

Dual Inhibition of mTOR and Estrogen Receptor Signaling *In vitro* Induces Cell Death in Models of Breast Cancer

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Abstract Purpose: RAD001 (everolimus), a mammalian target of rapamycin (mTOR) pathway inhibitor in phase II clinical trials in oncology, exerts potent antiproliferative/antitumor activities. Many breast cancers are dependent for proliferation on estrogens synthesized from androgens (i.e., androstenedione) by aromatase. Letrozole (Femara) is an aromatase inhibitor used for treatment of postmenopausal women with hormone-dependent breast cancers. The role of the mTOR pathway in estrogen-driven proliferation and effects of combining RAD001 and letrozole were examined *in vitro* in two breast cancer models.

Experimental Design: The role of the mTOR pathway in estrogen response was evaluated in aromatase-expressing MCF7/Aro breast cancer cells by immunoblotting. Effects of RAD001 and letrozole (alone and in combination) on the proliferation and survival of MCF7/Aro and T47D/Aro cells were evaluated using proliferation assays, flow cytometry, immunoblotting, and apoptosis analyses.

Results: Treatment of MCF7/Aro cells with estradiol or androstenedione caused modulation of the mTOR pathway, a phenomenon reversed by letrozole or RAD001. In MCF7/Aro and T47D/Aro cells, both agents inhibited androstenedione-induced proliferation; however, in combination, this was significantly augmented ($P < 0.001$, two-way ANOVA, synergy by isobologram analysis). Increased activity of the combination correlated with more profound effects on G₁ progression and a significant decrease in cell viability ($P < 0.01$, two-way ANOVA) defined as apoptosis ($P < 0.05$, Friedman test). Increased cell death was particularly evident with optimal drug concentrations.

Conclusion: mTOR signaling is required for estrogen-induced breast tumor cell proliferation. Moreover, RAD001-letrozole combinations can act in a synergistic manner to inhibit proliferation and trigger apoptotic cell death. This combination holds promise for the treatment of hormone-dependent breast cancers.

The estrogen receptor (ER) is an important predictive and prognostic marker in human breast cancer, being expressed in ~60% of breast cancers. ER is a member of a family of nuclear transcription factors exhibiting both ligand-dependent and ligand-independent transcriptional activity; 17 β -estradiol (E2) being the most potent ligand. In postmenopausal women, its biosynthesis is mediated by aromatase from androgenic substrates (1). Although therapeutics which interfere with ER function (antiestrogens, e.g., tamoxifen), have contributed to a

dramatic reduction in breast cancer mortality, at best 50% to 60% of ER-positive breast cancers respond (2). Consequently, a number of aromatase inhibitors (e.g., letrozole) that reduce estrogen biosynthesis itself have been developed (1). Indeed, letrozole, a potent, nonsteroidal aromatase inhibitor (registered as Femara) is indicated for first-line advanced metastatic and neoadjuvant therapy of breast cancers in postmenopausal women, after it was shown to be superior to the antiestrogen tamoxifen (3–5). Letrozole is also effective after tamoxifen failure in the second-line advanced metastatic disease setting (6, 7) and is efficacious in women who remained disease-free after receiving 5 years of prior adjuvant treatment with tamoxifen in the extended adjuvant indication (8).

Recently, it has become evident that estrogen/ER signaling is more complex than initially anticipated, exhibiting pleiotropic effects through nongenomic interactions with growth factor signaling pathways. In steroid-deprived MCF7 breast carcinoma cells, the ER is predominantly localized in the nucleus; however, upon E2 stimulation, a substantial proportion is translocated to the plasma membrane (9) contributing to growth factor receptor signaling (10, 11). Several levels of interaction between the estrogen/ER and growth factor pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt

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and the mitogen-activated protein kinase pathways, have been documented (10, 11). Moreover, the observation that long-term estrogen-deprived MCF7 cells (with increased estrogen sensitivity) exhibit up-regulation of ErbB2 and Erk1/2 (12), and increased Akt phosphorylation and mammalian target of rapamycin (mTOR) effector activation (13) also indicates that up-regulation of growth factor signaling is fundamental to the adaptation of breast cancer cells to low estrogen levels in cultured cells. In support of this hypothesis, tamoxifen treatment in the clinical setting resulted in increased tumor ErbB2 and p38 mitogen-activated protein kinase activation at relapse (14). It is feasible, therefore, that interactions between estrogen/ER and growth factor signal transduction pathways may contribute to both the failure of endocrine therapy as well as the development of resistance.

One strategy to improve the efficacy of aromatase inhibitors and potentially circumvent or delay the development of resistance is to concomitantly target the aromatase/estrogen/ER and growth factor pathways in breast cancer patients. In this respect, the PI3K/Akt pathway plays a major role in breast cancer, with up-regulation associated with a more aggressive clinical phenotype (15) and a worse clinical outcome for endocrine-treated patients (16). Furthermore, this pathway has been heavily implicated in resistance to antiestrogen therapeutics (10, 11, 17). An emerging mediator of PI3K/Akt activities relating to tumor cell growth and proliferation is the mTOR kinase (18, 19). The mTOR pathway is a central sensor for nutrient/energy availability, being further modulated by PI3K/Akt-dependent mechanisms (19). In the presence of mitogenic stimuli and sufficient nutrients and energy, mTOR relays a positive signal to the translational machinery by activating the 40S ribosomal protein S6 kinases (S6K1-2) and inhibiting the eukaryotic initiation factor 4E binding proteins (4E-BP1-3). The S6Ks have been implicated in the translational regulation of mRNAs that typically encode ribosomal proteins as well as components of the translational machinery. The mTOR-dependent phosphorylation of 4E-BP1 mediates its dissociation from the RNA cap-binding protein eIF-4E, thereby allowing reconstitution of a translationally competent initiation factor complex (eIF-4F). The eIF-4F complex also comprises eIF-4GI or eIF-4GII scaffold proteins and the eIF-4A RNA helicase, and activation results in the translation of proteins involved in G₁-S phase progression (19). The importance of mTOR signaling in tumor biology is now widely accepted (19, 20). Consequently, a number of agents that selectively target mTOR are being developed in the oncology indication (19).

RAD001 (everolimus) is an orally bioavailable, mTOR inhibitor currently in phase II clinical trials in cancer patients (19, 21). RAD001 potently inhibits tumor cell proliferation *in vitro* and exhibits antitumor activity in a range of animal models (18, 22–25). Irrespective of ER status, breast cancer cell lines seem particularly sensitive to RAD001 (and other rapamycins), with IC₅₀ values for *in vitro* antiproliferative activity in the sub- to low nanomolar range (24, 26, 27). As the PI3K/Akt pathway is heavily deregulated in breast cancer and up-regulation of this pathway is associated with increased sensitivity to mTOR inhibition (24, 27, 28), the application of RAD001 in this patient population is warranted.

We evaluated the potential for combining letrozole with RAD001 in two *in vitro* models of breast carcinoma (MCF7 and T47D). We show that estrogen-induced proliferation

is largely dependent on mTOR signaling. Furthermore, RAD001 in combination with letrozole has more profound effects on aromatase-mediated estrogen-induced proliferation in aromatase-expressing lines than either agent alone. In MCF7 cells, this translated at the molecular level to a greater modulation of key G₁ regulators. Strikingly, combinations of both agents triggered a more profound induction of programmed cell death in both models. These data are highly supportive of the combination of these agents for the therapy of endocrine-dependent breast cancers.

Materials and Methods

Cell culture

MCF7 and T47D human breast carcinoma lines (29) were cultured in MEM EBS (Amimed) or RPMI 1640 (Hyclone, Logan, UT), respectively. Supplements included 10% FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids (MCF7), 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.5 µg/mL insulin (T47D), and 0.5 (MCF7) or 0.3 (T47D) mg/mL G418. Cells were steroid deprived using phenol red-free medium supplemented with 10% charcoal-stripped FCS (Hyclone/Omega) for 3 days (T47D) or 5 days (MCF7), before E2 or androstenedione (Δ4A) treatment. Treatments were initiated either 2 days post-seeding (MCF7) or immediately (T47D), and cells were treated with ligands and inhibitors every other day for 6 days (except where otherwise mentioned).

Compounds and ligands

Both RAD001 (everolimus), a derivative of rapamycin [4-O-(2-hydroxyethyl)-rapamycin] and letrozole (Femara), a nonsteroidal aromatase inhibitor, were synthesized in the laboratories of Novartis Institutes for BioMedical Research (Basel, Switzerland) and were prepared in DMSO (20 mmol/L) and in ethanol (1 mmol/L), respectively. E2 and Δ4A (Sigma-Aldrich, St. Louis, MO) were prepared in ethanol at 1 and 10 mmol/L, respectively. Aliquots were stored at –20°C.

Cell proliferation assays

For E2 and Δ4A titrations, 5×10^3 MCF7/Aro and 6×10^3 MCF7 3 (1) cells were seeded (100 µL per well) into 96-well plates and steroid-deprived cells were treated every other day for 6 days. Effects on proliferation were analyzed using the YO-PRO DNA-binding fluorescent dye technique as previously described (22). To evaluate the antiproliferative effect of RAD001 and letrozole on MCF7/Aro, 10^5 cells were seeded into 6-well plates. Steroid-deprived cells were treated with 10 nmol/L Δ4A or 1 nmol/L E2 with RAD001 or letrozole (alone or in combination) every second day for 6 days. Cells were harvested by trypsinization, resuspended in PBS and counted using a CASY cell counter (Schärfe System, Reutlingen, Germany). For T47D/Aro, 6×10^4 cells were seeded into 6-well plates. Steroid-deprived cells were treated with 10 nmol/L Δ4A with RAD001 or letrozole (alone or in combination) every second day for 6 days. Cells were dissolved in 0.5 N NaOH and the protein concentration was determined.

Protein extraction and immunoblotting

To evaluate signaling pathways, 1.2×10^6 MCF7/Aro cells were seeded into 10-cm plates. Steroid-deprived cells were treated with vehicle or 1 nmol/L E2 with or without 30 minutes pretreatment with 20 nmol/L RAD001. Alternatively, cells were treated with 10 nmol/L Δ4A and concomitantly treated with 500 nmol/L letrozole or vehicle. Whole cell protein extracts were prepared as previously described (30), and supernatants were stored at –80°C.

To assess effects on cell cycle regulators, 8×10^5 and 10^6 MCF7/Aro cells were seeded into 10-cm plates, respectively. Steroid-deprived cells were treated with 10 nmol/L Δ4A and concomitantly treated with

vehicle, 100 nmol/L letrozole, and 2 nmol/L RAD001 (alone or in combination) for 4 hours. Floating cells were collected and adherent cells were harvested by scraping into PBS containing 1 mmol/L phenylmethylsulfonyl fluoride. Pooled cells were extracted and frozen as above.

Immunoblotting was done as previously described (23), with the following antibodies: anti-S6K1, anti-phospho-S6K1 (Thr³⁸⁹), anti-phospho-S6 (Ser²⁴⁰/Ser²⁴⁴), anti-eIF-4E, anti-phospho-eIF-4E (Ser²⁰⁹), anti-4E-BP1, anti-phospho-eIF-4G (Ser¹¹⁰⁸), anti-Akt, anti-phospho-Akt (Ser⁴⁷³), anti-Erk1/2, anti-phospho-Erk1/2 (Thr²⁰²/Tyr²⁰⁴), and anti-phospho-Rb (Ser⁷⁹⁵; Cell Signaling Technologies, Inc., Beverly, MA); anti-eIF-4G (Abcam, Cambridge, United Kingdom); anti-cyclin D1 (Novocastra, Newcastle, United Kingdom); anti-cyclin D2 and anti-cyclin D3 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-retinoblastoma (Calbiochem, La Jolla, CA); anti-actin (Chemicon, Temecula, CA); anti- β -tubulin (Tub2.1, Sigma, St. Louis, MO), and anti-S6 (provided by Dr. J. Mestan, Novartis Institutes for BioMedical Research).

Cell cycle analysis

To assess effects on the cell cycle, 5×10^5 MCF7/Aro cells were seeded into 6- or 10-cm plates. Steroid-deprived cells were treated with 10 nmol/L Δ 4A and simultaneously treated with vehicle, 100 nmol/L letrozole, and 0.2 or 2 nmol/L RAD001 (alone or in combination) for 24 hours. Floating cells were collected and adherent cells harvested by trypsinization. Cells were washed once with PBS and then resuspended in propidium iodide buffer [1 mmol/L sodium citrate (pH 4), 1.5 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.1% NP40, 4 μ g of propidium iodide/mL, and 175 μ g of RNase A/mL in PBS]. After 30 minutes of incubation in the dark on ice, cell cycle distribution was analyzed with a Becton Dickinson FACSCalibur flow cytometer.

Cell viability assays

YO-PRO. Cells (10^5) in 2 mL medium were seeded into 6-well plates. Steroid-deprived cells were treated with 10 nmol/L Δ 4A and concomitantly treated with vehicle, letrozole (alone or in combination with RAD001) every other day for 6 days. Effects on cell viability were analyzed using the YO-PRO DNA-binding fluorescent dye technique as previously described (22).

Terminal deoxynucleotidyl transferase-mediated nick-end labeling. For flow cytometry, 4×10^5 cells were seeded into 10-cm plates. Steroid-deprived cells were treated with 10 nmol/L Δ 4A and concomitantly treated with vehicle, letrozole (alone or in combination with RAD001) every second day for 6 days. Cells were stained according to the manufacturer (APO-DIRECT; BD Pharmingen, San Diego, CA). FITC-labeled cells were analyzed by flow cytometry using Cell Quest software. For microscopic analyses, 10^5 MCF7/Aro cells were seeded on gelatin-coated coverslips in 6-well plates. Steroid-deprived cells were treated as above. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining was done directly on the adherent cells in a humidified chamber as above. Cell nuclei were counterstained with Hoechst 33258 (Molecular Probes, Eugene, OR) in the dark. Cells were washed thrice with PBS and coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Apoptotic cells were analyzed by fluorescence microscopy (Leica DM IRB, 20 \times objective; Kodak DC290 Zoom digital camera).

Statistical analyses

Proliferation and cell viability data were statistically analyzed using two-way ANOVA (with Tukey test for pairwise comparisons) to test for the effect of RAD001 and letrozole as single agents and for interactions between the compounds. Calculations were made using SigmaStat 3.1 (Systat Software, Systat Software GmbH, Erkrath, Germany), and $P < 0.05$ was considered statistically significant. To further determine the nature of the letrozole-RAD001 interactions, partial isobolograms were constructed to permit estimating the coefficient g , which represents the value of the equation $Ac / (Am + Bc) / Bm$, where Ac (or Bc) is the dose of compound A (or B) in combination, Am (or Bm) is the dose of

compound A (or B) in monotherapy, that give equivalent activity (31, 32). When $g = 1$, the combination is additive in nature; when >1 , the combination is considered antagonistic; and when <1 , the combination may be considered synergistic. The activity of the known doses of letrozole or RAD001 in combination was taken as a reference point for determining, by interpolation of the concentration-response curves, the concentrations of letrozole and RAD001 in monotherapy that would produce the same activity. As some of the antiproliferative activities of the combinations fell outside of the single agent concentration-response curves, these combinations were not available for use in determining interactions. Of the data that satisfied these restrictions, all combinations showed synergy by this calculation

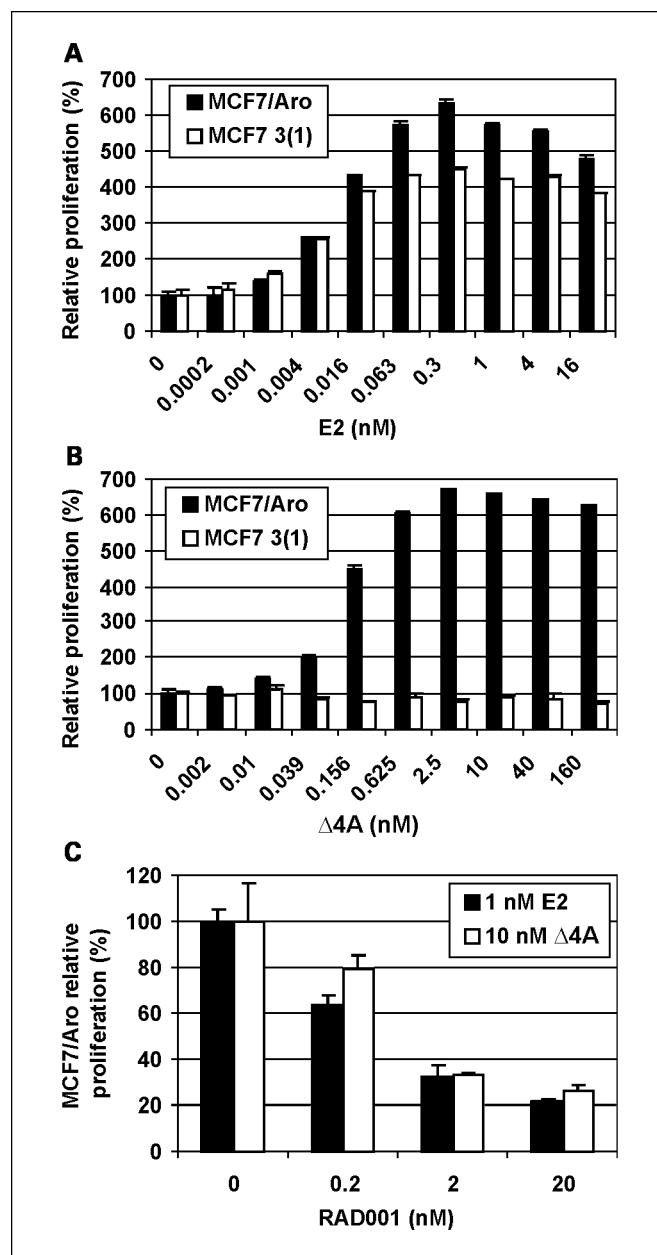


Fig. 1. Estrogen-driven proliferation of MCF7 cells is dependent on mTOR. Steroid-deprived MCF7 3(1) (A and B) and MCF7/Aro (A-C) cells were treated with increasing concentrations of E2 (A and C) or Δ 4A (B and C) in the absence (A and B) or presence (C) of increasing concentrations of RAD001 for 6 days. Relative proliferation was assessed using the YO-PRO DNA-binding fluorescent dye procedure as described in Materials and Methods (A and B), or by trypsinization and direct counting of the cells (C). Columns, means of triplicate values; bars, \pm SD.

(31, 32). Furthermore, two-way ANOVA was used to determine interactions between RAD001 and letrozole, which when positively interacting, according to Slinker (33) shows drug synergy. For flow cytometry/TUNEL analyses, the statistical significance was determined by the Friedman test for multiple comparison of ratios (34), using Systat V11.0 (Systat Software).

Results

Estrogen-driven proliferation of MCF7/Aro cells exhibits mammalian target of rapamycin dependency. To assess the estrogen sensitivity of MCF7 cells, we evaluated the relative proliferation of MCF7/Aro (stably expressing aromatase) and MCF7 3(1) vector control cells in the presence of E2 or the precursor Δ 4A, using the YO-PRO proliferation assay (Fig. 1A and B). Steroid-deprived cells were treated every other day for 6 days. Steroid deprivation completely abolished MCF7/Aro and MCF7 3(1) cell proliferation (data not shown) thus indicating that proliferation is estrogen dependent. Consistent with this observation, both E2 and Δ 4A stimulated the proliferation of MCF7/Aro cells in a concentration-dependent manner, while MCF73(1) cells responded only to E2 (Fig. 1A and B). Hence, MCF7/Aro cells can convert Δ 4A into estrogens. In agreement with previous reports (35–37), these experiments defined 1 nmol/L E2 and 10 nmol/L Δ 4A as effective concentrations for further experiments.

MCF7 parental cells cultured in complete medium are sensitive to mTOR inhibition (26, 27). In complete medium, we also observed that RAD001 potently inhibited MCF7 proliferation ($IC_{50} = 0.6 \pm 0.1$ nmol/L), and MCF7 3(1) and MCF7/Aro cells exhibited a similar response to optimal RAD001 concentrations (20 nmol/L RAD001 induces a ~20% increase in G_1 population after 24 hours of incubation; data not shown). To examine whether estrogen-driven proliferation of MCF7/Aro cells was dependent on mTOR signaling, steroid-deprived cells were stimulated with E2 or Δ 4A in the presence of increasing concentrations of RAD001 or vehicle (Fig. 1C). Interestingly, RAD001 dramatically impaired E2- and Δ 4A-induced proliferation of MCF7/Aro cells in a concentration-dependent manner, with maximal effects elicited at 2 to 20 nmol/L RAD001 (2 nmol/L, 68% and 67%; 20 nmol/L, 79% and 74% inhibition of E2- and Δ 4A-dependent proliferation, respectively), and partial inhibition at 0.2 nmol/L RAD001 (Fig. 1C). From these data, 0.2 and 2 nmol/L were defined as suboptimal and optimal RAD001 concentrations, respectively.

The effect of E2 on mTOR signaling was also analyzed. Steroid-deprived MCF7/Aro cells were either vehicle-treated or treated for up to 24 hours with E2, with or without a 30 minutes pretreatment with 20 nmol/L RAD001 (Fig. 2). Vehicle had little effect on mTOR pathway components. In contrast, E2 treatment induced prolonged phosphorylation of S6K1 and its substrate

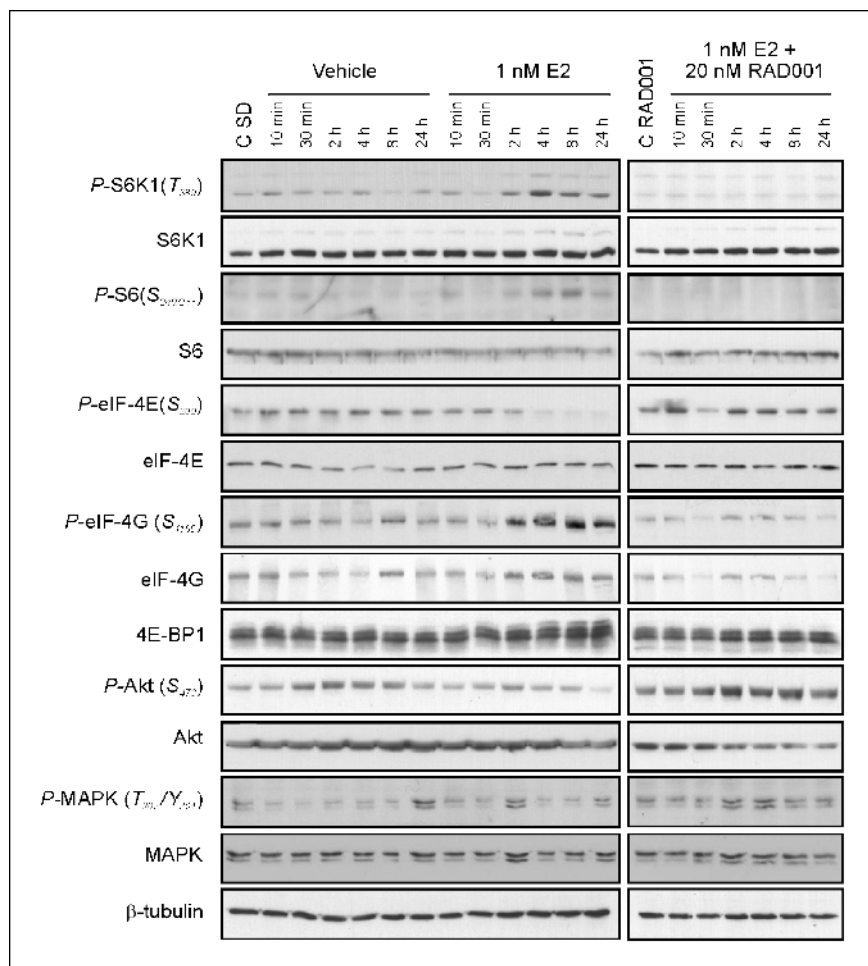
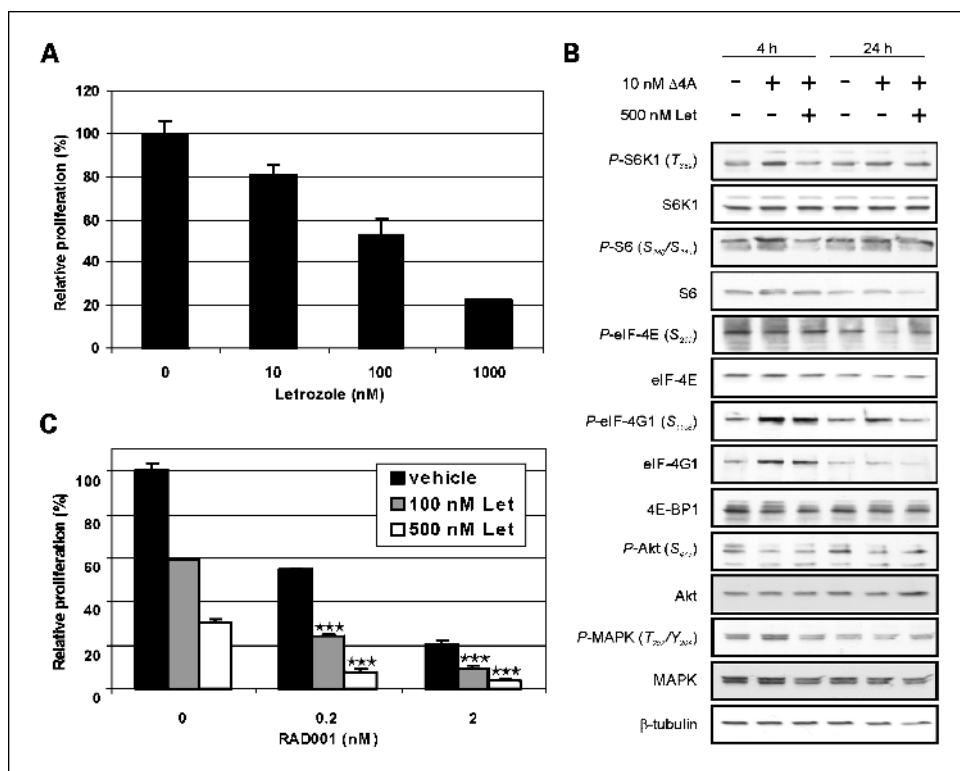


Fig. 2. Estradiol-stimulated MCF7 proliferation is associated with modulation of the mTOR pathway. Steroid-deprived MCF7/Aro cells were treated with vehicle or 1 nmol/L E2 without (left) or with (right) pretreatment with 20 nmol/L RAD001 for 30 minutes. At the times indicated, cells were extracted as described in Materials and Methods. Lane C SD, steroid-deprived control; Lane C RAD001, RAD001-pretreated control. Protein extracts were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with the indicated antibodies. β -Tubulin was used as a loading control.

Fig. 3. Combinations of RAD001 and letrozole result in enhanced antiproliferative activity in androstenedione-driven MCF7/Aro cells. Steroid-deprived MCF7/Aro cells were treated with 10 nmol/L Δ 4A and increasing concentrations of letrozole (Let, A) alone or in combination with 0.2 or 2 nmol/L RAD001 (C) for 6 days. Relative proliferation was assessed by direct cell counting. Columns, means of triplicate values; bars, \pm SD. Stars, $P < 0.001$, two-way ANOVA using Tukey's test for pairwise comparisons (synergistic drug interaction). B, cells were left untreated or treated with 10 nmol/L Δ 4A with concomitant treatment with 500 nmol/L letrozole or vehicle for 4 or 24 hours. Whole cell protein extracts were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with the indicated antibodies. β -Tubulin was used as a loading control.



the 40S ribosomal protein S6, which was evident after 2 hours and maintained for 24 hours (Fig. 2, left). Consistent with these changes being mTOR regulated, both responses were inhibited by RAD001 pretreatment (Fig. 2, right). Similarly, E2 led to decreased electrophoretic mobility of the translational repressor 4E-BP1 for 2 to 24 hours, a phenomenon indicative of increased phosphorylation and prolonged functional inactivation and also counteracted by RAD001 treatment (Fig. 2, compare left and right). Of particular interest, E2 treatment caused decreased eIF-4E and increased eIF-4G1 phosphorylation, events that paralleled effects on S6K1 and 4E-BP1 and antagonized by RAD001 (Fig. 2). Moreover, short-term treatment with RAD001 alone resulted in increased phosphorylation of eIF-4E, an observation not previously reported (Fig. 2, compare lane C SD with lane C RAD001). In the presence of RAD001, prolonged increased Akt phosphorylation was also observed, consistent with previous observations (38). However, although it has been previously reported that E2 induces transient induction (5-10 minutes) of Akt phosphorylation in MCF7 cells (39), analysis of Akt and mitogen-activated protein kinase phosphorylation suggested no E2-specific modulation of these signal transducers in our experimental setting, where the first measurement was after 10 minutes.

Altogether, these data provide a molecular basis explaining the observation that RAD001 inhibits E2- and Δ 4A-induced proliferation in MCF7/Aro cells, with specific modulation of elements downstream of mTOR highlighting a contribution of this pathway to estrogen response.

Combinations of RAD001 and letrozole result in enhanced antiproliferative activity in androstenedione-driven MCF7/Aro cells. Letrozole inhibits the conversion of Δ 4A into estrogens by inhibiting aromatase activity (1). Hence, treatment of MCF7/Aro cells with letrozole resulted in a concentration-

dependent inhibition of Δ 4A-driven proliferation (Fig. 3A). The IC₅₀ for letrozole was defined as \sim 100 nmol/L, with maximal inhibition observed at 500 to 1,000 nmol/L (Fig. 3C and A). Moreover, Δ 4A treatment also induced modulation of the mTOR pathway, an effect reversed by the presence of 500 nmol/L letrozole (Fig. 3B). Hence, 100 and 500 nmol/L were defined as suboptimal and optimal concentrations of letrozole, respectively.

The effect of combining suboptimal and optimal concentrations of RAD001 and letrozole on Δ 4A-driven proliferation of MCF7/Aro cells was evaluated. As expected (see Fig. 1C and Fig. 3A), treatment with RAD001 or letrozole alone inhibited MCF7/Aro proliferation in a concentration-dependent manner (Fig. 3C). Strikingly, combining suboptimal concentrations of RAD001 and letrozole significantly enhanced the antiproliferative activity as compared with either agent alone (0.2 nmol/L RAD001, 45%; 100 nmol/L letrozole, 41%; Combination, 76% inhibition of proliferation). A similar phenomenon was observed with optimal concentrations of the two agents (2 nmol/L RAD001, 80%; 500 nmol/L letrozole, 70%; Combination, 96% inhibition of proliferation). Statistical analysis indicated that highly significant interactions exist between RAD001 and letrozole ($P < 0.001$, two-way ANOVA), suggesting strong combination effects and, according to Slinker (33), synergistic drug interaction. Furthermore, using methods that facilitate determination of combination effects based upon limited isobologram data (31, 32), synergistic drug interactions were shown (see Materials and Methods).

Increased antiproliferative potential of RAD001/letrozole combinations correlates with more pronounced G₁ accumulation. Both mTOR and estrogen signaling pathways are known to regulate G₁-phase progression (19, 40). To investigate the effect of letrozole and RAD001 treatment on cell cycle distribution,

steroid-deprived MCF7/Aro cells were treated with $\Delta 4A$ in the absence or presence of 100 nmol/L (suboptimal) letrozole and 0.2 nmol/L (suboptimal) or 2 nmol/L (optimal) RAD001, alone or in combination. To observe immediate effects on cell cycle distribution, flow cytometry was done after 24 hours incubation (Fig. 4A). As expected (24), 2 nmol/L RAD001 induced an increase in the G₁ population, whereas 0.2 nmol/L RAD001 had no-to-minimal effects (Fig. 4A, top row and see legend). Consistent with a previous report (36), treatment with 100 nmol/L letrozole also affected G₁ progression; however, combinations of both agents triggered a more pronounced G₁ accumulation (Fig. 4A, bottom row and see legend). Strikingly, this occurred even with the suboptimal 0.2 nmol/L RAD001 concentration, which alone had little effect on the cell cycle.

To further analyze these cell cycle effects, analysis of proteins central to G₁-S phase progression was done. Steroid-deprived MCF7/Aro cells were treated for 4 hours with $\Delta 4A$ in the absence or presence of 100 nmol/L letrozole or 2 nmol/L RAD001, alone or in combination. RAD001 caused S6 dephosphorylation and decreased 4E-BP1 protein mobility (Fig. 4B, left). A minor effect of 100 nmol/L letrozole on these proteins was also observed, consistent with this suboptimal concentration (Fig. 4B and see Fig. 3A). Analysis of the

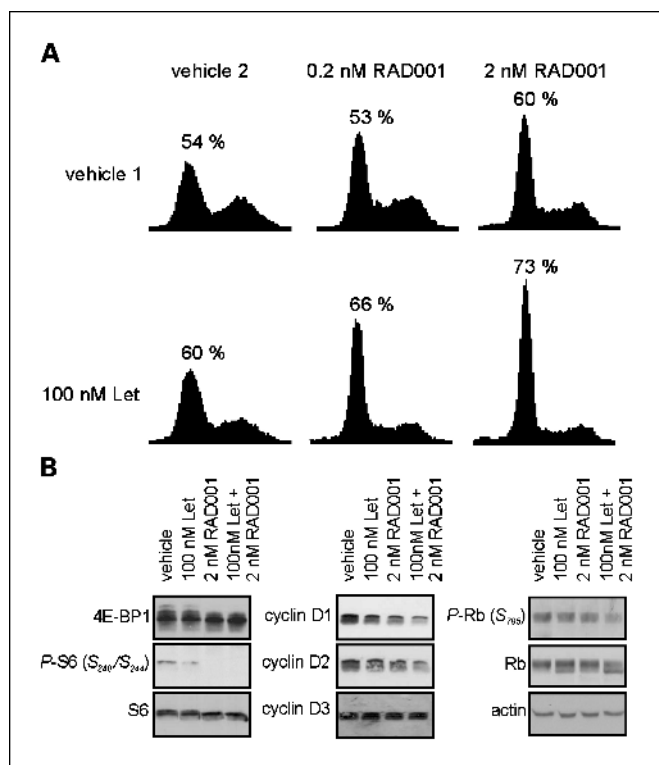


Fig. 4. Increased antiproliferative potential of RAD001/letrozole combinations correlates with more pronounced G₁ accumulation. Steroid-deprived MCF7/Aro cells were treated with 10 nmol/L $\Delta 4A$ in the absence or presence of 100 nmol/L letrozole (Let), alone or in combination with RAD001. After 24 hours, cell cycle distribution was analyzed by flow cytometry. Cell cycle profiles and the percentage of cells in the G₁ phase of a representative experiment are presented. A repeat experiment gave similar results: G₁ accumulation, 47%, 51%, and 71% for vehicle and 0.2 and 2 nmol/L RAD001 alone, respectively; and 54%, 64%, and 76% for letrozole alone or in combination with 0.2 and 2 nmol/L RAD001, respectively. Vehicle 1, ethanol; vehicle 2, DMSO. B, steroid-deprived MCF7/Aro cells were treated for 4 hours as indicated. Whole cell protein extracts were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with the indicated antibodies. Actin was used as a loading control.

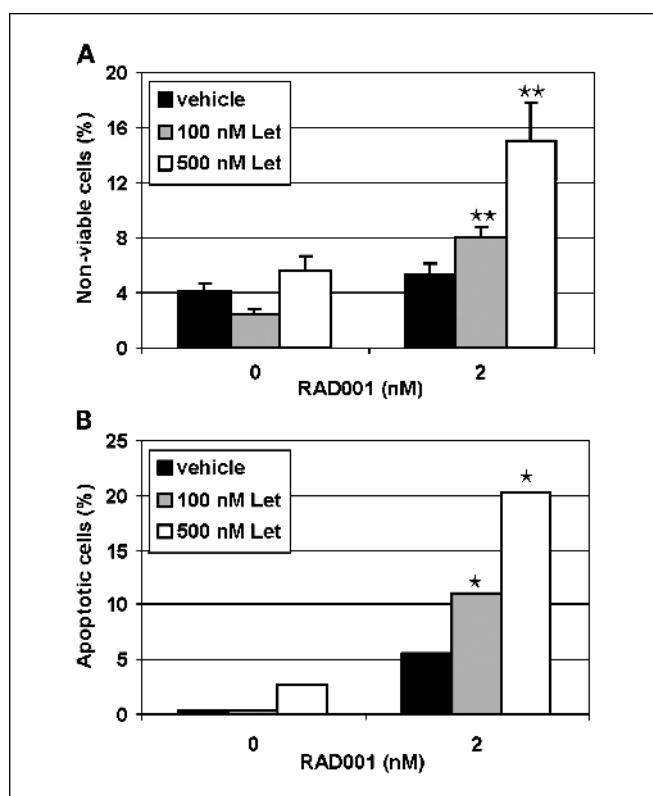


Fig. 5. Dual inhibition of mTOR and estradiol signaling induces apoptosis of MCF7/Aro cells. Steroid-deprived MCF7/Aro cells were treated with 10 nmol/L $\Delta 4A$ in the absence or presence of 100 or 500 nmol/L letrozole (Let), alone or in combination with 2 nmol/L RAD001 for 6 days. The numbers of nonviable and apoptotic cells were evaluated using the YO-PRO assay (A) or a cytometry-based TUNEL analysis (B), respectively, as described in Materials and Methods. Columns, means of triplicate values (A) or a single value (B); bars, \pm SD. A, stars, $P < 0.01$, two-way ANOVA using Tukey's test for pairwise comparisons. B, stars, $P < 0.05$, Friedman test.

expression of the D-type cyclins, essential subunits of G₁ cyclin-dependent kinases (Cdk4/6) suggested to be regulated through both mTOR and ER signaling (19, 40), showed that both RAD001 and letrozole similarly reduced cyclin D1 and D2 protein expression, with cyclin D3 levels unaffected. In combination, however, a further decrease in cyclin D1 and D2 protein was observed (Fig. 4B, middle). Cyclin D complexed with Cdk4 is essential for the phosphorylation of the retinoblastoma tumor suppressor protein, in particular on residue Ser⁷⁹⁵ (41). Indeed, RAD001 and letrozole caused a slight increase in retinoblastoma mobility, indicative of dephosphorylation and activation as a suppressor of proliferation, but this was accentuated with the combination; in which case dephosphorylation of Ser⁷⁹⁵ was observed (Fig. 4B, right). Taken together, these data indicate that dual inhibition of E2 and mTOR signaling can result in more profound effects on G₁ regulators, culminating in a more pronounced G₁ accumulation.

Dual inhibition of mammalian target of rapamycin and estradiol signaling induces apoptosis of MCF7/Aro cells. Although inhibition of G₁ progression could explain how RAD001 and letrozole interact to inhibit tumor cell proliferation, with optimal drug concentrations we observed that the combination resulted in reduced cell numbers than present at the time of treatment initiation (data not shown). To analyze effects on cell viability, the YO-PRO survival assay

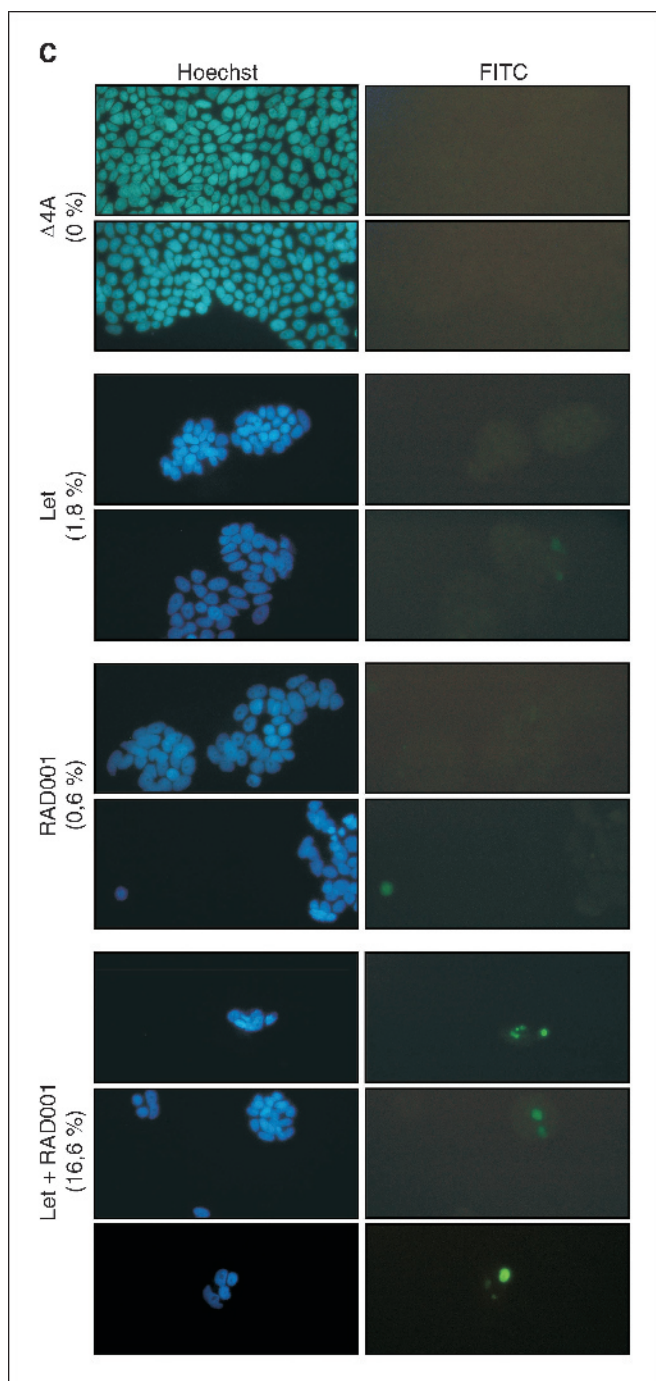


Fig. 5 continued. C, steroid-deprived MCF7/Aro cells seeded on gelatin-coated coverslips were treated with 10 nmol/L Δ 4A and 500 nmol/L letrozole, alone or in combination with 2 nmol/L RAD001 for 6 days. Adherent cells were stained for apoptotic cells by TUNEL as described in Materials and Methods and analyzed by fluorescent microscopy. Note that cell densities are in agreement with the antiproliferative effect of the single agent or combination treatments. Cells staining for FITC were quantified relative to the total number of cells (determined from the Hoechst dye). % Cells positive for TUNEL are shown in brackets (*left*).

was done. MCF7/Aro cells were treated with Δ 4A in the absence or presence of 2 nmol/L RAD001 or 100 or 500 nmol/L letrozole for 6 days. Treatment with RAD001 or letrozole alone had little effect on cell viability, indicating that both drugs are essentially cytostatic at the concentrations used in this study (Fig. 5A). However, combining the agents

resulted in a statistically significant decrease in cell survival ($P < 0.01$, two-way ANOVA). This was particularly evident with optimal (500 nmol/L) letrozole concentrations where a \sim 3-fold reduction in cell viability was observed with the combination versus the single agents (Fig. 5A). To establish whether reduced viability was due to programmed cell death (apoptosis), a flow cytometry-based TUNEL assay was done. Although 2 nmol/L RAD001 and 500 nmol/L letrozole alone had minimal effects on apoptosis, a significant potentiation of apoptotic cell death was observed with the combination ($P < 0.05$, Friedman test; Fig. 5B; ref. 34), again particularly evident with 500 nmol/L letrozole (apoptotic index: 5.6%, 2 nmol/L RAD001; 2.6%, 500 nmol/L letrozole; 20%, Combination). Induction of apoptosis was also confirmed using a fluorescence microscopy-based TUNEL assay. Treatment of MCF7/Aro cells with optimal concentrations of RAD001 or letrozole clearly reduced the number of cells per field, consistent with their antiproliferative effects (Figs. 1C, Fig. 3A, and Fig. 4A), but only 0.6% and 1.8% of the cells stained positively for TUNEL, respectively (Fig. 5C). In comparison, drug combination further reduced the number of cells per field and 16.6% of the cells stained positive for TUNEL. Taken together, these data show that combinations of RAD001 and letrozole trigger apoptotic cell death in MCF7/Aro cells.

Dual inhibition of mammalian target of rapamycin and estradiol signaling has enhanced antiproliferative activity and induces apoptosis in T47D/Aro cells. To investigate whether a similar interaction between RAD001 and letrozole occurs in another breast cancer model, steroid-deprived, aromatase-expressing T47D (T47D/Aro) cells were treated with Δ 4A in the absence or presence of increasing concentrations of RAD001 and letrozole, alone and in combination, for 6 days. As observed with the MCF7/Aro cells, estrogen-induced proliferation was inhibited in a concentration-dependent manner by both RAD001 and letrozole (Fig. 6A and B, respectively). Most importantly, combination of the two agents again exhibited increased antiproliferative potential (Fig. 6C). Two-way ANOVA indicated highly significant drug interactions ($P < 0.001$) and, according to Slinker (33), synergistic drug interaction. Determination of combination effects based upon limited isobologram data also indicated synergy (31, 32). Furthermore, TUNEL analysis again showed a significant potentiation of apoptosis with the combination ($P < 0.05$, Friedman test; Fig. 6D; ref. 34), which was particularly evident with higher letrozole concentrations (apoptotic index: 0.03%, 2 nmol/L RAD001; 0.04%, 200 nmol/L letrozole; 7.5%, Combination). Hence, data from this second model confirm the potential of this drug combination for the treatment of hormone-dependent breast cancer.

Discussion

With the development of targeted therapeutics, such as letrozole and RAD001, emerges a potential for combining these agents in rational, mechanism-based approaches to achieve a more potent antitumor effect in the patient. Clearly, in the case of the ER and mTOR signaling pathways, there is a large body of evidence suggesting that these pathways have distinct as well as overlapping signaling cascades and outputs (10, 16, 17, 26, 35, 39). With this in

mind, we have shown that E2-driven proliferation of MCF7 and T47D breast carcinoma cells is highly sensitive to the antiproliferative effects of RAD001; leading to a concentration-dependent accumulation of MCF7 in G₁ phase of the cell cycle. It has previously been reported that E2-driven proliferation of T47D cells in the absence of mitogenic support is almost completely abolished by rapamycin (35). Our data show that a similar phenomenon occurs in the presence of mitogens and are in agreement with previous work showing moderate effects of rapamycin treatment on E2-driven S-phase entry of MCF7 cells (39). This illustrates the central importance of the mTOR pathway and its dominance over mitogenic signaling in the context of estrogen response. In agreement with a dependency on mTOR, we further show that E2 treatment of MCF7/Aro cells resulted in rapid activation of the S6K1/S6 pathway and modulation of 4E-BP1/eIF-4E/eIF-4GI phosphorylation. This occurred within 4 hours of E2 addition, was also associated with Δ4A-induced proliferation, and was prevented by concomitant treatment with either RAD001 or letrozole. These data, therefore, provide the first report defining clear modulation of downstream elements of mTOR signaling in response to estrogen signaling. In addition, these data are consistent with a recent report that long-term estrogen deprivation enhances the sensitivity of MCF7 cells to the mitogenic effect of E2, a phenomenon associated with enhanced phosphorylation of S6K1 and 4E-BP1 (13). We have also defined a novel response of tumor cells to mTOR inhibition, characterized by increased phosphorylation of a major *in vivo* eIF-4E site (Ser²⁰⁹). We have observed a similar phenomenon with a number of tumor lines derived from prostate carcinoma and glioblastoma.³ The exact role of eIF-4E Ser²⁰⁹ phosphorylation is controversial (42); however, our data suggest a negative effect on translational events required for the proliferative response.

The major aim of our work was to evaluate the potential for combining RAD001 and letrozole in aromatase-expressing breast cancer cell lines. Strikingly, drug combinations significantly enhanced the antiproliferative activity compared with either agent alone, with statistical analysis indicating a synergistic interaction. Consistent with previous reports (24, 36), treatment of MCF7/Aro cells with letrozole or RAD001 induced G₁ accumulation; however, this was clearly increased with the combination. Both agents caused minor decreases in cyclin D1 and D2 expression after 4 hours of treatment, suggesting that these events are causative rather than a consequence of decreased proliferation. Effects on cyclin D were transient, as after 24 hours no effect was observed (data not shown), consistent with previous work (39, 43). Decreased cyclin D1 expression has also been reported after E2 ablation or letrozole treatment of MCF7 and aromatase-expressing MCF7 xenografts, respectively (36, 44, 45). Cyclin D-dependent kinases are essential regulators of retinoblastoma phosphorylation in early G₁ phase (41); indeed, retinoblastoma phosphorylation and inactivation as a cell cycle suppressor correlates with cyclin D1 induction in E2-stimulated steroid-deprived MCF7 cells (39, 43). Here, both letrozole and RAD001 treatment moderately increased retino-

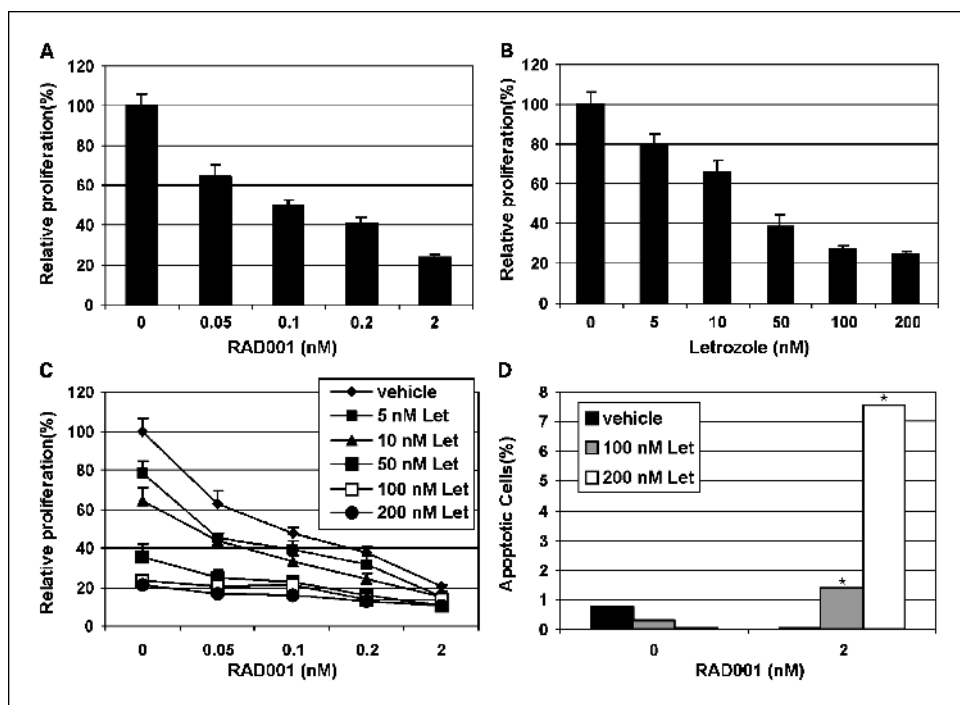
blastoma mobility indicative of decreased phosphorylation. Moreover, combination of both agents caused a more profound decrease in both cyclin D1 and D2 levels, accompanied by greater effects on retinoblastoma protein mobility/phosphorylation; fully supportive of the increased antiproliferative effect and G₁ accumulation observed. Taken together, our data show for the first time that concomitant inhibition of the mTOR pathway and estrogen signaling causes more profound effects on G₁ progression and key G₁ regulators.

The aim of cancer therapy is to eradicate tumors rather than purely delay or halt development. In this context, we further show potentiation of apoptotic cell death with combinations of both agents, which alone have minimal effects on cell viability. This potentiation occurred after prolonged treatment with the agents (i.e., 6 days). Estrogen withdrawal or letrozole treatment has been previously reported to induce apoptotic cell death in MCF7 xenografts (36, 44, 45). This observation was substantiated *in vitro*, where estrogen withdrawal, antiestrogens, and aromatase inhibitors were shown to induce apoptosis to varying degrees in aromatase-expressing MCF7 cells (36). In our study, letrozole alone had minimal effects on cell viability when used at optimal concentrations, contrary to the more profound effects reported by others following TUNEL assay (36). We have confirmed our results using two TUNEL assay approaches (fluorescence-activated cell sorting and fluorescence microscopy) and the YO-PRO survival assay and suggest that the differences observed between our data and that of Thiantanawat et al. (36) may be based on the supraoptimal letrozole concentrations and longer incubation periods (i.e., 8 days) used in the latter case. In the presence of mitogens and sufficient nutrients, rapamycins generally act cytostatically, as observed here for RAD001. However, under stress conditions, potentiation of cell death has been reported in certain cellular backgrounds (19, 22, 46, 47). Here we show that in a "stress" situation that recapitulates E2 deprivation (i.e., in the presence of letrozole), concomitant RAD001 treatment potentiates a significant induction of breast tumor cell death. Previous studies have indicated that E2 protects against cell death by increasing the levels of the antiapoptotic protein Bcl-2 (48). Similarly, it was reported that antiestrogens and aromatase inhibitors induce cell death *in vitro* by increasing the expression of the proapoptotic protein Bax and decreasing Bcl-2 expression, a phenomenon correlating with increased caspase activity (36). Although we also looked for specific effects on apoptotic regulators in the combination-treated cells, the analysis was complicated by a general reduction in protein expression coinciding with the induction of cell death/apoptosis (data not shown). Hence, although reduced protein expression is not surprising considering the role of the mTOR pathway in the regulation of global protein translation (22), this hindered a concrete analysis of the molecular basis of the increased cell death observed with the combination.

Taking all these data together, the more profound effects of RAD001/letrozole combinations on both cell cycle progression and survival, in breast lines sensitive to both agents alone, point to a clear potential for combining these agents for the treatment of ER-positive breast cancers. However, although endocrine therapy is one of the most effective systemic therapies for hormone receptor-positive breast cancer patients, efficacy is

³ Unpublished data.

Fig. 6. Dual inhibition of mTOR and estradiol signaling has enhanced antiproliferative activity and induces apoptosis in T47D/Aro cells. Steroid-deprived T47D/Aro cells were treated with 10 nmol/L Δ 4A and increasing concentrations of RAD001 or letrozole (Let) alone (A and B, respectively) or in combination (C) for 6 days. Relative proliferation was assessed by extraction and protein determination. Columns, means of triplicate values; bars, \pm SD. Two-way ANOVA indicated highly significant ($P < 0.001$), synergistic drug interactions. D, steroid-deprived T47D/Aro cells were treated with 10 nmol/L Δ 4A in the absence or presence of 100 or 200 nmol/L letrozole, alone or in combination with 2 nmol/L RAD001. Numbers of apoptotic cells were evaluated using a cytometry-based TUNEL analysis as described in Materials and Methods. Stars, $P < 0.05$, Friedman test.



often limited by the presence/onset of resistance (49). Aromatase inhibitors have a different mode of action than selective estrogen receptor modulators (1, 5), and indeed, agent-selective resistance seems to exist (49), a situation highlighted by the observation that patients who relapse after previous response to tamoxifen can subsequently respond to aromatase inhibitors (6, 7). Whether resistance is defined as agent-selective, pan, intrinsic or acquired, there is compelling evidence that up-regulation of signal transduction pathways (exemplified by increased ErbB2 signaling in tamoxifen-resistant breast cancer cells) plays a major role in resistance to endocrine therapies. Thus, clinical trials are ongoing, to examine combinations of endocrine agents with ErbB receptor inhibitors (49). Although estrogen deprivation may be more effective than antagonizing the ER in terms of levels of inherent or acquired resistance, it is conceivable that resistance will eventually develop. In this context, Akt is an essential mediator of ErbB-dependent antiestrogen resistance (17), activation predicts a worse clinical outcome among endocrine-treated patients (16) and mTOR inhibition has been observed to restore tamoxifen response in a

breast cancer xenograft model expressing a constitutively active allele of Akt (50). Increased Akt phosphorylation and mTOR effector activation have also been shown in long-term estrogen-deprived cells (13), indicating that up-regulation of Akt/mTOR signaling is also fundamental to the adaptation of breast cancer cells to low E2 levels in cultured cells, a situation that could be said to mimic therapy with aromatase inhibitors. We propose that the use of mTOR inhibitors (such as RAD001) in combination with letrozole provides a rational approach not only in breast tumors sensitive to both agents alone (as shown here) but may also have potential as an approach to circumvent/ combat endocrine resistance in the clinic.

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