Supplementary Data

Supplemental Materials and Methods

Growth Assays with BT-20 Cells

BT-20 was passaged in Modified Eagle Medium (MEM) supplemented with 10% FBS and and 1% penicillin/streptomycin. When hormone-starved, cells were cultured in phenol red- and glutamine-free MEM supplemented with 5% charcoal-stripped FBS and 2 mM L-glutamine. All liquid cell culture reagents were from Gibco (Billings, MT). For growth assays, 10^5 BT-20 cells were cultured and assayed as described in the main body of the paper.

Immunoprecipitation and Western Blot Analysis of MCF-7 Lysates

MCF-7 cells were lysed and their proteins quantitated as described within the manuscript. To immunoprecipitate proteins, protein G beads (Millipore, Temecula, CA) were prepared one day prior to lysis by combining 40 μl beads in 500 μl PBS with the immunoprecipitating antibody or isotype-specific IgG control antibody for 30 min at 4°C, with gentle agitation. Immunoprecipitations of ER-α were carried out with anti-ER-α, clone 1D5 (Vector Labs, Burlingame, CA) at 1 μg antibody/mg lysate, of AR with PG-21 (Millipore) at 0.5 μg/mg, and of c-Src with 2-17 (S. Parsons lab) at 1 μg/mg. Isotype-specific IgG control antibodies were used at the same concentration as the precipitating antibodies. Beads were pelleted and resuspended in 500 μl of a 1% bovine serum albumin (BSA, Roche Diagnostics GmbH, Mannheim, Germany) solution in PBS and incubated overnight at 4°C with gentle agitation. The next day beads were washed once in NP-40 lysis buffer and incubated with lysate overnight at 4°C with gentle agitation. Beads were washed three times in NP-40 buffer, resuspended in sample buffer, and boiled for 5 min to denature proteins. Because multiple co-precipitating proteins were analyzed per immunoprecipitation, 6 mg lysate was immunoprecipitated per treatment group and this was subdivided into three portions for SDS-PAGE analysis. When blotting immunoprecipitates, cross-species antibodies were used to detect proteins, and dilutions were adjusted to optimize detection. All other Western

blotting procedures on immunoprecipitates and whole cell lysates were performed as described within the manuscript.

AlphaEaseFC version 3.1.2 (Alpha Innotech Corp., San Leandro, CA) was used to quantitate scanned protein bands on film. All total protein levels were normalized to the loading control. Relative associations of immunoprecipitated proteins reflect the proportion of a protein within a cell associating with another protein. This was calculated by first dividing the immunoprecipitated and associated proteins by their respective normalized protein levels in the whole cell lysate. The normalized associated protein was then divided by normalized immunoprecipitated protein. All conditions were divided by the value for the untreated control and expressed as the fold change of the control. Comparisons between treatment groups were carried out using ANalysis Of VAriance between groups (ANOVA) corresponding to factorial experiments done in randomized blocks after transforming values to the log scale (to account for experiment-to-experiment variation in growth). All error bars indicate standard errors of the mean (SEM).

Growth Assay of MCF-7 Cells

Cells were treated and assayed as described within the main body of the paper. SU6656 (1 μM) was obtained from Calbiochem, LY 295002 (25 μM) from Cayman Chemical (Ann Arbor, MI), and bicalutamide (3 μM) from Sigma-Aldrich Corporation (Saint Louis, MO). All inhibitors were suspended in dimethyl sulfoxide (DMSO; Fisher Scientific) except fulvestrant, which was mixed with ethanol. The final concentration of inhibitors contained no more than 1% DMSO or ethanol. Unless otherwise specified, all other chemical reagents were purchased from Fisher Scientific (Waltham, MA).

LNCaP Culture and Treatment with Bicalutamide

The human prostate cancer cell line, LNCaP, was passaged and seeded in growth medium: T medium supplemented with 5% FBS, 2 mM L-glutamine solution, and 1% penicillin/streptomycin solution. For

assaying, 2.5 x 10^6 cells were seeded per 10 cm dish for each group and incubated overnight. The next day plates were washed three times with DPBS and depleted of hormones by addition of starvation medium (phenol-red-free RPMI, 5% charcoal-stripped FBS). After 24 hrs, DMSO or 1 nM R1881 (PerkinElmer, Waltham, MA) was added to half the plates, and all were incubated overnight. Thirty micromolar bicalutamide was then added and incubated 0.5, 1, 2, 4, or 6 hrs. Controls untreated with bicalutamide for 6 hrs were also included (0 time point). Cells were lysed, and proteins were separated and analyzed by Western blotting as described.

MCF-7 Treatment with Bicalutamide

Cells were treated in cell culture as described for the cell growth assays with inhibitors and then lysed and analyzed as detailed in the Western blot analysis section.

Silencing of Androgen Receptor

For the the mock control, non-specific (NS) siRNA control, and AR siRNA transfected groups, siRNA resuspension buffer (Dharmacon 5x siRNA Buffer, ThermoScientific), 100 μM NS siRNA stock [1], or 100 μM AR siRNA stock of On-Target Plus Smartpool human AR siRNA (Thermoscientific) was diluted to 1 pmol/ml final volume in Opti-MEM I Reduced-Serum Medium (250 μl/ml final volume or 1/3 cell suspension volume, Gibco). RNAiMAX (Invitrogen, Paisley, PA) was then added to the siRNA solution at a concentration of 0.625 μl/ml final volume and incubated 20 min at room temperature. Cells were detached with trypsin and diluted in antibiotic-free growth medium (DMEM supplemented with 10% fetal bovine serum and 1% 100 mM sodium pyruvate solution) to a density of 2.5 x 10^4 cells/ml. Cells were then added to the siRNA suspensions and immediately aliquoted into tissue culture dishes, which were incubated overnight. The final volume of the transfection-cell mixture was calculated by dividing the total cell number by 1.875 x 10^4. Transfected cells were then analyzed for estrogen and/or doxorubicin effects on net proliferation as in Figures 2, 7, and 8.

Additional List of Abbreviations

Bic: bicalutamide, AR-targeted drug; LY: LY 294002, PI3K inhibitor; SU: SU6656, SFK inhibitor

Supplemental Data 1. Estrogen and doxorubicin responses in an ER- cell line. Cells were treated as described in Figure 1 and analyzed by cell counting. A. ER- BT-20 cells are sensitive to doxorubicin, but not estrogen. Cells were treated with 0, 10, 25,100, 1000, 3000, or 10,000 nM doxorubicin in the presence (dashed line) or absence (solid line) of 10 nM estrogen as described in Supplemental Materials and Methods. The results are expressed as the raw cell number ± SEM for three experiments. There are no statistically significant differences between groups treated with and without estrogen. B. ER- BT-20 cells are more sensitive to doxorubicin than the ER+ cell lines, MCF-7 and T47-D. MCF-7 (black bars), T47-D (grey bars), and BT-20 (white bars) cells were treated with 0, 25, or 100 nM doxorubicin in the absence of estrogen and the total cell number graphed as the fold change of each cell line's untreated control. *Indicates a significant change between groups of the same cell line treated with and without doxorubicin ($p \leq 0.05$), †denotes a statistically significant difference between the sensitivity of two cell lines at the same doxorubicin concentration.

Supplemental Data 2. Prolonged estrogen and/or doxorubicin treatment cause(s) changes at the molecular level in MCF-7 cells.

Supplemental Data 3. Prolonged estrogen exposure alters the relative association of c-Src with ER-α. MCF-7 cells, cultured for 72 hrs in the presence or absence of estrogen, were lysed and analyzed by immunoprecipitation and Western blotting. Within each panel, the Western blots are representative of nine or more experiments that were quantitated and shown in the graph. For each graph, the untreated control was set to 1, and the estrogen-treated group was expressed as the mean fold change of the

untreated control ± SEM, as described in Materials and Methods. *Indicates statistical significance between groups treated with and without estrogen ($p \leq 0.05$). A. The relative association of c-Src with immunoprecipitated ER-α decreased with prolonged estrogen treatment. B. In the presence of estrogen, doxorubicin reduced the relative association of c-Src with immunoprecipitated ER-α from levels seen with doxorubicin alone. Additional interactions between the ER-α, AR, MNAR, and c-Src were also analyzed in all conditions, with the aforementioned exceptions, no significant changes to relative associations were seen between estrogen-treated and -untreated groups (data not shown).

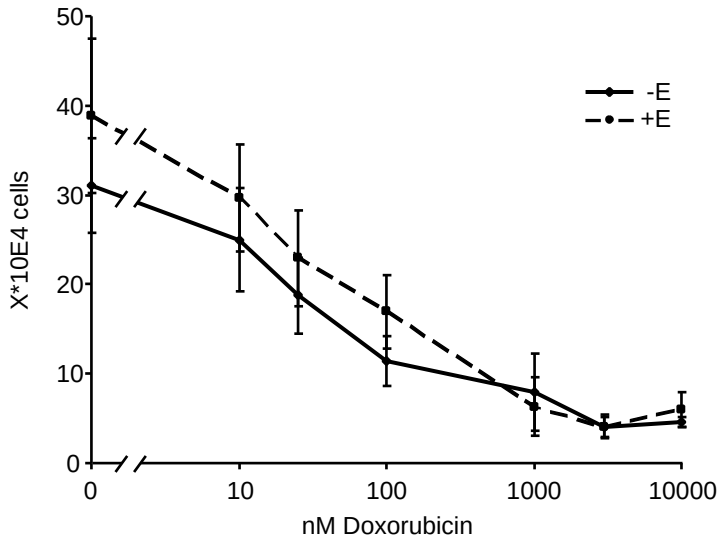
Supplemental Data 4. SFK, PI3K, and AR-targeted drugs are effective inhibitors of estrogen-induced MCF-7 cell growth that act independently of doxorubicin. MCF-7 cells were treated and analyzed as in Figure 7. *Indicates that the addition of estrogen has significantly altered ($p \leq 0.05$) the level from that of its non-estrogen treated counterpart (e.g. DMSO - Dox - E vs. DMSO - Dox + E), †denotes a significant change with doxorubicin-treatment from the level of its non-doxorubicin treated counterpart (e.g. DMSO - Dox - E vs. DMSO + Dox - E), and ‡ signifies a difference between groups treated with DMSO and an inhibitor (e.g. DMSO - Dox + E vs. inhibitor - Dox + E). Cells treated with 1 μM SU6656 (SU, a SFK inhibitor, panel A), 25 μM LY 294002 (LY, a PI3K inhibitor, panel B), or 3 μM bicalutamide (Bic, an AR-targeted drug, panel C) demonstrated inhibitor-dependent decreases in estrogen-induced proliferation in both the absence and presence of doxorubicin.

Supplemental Data 5. AR modulation in MCF-7 cells. A. Bicalutamide treatment reduced AR protein levels in LNCaP prostate cancer cells in the presence or absence of a synthetic androgen, R1881. Cells were treated and analyzed as described in Materials and Methods. B. In contrast to LNCaP cells, bicalutamide caused increased AR levels and phosphorylation in the absence of estrogen in MCF-7 cells, while having no effects on these events in the presence of estrogen. MCF-7 cells were treated with or

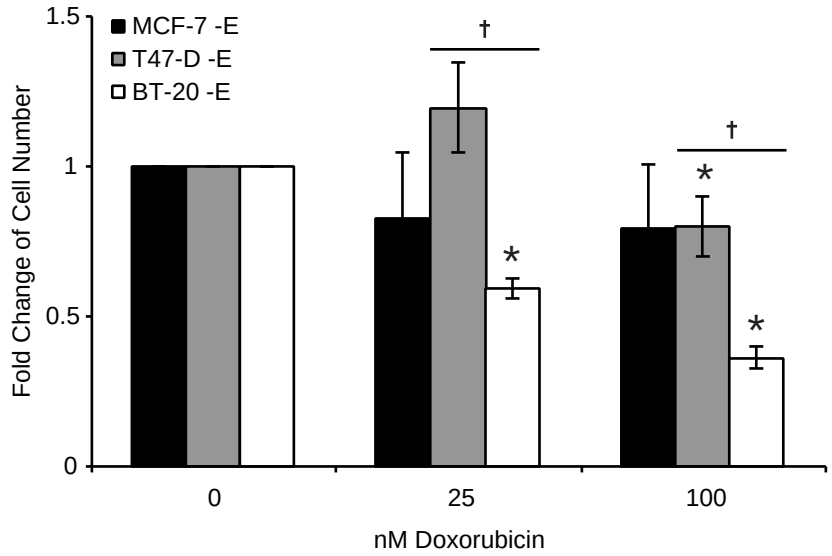
without estrogen and/or doxorubicin and analyzed as described in Materials and Methods. C. AR silencing in MCF-7 cells had no significant net effects on estrogen or doxorubicin modulation of cell proliferation. The graph depicts the results of three experiments carried out as described in Materials and Methods. Results are expressed as the mean fold change in cell number ± SEM for each treatment group. *Indicates - E vs. + E ($p \leq 0.05$); †denotes - Dox vs. + Dox. The inset Western blot demonstrates specific knock-down with an AR siRNA. The UT group was an untransfected control.

1 Gioeli D, Black BE, Gordon V, Spencer A, Kesler CT, Eblen ST, Paschal BM, Weber MJ: Stress kinase signaling regulates androgen receptor phosphorylation, transcription, and localization. Mol Endocrinol 2006;20:503-515.

A. BT-20


50
40
30
20
10
0
X*10E4 cells
-E
+E
0
10
100
1000
10000
nM Doxorubicin

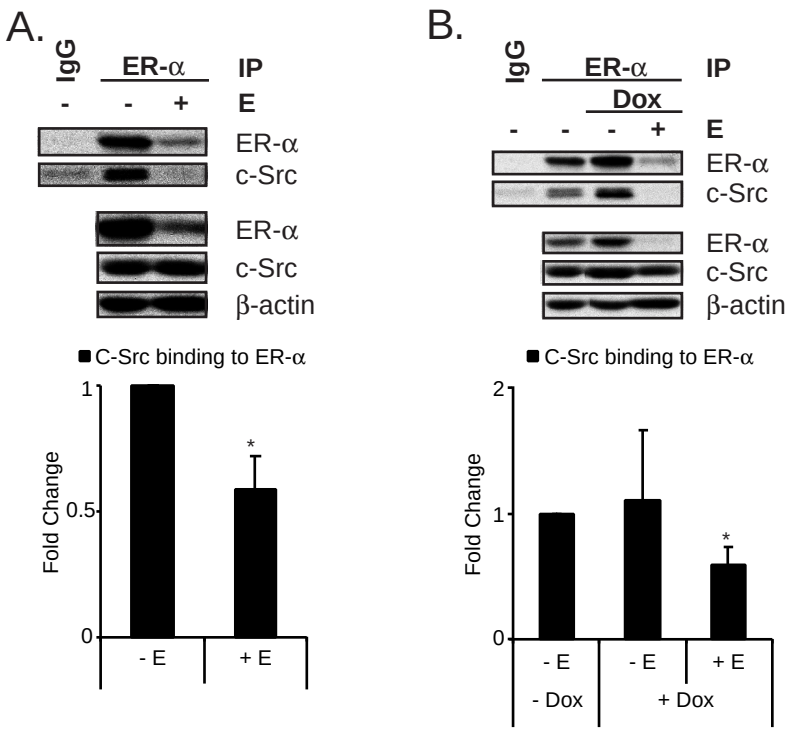
B.


1.5
1
0.5
0
Fold Change of Cell Number
MCF-7 -E
T47-D -E
BT-20 -E
†
†
*
*
*
0
25
100
nM Doxorubicin

Supplementary Data 2. Prolonged estrogen and/or doxorubicin treatment cause(s) changes at the molecular level in MCF-7 cells.

	- Dox		+ Dox	
	- E	+ E	- E	+ E
AR	1	**3.21 +/- 0.33**	*1.57 +/- 0.12*	***4.87 +/- 0.59***
pS210/S213	1	0.99 +/- 0.22	0.79 +/- 0.11	0.86 +/- 0.25
pY534	1	1.57 +/- 0.44	0.91 +/- 0.10	1.20 +/- 0.18
EGFR	1	**0.69 +/- 0.09**	*2.19 +/- 0.39*	**0.96 +/- 0.19**
ER-α	1	**0.31 +/- 0.03**	*1.47 +/- 0.13*	**0.38 +/- 0.06**
pS118	1	**4.06 +/- 0.43**	1.01 +/- 0.09	**4.26 +/- 0.60**
pS167	1	**14.15 +/- 2.23**	*0.75 +/- 0.06*	**16.59 +/- 2.61**
pY537	1	**2.91 +/- 0.35**	1.02 +/- 0.10	**3.99 +/- 0.68**
ER-β	1	1.02 +/- 0.35	1.04 +/- 0.17	0.83 +/- 0.26
HER2	1	**0.30 +/- 0.04**	*1.84 +/- 0.23*	**0.38 +/- 0.05**
pY877	1	**5.48 +/- 1.43**	1.39 +/- 0.36	***6.79 +/- 1.59***
ERK 1/2	1	1.10 +/- 0.10	1.08 +/- 0.13	1.16 +/- 0.22
pT183/Y185	1	**0.62 +/- 0.15**	1.07 +/- 0.19	0.92 +/- 0.19
MNAR	1	**0.82 +/- 0.07**	1.07 +/- 0.09	***0.64 +/- 0.06***
PI3K	1	**2.16 +/- 0.21**	1.11 +/- 0.11	*1.64 +/- 0.24*
c-Src	1	**0.66 +/- 0.09**	1.08 +/- 0.12	***0.51 +/- 0.09***
pY418	1	**1.51 +/- 0.15**	1.16 +/- 0.12	***2.07 +/- 0.26***

All values are obtained from analyses of data depicted in Figs. 3-6 and expressed as the mean fold change in relationship to the untreated (- Dox, - E) control.
Bold font denotes $p < 0.05$ when compared to - E counterpart
Underlined italic font denote $p < 0.05$ when compared to - Dox counterpart


A.
IgG
ER-α
IP
- - +
E
ER-α
c-Src
ER-α
c-Src
β-actin
C-Src binding to ER-α
Fold Change
1
0.5
0
*
- E
+ E
B.
IgG
ER-α
IP
Dox
- - - +
E
ER-α
c-Src
ER-α
c-Src
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Fold Change
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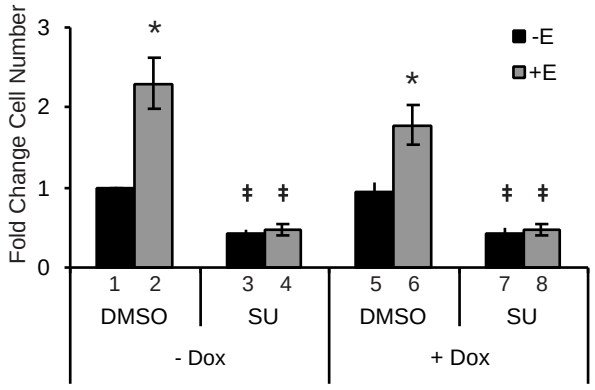
1
0
*
- E
- E
+ E
- Dox
+ Dox

A.


Fold Change Cell Number
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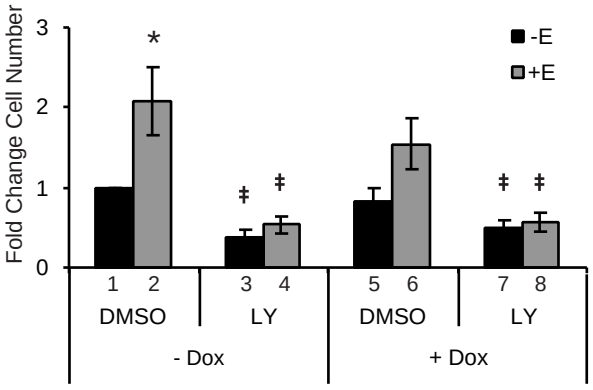
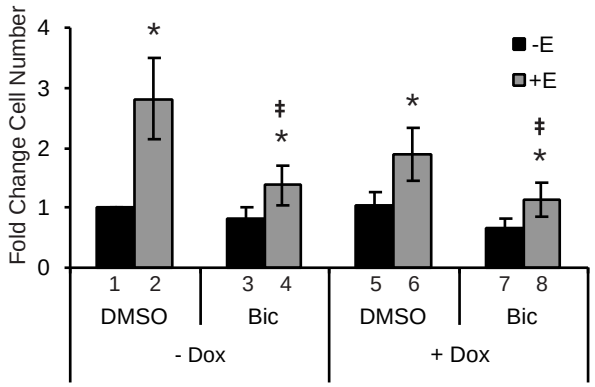
2
3
-E
+E
1 2 3 4 5 6 7 8
DMSO
SU
DMSO
SU
- Dox
+ Dox

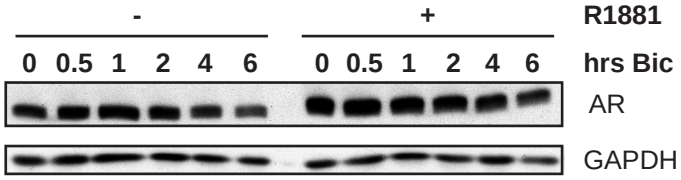
B.


Fold Change Cell Number
0
1
2
3
-E
+E
1 2 3 4 5 6 7 8
DMSO
LY
DMSO
LY
- Dox
+ Dox

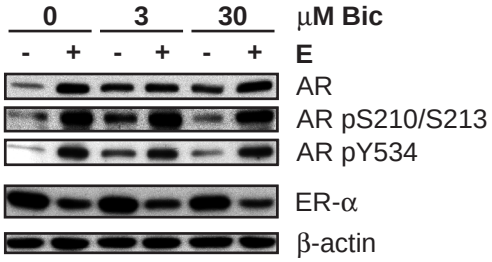
C.


Fold Change Cell Number
0
1
2
3
4
-E
+E
1 2 3 4 5 6 7 8
DMSO
Bic
DMSO
Bic
- Dox
+ Dox

A. LNCaP



B. MCF-7



C. MCF-7