The farnesyltransferase inhibitor R115777 (tipifarnib) in combination with tamoxifen acts synergistically to inhibit MCF-7 breast cancer cell proliferation and cell cycle progression in vitro and in vivo

Lesley-Ann Martin,1 Julia E. Head,2 Sunil Pancholi,2 Janine Salter,2 Emma Quinn,3 Simone Detre,3 Stan Kaye,1 Angela Howes,5 Mitch Dowsett,2,3 and Stephen R.D. Johnston4

1Section of Medicine, Institute of Cancer Research, Sutton, United Kingdom; 2Breakthrough Breast Cancer Centre and Department of Academic Biochemistry, Institute of Cancer Research; 3Breast Unit, Royal Marsden Hospital, London, United Kingdom; and 4Oncology Drug Development, Johnson and Johnson PRD, High Wycombe, United Kingdom

Abstract
Cross-talk between receptor tyrosine kinases and estrogen receptor is at least partly responsible for the development of acquired resistance to endocrine therapies. Hence, targeting receptor tyrosine kinases and their downstream partners with inhibitors/antagonists may reverse this resistance. Although ras mutations are rare in breast cancer (2%), aberrant function of Ras signal transduction pathways is common. We therefore investigated the efficacy of the farnesyltransferase inhibitor (FTI) R115777 (tipifarnib) in combination with tamoxifen in MCF-7 human breast cancer models both in vitro and in vivo. There was a synergistic antiproliferative interaction between R115777 and 4-hydroxy-tamoxifen in vitro as calculated by median effect analysis. The combination resulted in a significantly greater G1 arrest than either drug alone and this was associated with marked inhibition of cyclin D1 and induction of the cell cycle inhibitor p27kip1. Combining R115777 with either tamoxifen or estrogen withdrawal in vivo produced a significantly greater inhibition of tumor growth and lower xenograft cell proliferation than either therapy alone. These results suggest that the combination of this FTI with endocrine therapy may be of therapeutic benefit in the treatment of breast cancer. Enhanced G1 arrest due to modulation of cell cycle regulatory proteins may be the underlying mechanism for the positive interaction between FTIs and tamoxifen. [Mol Cancer Ther 2007;6(9):2458–67]

Introduction
Ras plays a crucial role in linking tyrosine kinase activation to downstream cytoplasmic and nuclear targets (1), ultimately leading to cell proliferation and cell survival. Although Ras is not mutated in breast cancer, abnormal expression or signaling through Ras is common (2); therefore, Ras has been a target for the development of signal transduction inhibitors in breast cancer.

For the Ras protein to become activated prenylation-dependent association with the cell membrane is necessary (3) This posttranslational processing of Ras involves the enzyme farnesyltransferase, which transfers a 15-carbon farnesyl isoprenoid group from farnesyl diphosphate to the COOH-terminal tetrapeptide CAAX sequence. Farnesyltransferase inhibitors (FTI) were thus developed as a novel therapy to target aberrant Ras function in cancer. R115777 (tipifarnib, Zarnestra) is a methyl quinolone and is one of the most advanced FTIs now in clinical development.

A critical observation was the finding that the sensitivity of various human tumor cell lines to FTIs did not correlate with the presence of oncogenic Ras mutations (4). Indeed, breast cancer cells that lacked Ras mutations but had the active protein kinase were sensitive to FTIs (5). Interest has surrounded the potential for FTIs to be combined with endocrine agents, to overcome or prevent endocrine resistance. It has become clear that estrogen receptor (ER) signaling and growth factor pathways cross-talk to modulate endocrine response in breast cancer cells during prolonged therapy (6, 7). Various signal transduction inhibitors including tyrosine kinase and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitors are effective in treating or even preventing endocrine-resistant growth in both in vitro and in vivo experimental models (8, 9). We have shown previously that the FTI R115777 delays the growth of hormone-dependent MCF-7 breast cancer xenografts in vivo (10). Clinical trials of R115777 have shown that at 300 mg twice daily, maximum plasma concentrations in the order of 600 to 900 ng/mL are achievable (11, 12) and R115777 can induce objective tumor regressions together with stable disease ≥24 weeks in 25% of patients with advanced breast cancer (13).
The aim of our currently reported studies was to determine the efficacy of the combination of R115777 and tamoxifen in MCF-7 breast cancer models both in vitro and in vivo and to examine the possible mechanisms for any interaction observed.

Materials and Methods

Cell Culture

Wild-type MCF-7 cells were maintained in phenol red–free RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies) and 1 nmol/L estradiol (E2; Sigma). SKBR3 cells were grown in phenol red–containing RPMI 1640 supplemented with 10% fetal bovine serum. For all experiments, cell lines were stripped of steroids for 3 days before seeding by culturing in phenol red–free RPMI 1640 supplemented with 10% (v/v) dextran-coated charcoal-stripped fetal bovine serum (14). Cells were treated with 1 nmol/L E2 plus R115777 (Johnson and Johnson R&D), 4-hydroxy-tamoxifen (4-OH-tamoxifen; Calbiochem), or both drugs concomitantly. R115777 was dissolved in acidified H2O. 4-OH-tamoxifen was dissolved in 100% ethanol, and the final concentrations of ethanol and HCl were 0.1% (v/v) and had no effect on the rate of cell proliferation.

Cell Growth Assays

Steroid-depleted cells were seeded into 12-well plates at a density of ~1 × 10⁴ cells per well or into 96-well plates at a density of 4,000 cells per well, in dextran-coated charcoal medium. After 24 h, monolayers were treated with E2 plus inhibitors either alone or in combination. The 12-well plates were treated for 6 days with daily changes. Cell number was then determined using a Z1 Coulter counter (Beckman Coulter). The 96-well plates were treated with a single dose and left for 96 h at which time cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (15). The interaction between R115777 and 4-OH-tamoxifen was analyzed by the median effect plot method described by Chou and Talalay (16). Calculation of the combination index took into account a nonfixed drug ratio and was based on the assumption that the action of the two drugs was mutually nonexclusive for the strict detection of synergism. A combination index < 1 indicates synergism, combination index = 1 indicates additivity, and a combination index > 1 indicates antagonism. Experiments were repeated thrice.

Cell Cycle Analysis

MCF-7 cells, depleted previously of steroids for 3 days, were seeded into 10-cm dishes and treated with vehicle (0.01% v/v ethanol), E2, R115777, 4-OH-tamoxifen, or a combination of agents at the concentrations indicated. Attached and detached cells were harvested 48 h after treatment. Attached cells were collected by centrifugation (100 × g, 5 min). Attached cells were harvested by trypsinization. Cell pellets for both attached and detached cells were washed with PBS and then fixed in ice-cold 70% ethanol. For cell cycle analysis, cells were recentrifuged and stained with propidium iodide (Sigma). DNA content was determined by flow cytometry (Coulter EPICS Elite ESP system, Beckman Coulter). The proportion of cells in S and G2-M phase of the cell cycle was calculated from DNA histograms using WinMDI version 2.8. Statistical analysis was done using the Student’s-Newman-Keuls’ test.

Western Blot Analysis

MCF-7 cells were stripped of steroids for 3 days and then seeded at a density of 2 × 10⁴ cells per 10-cm dish. The next day, monolayers were serum starved for 24 h. Cells were subsequently treated for 48 h, unless otherwise stated, with vehicle, E2, 4-OH-tamoxifen, R115777, or a combination of agents. Cell monolayers were washed with ice-cold PBS and then lysed in extraction buffer as described previously (8). Equal amounts of protein (50 μg) were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. Proteins were detected using enhanced chemiluminescence plus reagent (Amersham Pharmacia) after incubation overnight at 4°C for primary or 2 h at room temperature for secondary antibodies. The following primary antibodies were used: phosphorylated ERK1/2 (pERK1/2) and total ERK1/2, phosphorylated (pAKT) and total AKT, p21, cyclin D1, and phosphorylated Erα Ser118 (all of which were purchased from Cell Signaling, Inc.); total ER (6F11; Novacastra Laboratories Ltd.); HDJ2 (Neomarkers); and actin (AC-20; Sigma). Secondary antibodies (anti-mouse and antirabbit horseradish peroxidase) were obtained from Amersham Pharmacia.

ER Transcriptional Analysis

MCF-7 cells stripped previously of steroids for 3 days were seeded in 24-well plates and transfected by Lipofectin (Invitrogen) with 0.25 μg EREIItkluc (luciferase reporter plasmid) and 0.25 μg pCH110 (β-galactosidase for normalizing luciferase data) for 4 h as described previously (8). Monolayers were treated for 24 h with E2, 4-OH-tamoxifen, R115777, or a combination of agents. Luciferase and β-galactosidase activity were measured using a luminometer.

Immunofluorescence

Cells were stripped of steroid for 2 days and then plated at a density of 5 × 10⁴ cells on glass coverslips. Monolayers were treated with the drug combinations indicated in the figure legends. After 16 h of treatment, coverslips were washed in PBS. Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Cells were incubated in monoclonal antihuman p27 antibody (DakoCytomation) diluted 1:200 and then lysed in extraction buffer as described previously (8). Monolayers were treated for 24 h with E2, 4-OH-tamoxifen, R115777, or a combination of agents. Luciferase and β-galactosidase activity were measured using a luminometer.

Figure Legends

1. Immunofluorescence

2. Western Blot Analysis

3. Cell Cycle Analysis

4. Cell Growth Assays

5. Materials and Methods

6. Results

7. Conclusions

8. Acknowledgments

9. References
**Terminal Deoxynucleotidyl Transferase – Mediated dUTP Nick End Labeling**

Cells were treated as described for immunofluorescence. After 48 h of treatment with the drug combinations indicated in the figure legends, apoptosis was detected using the TACS TdT *in situ* Apoptosis Detection kit (R&D Systems) following manufacturer’s protocols. Cells on coverslips were mounted onto glass slides using Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole. Images were collected sequentially in two channels on a Leica TCS SP2 confocal microscope.

**Human Tumor Xenografts**

All experiments were carried out in accordance with Home Office guidelines and Institute of Cancer Research Ethics Committee. Female ovariectomized Ncr foxhead nude mice were kept under sterile conditions with free access to food and water. MCF-7 xenografts were initiated by implantation of 2-mm diameter tumor fragments from established tumors. Tumor growth was maintained by E2 supplementation through i.d. injection of 17β-estradiol pellets (dose 1.7 mg over 60 days; Innovative Research of America). Once tumors reached a diameter of ~7 mm, mice were randomized to receive vehicle [20% w/v β-cyclodextrin (pH 2.5) for R115777, 50% PEG 300, 50% H2O + 1 drop 1N HCl per 3 mL for tamoxifen], R115777 (50 mg/kg twice daily), tamoxifen (20 mg/kg), or a combination of both R115777 and tamoxifen. Two further treatment arms were used to assess the effect of E2 withdrawal (removal of the E2 pellet) or E2 withdrawal combined with R115777 (50 mg/kg twice daily). All drugs were given by oral gavage daily for 5 consecutive days followed by a 2-day rest period, for a total of 19 days. The experiment was done twice giving similar results; therefore, the growth data were combined for statistical analysis. There were six tumor-bearing animals in each group and all tumors were harvested on day 19. Tumor volumes were calculated using the formula \( a^2 \times \frac{b \times \pi}{6} \), where \( a \) and \( b \) are orthogonal tumor diameters and expressed as a percentage of the volume at the start of treatment (relative tumor volume). Overall statistical difference was calculated using the Kruskal-Wallace test and statistical differences between individual treatment arms were calculated using the Mann-Whitney test.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Growth response of MCF-7 to R115777 in combination with 4-OH-tamoxifen reveals synergy. MCF-7 cells were treated with increasing doses of 4-OH-tamoxifen (A) in the presence 1 nmol/L E2 or R115777 (B). Data are expressed as fold change compared with control. C and D, duplicate experiments (as described in A and B) were carried and assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay after 96 h of treatment. Points, fold change relative to control; bars, SE.
Xenograft Proliferation and Apoptosis

Markers for proliferation and apoptosis were assessed by immunohistochemistry and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling, respectively, on formalin-fixed paraffin-embedded xenograft tissue taken on day 19. Briefly, 4-μm sections were dewaxed and rehydrated and antigens were retrieved using citrate buffer (pH 6.0) before endogenous peroxidase activity was blocked. Proliferation was shown using an antibody against Ki-67 (MIB-1; DakoCytomation) at a dilution of 1:50. Antigen expression was visualized using the Strept ABC system (DakoCytomation) and diaminobenzidine (Sigma). All immunohistochemistry slides were examined by light microscopy. Apoptosis was determined by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay as described previously (18). MIB-1 was scored as percentage of tumor cells positive in 10 high-powered fields (>×100). Apoptosis was assessed as percentage of positive apoptotic bodies in a minimum of 3,000 tumor cells, excluding areas of necrosis. A proliferation/apoptosis index (CTI) was also calculated for each individual tumor (19).

Results

Synergistic Interaction between R115777 and 4-OH-Tamoxifen in MCF-7 Cells

MCF-7 cell proliferation assays showed that the IC₅₀ for 4-OH-tamoxifen in the presence of E₂ (1 nmol/L) was between 10 and 100 nmol/L (Fig. 1A). Combination experiments with R115777 were therefore carried out with a fixed suboptimal 10 nmol/L dose of 4-OH-tamoxifen so that the effects of the FTI were not masked. Increasing doses of R115777 in the presence of E₂ inhibited growth in MCF-7 cells in vitro with an IC₅₀ of ~400 nmol/L (Fig. 1B). Combining R115777 with 10 nmol/L 4-OH-tamoxifen in the presence of E₂ reduced the IC₅₀ 8-fold from 400 to 50 nmol/L (Fig. 1B). The experiment was repeated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and similar results were observed (Fig. 1C and D). Further analysis using the median effect method of Chou and Talalay (14) revealed combination indices <1 for each dose of R115777 tested, indicating a synergistic relationship between the FTI and 4-OH-tamoxifen in MCF-7 cells (Supplementary Table S1).

As expected, 4-OH-tamoxifen had no inhibitory effect on the ER-negative cell line SKBR3 or any effect on the IC₅₀ of R115777 in these cells (Supplementary Fig. S1).

The Interaction between R115777 and 4-OH-Tamoxifen Does Not Involve ER/ERE–Directed Transcription

To assess the effects of R115777 alone or in combination with 4-OH-tamoxifen on ER/ERE transcription, MCF-7 cells were transfected with a reporter construct consisting of two copies of an ERE upstream of a luciferase reporter gene. Treatment with escalating doses of R115777 alone had no effect on ER/ERE transactivation compared with the vehicle-treated control (Fig. 2A). Similarly, whereas addition of E₂ resulted in a 8-fold increase in ERα transactivation compared with the vehicle-treated control (Fig. 2B), addition of increasing doses of R115777 had no added effect. Treatment with 10 nmol/L 4-OH-tamoxifen

---

*Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
inhibited the stimulatory effect of E2 by ~80% (Fig. 2C). R115777 in combination with 4-OH-tamoxifen, however, did not enhance this, suggesting that the synergistic interaction between the two drugs does not involve direct interaction with ER/ERE–directed transcription.

The Interaction between R115777 and 4-OH-Tamoxifen on ER, ERK1/2, and AKT Signal Transduction Pathways in MCF-7 Cells

The ERK1/2 and AKT signal transduction pathways are known to cross-talk with ER modulating endocrine sensitivity (reviewed in ref. 20). We therefore investigated the interaction between R115777 and 4-OH-tamoxifen on pERK1/2, pAKT, and phosphorylated ERα Ser118. Western blot analysis of cells treated for 48 h with the agents alone or in combination showed that E2 decreased total ERα while increasing expression of phosphorylated ERα Ser118, pAKT, and pERK1/2 compared with the vehicle control (Fig. 3). Addition of 4-OH-tamoxifen had little effect on the expression of the phosphorylated proteins compared with E2 alone. However, R115777 in combination with E2 caused a further decrease in total ER compared with E2 alone and a slight elevation in phosphorylated ERα Ser118. pERK1/2 revealed a minimal decrease, whereas pAKT was reduced almost to control levels (Fig. 3). A combination of R115777 and 4-OH-tamoxifen resulted in a marked increase in phosphorylated ERα Ser118 compared with E2 or E2 plus 4-OH-tamoxifen. Similarly total ER seemed elevated with this combination compared with E2 alone and pERK1/2 levels were similar to E2 alone and higher than those seen with R115777 plus E2. pAKT expression remained suppressed similar to the levels seen in cells treated with E2 plus R115777. Total protein levels for ERK1/2 and AKT remained comparable for each treatment. To monitor the efficiency of the FTI, whole-cell extracts from each treatment were probed for HDJ2 (21). Only in the presence of R115777 alone or in combination was an unpreylnated form of HDJ2 detected.

The Combination of R115777 and 4-OH-Tamoxifen Results in a Greater G1 Arrest than Either Drug Alone

Tamoxifen and FTIs are both known to induce G1 cell cycle arrest (22, 23). We therefore examined the effects of the drugs alone and in combination on the MCF-7 cell cycle. MCF-7 cells were treated with E2 (1 nmol/L), 4-OH-tamoxifen (10 nmol/L), R115777 (100 nmol/L), or a combination. Initial cell cycle analysis at 24, 48, 72, and 96 h showed maximal effects for the drugs alone and in combination at 48 h (data not shown) in keeping with previous studies (24). Therefore, a 48-h time point was chosen for all subsequent experiments. Analysis of the vehicle-treated control showed 8% of cells in S phase and 87% cells in G1 (Fig. 4A). Treatment with E2 increased the number of cells entering S phase from 8% to 30%, with a concomitant reduction in the number of cells in G1 phase from 87% to 58%. 4-OH-tamoxifen in the presence of E2 minimally inhibited the cells entering into S phase (30% to 27%) and caused a small increase in the number of cells in G1 (56–64%). This minimal effect was anticipated as only a low dose of 4-OH-tamoxifen was used so as not to mask any effects seen with the combination. R115777 had a similar effect to 4-OH-tamoxifen with 20% of cells moving into S phase and 66% of cells remaining in G1. There was no effect of either drug on the percentage of cells in G2-M phase (data not shown). The combination, however, resulted in a greater G1 arrest characterized by only 15% of cells in S phase and 76% of cells in G1, giving a cell cycle profile closely resembling the vehicle-treated controls (Fig. 4A). The increase of cells in G1 phase and decrease of cells in S phase were statistically significant (P < 0.05) when the combination arm was compared with either R115777 or 4-OH-tamoxifen alone.

Although our growth progression (data not shown) and cell cycle analysis suggested that the drug combination caused a cytostatic rather than cytotoxic effect, several studies in various tumor types have shown that FTIs can increase apoptosis (4, 25–28). To assess this, MCF-7 cells were treated for 48 h with R115777 alone or in combination with 4-OH-tamoxifen. Analysis using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining showed no increase in apoptosis (Fig. 4B).

R115777 and 4-OH-Tamoxifen Inhibit Cyclin D1 Activity and Induce p27kip1

Based on the above data, we investigated whether the expression of cyclin D1, p21waf1/cip1, or p27kip1 altered with treatment. No consistent changes were seen in the expression of p21waf1/cip1 with any treatment (data not shown). However, control cells shown previously to be in G1 arrest had high expression of p27kip1 at 16 h and a low expression of cyclin D1 at 48 h (Fig. 5A). The addition of...


E2 to the medium decreased the expression of p27kip1 at 16 h and increased the expression of cyclin D1 at 48 h. Individually, R115777 and 4-OH-tamoxifen reduced the E2-stimulated expression of cyclin D1 at 48 h and had no effect on p27kip1 levels at 16 h. When the two inhibitors were given together, there was a marked decrease in cyclin D1 expression at 48 h with a parallel increase in p27kip1 at 16 h of a similar intensity to the vehicle-treated controls (Fig. 5A). Phosphorylation of p27kip1 is widely recognized as the major regulatory mechanism influencing protein abundance. Phosphorylation of threonine 187 (p27kip1Thr187) by cyclin-dependent kinase 2 results in the association of p27kip1 with ubiquitin ligase SCF SKP2 leading to proteosomal degradation (29–31). Conversely, phosphorylation of Ser10 (p27kip1Ser10) by human kinase interacting stathmin stabilizes the p27kip1 protein in G1 (32, 33). We therefore assessed the phosphorylation of these two common sites on p27kip1 using phospho-specific antibodies. As shown in Fig. 5B, control cells in G1 arrest showed high levels of p27kip1Ser10 and corresponding low levels of p27kip1Thr187. Addition of E2 caused a decrease in p27kip1Ser10 and increase in p27kip1Thr187. Treatment with E2 and 4-OH-tamoxifen showed a similar profile to E2 alone. However, R115777 alone showed an increase in both p27kip1Thr187 and p27kip1Ser10, whereas the combination of R115777 with 4-OH-tamoxifen significantly decreased p27kip1Thr187 and increased p27kip1Ser10 in keeping with the levels seen in the G1 arrested control.

Recent studies have suggested that AKT phosphorylates p27 on Thr157 (p27kip1Thr157) suppressing nuclear import and subsequent p27-driven G1 arrest (34). We therefore assessed p27kip1Thr157 and showed that 4-OH-tamoxifen in combination with R115777 reduced phosphorylation to a similar degree as seen in the G1 arrested control. Evaluation of p27kip1 by confocal microscopy (Fig. 5C) showed predominantly nuclear staining in the control cells under G1 arrest. Addition of E2 significantly reduced this. Treatment with 4-OH-tamoxifen increased p27kip1 compared with E2 as did R115777. The combination of 4-OH-tamoxifen and R115777 seemed to enhance expression to a greater extent compared with either monotherapy.

Figure 4. R115777 in combination with 4-OH-tamoxifen (4-OHT) enhances G1 arrest compared with 4-OH-tamoxifen alone but does not induce apoptosis. A and B, synchronized MCF-7 monolayers were treated with vehicle, E2 (1 nmol/L), or R115777 (100 nmol/L) ± 4-OH-tamoxifen (10 nmol/L). Cell cycle was monitored by fluorescence-activated cell sorting analysis of cells stained with propidium iodide after 48 h of treatment. DNA histogram was analyzed to compare the percentage of cells in S phase (A) and G1 phase (B) of the cell cycle. Columns, % of attached cell number; bars, SE. *, P < 0.05 combination compared with 4-OH-tamoxifen or R115777 alone. C, synchronized MCF-7 cells treated as described above were assayed for apoptosis using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL). 4-OH-T and 4-OH-tamoxifen.
Combining R115777 with Either Tamoxifen or E2 Withdrawal Produces Significantly Greater Inhibition of Tumor Growth than Either Therapy Alone

We have shown previously that MCF-7 human breast cancer xenografts are growth inhibited by continuous daily treatment with R115777 (10). In light of our in vitro data and the suggestion of a synergistic interaction between R115777 and 4-OH-tamoxifen, we studied the antitumor activity of R115777 and tamoxifen in mice bearing subcutaneous MCF-7 breast cancer xenografts. We also investigated the effect of the FTI in combination with E2 withdrawal. Combined therapy with tamoxifen and R115777 produced greater tumor growth inhibition when compared with either drug alone (Fig. 6A) and, similarly, E2 deprivation and R115777 in combination resulted in greater growth inhibition than either E2 deprivation or R115777 alone (Fig. 6B). R115777 had only a modest effect compared with E2 supplemented controls with a mean relative tumor volume of 4.85 versus 5.06 (Supplementary Table S2), whereas tamoxifen seemed to induce tumor stasis (mean relative tumor volume, 1.9). The combination of tamoxifen and R115777 (mean relative tumor volume, 0.7) produced a statistically greater tumor regression when compared with tamoxifen alone ($P < 0.05$) or R115777 alone ($P < 0.001$). Withdrawal of E2 alone induced significant tumor regression (mean relative tumor volume, 2.01) and this effect was enhanced with R115777 addition (mean relative tumor volume, 0.9; $P < 0.05$). No differences between the control and treatment arms in the number of deaths were noted or any adverse effects on body weight (Fig. 6C).

The Combination of R115777 and Tamoxifen or E2 Withdrawal Resulted in Significantly Lower Xenograft Cell Proliferation than Either Treatment Alone

Tumors were harvested from each group both at day 5 and day 19. Formalin-fixed tissue was analyzed for cell proliferation by Ki-67 immunostaining. Ki-67 was lower in the tumors treated with E2 withdrawal plus R115777 compared with E2 withdrawal alone (mean, 7.6% versus 7.6%; $P = 0.029$; Supplementary Table S2). Similarly, the combination of tamoxifen and R115777 resulted in significantly lower Ki-67 compared with either tamoxifen or R115777 alone (mean of 5% versus 16.9% ($P = 0.11$) and 67.3% ($P = 0.029$), respectively). In contrast, no significant difference in apoptotic scores was seen between the
treatment groups. Analysis of the CTI showed the control group to be ~25, whereas treatment with tamoxifen or E2 withdrawal reduced the CTI to 3. R115777 alone also reduced the CTI compared with control. The combination of tamoxifen and R115777 or R115777 coupled with E2 withdrawal was most effective at lowering the CTI (0.8 and 0.7, respectively), which may account for the decrease in tumor volume.

Discussion
R115777 is one of the most advanced FTIs in development and has already reached phase II/III trials as monotherapy and in combination with cytotoxics or endocrine therapy. We have shown previously that R115777 can inhibit growth in ER-positive breast cancer cells in vitro and in vivo (10) and that the drug is efficacious as monotherapy in patients with advanced breast cancer (13). Based on our increasing knowledge of the interactions between ER and growth factor receptor signaling, there is a rationale for combining signal transduction inhibitors with endocrine therapy in breast cancer (35). The aim of this study was to investigate the effects of the FTI R115777 in combination with tamoxifen in vitro and in vivo, in breast cancer models.

Our results reveal that submicromolar concentrations of R115777 (>50 nmol/L) induce significant growth inhibition of MCF-7 cells in vitro when given in a single dose. The IC50 was ~400 nmol/L and the dose-response curve was relatively flat compared with those obtained with cytotoxics, in keeping with previously published data (10). However, we have shown that the addition of a suboptimal dose of 4-OH-tamoxifen (10 nmol/L) enhanced the efficacy of R115777 in MCF-7 cells, reducing the IC50 ~8-fold. Further analysis by the median effect method of Chou and Talalay (16) showed a strong synergistic interaction between R115777 and 4-OH-tamoxifen. In support of this, recently published data with FTI-277 have also shown either synergistic or additive effects when combined with tamoxifen (24, 36). The lack of any interaction between R115777 and 4-OH-tamoxifen in the ER-negative SKBR3 breast cancer cell line suggests that the synergy seen in the ER-positive MCF-7 cells might be explained by an interaction at the level of the ER. However, R115777 in the presence or absence of E2 had no effect on MCF-7 cell ERE/ERα–directed transcription. 4-OH-tamoxifen in the presence of E2 reduced the transcriptional activity as expected, but the addition of R115777 did not enhance this effect. This is in contrast to recently published data that showed that FTI-277 increased ERα transcriptional activity in both the presence and the absence of E2 (37). However, this effect was seen in MCF-7 cells that were stably, not transiently, transfected with a luciferase reporter and required preincubation with the FTI for 24 h before addition of E2.

Tamoxifen and FTIs are both known to induce cell cycle arrest (22, 23) and many prenylated proteins are components of signal transduction pathways stimulated by E2. We therefore studied, in the presence of E2, the effect of R115777 and 4-OH-tamoxifen alone or in combination on MCF-7 cell cycle progression. Both inhibitors at suboptimal concentrations inhibited E2-stimulated cell cycle progression inducing a G1 cell cycle arrest but the combination had a significantly greater effect than either drug alone. This suggests that farnesylated proteins are involved in the mitogenic response of MCF-7 cells to estrogen and that a FTI in conjunction with endocrine therapy could be a more effective way to inhibit E2-stimulated growth. These data concur with recently published cell cycle data using the FTI-277 in combination with tamoxifen (24).

Antiestrogens modulate the cell cycle through a decrease in cyclin D1 expression and an increase in the cell cycle

Figure 6. R115777 in combination with tamoxifen or E2 withdrawal was more effective at reducing tumor volume compared with either treatment alone. A, MCF-7 xenografts were grown in the presence of E2. Once tumors reached the desired size, animals were randomized to receive vehicle, E2, tamoxifen, R115777, or a combination of agents. B, once tumors had reached the desired size, animals were randomized into groups and treated with vehicle, E2, withdrawal of the E2 support, R115777, or a combination of E2 withdrawal and R115777 addition. Tumor volumes were measured at the intervals indicated. Bars, SE. C, adverse effects on body weight were monitored during the course of treatments shown in (A).
inhibitor p21\textsuperscript{waf/cip1} (38). It has been shown that the cell cycle inhibitor p27\textsuperscript{kip1} is also essential for tamoxifen-induced G\textsubscript{1} arrest (39). In MCF-7 cells, FTIs have been shown to increase p21\textsuperscript{waf/cip1} transcription (22) and to increase the association of p27\textsuperscript{kip1} with cyclin E-cyclin-dependent kinase 2 complexes, leading to G\textsubscript{1} arrest (24). We have shown in vitro that R115777 in combination with 4-OH-tamoxifen increased p27\textsuperscript{kip1} expression to a similar level seen in the G\textsubscript{1} arrested control. The combination decreased threonine phosphorylation of p27\textsuperscript{kip1} at position 187 and increased serine phosphorylation at position 10. This suggests that up-regulation of p27\textsuperscript{kip1} in response to the combination of R115777 with 4-OH-tamoxifen may be the result of posttranslational mechanisms.

In conclusion, these results add to the increasing body of evidence that combinations of signal transduction inhibitors with endocrine therapy may be of therapeutic benefit in the treatment of breast cancer. Our data, together with others, suggest that enhanced G\textsubscript{1} arrest due to modulation of cell cycle regulatory proteins may be the underlying mechanism for the synergistic/additive interaction of FTIs with tamoxifen. Several small phase I/II trials have been initiated with FTIs in combination with endocrine therapies, including tamoxifen, fulvestrant, or an aromatase inhibitor (44). The ultimate clinical test for the hypothesis that FTIs may enhance the efficacy of endocrine therapy is the randomized controlled trial and results from a recently initiated with FTIs in combination with endocrine therapy is the randomized controlled trial and results from a recently completed study with letrozole ± R115777 are awaited. Preclinical evaluation remains critical in developing logical combinations for novel therapeutics such as FTIs. The current data support the view that such therapies could further enhance the benefit of endocrine therapy in the treatment of breast cancer.

Acknowledgments

We thank Lisa Patterson, Melanie Valenti, and Bhavani Krishnan (Section of Medicine, Institute of Cancer Research, Sutton, United Kingdom) for their expert technical assistance.

References


Molecular Cancer Therapeutics

The farnesyltransferase inhibitor R115777 (tipifarnib) in combination with tamoxifen acts synergistically to inhibit MCF-7 breast cancer cell proliferation and cell cycle progression \textit{in vitro} and \textit{in vivo}

Lesley-Ann Martin, Julia E. Head, Sunil Pancholi, et al.

\textit{Mol Cancer Ther} 2007;6:2458-2467.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/6/9/2458

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2011/08/10/6.9.2458.DC1

Cited articles
This article cites 44 articles, 23 of which you can access for free at:
http://mct.aacrjournals.org/content/6/9/2458.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/6/9/2458.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.