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Estrogen-related receptor-α antagonist inhibits both estrogen receptor–positive and estrogen receptor–negative breast tumor growth in mouse xenografts

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Abstract

Estrogen-related receptors (ERR) are orphan members of the nuclear receptor superfamily most closely related to estrogen receptors (ER). Although ERα is a successful target for treating breast cancer, there remains an unmet medical need especially for estrogen-independent breast cancer. Although estradiol is not an ERR ligand, ER and ERR share many commonalities and overlapping signaling pathways. An endogenous ERR ligand has not been identified; however, novel synthetic ERRα subtype–specific antagonists have started to emerge. In particular, we recently identified a novel compound, *N*-[(2*Z*)-3-(4,5-dihydro-1,3-thiazol-2-yl)-1,3-thiazolidin-2-yl idene]-5H dibenzo[*a,d*][7]annulen-5-amine (termed compound A) that acts specifically as an ERRα antagonist. Here, we show that compound A inhibited cell proliferation in ERα-positive (MCF-7 and T47D) and ERα-negative (BT-20 and MDA-MD-231) breast cancer cell lines. Furthermore, we report the differential expression of 83 genes involved in ERRα signaling in MCF-7 and BT-20 breast cancer cell lines. We show that compound A slowed tumor growth in MCF-7 and BT-20 mouse xenograft models, and displayed antagonistic effects on the uterus. Furthermore, a subset of genes involved in ERRα signaling *in vitro* were evaluated and confirmed *in vivo* by studying uterine gene expression profiles from xenograft mice. These results suggest for the first time that inhibition of ERRα signaling via a subtype-specific antagonist may be an effective therapeutic strategy for ER-positive and ER-negative breast cancers. [Mol Cancer Ther 2009;8(3):672–81]

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Introduction

Estrogen-related receptor (ERR)-α is an orphan member of the steroid/nuclear receptor superfamily. The ERR subfamily consists of three members, ERRα, ERRβ, and ERRγ, which share a high degree of amino acid homology to the estrogen receptors (ER) but do not bind natural estrogens or any other endogenous ligand (1, 2). ERRα was found by using the DNA-binding domain of ERα as a hybridization probe to screen recombinant DNA libraries (1). ERRα mRNA is expressed in the mouse embryo developing heart, intestine, brain, spinal cord, brown fat, and bone (3). ERRα is ubiquitously expressed in adult tissues with higher expression in tissues in which a high level of metabolism and fatty acid β-oxidation occurs, including skeletal muscle, kidney, heart, liver, and adipose (1, 4, 5). The ERRα-knockout phenotype is viable, fertile, and does not exhibit any apparent anatomic alterations except reduced body weight and smaller peripheral fat deposits (5). ERRs and ERs share common target genes previously shown to be important for breast cancer such as pS2 (6), lactoferrin (7, 8), aromatase (9), and osteopontin (10) and exhibit cross-talk. Whereas many other members of the steroid receptor superfamily are activated by ligands (including ERs), ERRs are constitutively active without the addition of a specific ligand (11).

Three ERRα subtype–selective inverse agonists or antagonists have been reported in addition to the ERRα subtype–selective antagonists we have previously described (12). XCT790 is a selective inverse agonist reported to have an IC_{50} of 0.37 μmol/L in a GAL4-ERRα cell-based transfection assay (13). Cyclohexylmethyl-(1-*p*-tolyl-1H-indol-3-ylmethyl)-amine has been cocrystallized with the human ERRα LBD and a novel molecular mechanism of action for inverse agonism of ERRα has been proposed (14). The synthetic estrogen diethylstilbestrol (DES) was shown in a fluorescence resonance energy transfer assay to be an antagonist for the ERRs at high concentrations (2).

There are strong supporting data in the literature to consider the use of an ERRα-specific antagonist to treat breast cancer. ERRα is expressed in multiple human breast cancer cell lines, breast tumors, and in breast adipose tissue (6, 15, 16). ERRα expression in clinical breast cancer tumors correlates with an increased risk of disease recurrence and adverse clinical outcome (16). Moreover, ERRα expression is associated with an unfavorable, aggressive tumor phenotype correlating with ErbB2 (HER2, Neu)

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overexpression (15). Furthermore, the ERRα ligand DES inhibits breast cancer proliferation at high concentrations (6), and in the past, has been used in the clinic to treat breast cancer (17).

The rationale for an ERRα antagonist to treat breast cancer plus our recent discovery of new ERRα subtype–specific ligands has led to an effort to study the effects of ERRα antagonists on breast tumor growth. Specifically, *N*-[(2*Z*)-3-(4,5-dihydro-1,3-thiazol-2-yl)-1,3-thiazolidin-2-yl idene]-5H dibenzo[*a*,*d*][7]annulen-5-amine (or for simplicity, ''compound A'' in the present study) has the strongest antagonistic effect on the constitutive interaction between ERRα and nuclear coactivators among the ERRα ligands we identified (12). Our experimental results show that compound A slows cell proliferation in ERα-positive (MCF-7 and T47D) and ERα-negative (BT-20 and MDA-MD-231) breast cancer cell lines. We describe changes in the expression of genes involved in ERRα signaling in MCF-7 and BT-20 breast cancer cells treated with compound A. We further show that compound A inhibits tumor growth in MCF-7 and BT-20 mouse xenograft models while showing antagonistic effects on the uterus. Lastly, a subset of genes determined to be involved in ERRα signaling *in vitro* were validated *in vivo* by studying the gene expression profile in the uteri from treated mice.

Materials and Methods

Cell Proliferation Assays

Human breast cancer cell lines MCF-7, T47D, BT-20, and MDA-MB-231 were obtained from American Type Culture Collection and maintained as described in their product information sheets. Cells were grown in estrogen-free medium for 4 days prior to each experiment and were seeded into six-well plates. MCF-7 cell lines were seeded at 10,000 cells/well, whereas BT-20 cells were seeded at 20,000 cells/well. The following day, medium containing DMSO (vehicle), 1 μmol/L of compound A (Merck & Co.), 10 nmol/L of 17β-estradiol (E2; Sigma), 3 pmol/L of E2, or 1 μmol/L of compound A/3 pmol/L of E2 were added. MCF-7 cells were counted on days 2 to 6, 8, 10, and 12. BT-20 cells were counted on days 2, 7, and 10 to 14. MCF-7/shGFP, MCF-7/shERRα2, and MCF-7/shERRα3 RNAi cell lines[3] were counted on days 3 and 6 to 10. For dose titrations, cells were seeded into 96-well plates. MCF-7 and T47D cell lines were plated at 3,000 cells/well, BT-20 cell line at 6,000 cells/well, and MDA-MB-231 at 5,000 cells/well. On days 1, 4, and 8, cells were treated with vehicle (DMSO), compound A, or DES at the indicated concentrations (100 pmol/L–100 μmol/L) in medium with 3 pmol/L of E2. On day 9, DNA contents were measured using the CyQuant Cell Proliferation Assay Kit (Invitrogen) according to the protocols of the manufacturer.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from MCF-7 and BT-20 cells treated with vehicle (DMSO), 3 pmol/L of E2, 5 μmol/L of compound A, or 3 pmol/L of E2/5 μmol/L of compound A for 2, 24, or 48 h, respectively. The RNA samples were treated with DNase I (Ambion, Inc.) and cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems).

Quantitative Real-time Reverse Transcription-PCR

Two hundred and twenty-six genes involved in ER, cancer, and ERRα signaling (Supplementary Fig. S1)[4] were assayed using TaqMan Low-Density Custom Array cards (Applied Biosystems). Four to eight copies per gene (depending on card configuration) were present on multiple arrays. Genes assayed were selected based on the published literature. Real-time reverse transcription-PCR was done with an ABI 7900 HT sequence detection system (Applied Biosystems). The expression levels of target genes were normalized to 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase as endogenous quantitative controls. Relative gene expression was calculated based on the ΔΔCt method as outlined in the Applied Biosystems User Guide. Relative gene expression values down-regulated ≥1.2-fold or up-regulated >1.2-fold ($P < 0.05$) were reported and analyzed using Ingenuity Pathway Analysis (Ingenuity Systems). Genes showing significant differential expression were retested in the 96-well 7900 HT FAST real-time reverse transcription-PCR format to confirm low-density array results.

Pharmacokinetic Study of Compound A

Five- to 6-week-old female ovariectomized athymic nude mice (NCr; Taconic) were implanted s.c. with 0.36 mg (60-day release) E2 pellets (Innovative Research of America). Mice were divided into two groups of 15 mice treated with either 15 or 30 mg/kg of compound A, and one group of 3 mice dosed with vehicle (10% DMSO/90% sesame oil).

Three days post-E2 implantation, mice received 5 consecutive days of compound A or vehicle (day 1–5). At 6, 12, 24, 36, and 48 h post-day 5, three mice from each treatment group were euthanized and blood was collected by cardiac puncture using Capiject (K2-EDTA) blood collection tubes (Terumo). Blood was also collected from three mice dosed with vehicle at the last time point. Plasma was isolated from the samples and sent to Charles River Laboratories for compound A plasma concentration determination using API-5000 liquid chromatography-tandem mass spectrometry (Applied Biosystems) following protein precipitation. Watson Bioanalytical LIMS (Thermo Scientific, version 7.2.0.02) was used to determine pharmacokinetic parameters based on plasma concentrations over time.

Mouse Xenograft Models

MCF-7 (1×10^7) cells/site were injected (s.c.) into the axillary mammary fat pads of 5- to 6-week-old female

[3] Chisamore MJ, Wilkinson HA, Flores O, Chen JD. Characterization of a novel subtype specific estrogen related receptor α antagonist in MCF-7 breast cancer cells. Endocr Relat Cancer. Submitted 2008.

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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ovariectomized athymic nude mice (NCr; Taconic) that were also implanted with an E2 pellet (same as described above). After 5 weeks, mice were divided into three treatment groups consisting of 10 mice/group: (*a*) control vehicle (10% DMSO/90% sesame oil), (*b*) 15 mg/kg of compound A, and (*c*) 5 mg/wk of ICI-182,780 (AstraZeneca). BT-20 (5×10^6) cells/site were injected (s.c.) into the axillary mammary fat pads of 5- to 6-week-old female athymic nude mice. After 8 weeks, mice were divided into two treatment groups consisting of 11 to 15 mice/group: (*a*) control vehicle (10% DMSO/90% sesame oil) and (*b*) 30 mg/kg of compound A. Both MCF-7 and BT-20 xenograft models received s.c. injections every 3 days of either vehicle or compound A. The MCF-7 xenograft ICI-182,780 treatment group received one s.c. injection weekly of 5 mg/animal. At the termination of the experiments, the animals were euthanized, whole body weight was measured, blood was drawn to determine compound A plasma concentration and uteri were weighed. Uteri were snap-frozen in liquid nitrogen and sent to Cogenics where total RNA was extracted. RNA samples from each treatment group were pooled, cDNA was synthesized, and 46 genes known to be involved in ERRα signaling were assayed using TaqMan Low-Density Custom Array cards (Applied Biosystems). Genes with significant differential expression were re-tested in the 96-well 7900 HT FAST real-time reverse transcription-PCR format to confirm low-density array results.

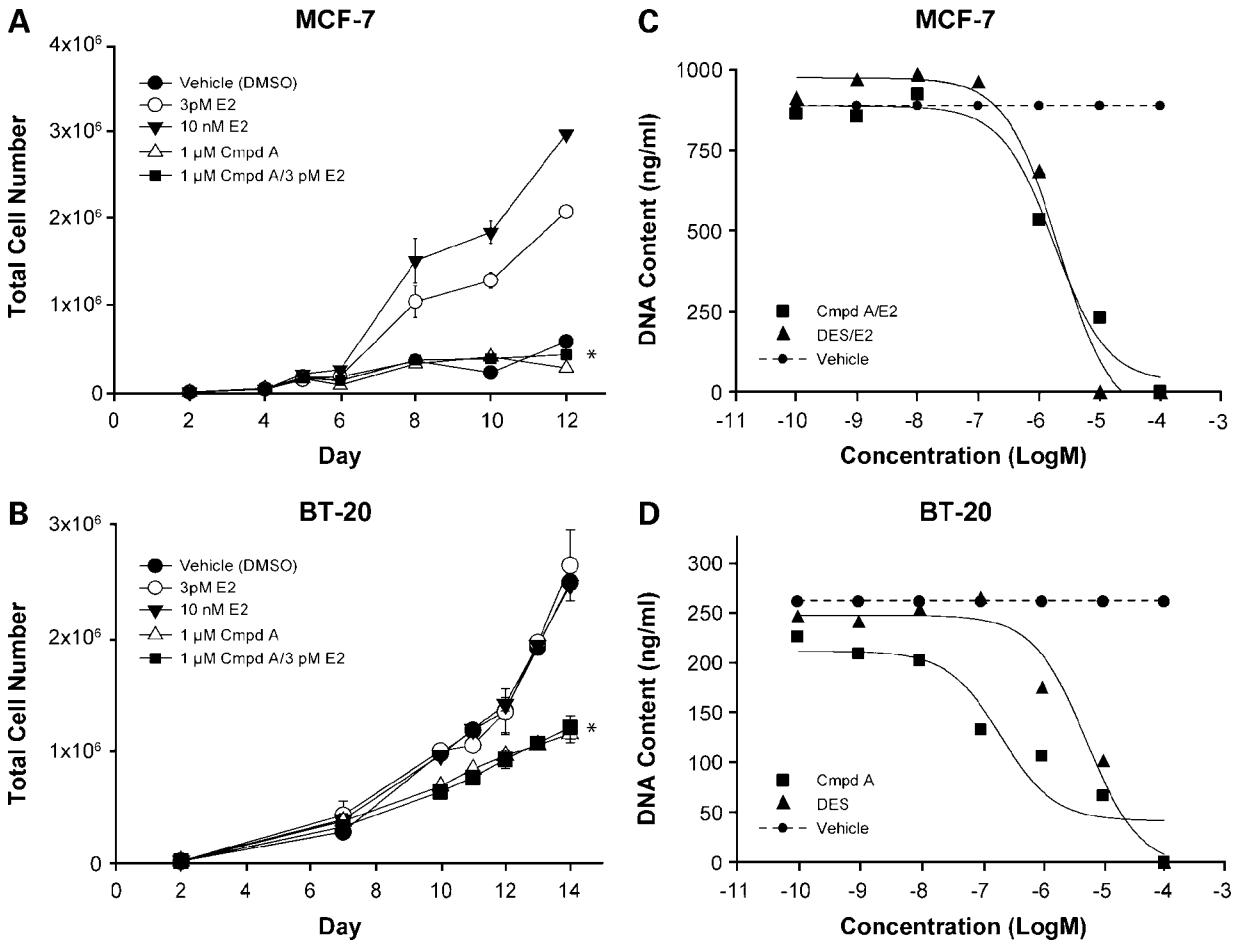

Figure 1. ERRα-specific antagonist compound A inhibits breast cancer cell proliferation. **A,** proliferation rate of MCF-7 cells grown in E2-free medium treated with vehicle (●), 1 μmol/L of compound A (○), 10 nmol/L of E2 (▼), 3 pmol/L of E2 (△), or 1 μmol/L of compound A/3 pmol/L of E2 (■). **B,** proliferation rate of BT-20 cells grown in E2-free medium treated with vehicle (●), 3 pmol/L of E2 (○), 10 nmol/L of E2 (▼), 1 μmol/L of compound A (△), or 1 μmol/L of compound A/3 pmol/L of E2 (■). *, $P < 0.001$. Differences in mean total cell number between groups [3 pmol/L E2 vs. 1 μmol/L compound A (*Cmpd A*) or 1 μmol/L compound A/3 pmol/L E2 for MCF-7 and vehicle vs. 1 μmol/L compound A or 1 μmol/L compound A/E2 for BT-20] were measured by ANOVA followed by a Student's *t* test with a 0.05 significance level. Proliferation rate was determined as outlined in Materials and Methods. *Points,* mean total cell number; *bars,* SE. **C** and **D,** growth curve of MCF-7 (**C**) and BT-20 (**D**) cells treated with vehicle (●), compound A (■), or DES (▲) at the indicated concentration (100 pmol/L – 100 μmol/L) in medium that also contained 3 pmol/L of E2. After 9 d of treatment, DNA content was measured as outlined in Materials and Methods. The titrated dose-response curves were graphed by using GraphPad Prism, version 4.01 (GraphPad) nonlinear regression (curve fit) program.

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Table 1. IC_{50} values of MCF7, T47D, BT-20, and MDA-MB-231 breast cancer cell lines treated with compound A or DES

Cell line	IC_{50} (μmol/L)	
	Compound A	DES
MCF-7 [ER (+)]	0.97	2.27
T47D [ER (+)]	1.02	1.67
BT-20 [ER (−)]	0.225	4.3
MDA-MB-231 [ER (−)]	0.65	2.2

NOTE: Compound A shows lower IC_{50} values in the MCF-7, T47D, BT-20, and MDA-MB-231 cells in comparison to DES. GraphPad Prism, version 4.01 (GraphPad Software) was used to calculate values.

Results

ERRα-Specific Antagonist Compound A Inhibits Breast Cancer Cell Proliferation

We examined the effects of treatment with compound A on cell proliferation of the ER-positive MCF-7 and ER-negative BT-20 breast cancer cell lines. The estrogen-dependent MCF-7 cells treated with 10 nmol/L of E2 showed the most growth between days 5 and 11, followed by 3 pmol/L of E2 (Fig. 1). MCF-7 cells treated with DMSO showed little growth whereas cells treated with compound A, with or without E2, exhibited significantly reduced proliferation ($P < 0.001$) versus MCF-7 cells treated with E2. Likewise, the BT-20 breast cancer cells, in which E2 does not have any effect on cell proliferation (18–20), showed significantly reduced cell proliferation ($P < 0.001$) when treated with compound A compared with the vehicle group (Fig. 1B). Titrated dose-response growth curves were also carried out and reduced cell growth was shown in both MCF-7 and BT-20 cell lines (Fig. 1C and D), along with T47D and MDA-MB-231 (data not shown). After 9 days of treatment at 100 μmol/L of compound A or DES, cytotoxicity was observed. IC_{50} for all four cell lines in response to compound A and DES were calculated (Table 1). These data show that compound A exhibits lower IC_{50} values for all four cell lines in comparison with DES, and suggests that compound A is a more potent inhibitor of breast cancer cell proliferation.

Differentially Expressed Genes in Breast Cancer Cells Treated with ERRα Antagonist

To study ERRα signaling in breast cancer, 226 genes known to be involved in ER, cancer, and ERRα signaling (Supplementary Fig. S1)[4] were assayed using TaqMan Low-Density Custom Array cards (Applied Biosystems). MCF-7 and BT-20 breast cancer cells were treated with vehicle (DMSO) or compound A for 2, 24, and 72 hours. Relative gene expression values (83 genes in total) that were down-regulated or up-regulated by >1.2-fold ($P < 0.05$) are reported (Fig. 2).

In the MCF-7 cell line, after 2 hours of treatment with compound A, 20 genes were down-regulated, 4 were up-regulated, and 1 gene (*ELF3*) that was down-regulated when treated with compound A was also up-regulated when treated with E2. After 24 hours of treatment with compound A, 31 genes were down-regulated whereas 6 were up-regulated. In addition, 4 genes increased whereas 9 genes decreased expression when treated with E2. Nine of the 20 genes that were down-regulated at 2 hours when treated with compound A were also down-regulated at 24 hours. After 72 hours, 30 genes decreased whereas 24 increased expression when treated with compound A. Furthermore, 12 genes were down-regulated, whereas 22 were up-regulated when treated with E2. Twenty of the 31 genes down-regulated at 24 hours were also down-regulated at 72 hours when treated with compound A. Finally, there were 7 genes that showed decreased expression at all three time points in MCF-7 cells when treated with compound A.

In the ER-negative BT-20 cells treated with compound A, 18 genes were down-regulated at 2 hours; at 24 hours, 18 genes were down-regulated whereas 3 were up-regulated. Eight of the 18 genes down-regulated at 2 hours were also decreased at the 24-hour time point. At 72 hours, 20 genes were down-regulated whereas 6 were up-regulated. In addition, 14 genes that were down-regulated and 1 gene that was up-regulated were the same genes at both the 24-hour and 72-hour time points. Finally, 7 genes were down-regulated at all three time points. Among these 7 genes, one (*HSD3B1*) was also down-regulated at all three time points in MCF-7 cells treated with compound A.

Subsequently, using gene expression data from both MCF-7 and BT-20 cell lines, Ingenuity Pathway Analysis was used to model potential ERRα signaling pathways affected by compound A. At 2 hours of treatment, all seven genes that were decreased in both cell lines (including two that were decreased by <1.2-fold) were built into one signaling pathway (Fig. 3A). Except for phosphorylation of ERRα by mitogen-activated protein kinase 3, no signaling directly involves ERRα (ESRRA) at this short time point. At 24 hours, 13 genes were commonly decreased in both cell lines, and 11 were modeled into a single network involving energy metabolism (Fig. 3B). These include the key enzyme ACAMD involved in the mitochondrial β-oxidation of fat, the nuclear receptor coactivator PGC-1α, and carnitine acetyltransferase which is critical for energy homeostasis and fat metabolism (21). At 72 hours of treatment, 14 out of the 19 genes affected in both cell lines also fit into a single network involving ERRα signaling (Fig. 3C). Similar to the 24-hour time point, ACADM, PGC-1α, and SPP1 are again shown to have a direct relationship with ERRα. Additionally, HMGB2, a DNA-binding protein, known to bind ERα and ERβ (22), was up-regulated. Thus, along with energy metabolism, disrupting ERRα signaling in breast tumors may alter other relevant gene functions and pathways.

Compound A Inhibits the Growth of Breast Tumors in Mouse Xenograft Models

To study the efficacy of compound A *in vivo* for treating breast cancer, we first determined the pharmacokinetic parameters of compound A in ovariectomized athymic nude mice implanted with E2 pellets and treated with compound A. Plasma concentrations of compound A were

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Gene	MCF-7/Cmpd A			MCF-7/E2			BT-20/Cmpd A		
	2h	24h	72h	2h	24h	72h	2h	24h	72h
ACADM		-8.55	-1.45					-5.56	-1.67
ACSS2		-1.50							
ADRB3	-1.33	-2.08	-1.59						
APC			1.25						
BCAR1			-1.80			-1.75			
BCAR3			-1.72			-1.68			
BRCA1			1.25			1.71			
BRMS1			1.36			1.48			
CD44		1.30	1.72		3.58	5.00			-2.31
CEBPB			2.12			2.06			
CEBPG			1.76			1.42			1.22
CRAT		-5.20	-3.55				-1.92	-5.63	-21.69
CREM	-1.28								
CTBP1			1.49				-1.85		
CYP1B1			1.32			1.88			
CYP19A1	-2.04	-1.64						-4.74	-4.84
DCC			4.23						
ECH1		-1.49	-1.20		-1.95	*			
ELF3	-1.27			2.06					
ELOVL3	-1.23	-1.46	-2.39						
ESRRA									-1.56
ESR2								1.31	2.44
ESRRG			-4.78			-2.59		1.66	
FASN		-1.84	-2.65			1.37	-2.23		
FGF2			3.52			6.18			
FOLH1	-1.34	-2.16		*	-2.56				
FOS	1.78			4.50				1.52	
FXYD5			-1.81			-1.87			
GAMT	-1.20								
GTF2H1	*	-1.42					-1.67		
HDAC6	*	-1.35					-1.63		
HGF	-1.95						-2.37	-2.03	-8.63
HMGB2		1.60	1.50		2.32	2.39			1.25
HSD17B2	1.78	2.12		1.78					-2.85
HSD3B1	-2.26	-2.95	-17.74		-2.41	-3.65	-3.61	-1.44	-4.01
IGF1	1.39	2.87							
IL18		-1.37							1.52
ISGF3G		-1.77	-1.88		-2.23	*	-2.00		
KISS1							-2.28		
LYPD3			-1.52			1.33		-2.05	-3.43
MMP2		-2.77	***					-1.75	-2.36
MMP9	-1.20								
MMP10	-1.48								
MORF4L2		-1.31	*					-1.47	-1.48
MTHFD2			2.83			2.40	*	-1.44	
MYC			2.27			5.18		-2.84	*
NCOA2		-1.32	-1.22						
NCOR1		-1.29	*					-1.48	
NFATC4		-1.95	-2.38		-2.78	*	-2.55	-1.37	-2.77
NOS3		1.20	2.12		-2.25	-2.25			1.44
NRG1	-2.26								
NR0B2		-2.40					-6.71		
NR4A3			1.32						1.26
OVGP1			1.20						
PECAM1	-2.00						-4.20	-1.45	-12.96
PLG	1.44		-1.79						
PPARGC1A	-2.59	-2.34	-57.73					-1.85	-9.35
PSCA	-1.47								-3.17
RERG	**	1.24			9.65				
RHOC	-1.21								
S100A4		-1.56	-2.35			1.47	-1.94	-1.27	-4.03
SERPINB5		-1.77	-2.59			1.25			-2.17
SERPINE1	-1.24	-1.29	-2.28			-2.82	-2.50	-1.90	
SET			1.33			1.40	-1.70		
SMAD2			1.30						
SMAD4			1.31						
SNCG		-1.42	-1.96		-1.86	-1.48		-2.32	-2.83
SPP1		-2.59	-6.74				-2.22	-1.76	-8.12
SSTR2			1.34			1.73	-1.95		
SULT2A1		-5.87	***						
SULT1E1	-7.91						-2.23		
TACSTD1			1.37						
TFF1		-2.21	-2.44		20.13	158.9			
TFRC			1.34			1.30			
TNFSF10			-2.14						
TWIST1	-1.93	-2.32	-1.93		-1.97	-4.85			
UBC			1.34			1.29			
UGT1A3			-3.38			-2.00			
UGT2B15			-3.06			8.37			
VIP			-4.57			6.13			
WISP1	-1.93	-2.57	-2.25		-2.79	-2.13			
XDH		-4.02	-2.97			-1.21			-6.40
YWHAZ			1.41			1.78			

determined between 6 and 48 hours after the last treatment and a steady state of ~20 ng/mL was reached at 24 hours (Fig. 4A). The area under the curve (μmol × hours / L) was 3.5 and 4.1 for 15 and 30 mg/kg of compound A treatments, respectively. At 6 hours, the C_{max} was reached: 0.13 μmol/L for the 15 mg/kg and 0.21 μmol/L for the 30 mg/kg doses. The maximum achievable dose was determined to be 49.7 and 78.4 ng/mL (15 and 30 mg/kg, respectively) at 6 hours.

To determine if compound A would inhibit breast tumor growth, breast tumor xenografts were established in ovariectomized athymic nude mice. For the E2-dependent breast tumor model, MCF-7 cells were injected into the mammary fat pads of ovariectomized athymic nude mice implanted with an E2 pellet. After 5 weeks, tumor cross-sectional area reached 0.28 cm^2, at which time, mice were treated with compound A or ICI-182,780 (Fig. 4B). After 2 weeks of treatment, a decrease in growth was observed in both treatment groups (Fig. 4B). Comparison of tumor size after 2, 3, and 4 weeks of treatment suggests that growth stabilization occurred in both the ICI-182,780 and compound A groups. The E2 control group tumors increased in size at a steady rate over time throughout the entire experiment reaching 0.6 cm^2. At the final measurement, the compound A group tumor area was 0.3 cm^2 ($P = 0.004$) whereas the ICI-182,780 tumor area was 0.2 cm^2 ($P < 0.001$).

For the E2-independent breast tumor model, BT-20 tumors were similarly established in the axillary mammary fat pads of athymic nude mice. After 8 weeks, tumor cross-sectional area reached 0.2 cm^2 and the mice were treated with control vehicle or compound A (Fig. 4C). By the second week of treatment, inhibition of tumor growth by compound A was clearly seen. After 5 weeks of treatment, the vehicle group tumor size reached 0.5 cm^2, whereas compound A treatment group tumor size was significantly smaller at 0.3 cm^2 ($P = 0.007$). At the conclusion of the xenograft experiments, 4 days after the final treatment administration, the plasma concentration of compound A was determined to be 15.1 ng/mL in the MCF-7, and 17.9 ng/mL in the BT-20 xenograft model, consistent with the pharmacokinetic data (Fig. 4A).

Compound A Exhibits Antagonistic Effects on the Mouse Uterus

Uterine weight has been extensively used to monitor estrogen-mediated proliferative effects on the uterus (23). Therefore, at the end of the MCF-7 and BT-20 xenograft experiments, uterine weights were measured. As expected, the pure antiestrogen ICI-182,780 significantly decreased

Figure 2. Gene expression profiles of breast cancer cells treated with ERRα antagonist compound A. MCF-7 and BT-20 cells were treated with 5 μmol/L of compound A, 3 pmol/L of E2, or vehicle control for 2, 24, and 72 h. Gene expression profiles were determined using low-density array card. Out of 226 genes assayed, 83 were down-regulated by ≥1.2-fold or up-regulated by >1.2-fold ($P < 0.05$). *Green,* down-regulation; *red,* up-regulation. *, a decrease in expression, but ≤1.2; **, an increase in expression, but <1.2. Values were included due to a trend seen in comparison to other time points. ***, expression was measured in vehicle sample, but upon treatment, amplification was not detectable. Comparison of relative gene expression (vehicle vs. treatment) was done using ANOVA followed by a Student's *t* test with a 0.05 significance level.

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($P < 0.001$) uterine weight in the MCF-7 xenograft study (Fig. 5A), whereas the ERRα antagonist compound A group also showed a slight decrease ($P = 0.303$). Moreover, compound A significantly decreased ($P = 0.013$) uterine weight in the BT-20 xenograft study (Fig. 5B). These data suggest that the ERRα antagonist compound A exhibits antagonistic effects on uterine growth.

To investigate the antagonistic effect caused by compound A in the uterus, 46 genes known to play a role in ERRα signaling (shown in bold in Fig. S1) were assayed using TaqMan Low-Density Custom Array cards. In this assay, we found that 18 genes were down-regulated ≥1.2 ($P < 0.05$; Supplementary Fig. S2A).[4] Of these 18 genes down-regulated in the mouse uteri, 10 were also down-regulated in the MCF-7 and BT-20 cell lines treated with compound A (Fig. 2). Six genes (*ESRRG, FASN, LYPD3, MTHFD2, MYC*, and *SET*) that were up-regulated or down-regulated depending on the cell line and time point were all down-regulated in the mouse uteri. *CYP1B1* and *NR4A3* were up-regulated in both the MCF-7 and BT-20 cell lines but were both down-regulated in the mouse uteri. Importantly, 14 out of the 18 genes down-regulated in the mouse uteri by compound A were built into one signaling pathway involving ERRα (Supplementary Fig. S2B).[4] These data suggest that compound A also affects ERRα signaling in mouse uterus.

We have previously shown that compound A induces ERRα protein degradation in MCF-7 cells through the ubiquitin proteasome pathway,[3] and postulated that ERRα degradation leads to breast cancer cell growth inhibition. Thus, we established MCF-7/shERRα RNAi stable knockdowns[3] by transfecting MCF-7 cells with short hairpin RNA expression plasmids (SuperArray Bioscience Corporation) specific for ERRα. A short hairpin RNA expressing a scrambled artificial sequence that does not match any human, mouse, or rat gene was used as a negative control. Cell proliferation assays were done and the results show that knocking down ERRα led to the inhibition of breast cancer cell proliferation (Supplementary Fig. S3).[4] Additionally, knocking down ERRα led to the down-regulation of ACADM, CYP19A1, PGC-1α, SPP1, and TFF1.[3] Likewise, ACADM, CYP19A1, PGC-1α, SPP1, and TFF1 were down-regulated when MCF-7 cells were treated with compound A (Fig. 2).

Discussion

This is the first demonstration that an ERRα-selective antagonist could inhibit the proliferation of breast cancer

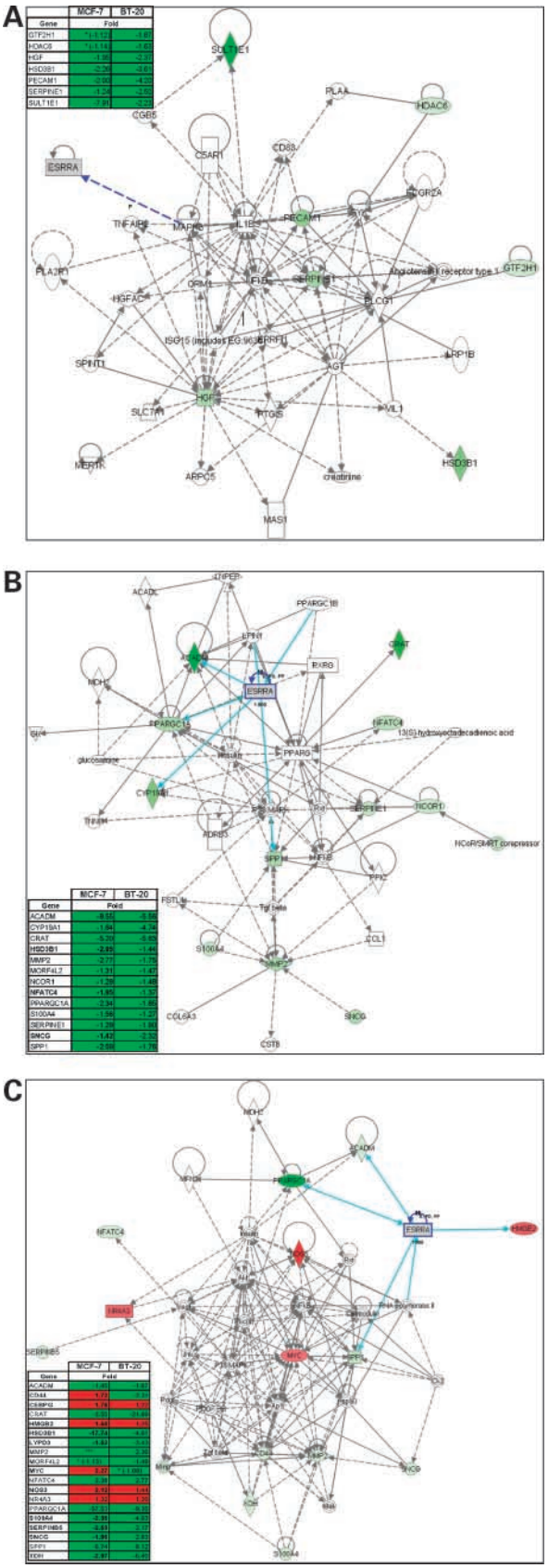

Figure 3. Signaling pathways affected by ERRα antagonist compound A in breast cancer cells. Relative gene fold expression data from MCF-7 and BT-20 cells treated with compound A for 2, 24, and 72 h were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity Systems). Differentially expressed (vehicle vs. compound A) genes from both MCF-7 and BT-20 were analyzed at 2 h (**A**), 24 h (**B**), and 72 h (**C**). Genes in boldface (*left*) also responded to E2 treatment. Solid line, direct interaction (*turquoise blue solid line,* direct interaction with ERRα); *dotted line,* indirect interaction. *Green nodes,* down-regulated; *red nodes,* up-regulated (for more information, see also http://www.ingenuity.com/).

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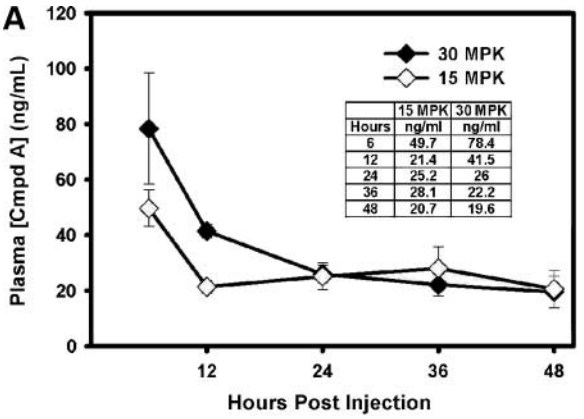
cells *in vitro* (Fig. 1) and slow tumor growth in mouse xenograft models (Fig. 4B and C). We have also investigated the molecular basis for this observation by examining changes in gene expression in breast cancer cell lines and tissue in response to the ERRα antagonist treatment (Figs. 2 and 3).

Is antagonizing ERRα leading to breast cancer cell and tumor inhibition mediated by the disruption of energy metabolism? We found that 11 genes that were down-regulated at 24 hours of treatment with compound A fit into a network involving energy metabolism (Fig. 3B).



Hours	15 MPK ng/ml	30 MPK ng/ml
6	49.7	78.4
12	21.4	41.5
24	25.2	26
36	28.1	22.2
48	20.7	19.6

Compound A down-regulates ACADM, which catalyzes the initial step in mitochondrial fatty acid oxidation. It has been well-established that ERRα is a direct regulator of ACADM (3, 24, 25). Likewise, the nuclear coactivator PGC-1α was down-regulated. PGC-1α is a key regulator of genes that control many important aspects of metabolism including glucose uptake, gluconeogenesis, mitochondrial biogenesis, and adaptive thermogenesis (26). PGC-1α interacts with ERRα and potentiates transcriptional activity (4, 24, 27, 28). Moreover, a pathway describing coregulation of ERRα and PGC-1α along with up-regulating PPARα, which in turn regulates ACADM, has also been described (29). Compound A treatment led to the down-regulation LPIN1, which is a PGC-1α target gene, and is known to be involved in pathways that lead to the expression of genes involved in mitochondrial fatty acid oxidative metabolism.

Interestingly, LPIN1 has also been shown to interact with ERRα (30). Also, other genes identified in this network involved in energy metabolism include malate dehydrogenase 2, which is found in the mitochondria where it plays a critical role in the malate-aspartate shuttle that catalyzes the oxidation of malate to oxaloacetate in the citric acid cycle (31). ACADL (long chain acyl-CoA dehydrogenase) takes part in catalyzing (along with ACADM) the initial step of mitochondrial fatty acid β-oxidation (32). Carnitine acetyltransferase catalyzes the transfer of acyl groups from acyl-CoA thioester to carnitine. It is a key enzyme for energy homeostasis and fat metabolism through the regulation of the ratio of acyl CoA/CoA in subcellular compartments (21). Therefore, given the key genes involved in energy metabolism that are disrupted upon treatment with compound A for 24 hours, it seems as though antagonizing ERRα in breast tumors can lead to tumor growth inhibition at least partly through affecting energy metabolism.

Figure 4. ERRα antagonist compound A inhibits breast tumor growth in mouse xenograft models. **A,** pharmacokinetic study of compound A in athymic nude mice. Ovariectomized, athymic nude mice implanted with an E2 pellet received 15 mg/kg of compound A, 30 mg/kg of compound A, or vehicle control for 5 consecutive days. Compound A plasma concentration was measured at 6, 12, 24, 36, and 48 h after day 5 article administration. **B,** compound A inhibits the growth of MCF-7 xenograft breast tumor. MCF-7 (1×10^7) cells/site were injected (s.c.) into the axillary mammary fat pads of ovariectomized, athymic nude mice that were also implanted with an E2 pellet. After 5 wk, mice were divided into three treatment groups: control vehicle, 15 mg/kg of compound A, and 5 mg/wk of ICI-182,780. Tumor cross-sectional area was determined weekly by Vernier calipers and calculated using the formula: length / 2 × width / 2 × π. In comparison to the E2 control group, significant inhibition of growth was seen in both the ICI-182,780 and compound A groups. At week 9, the compound A group tumor area was 0.3 cm^2 (*, $P = 0.004$) whereas ICI-182,780 was 0.2 cm^2 (*, $P < 0.001$). **C,** compound A inhibits the growth of BT-20 xenograft breast tumor. BT-20 (5×10^6) cells/site were injected (s.c.) into the axillary mammary fat pads of athymic nude mice. After 8 wk, mice were divided into two treatment groups: control vehicle and 30 mg/kg Cmpd A. At week 12, the vehicle group tumor size had reached 0.5 cm^2 whereas compound A treatment group was significantly reduced by 0.3 cm^2 ($P = 0.007$). Differences in mean tumor area between groups were measured by ANOVA followed by a Student's *t* test with a 0.05 significance level.

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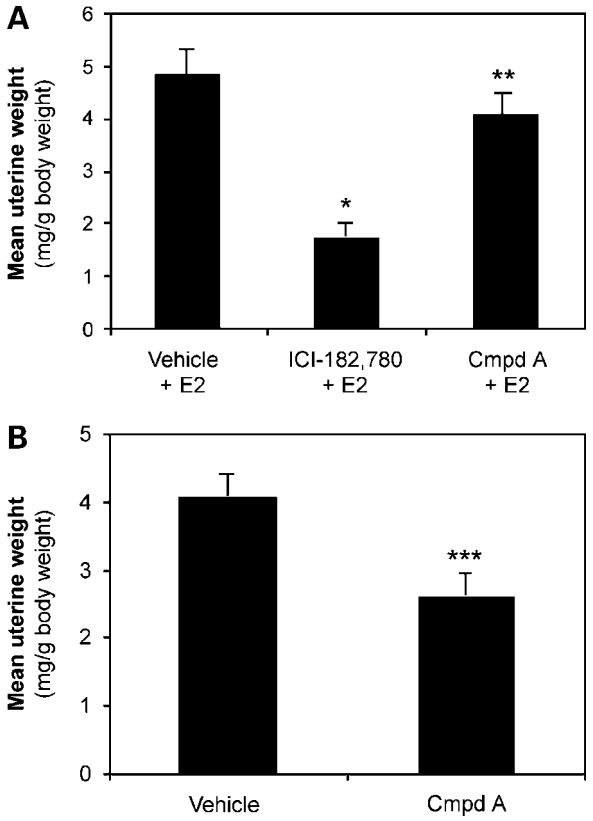

Figure 5. ERRα antagonist compound A reduces uterine weights of mice from breast cancer xenograft models. Whole body weights of mice were weighed; subsequently, uteri were excised, cleaned, and weighed from the same mice (from Fig. 4B and C) treated with (**A**) MCF-7 xenograft: vehicle, 5 mg/wk of ICI-182,780, or 15 mg/kg of compound A, or (**B**) BT-20 xenograft: vehicle or 30 mg/kg. *, $P < 0.001$; **, $P = 0.303$; and ***, $P = 0.013$. Differences in uteri/body weight between groups were measured by ANOVA followed by a Student's *t* test with a 0.05 significance level.

Along with the genes discussed above, compound A also down-regulated CYP19A1 and SPP1 in both MCF-7 and BT-20 cells treated for 24 hours (Figs. 2 and 3B). CYP19A1 (aromatase) is the estrogen synthetase that converts androgens to estrogens including E2, the potent natural ligand of ER. CYP19A1 is an ERRα target gene; it regulates the expression of aromatase (9). DES (at low concentrations that activate ERα) and E2 treatment results in an increase of ERRα in the mouse uterus (33). Furthermore, ERRα increases aromatase expression in breast tissue (9), ERRα is a direct target of ERα activity, and ERα induces ERRα expression in the breast (33, 34). Moreover, ERRα stimulates the estrogen-responsive gene, *SPP1* (osteopontin; ref. 10). At 72 hours of treatment by compound A, 14 genes fit into a network involving ERRα signaling (Fig. 3C). In particular, the up-regulated HMGB2 is reported to have a broad architectural function in the assembly of nucleoprotein complexes involved in the regulation of transcription (22).

Additionally, it has been reported that ERRα increases the expression of HMGB2 in rat neonatal myocytes (29). Other interesting genes to note, the transcription factor MYC, has been shown to bind the osteopontin promoter and promote up-regulation in astrocytic cells (35). Additionally, S100A4 (S100 calcium binding protein A4), shows elevated expression in cancer and contributes to tumor cell motility and metastatic progression (36). Moreover, S100A4 increases osteopontin (SPP1) expression (37) and SNCG. Therefore, antagonizing ERRα activity with compound A could result in the inhibition of ERα signaling and other estrogenic responses, which might also account for its inhibition of breast cancer cell lines and tumors.

It is of interest to point out that the networks built by Ingenuity Pathway Analysis (Fig. 3) did not include ER, which implies that these pathways are estrogen-independent. In the future, it will be interesting to compare gene expression data from multiple ER-negative breast cancer cell lines (MDA-MB-231, BT-20, and SKBR3) treated with an ERRα subtype–specific antagonist in order to identify key genes involved in ERRα-dependent/ER-independent signaling in breast cancer.

Due to the multiple interconnections between ERα and ERRα, we also looked at the effects of E2 treatment on genes known to be differentially expressed in breast cancer cells upon treatment with an ERRα subtype–specific antagonist yet were also estrogen responsive (Fig. 2). Although seven genes were differentially expressed in both MCF-7 and BT-20 breast cancer cells when treated for 2 hours with compound A, no effect was seen (on the same genes) after 2 hours of treatment with E2 (Figs. 2 and 3A). At 24 hours, although 11 genes were differentially expressed when treated with compound A and were built into one pathway; only 2 (*NFATC4* and *SNCG*) out of the 11 genes responded to E2 (Figs. 2 and 3B). NFATC4 (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4) is a transcriptional coactivator that regulates PPARγ and is involved in fat cell differentiation (38). Although PPARγ does not directly interact with ERRα, it interacts with LPIN1, ACADM, and PGC-1α which all directly interact with ERRα. SNCG (synuclein-γ; breast cancer–specific protein 1) is not a well-understood protein that regulates matrix metalloproteinases, and is reported to be overexpressed in advanced infiltrating breast cancer. Furthermore, this overexpression increases invasion and metastasis in breast cancer. Additionally, although there have been no reports that SNCG interacts directly with ERRα, it has been reported that SNCG increases the activity of matrix metallopeptidase 2 (39). Moreover, it has been shown that osteopontin (SPP1) stimulates matrix metallopeptidase 2 activation (40). At 72 hours, 14 genes differentially expressed in breast cancer cells treated with compound A were modeled into a pathway involving ERRα signaling; 8 of the 14 genes modeled also responded to E2 (Figs. 2 and 3C). Of the eight genes that responded to compound A and E2 treatment, only one (*HMGB2*, described above) directly interacted with ERRα. Therefore, whereas an increase in genes that were both differentially expressed by compound A and E2 was observed over time, only one gene (*HMGB2*) directly interacted with ERRα when modeled by Ingenuity Pathway Analysis.

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Given that ERRα can stimulate ERα target genes in the absence of estrogen, is it possible that ERRα stimulates and/or controls ER-independent cancer growth? Our BT-20 and MDA-MB-231 cell proliferation data (Fig. 1), along with our BT-20 xenograft results (Fig. 4), show that there are ERRα signaling pathways acting independent of ERα. By studying differential gene expression between the vehicle and compound A, we were able to focus on studying ERRα signaling that is independent of estrogen.

Additionally, growth inhibition by compound A in known ERRα-positive cells: CaCo-2 (colon), C2C12 (muscle), HEP G2 (liver), MG-63 (bone), RD (muscle), RL95-2 (uterus), and SAOS-2 (bone) were examined. Decreased proliferation of RL95-2 cells with compound A was exhibited (IC_{50} = 250 μmol/L), whereas no or little change on the other cell lines proliferation rates were affected by compound A (data not shown). Therefore, except at high concentrations in the endometrial RL95-2 cells, compound A exhibits a minimal affect on nonreproductive cells.

We have shown that compound A does not bind ERα in an ERα/coactivator homogenous time-resolved fluorescence assay (12). It is also known that ERα mRNA and protein are not significantly affected when MCF-7 cells are treated with compound A.[3] Yet interestingly, compound A acts as an antagonist in the uterus (Fig. 5). High levels of ERRα are known to be present in the uterus and it has been well-established that compounds exhibiting agonistic or antagonistic ERα activity can be monitored in the uterus (23). We have further analyzed the expression of 46 genes, which showed a robust effect in response to compound A treatment *in vitro*, in uteri from mice that were treated with ERRα antagonist. Eighteen genes were down-regulated in the mouse uteri, and 10 of them were also down-regulated in the MCF-7 and BT-20 cell lines treated with compound A (Supplementary Fig. S2).[4] These data suggest that compound A may affect ERRα signaling in the uterus. Furthermore, we have established that knocking-down ERRα in breast cancer cells led to inhibition of cell proliferation (Supplementary Fig. S3).[4] Future studies to further investigate these genes and the role of ERRα signaling in the uterus are of interest. Our data also imply an ERRα-dependent proliferative pathway that may be independent of ERα.

The ERRα knockout mouse is reported to have a reduced body weight, altered peripheral fat deposit and morphology, and show reduced lipogenesis (5). The knockout also exhibits significant fat malabsorption and steatorrhea (41). Interestingly, compound A did not significantly affect whole mouse body weights. In the MCF-7 xenograft model, the vehicle group weighed 22.5 ± 1.5 g, the ICI-182,780 group weighed 24.3 ± 0.61 g, and the compound A group weighed 24.5 ± 0.43 g. Whereas in the BT-20 xenograft model, the vehicle group weighed 25.9 ± 0.57 g and the compound A group weighed 25.2 ± 0.44 g. Additionally, there was no observable toxicity seen in mice treated with compound A.

The success of targeting ERα to treat breast cancer with such drugs as tamoxifen and ICI-182,780 (faslodex) has been well documented (42, 43). Future studies should include treating ERα-positive cells and mouse xenografts with combinations of tamoxifen, faslodex, or an aromatase inhibitor with compound A. Treating tamoxifen-resistant cells with compound A would also be of great interest. Although there are many successful therapies to breast cancer, tragically more than 40,000 women will still die this year. Consequently, there continues to be an unmet medical need for breast cancer treatments. Importantly, our *in vitro* and *in vivo* studies suggest that ERRα could be an effective target to treat breast cancer, independent of ER status.

Disclosure of Potential Conflicts of Interest

M.J. Chisamore, H.A. Wilkinson, and O. Flores are employees of Merck Research Laboratories. No other potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Estrogen-related receptor-α antagonist inhibits both estrogen receptor–positive and estrogen receptor–negative breast tumor growth in mouse xenografts

Michael J. Chisamore, Hilary A. Wilkinson, Osvaldo Flores, et al.

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