). Figure 6C and D...
show that siNR4A1 or treatment with DIM-C-pPhCO2Me decreased expression of survivin, bcl2 and EGFR in MDA-MB-231, MCF-7, and SKBR3 cells. However, Sp protein levels were unchanged. Molecular analysis of NR4A1-dependent regulation of survivin showed that in pancreatic cancer cells, NR4A1 and p300 cooperatively activated survivin expression by interacting with Sp1 bound to the proximal GC-rich region of the survivin promoter (Lee et al. 2010). Regulation of growth-promoting and survival genes which contain GC-rich promoters by NR4A1 is consistent with the growth inhibitory and apoptotic effects of siNR4A1 and C-DIM/NR4A1 antagonists on breast cancer cells and tumors. It is comparable to that observed in lung, colon and pancreatic cancer cell lines (Lee et al. 2010, 2012, 2014a,b).

A recent study showed that NR4A1 maintains low levels of oxidative and ER stress in pancreatic cancer cells by regulating expression of TXNDC5 and IDH1 which maintain cellular levels of reducing equivalents (Lee et al. 2014a). DIM-C-pPhCO2Me or siNR4A1 decreased expression of TXNDC5 and IDH1 in breast tumors (in vivo; Supplemental Figure S2) and in MCF-7, MDA-MB-231, and SKBR3 cells. This was accompanied by increased levels of ROS and induction of markers of ER stress (Figs 4 and 5). These results are consistent with previous studies in pancreatic cancer cells. There is also emerging evidence that both TXNDC5 and IDH1 are overexpressed in breast cancer cells and tumors (Xu et al. 2010, Chang et al. 2013). Their expression and functions in breast cancer cells are currently being investigated.

In summary, results of this study are consistent with a pro-oncogenic role for NR4A1 in breast cancer as an important regulator of cell growth and survival. NR4A1-regulated pro-oncogenic pathways and genes are similar to those observed in pancreatic, lung, and colon cancer cells and tumors (Lee et al. 2010, 2012, 2014a,b). This study also demonstrated the effectiveness of the NR4A1 antagonists DIM-C-pPhCO2Me and DIM-C-pPhCN as inhibitors of breast cancer cell and tumor growth as well as survival. Current structure-activity studies are focused on identifying the most effective C-DIM/NR4A1 antagonists for future clinical applications in breast cancer chemotherapy, including the inhibition of breast invasion (Zhou et al. 2014) through inhibition of nuclear NR4A1 by receptor antagonists.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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