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# Structurally Novel Antiestrogens Elicit Differential Responses from Constitutively Active Mutant Estrogen Receptors in Breast Cancer Cells and Tumors

Yuechao Zhao[1], Mary J. Laws[1], Valeria Sanabria Guillen[1], Yvonne Ziegler[1], Jian Min[2], Abhishek Sharma[2], Sung Hoon Kim[2], David Chu[3], Ben Ho Park[3], Steffi Oesterreich[4], Chengjian Mao[5], David J. Shapiro[5], Kendall W. Nettles[6], John A. Katzenellenbogen[2], and Benita S. Katzenellenbogen[1]

## Abstract

Many estrogen receptor α (ERα)–positive breast cancers develop resistance to endocrine therapy via mutation of ERs whose constitutive activation is associated with shorter patient survival. Because there is now a clinical need for new antiestrogens (AE) against these mutant ERs, we describe here our development and characterization of three chemically novel AEs that effectively suppress proliferation of breast cancer cells and tumors. Our AEs are effective against wild-type and Y537S and D538G ERs, the two most commonly occurring constitutively active ERs. The three new AEs suppressed proliferation and estrogen target gene expression in WT and mutant ER-containing cells and were more effective in D538G than in Y537S cells and tumors. Compared with WT ER, mutants exhibited approximately 10- to 20-fold lower binding affinity for AE and a reduced ability to be blocked in coactivator interaction, likely contributing to their relative resistance to inhibition by AE. Comparisons between mutant ER–containing MCF7 and T47D cells revealed that AE responses were compound, cell-type, and ERα-mutant dependent. These new ligands have favorable pharmacokinetic properties and effectively suppressed growth of WT and mutant ER–expressing tumor xenografts in NOD/SCID-γ mice after oral or subcutaneous administration; D538G tumors were more potently inhibited by AE than Y537S tumors. These studies highlight the differential responsiveness of the mutant ERs to different AEs and make clear the value of having a toolkit of AEs for treatment of endocrine therapy–resistant tumors driven by different constitutively active ERs. *Cancer Res; 77(20); 5602–13. ©2017 AACR.*



## Introduction

Estrogen receptor α (ER)–containing breast cancers account for about three fourths of all breast cancers, and many of these primary breast cancers are effectively treated with antiestrogens (AE) or aromatase inhibitors (1–4). Although these endocrine therapies have been mainstays in breast cancer treatment, breast cancers often become resistant to these initial treatments and recur. Therefore, second-line endocrine treatments have been developed. These include most prominently, the selective ER modulator (SERM) and downregulator (SERD), fulvestrant (ICI 182,780), which is not orally active and must be administered as a large volume intramuscular injection (5). Although two new orally active SERDs have been reported recently [GDC-0810 (6) and AZD9496 (7)], they have some troubling side effects, including causing diarrhea in one third of patients (8), which could limit their clinical implementation and suggests the need for developing other new AEs.

Of the ER-positive breast cancers that recur as metastatic cancers, often many years after the initial cancer diagnosis and treatment, approximately 40% have been found to possess mutations in the ER protein that confer constitutive activity and hormone-independent growth, and are associated with shorter patient overall survival (3, 9–17). These ER mutations are all in the ligand-binding domain of the receptor, and most frequently, they result in changes at amino acid Y537 or D538 to generate Y537S (or N) or D538G constitutively active receptors (18, 19). Because there is now a clinical need for new AEs that will effectively work against wild-type (WT) and constitutively active mutant ERs, we describe herein studies in which we have developed and now characterize three chemically

[1]Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, Illinois. [2]Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois. [3]Department of Oncology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University School of Medicine, Baltimore, Maryland. [4]Department of Pharmacology and Chemical Biology, Womens Cancer Research Center, Magee Womens Research Institute, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania. [5]Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois. [6]Department of Cancer Biology, Scripps Research Institute, Jupiter, Florida.

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Current address for A. Sharma: Department of Biomedical Engineering, Chemistry and Biological Sciences, Stevens Institute of Technology, Hoboken, NJ.

**Corresponding Author:** Benita S. Katzenellenbogen, Department of Molecular and Integrative Physiology, University of Illinois, 524 Burrill Hall, 407 S. Goodwin Ave., Urbana, IL 61801. Phone: 217-333-9769; Fax: 217-333-1133; E-mail: katzenel@illinois.edu

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novel AEs that can suppress the proliferation of breast cancer cells and tumors driven by WT ER and these mutant constitutively active ERs. These AE ligands might ultimately prove to be useful in treatment of breast cancer patients with endocrine therapy–resistant tumors driven by constitutively active ERs.

## Materials and Methods

### Cell cultures, reagents, and ligands

17β-Estradiol (E2), 4-hydroxytamoxifen (4-OHT), and fulvestrant (ICI 182780) were from Sigma-Aldrich. AZD9496 was from MedChemExpress. The preparations of the AEs K-07, K-09, and K-62 have been reported (20). MCF7 and T47D cells from the ATCC were maintained and cultured as described previously (21, 22). Cell lines were authenticated at the Genetics Core of the University of Arizona (Tucson, AZ). T47D cells with both mutant ERα alleles were generated by CRISPR-Cas9 technology, and mutation status of the clonal cell lines was verified by genotyping as described before (21). MCF7 cells or T47D cells containing 50% mutant Y537S- or D538G-ERα and 50% WT ERα, determined by DNA sequencing and digital drop PCR analyses as detailed previously (17, 23), were generated by adenovirus-associated viral infection (MCF7) or CRISPR-Cas-9 methodology (T47D; refs. 17, 21, 23, 24). These cells were cultured as described previously (17, 21, 23, 24). Serum used in all cell cultures was premium grade FBS (VWR Life Science Seradigm, cat# 97068-085). Cells were used in experiments within the first five passages after thawing. Cells were sometimes transferred to phenol red-free tissue culture media supplemented with 5% charcoal-stripped FBS for 5 days so as to be in an estrogen-depleted condition before use in some experiments as noted. All cells were tested for mycoplasma using Real-Time PCR Mycoplasma Detection Kit (Akron Biotech).

### Cell proliferation assay

WST-1 assay (Roche) was used to quantify cell viability as described previously (25). Absorbance was measured at 450 nm using a VICTOR X5 PerkinElmer 2030 Multilabel Plate Reader. All assays were performed in triplicate.

### In-cell Western and Western blot assays

Cells were cultured in 96-well plates at 3,000 cells per well and treated with compound for 24 hours. Cells were washed twice in PBS, fixed with 4% formaldehyde (Thermo Fisher Scientific) solution in PBS, permeabilized in 0.1% Triton X-100 in PBS, blocked with Odyssey Blocking Buffer (LI-COR), and incubated with rabbit HC-20 ERα antibody (Santa Cruz Biotechnology, cat# SC-543) or mouse F10 ERα antibody (Santa Cruz Biotechnology, cat# SC8002) at 4°C overnight. Both IRDye 800 CW goat anti-rabbit secondary antibody (LI-COR, cat# 926-32211) and Cell Tag 700 (LI-COR, cat# 926-41090) were diluted (1:600) for incubation with cells. Plates were washed and ERα staining signals were quantified and normalized with Cell Tag signals (to control for any differences in cell number per well) using a LI-COR Odyssey infrared imaging system. Fold changes of ERα protein levels were calculated relative to the vehicle-treated WT samples. Results are the average ±SD from at least three independent experiments, each performed with 4 wells per treatment condition. Western immunoblots for GREB1 protein used rabbit polyclonal antibody (Sigma, HPA024616 at 1:1,000 dilution) and for ERα used mouse monoclonal F10 antibody (Santa Cruz Biotechnology cat# SC-8002 at 1:1,000 dilution). β-Actin was detected with mouse mAb (Sigma-Aldrich at 1:10,000 dilution).

### IHC

IHC was performed on paraffin-embedded tissue sections as before (26). A mouse monoclonal anti-human ERα antibody (Novacastra, cat# NCL-L-ER-6F11) was used at 1:300 dilution.

### RNA isolation and real-time PCR

Total RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using MMTV reverse transcriptase (New England BioLabs). Real-time PCR was performed using SYBR Green PCR Master Mix (Roche) as described previously (27). Relative mRNA levels of genes were normalized to the housekeeping gene 36B4, and fold change calculated relative to the vehicle-treated samples. Results are the average ±SD from at least two independent experiments carried out in triplicate.

### Receptor ligand binding and coregulator interaction assays

Binding of E2 to WT and mutant ERs was determined by direct Scatchard binding assay using tritiated E2 (28). Binding of other ligands was determined by a competitive radiometric assay with tritiated E2 and increasing concentrations of ligand (28).

A time-resolved fluorescence resonance energy transfer (FRET) assay was used to determine the ability of AE compounds to reduce the interaction of WT or mutant ERs with the coactivator SCR3/AIB1, which is often amplified and expressed at high levels in ER-positive breast cancers. The assay used site-specific labeled biotin-streptavidin/terbium WT, Y537S, or D538G ER ligand-binding domain, fluorescein-labeled SRC3 nuclear receptor interaction domain, and E2 and increasing concentrations of K-07, K-09, K-62, or trans-hydroxytamoxifen, and FRET was monitored as described previously (11, 29).

### Pharmacokinetic studies

All experiments involving animals were conducted in accordance with NIH (Bethesda, MD) standards for the care and use of animals, with protocols approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC). The pharmacokinetics of compounds K-09, K-07, and K-62 were monitored after single dose administration into female CD1 mice (7–9 weeks of age) via subcutaneous injection or oral gavage. For subcutaneous injection, each compound was dissolved in DMSO and then mixed with corn oil for a total injection volume of 100 μL (10% DSMO + 90% corn oil) per mouse. For oral gavage, compounds were administered in a 200 μL formulation of 9/0.5/0.5/90 parts of PEG400/Tween80/Povidone/0.5% carboxymethylcellulose. Multiple plasma samples were collected from each mouse ($n = 4$ for each experiment) over the course of 48 hours after compound administration. Compounds were quantified by LC/MS-MS at the University of Illinois Metabolomics Core Facility. The data were fitted to a noncompartment model.

### *In vivo* breast cancer xenograft studies

For examination of the growth of tumors containing WT ER, female NOD/SCID-γ (NSG) mice were ovariectomized at 7 to 8 weeks of age, and 2 weeks after ovariectomy,

animals were supplemented with 0.36 mg E2-pellets (60-day release, Innovative Research of America) to support ER-positive tumor growth. Cell suspensions of WT MCF7 cells ($2 \times 10^6$ cells/mouse) were injected orthotopically into the right axial mammary gland. When tumors reached 100 to 150 $mm^3$ in size, mice were randomized and received compound or control vehicle (corn oil) by daily subcutaneous injection or oral gavage (PEG400/PVP/Tween/CMC as vehicle) per day, for approximately 26 days, until vehicle-treated tumors grew to approximately 1,000 $mm^3$. Tumor volume (length $\times$ $width^2/2$) and animal body weights were monitored over time.

For examination of the growth of tumors containing mutant ERs, female NSG mice were ovariectomized but received no E2 pellets, to mimic the low estrogen environment of postmenopausal women. At 3 weeks after ovariectomy, cells were injected orthotopically into the axial mammary gland. Animals received daily subcutaneous injection or oral gavage of vehicle or compound, and tumor growth and animal body weights were monitored over time.

### Ethical approval

All animal experiments were performed in accordance with institutional protocols approval by the University of Illinois IACUC and the NIH Guide for the Care and Use of Laboratory Animals guidelines. This article does not contain any studies with human participants performed by any of the authors.

## Results

### Structurally novel AEs suppress growth of breast cancer cells with constitutively active mutant ERα

We first examined the effects of three new AEs developed in our laboratory (Fig. 1A) in T47D cells containing all WT ERs or all mutant ERs (Y537S or D538G). The mutant ERs were introduced by homology-directed repair using CRISPR-Cas9 technology (21). The mutants examined, Y537S and D538G, are the two most commonly occurring mutant ERαs in humans with therapy-resistant breast cancers. The three AEs have an adamantyl core and were selected through structure–activity relationship studies on breast cancer cells with WT ER (20). Hence, the current studies are the first to examine their effects on cells and tumors with growth driven by constitutively active ERs.

As seen in Fig. 1B, cells containing the Y537S and D538G ER mutants showed high constitutive activity in the absence of estrogen, having 11 times and 4 times higher proliferative activity, respectively, than that of cells containing WT ER. Treatments with the known AEs fulvestrant or AZD9496 resulted in marked suppression of this constitutive activity in both mutants. All three of our new compounds fully suppressed proliferative activity of the D538G cells, whereas in Y537S cells, good suppression was effected by K-62, but to only a more limited extent by the K-07 and K-09 compounds. In the presence of E2 (Fig. 1C), proliferation of WT and D538G cells was increased, whereas cells with Y537S ER

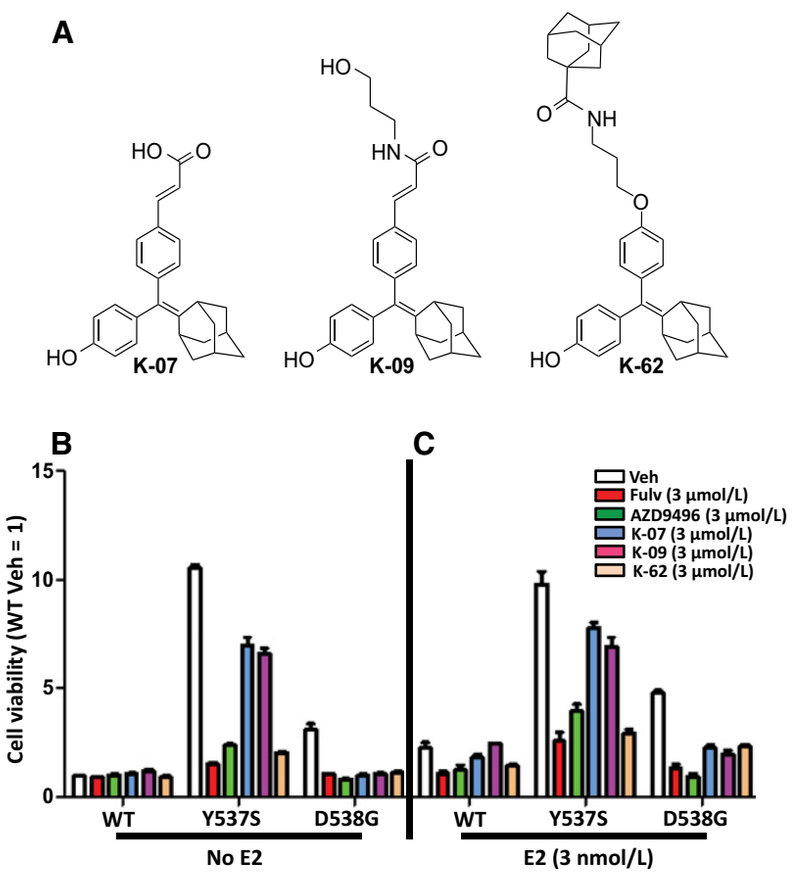


**Figure 1.**
Proliferation of T47D cells harboring homozygous mutant ERα (introduced by CRISPR-Cas9) is suppressed by AE. **A,** Structures of the three AEs studied. T47D cells with WT-ERα, Y537S-ERα, or D538G-ERα were cultured in E2-deprived conditions (**B**) or with E2 ($3 \times 10^{-9}$ mol/L; **C**) and compounds at $3 \times 10^{-6}$ mol/L for 6 days to evaluate the impact of compounds on cell viability. Values are the mean ± SD of three determinations from three separate experiments.

proliferated rapidly in the absence of added E2 and showed no further increase with E2. Again, all five compounds suppressed proliferation of the mutant ER–containing cell lines, with D538G cell proliferation being more effectively suppressed by the AEs than Y537S cell proliferation. In cells with WT and Y537S ER, K-62 was as inhibitory as fulvestrant and AZD, with K-07 and K-09 being less effective antiproliferative agents.

Because the studies in Fig. 1 were conducted at only one high concentration of each compound (3 μmol/L), we carried out proliferation dose–response studies with each of these compounds in the three cell types (i.e., containing WT or the two mutant ERs). As seen in Fig. 2A–E, the mutants again showed strong constitutive proliferative ER activity, whereas E2 increased WT ER cell proliferation to that of the constitutively active mutant ER cells. With all five compounds, dose-dependent inhibition of cell proliferation revealed that mutant Y537S-ER and D538G-ER cells showed more resistance to suppression by all of the AEs, with Y537S cells requiring the highest concentrations of AEs to bring about growth inhibition. Approximately 10 to 100 times higher concentrations of

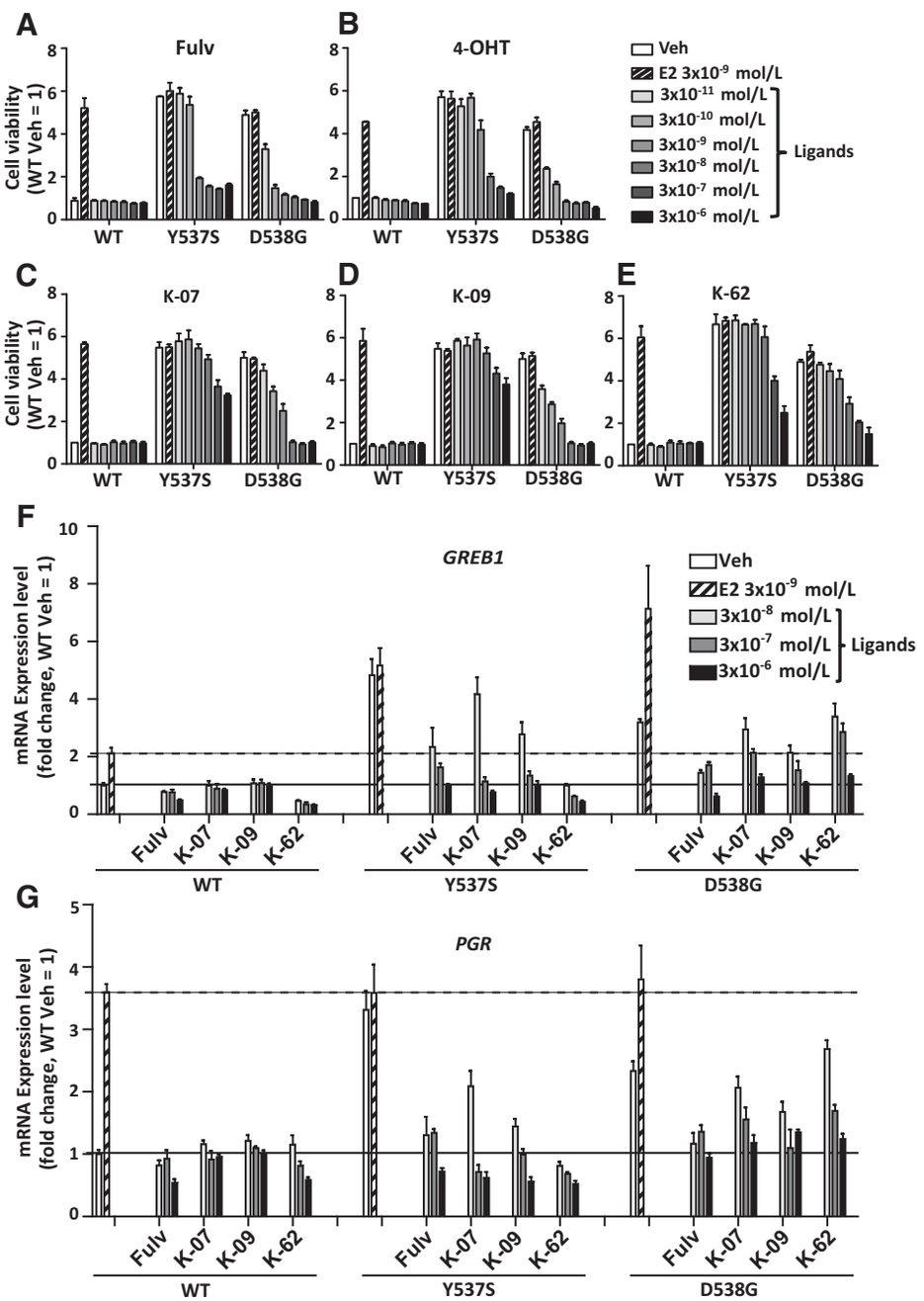


**Figure 2.**

Dose-dependent inhibition of T47D cell proliferation and gene expression by AEs. D538G-ERα is more effectively suppressed by AEs than Y537S-ERα. **A–E,** T47D cells with WT-ERα, Y537S-ERα, or D538G-ERα were cultured under estrogen-deprived conditions (in phenol red-free medium with 5% charcoal dextran-treated serum). They were treated with ligands at the concentrations indicated ($3 \times 10^{-11}$–$3 \times 10^{-6}$ mol/L), and cell proliferation was monitored after 6 days. Values are mean ± SD of three determinations from three separate experiments. **F** and **G,** Effects of E2 and ligands on expression of the ER target genes *GREB1* and *PGR* in T47D cells were monitored after treatments with vehicle, E2, and ligands for 24 hours, followed by RNA extraction and qPCR analysis. Black horizontal line, mRNA expression in vehicle-treated WT T47D cells (set as 1); dashed horizontal line, mRNA expression level in E2-treated cells. Values are the mean ± SD of three determinations from two separate experiments.

**Table 1A.** Ligand binding to WT and mutant ERs[a]

| | $K_i$ (nmol/L; fold increase over WT) | | |
|---|---|---|---|
| **Ligand** | **WT-ER** | **Y537S-ER** | **D538G-ER** |
| E2 (Kd) (nmol/L) | 0.22 ± 0.11 | 1.40 ± 0.54 (6.4×) | 1.77 ± 0.66 (8.0×) |
| Trans-hydroxytamoxifen | 0.12 ± 0.01 | 2.64 ± 0.4 (22.0×) | 3.28 ± 0.7 (27.3×) |
| Fulvestrant | 0.13 ± 0.03 | 3.68 ± 0.77 (28.3×) | 5.06 ± 1.16 (38.9×) |
| AZD 9496 | 0.19 ± 0.05 | 5.20 ± 1.16 (26.9×) | 7.52 ± 1.9 (39.0×) |
| K-07 | 0.30 ± 0.01 | 3.94 ± 1.10 (13.1×) | 4.60 ± 1.28 (15.3×) |
| K-09 | 0.45 ± 0.07 | 2.88 ± 0.37 (6.4×) | 7.00 ± 1.36 (15.6×) |
| K-62 | 0.47 ± 0.02 | 5.35 ± 1.37 (11.4×) | 10.31 ± 2.93 (21.9×) |

**Table 1B.** Reversal of coactivator SRC3/AIB1 binding to WT and mutant ERs

| | | **WT-ER** | **Y537S-ER** | **D538G-ER** |
|---|---|---|---|---|
| E2 Priming conc. (nmol/L)[b] | | 9 | 0.3 | 2.5 |
| E2 ($K_d$; nmol/L) | | 0.22 ± 0.11 | 1.40 ± 0.54 | 1.77 ± 0.66 |
| Cheng–Prusoff correction: 1 + [(E2)/$K_d$][c] | | 42 | 1.2 | 2.4 |
| **Ligand** | | **$K_i$ (nmol/L; fold increase over WT)** | | |
| | $IC_{50}$ nmol/L[d] | 11 | 3.4 | 6.0 |
| Trans-hydroxytamoxifen | $K_i$ nmol/L[e] | 0.26 | 2.8 | 1.3 |
| | (fold change from WT)[f] | (1×) | (11×) | (5×) |
| | $IC_{50}$ nmol/L[d] | 32 | 49 | 38 |
| K-07 | $K_i$ nmol/L[e] | 0.76 | 41 | 16 |
| | (fold change from WT)[f] | (1×) | (54×) | (21×) |
| | $IC_{50}$ nmol/L[d] | 50 | 29 | 26 |
| K-09 | $K_i$ nmol/L[e] | 1.2 | 24 | 11 |
| | (fold change from WT)[f] | (1×) | (20×) | (9.2×) |
| | $IC_{50}$ nmol/L[d] | 54 | 53 | 36 |
| K-62 | $K_i$ nmol/L[e] | 1.3 | 44 | 16 |
| | (fold change from WT)[f] | (1×) | (34×) | (12×) |

[a]Binding of E2 was determined by direct Scatchard binding assay using tritiated E2 (28). Binding of other ligands was determined by a competitive radiometric assay using tritiated $E_2$ and increasing concentrations of ligand (28).
[b]The concentration of E2 needed to recruit SRC3 binding to about 50% of maximum to each receptor. It differs from WT versus mutant ERs because of different levels of constitutive activity and E2-binding affinity of these ERs.
[c]This factor is required to convert $IC_{50}$ values to $K_i$ values.
[d]$IC_{50}$ values are read from the inhibition curves shown in Supplementary Fig. S2.
[e]$K_i$ values are determined by dividing the $IC_{50}$ values by the Cheng–Prusoff correction factor.
[f]Fold change from WT is determined for each AE by dividing the $K_i$ value for the mutant-ER by the $K_i$ value for WT-ER.

compounds were needed for equal suppression of growth in Y537S cells compared with D538G cells. Comparisons of cell proliferation conducted in WT and mutant ERα-expressing T47D cells in estrogen-replete conditions (i.e., full medium with 5% FBS) again showed high resistance of Y537S ER-containing cells to growth suppression by all AEs (Supplementary Fig. S1A–S1E).

### Reduced binding affinities and lower potencies in reversing coactivator binding by AE ligands for mutant ERs versus WT ERs

Table 1A summarizes the binding affinity of these ligands to WT, Y537S, and D538G ERs. Of note, AEs bound about 10 to 40 times less well to these mutant ERs versus WT ERs. E2 was also found to bind 7 times less well to the mutants. These differences in ligand binding to the mutant ERs could in part explain their relative resistance to inhibition of proliferation by the AE compounds, but not the greater resistance of the Y537S mutant compared with the D538G mutant. In assays monitoring the reversal of coactivator SRC3/AIB1 binding to WT and mutant ERs, we again found that 10 to 50 times higher concentrations of AE ligands were required with mutant ERs than with WT ERs, but in this assay, the interaction of SRC3 with the Y537S mutant was more difficult to reverse than with the D538G mutant (Table 1B; Supplementary Fig. S2), better reflecting the results from the proliferation inhibition assays.

### The constitutive gene expression activity of mutant ERs is suppressed by novel AE compounds

We observed constitutive expression of the ER target genes *GREB1* and *PGR* in cells with mutant ERs, and consistent with our findings of anti-proliferative activities of fulvestrant and our three new compounds, we found that fulvestrant, K-07, K-09, and K-62 effectively suppressed the expression of these genes in a dose-dependent manner (Fig. 2F and G). Stimulation was most effectively turned off in the WT cells, with both *GREB1* and *PGR* gene expression also being suppressed in both mutants by all compounds; however, higher concentrations of AEs were needed for suppression of gene expression in mutant ER- versus WT ER-containing cells. The lower potency of the AEs in suppressing gene expression in the mutant ERs is again consonant with their lower binding affinity and reduced potency in reversing coactivator binding to the mutants than WT ER (Table 1).

### Antiestrogenic compounds differentially downregulate WT and mutant ERα proteins in cells

We next compared the abilities of the compounds to elicit downregulation of WT ERα and mutant ERα proteins in the T47D cells. Levels of WT- and D538G-ERα were markedly reduced by fulvestrant and by our three AEs, whereas the Y537S-ERα protein was very resistant to downregulation by all ligands (Fig. 3A–E). Of note, 4-OHT did not downregulate

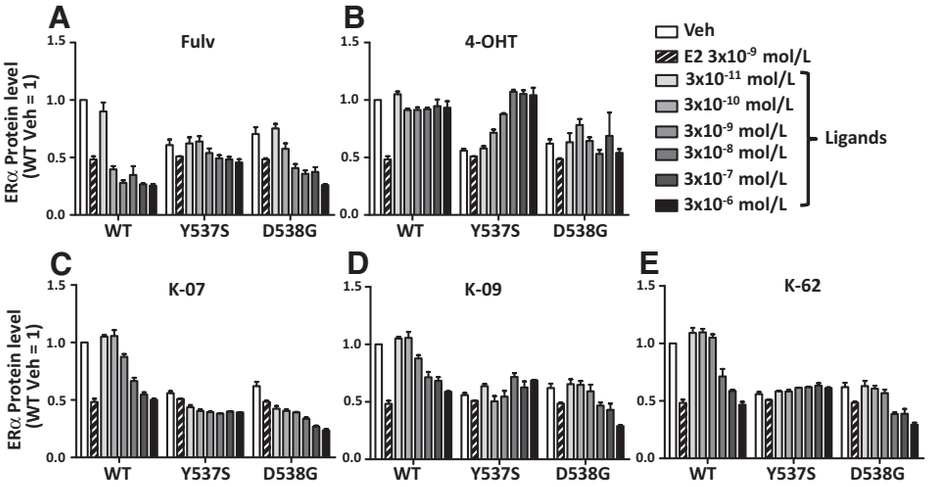


**Figure 3.**
Differences in the ability of compounds to induce the downregulation of WT or mutant ERα protein in cells. T47D cells were treated with vehicle control, E2, or compounds [fulvestrant (**A**), 4-OHT (**B**), K-07 (**C**), K-09 (**D**), K-62 (**E**)] alone for 24 hours at the concentrations indicated, and cells were subjected to in-cell Western blot analysis for evaluation of ERα protein levels.

mutant or WT-ERα (Fig. 3B), although it reduced proliferation of WT and mutant ER-expressing cells (Fig. 2B). In fact, 4-OHT reproducibly and dose dependently upregulated Y537S ERα levels, implying a possible change in the dynamics of turnover of this particular mutant ER protein.

### Comparison of ligand effects in T47D and MCF7 cells containing both WT and mutant ERs

We also carried out comparison studies using MCF7 cells and T47D cells containing 50% mutant ER and 50% WT ER (Supplementary Fig. S3). In these cells, we found that high concentrations of fulvestrant, 4-OHT, and K-07, K-09, and K-62 were again needed to obtain suppression of cell proliferation, similar to that observed in the T47D cells containing only mutant ERs (Supplementary Fig. S1). This suggests that the mutant ERα is dominant in determining cell phenotypic behavior when both receptors are present, as also seems to be the case in human metastatic breast tumors with these mutant ERs. Of interest, some differences were seen in ligand potency and efficacy when monitored in MCF7 or T47D cells. Hence, K-07 and K-09 worked more effectively as growth suppressors in MCF7 cells with mutant ERs, whereas fulvestrant was somewhat more potent in T47D cells with mutant ERs (Supplementary Fig. S3A and S3B). Likewise, our compounds and fulvestrant could reduce the ER protein level in these MCF7 and T47D cells, with fulvestrant generally eliciting a somewhat greater magnitude of downregulation (Supplementary Fig. S4A and S4B).

When the expression of estrogen target genes GREB1 and PGR was assessed in MCF7 and T47D cells with 50% mutant and 50% WT ER, gene expression was constitutively high (Supplementary Fig. S5) and increased only little with added E2, similar to the response in cells with only mutant ER. Gene expression was generally more fully suppressed by compounds in MCF7 cells versus T47D cells. Of note, the D538G ER was more fully inhibited than Y537S ER, regardless of the cell background. These findings suggest that beyond the status of ER itself (WT or mutant), other genomic and cellular alterations in the different cell backgrounds may well contribute to differences in responsiveness to treatment with different AEs.

### Pharmacokinetic properties of AEs K-07, K-09, and K-62

Prior to *in vivo* xenograft tumor studies, we monitored the pharmacokinetic properties of AEs K-07, K-09, and K-62 after subcutaneous and oral administration in mice (Fig. 4A–F). Overall, K-07 showed the most optimal pharmacokinetic properties by both subcutaneous and oral routes, having the highest blood levels and a very long terminal half-life in blood ($t_{1/2}$ = 14.0 hours after subcutaneous injection or $t_{1/2}$ = 97.2 hours after oral administration). Although K-09 and K-62 had long half-lives after subcutaneous injection, their blood levels were much lower compared with those of compound K-07 (note changes in *y*-axis). Because of its especially high blood levels and long half-life after oral administration, we also examined the oral efficacy of K-07 in subsequent tumor growth experiments.

### Structurally novel compounds inhibit tumor growth of WT and mutant ER–containing breast cancer cells *in vivo*

Because our new compounds suppressed cell proliferation and ER target gene expression, we investigated the ability of K-07, K-09, and K-62 to suppress growth of ER-positive breast cancer xenografts in NSG mice. Studies were first conducted in MCF7 xenografts containing WT ERα. We did not study T47D xenografts containing WT ERα because we found that WT T47D cells with E2 supplementation and mutant ER T47D cells without E2 pellets formed only very small tumors, whereas MCF7 cells formed much larger and faster-growing tumors. Mice receiving E2 pellet supplementation were injected with WT MCF7 cells orthotopically into the mammary fat pad, and when tumors reached 100 to 150 $mm^3$ in size, animals were randomized to groups and treated with compounds or fulvestrant administered daily subcutaneously at 80 mg/kg per day (Fig. 5). All compounds reduced tumor growth, with K-09 and K-62 being as effective as fulvestrant, and K-07 being the most effective in growth suppression (Fig. 5A). The favorable pharmacokinetics profile of K-07 likely underlies the observation that K-07 was most efficacious in inhibiting tumor growth. All compounds greatly reduced expression of the *GREB1* and *PGR* genes monitored in tumors harvested at day 26 (Fig. 5B).

### K-07 is a potent oral inhibitor of tumor progression *in vivo*

Orally administered K-07 very effectively reduced tumor growth within a few days of the start of treatment (Fig. 5C). ER target gene (*GREB1*, *PgR*) expression was also fully suppressed in tumors harvested from these animals (Fig. 5D). Analysis of GREB1 and ERα protein showed almost complete loss of GREB1

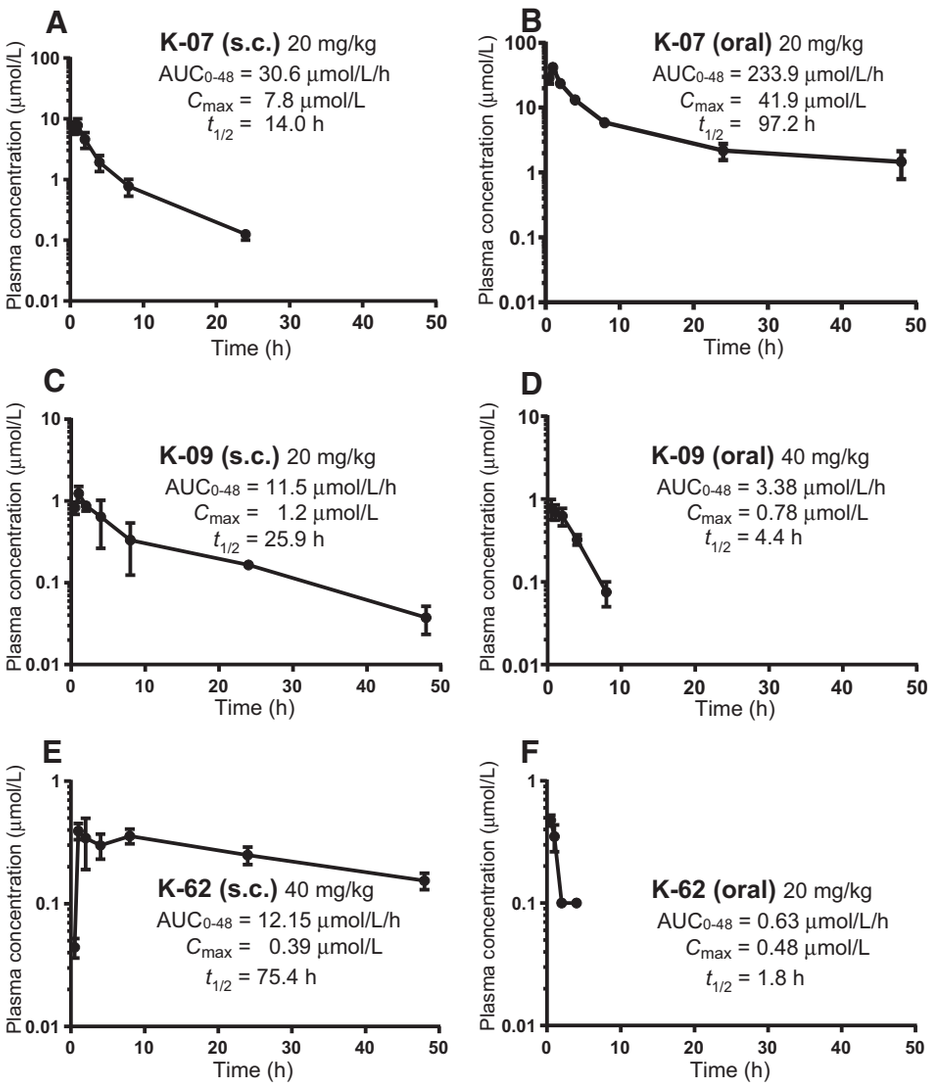


**Figure 4.**
Pharmacokinetics and half-lives of new AEs in mice after subcutaneous or oral administration. **A** and **B,** Pharmacokinetics of K-07 after single-dose administration via subcutaneous injection (20 mg/kg; **A**) or oral gavage (20 mg/kg; **B**). **C** and **D,** Pharmacokinetics of K-09 after single-dose subcutaneous injection at 20 mg/kg (**C**) or oral gavage at 40 mg/kg (**D**). **E** and **F,** Pharmacokinetics of K-62 after single-dose subcutaneous injection at 40 mg/kg (**E**) and oral gavage at 20 mg/kg (**F**). After compounds were administered, multiple plasma samples were collected from each mouse ($n = 4$ for each experiment) over the course of 48 hours. Compounds were quantified using LC/MS-MS. The data were fitted to a noncompartment pharmacokinetics model.

protein expression and a 51% reduction of ERα in the tumors of K-07–treated animals (Fig. 5E and F). Although K-07, K-09, and K-62 by subcutaneous and K-07 given by oral routes suppressed tumor growth substantially in these animals supplemented with E2 pellets, there was no effect on animal body weight compared with vehicle control animals over the time course of the treatments (Supplementary Fig. S6A and S6B).

**Y537S-ER and D538G-ER–containing cells form tumors in the absence of estrogen and are arrested by K-07 treatment**

To mirror the low estrogen environment in postmenopausal women, tumor xenograft studies with Y537S and D538G-containing MCF7 cells were conducted in ovariectomized NSG mice in the absence of any added E2. Y537S and D538G tumors grew well under these conditions (Fig. 6), and growth of these constitutively active tumors was acutely arrested by subcutaneous treatment with K-07, as effectively as by fulvestrant (Fig. 6A and B). Notably, oral K-07 was also very effective in arresting growth of the mutant ER tumors (Fig. 6C), and D538G tumors were more fully suppressed by K-07 treatment compared with Y537S tumors, consistent with the greater resistance of Y537S cells to antiproliferative effects of AEs that we found in cell cultures *in vitro*. Expression of estrogen-regulated genes, monitored in the small mutant ER–containing tumors at the end of the study, was almost fully suppressed by K-07 in both Y537S and D538G tumors (Fig. 6D and E). Analysis of ERα protein by Western immunoblot in the small tumors harvested at the end of the study revealed a 60% and 75% decrease in ERα in Y537S and D538G tumors, respectively (Fig. 6F). This substantial decrease in ERα in Y537S and D538G tumors receiving K-07 versus vehicle treatment was also seen by IHC of tumors as a marked decrease in nuclear ERα staining (Fig. 6G).

## Discussion

Our studies show that three AEs with novel chemical structures have efficacy in suppressing growth of breast cancer cells and tumors containing WT ERs and that, at higher concentrations, these compounds can also inhibit ER-regulated gene expression and proliferation of breast cancer cells

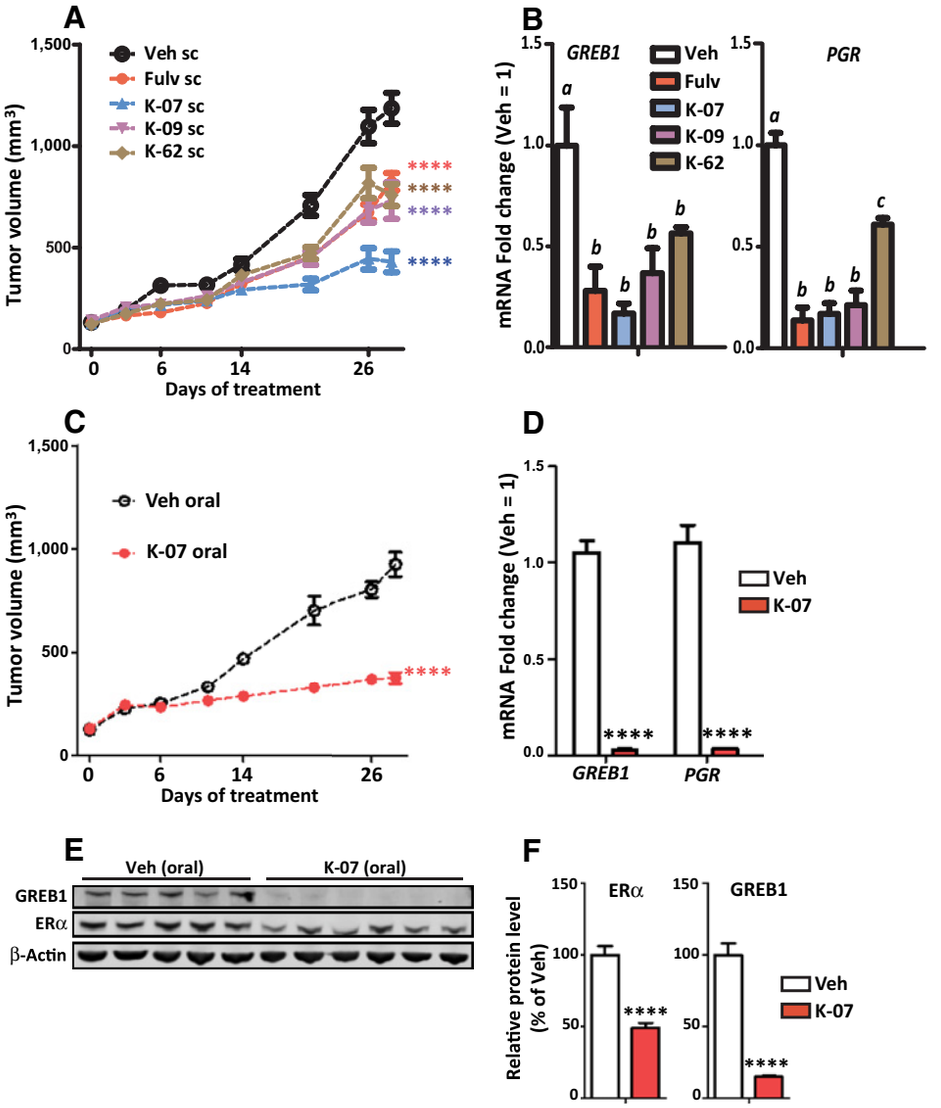


**Figure 5.**

New AEs show good growth suppression of MCF7 xenograft tumors and inhibition of estrogen target gene expression. NSG mice were supplemented with E2 (0.36 mg, 60-day release) pellets, injected with WT MCF7 cells to generate xenograft tumors, and dosed with 80 mg/kg of fulvestrant, K-07, K09, or K-62 by daily subcutaneous injection. **A,** Tumor volume was monitored over time (two-way ANOVA, Bonferroni posttest, ****, $P < 0.0001$; $n = 8$–9 per group). **B,** Harvested tumors were analyzed by qPCR for *GREB1* and *PGR* RNA levels (one-way ANOVA, Tukey posttest; $n = 8$–9/group). **C,** E2-supplemented mice bearing MCF7 tumors were dosed daily with vehicle or 80 mg/kg of K-07 by oral gavage. Tumor volumes of vehicle and K-07–treated tumors were monitored (two-way ANOVA, Bonferroni posttest, ****, $P < 0.0001$; $n = 8$ per group). **D,** After 28 days, tumors were harvested at 24 hours after the last control vehicle or K-07 oral treatment for gene expression analysis (*t* test, ****, $P < 0.0001$; $n = 8$ per group). **E,** Western blot examination of GREB1 and ERα protein in individual tumors. β-actin served as the loading control. **F,** Quantitation of ERα and GREB1 protein in vehicle and K-07–treated tumors (*t* test, ****, $P < 0.0005$; $n = 5$ vehicle and $n = 6$ K-07 tumors).

containing constitutively active mutant ERs. K-07 had the most optimal pharmacokinetic profile, and it was the most effective in suppression of WT and Y537S and D538G mutant tumor growth *in vivo*.

The studies provide new insights about mutant ERs that drive hormone-independent constitutive activity. Of note, we observed that the nature of the mutant ER (D538G or Y537S), the cell background (T47D or MCF7), and the chemical structure of the AEs (K-07 vs. K-09 vs. K-62 vs. fulvestrant) all affected response to AE ligands. Thus, these mutant ERs showed differential responsiveness to chemically distinct AEs, with cells containing the mutant Y537S-ER being more resistant to the AEs compared with the mutant D538G receptor and requiring higher compound concentrations for growth suppression. Other key regulators in breast cancer, such as the histone methyl transferase EZH2, also have various activating mutations that differ in their sensitivities to different EZH2 inhibitors (30). In our studies, the cell background was also very crucial. Thus, in T47D cells, Y537S elicited a more endocrine-resistant phenotype, whereas in the MCF7 cell background, the Y537S and D538G mutant ERs were more similar to each other in their response to AEs. This suggests that cell context, including alterations in genomic and cell signaling pathways, may work with ERs to confer different cell behaviors and responsiveness or resistance to treatment with different endocrine agents (23, 31, 32). This is not surprising, as it is known, for example, that among other differences, MCF7 and T47D cells carry different mutant forms of PI3K. Also, GATA3, an important factor for ER activity, is often mutated in breast cancers (16) and could influence responsiveness of WT and mutant ERs to AEs.

Our studies also document that the mutant ER allele fraction is crucial in the extent of endocrine treatment resistance observed. In fact, T47D cells with both alleles homozygous for the mutant ER showed greater resistance to AEs than did cells with 50% mutant and 50% WT ER. Notably however, even 50% mutant ER conferred a dominant AE-resistant phenotype. This is of importance because metastatic breast cancers usually contain a mixture of mutant and WT ERs (1, 3, 14–19, 32).

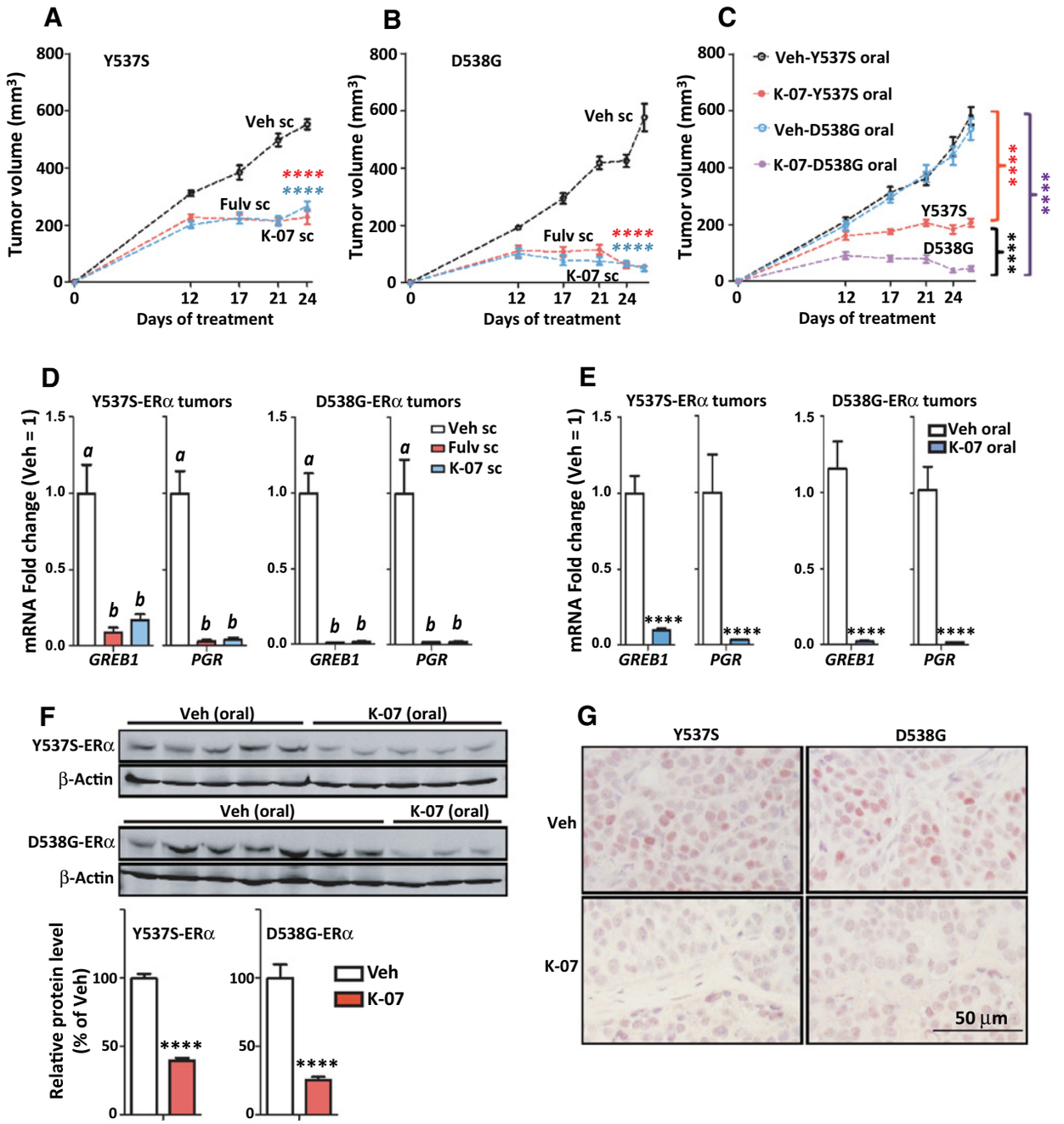


**Figure 6.**

Y537S ERα- and D538G ERα-containing tumors grow in the absence of estrogen and are arrested by K-07 treatment. NSG mice were ovariectomized and 3 weeks later received MCF7 cells containing half Y537S ER or D538G ER and half WT ER. **A** and **B,** Mice then received daily subcutaneous injection with vehicle or 80 mg/kg of K-07 or fulvestrant, and tumor volumes were monitored over time (two-way ANOVA, Bonferroni posttest, ****, $P < 0.0001$; $n = 8$ per group). **C,** Mice received daily treatment with oral vehicle or oral K-07 at 80 mg/kg, and tumor volumes were monitored over time (two-way ANOVA, Bonferroni post-test, $P < 0.0001$; $n = 8$ per group). **D** and **E,** Tumors harvested at day 26 were analyzed for expression of the estrogen target genes *GREB1* and *PGR*. **D,** One-way ANOVA, Tukey posttest; $n = 8$ per group. **E,** *t* test, ****, $P < 0.0001$; $n = 8$ per group. **F** and **G,** Harvested tumors were also analyzed for ERα protein by Western blot analysis of tumor lysates (some D538G tumors were too small for Western analyses so only three tumors are shown here; **F**) and by IHC for ERα in tumor tissue sections (**G**).

The pharmacokinetic properties of the AEs proved to be very important in their tumor-suppressive efficacies *in vivo*. K-07 displayed the best pharmacokinetic properties by either subcutaneous or oral routes. Despite the fact that all three AEs showed good antiproliferative and target gene-inhibitory activities in cells in culture, K-07, which displayed the best pharmacokinetic

properties, was the most effective growth inhibitor in breast tumor xenografts *in vivo*.

It is now recognized that approximately 40% of ER-positive breast cancers that become resistant to endocrine treatment and recur contain ER mutations (1, 3, 17–19). Most of these mutations are found in the C-terminal transactivation domain in the ligand-binding domain of the ER and result in changes at amino acids Y537, D538, L536, P535, or V534 and also at E380. Several large analyses have shown that the two most common mutations are at Y537 (changing Y to S, but also less commonly to N or C) and at D538 (always changed to G). These arise from single-nucleotide changes in the codons for these amino acids, resulting in the alteration of one amino acid. X-ray crystallography and molecular modeling have shown that Y537 and D538 are present at key locations in the activation function-2 region of the ligand-binding domain that determine the three-dimensional structure of helix 12 of the ER that is key in interactions with coregulators. These changes in ER structure (11, 33) result in ligand-independent interaction with coactivators that is normally seen in WT receptor only in the presence of estrogenic hormones (34–37). Notably, we report here that these changes in ER also reduce the receptor-binding affinity for AE ligands. This reduced binding affinity, shown in Table 1A, likely contributes to the relative resistance of these ER-mutant cells to AEs. However, as Y537S-ER was always more resistant to the suppressive effects of ligands compared with D538G-ER even though both mutant ERs showed similar reductions in their binding affinities for AEs, other factors are clearly involved, such as the more greatly reduced potency of the AEs in suppressing coregulator interaction to Y537S-ER than to D538G-ER (Table 1B). As we and others (38) have observed, tumors with mutant Y537S ERs are more AE resistant than those with D538G ERs.

When breast cancer patients no longer respond to treatment with tamoxifen or aromatase inhibitors, second-line treatment with fulvestrant is often used (39). However, fulvestrant is not orally available, so large volumes of fulvestrant in an oil-containing excipient are administered intramuscularly, which can be painful and is generally not liked by patients (4). Therefore, there is considerable interest in the development of new orally active AEs for treatment of these recurrent, usually metastatic, breast cancers. Recently, two orally available AEs have been reported, AZD9496 from AstraZeneca and GDC-0810 from Genentech/Seragon (6, 7). Although effective, each is not fully optimal, and some troubling side effects that may limit their clinical utility have been noted (8). RAD-0910 (40, 41) and boronic acid derivatives of fulvestrant and GW7604 (42, 43) show promise, but clinical evaluations are at very early stages. Thus, there is an unmet need for orally active AEs with an improved clinical profile and reduced side effects. Compound K-07 appears to be a potential alternative orally effective AE. Although in our studies, no impact on overall animal health was observed with this compound, or with K-09 or K-62, further investigations of ultimate safety and clinical effectiveness will be needed with K-07 or related AEs.

Because clinical studies have shown that patients with recurrent ER-positive breast cancer can respond to second- and often third-line endocrine treatments, having a toolkit of new AEs of different chemical classes should increase the beneficial options for such patients. It could bring therapeutic advances and result in inhibitors better matched for effectiveness in subsets of patients with breast tumors carrying differing ER mutations. We found differences in AE response of MCF7 and T47D cells containing mutant ERs, implying that different AEs may work better in different cell contexts. In this regard, it is noteworthy that in the BOLERO-2 trial, only patients with breast tumors carrying D538G ER mutations, but not Y537S mutations, showed increased progression-free survival when treated with the mTOR inhibitor everolimus plus the aromatase inhibitor exemestane, indicating that patients with breast cancers driven by different mutant ERs may benefit differentially from certain cancer treatments (9, 12). Patients with ER-positive tumors containing breast cancer cells with mutant ERs present in different tumor microenvironments may also differentially benefit, as it is now appreciated that breast tumors are quite heterogeneous in their cellular compositions (44–46), which can impact clinical outcomes of patients to endocrine treatments with AEs.

## Disclosure of Potential Conflicts of Interest

J.A. Katzenellenbogen is a consultant at Radius Health. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

**Conception and design:** Y. Zhao, M.J. Laws, A. Sharma, K.W. Nettles, J.A. Katzenellenbogen, B.S. Katzenellenbogen

**Development of methodology:** Y. Zhao, M.J. Laws, V.S. Guillen, A. Sharma, J.A. Katzenellenbogen, B.S. Katzenellenbogen

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Y. Zhao, M.J. Laws, V.S. Guillen, Y. Ziegler, A. Sharma, D. Chu, B.H. Park, J.A. Katzenellenbogen, B.S. Katzenellenbogen

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Y. Zhao, M.J. Laws, V.S. Guillen, Y. Ziegler, B.H. Park, K.W. Nettles, J.A. Katzenellenbogen, B.S. Katzenellenbogen

**Writing, review, and/or revision of the manuscript:** Y. Zhao, M.J. Laws, V.S. Guillen, Y. Ziegler, A. Sharma, D. Chu, B.H. Park, D.J. Shapiro, K.W. Nettles, J.A. Katzenellenbogen, B.S. Katzenellenbogen

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Y. Zhao, M.J. Laws, J. Min, S.H. Kim, S. Oesterreich, J.A. Katzenellenbogen, B.S. Katzenellenbogen

**Study supervision:** Y. Zhao, M.J. Laws, J.A. Katzenellenbogen, B.S. Katzenellenbogen

**Other (generation and basic characterization of genome-edited ESR1-mutant breast cancer cell lines, which were used in the studies for this article):** S. Oesterreich

**Other (provided materials):** C. Mao, D.J. Shapiro

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