Enhancement of the antitumor activity of tamoxifen and anastrozole by the farnesyltransferase inhibitor lonafarnib (SCH66336)

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Lonafarnib is an orally bioavailable farnesyltransferase inhibitor. Originally developed to block the membrane localization of Ras, subsequent work suggested that farnesyltransferase inhibitors mediate their antitumor activities by altering the biological activities of additional farnesylated proteins. Breast tumor models that express wild-type Ras have been shown to be sensitive to farnesyltransferase inhibitors. We have determined the effects of combining lonafarnib with the antiestrogen 4-hydroxy tamoxifen on hormone-dependent breast cancer cell lines *in vitro*. The effects of combining lonafarnib with tamoxifen or the aromatase inhibitor anastrozole on the growth of two different MCF-7 breast tumor xenograft models were also evaluated. In four of five human breast cancer cell lines, lonafarnib enhanced the antiproliferative effects of 4-hydroxy tamoxifen. The combination prevented MCF-7 cells from transitioning through the G_1 to S phase of the cell cycle and augmented apoptosis. This was associated with reduced expression of E2F-1 and a reduction in hyperphosphorylated retinoblastoma protein. Lonafarnib plus 4-hydroxy tamoxifen also inhibited the mammalian target of rapamycin signal transduction pathway. In nude mice bearing parental MCF-7 or aromatase-transfected MCF-7*Ca* breast tumor xenografts, lonafarnib enhanced the antitumor activity of both tamoxifen and anastrozole. These studies indicate that lonafarnib enhances the efficacy of endocrine agents clinically used for treating hormone-dependent breast cancer. *Anti-Cancer Drugs* 18:923–931 © 2007 Lippincott Williams & Wilkins.

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Introduction

In hormone-dependent breast cancer the acquisition of resistance to endocrine therapies is associated with the activation of growth factor receptor signal transduction pathways [1–4]. These pathways confer an estrogen-independent growth advantage to cells being challenged with an endocrine therapy and activate estrogen receptor (ER)-α independently of 17β-estradiol (E2) binding [5–8]. Both the extracellular-signal related kinase (ERK, p42/p44 mitogen-activated protein kinase) and AKT can activate ER-α by phosphorylating Ser^{118} [5,6] and Ser^{167} [7,8], respectively, leading to its E2-independent activation.

Growth factor receptor activation of ERK and AKT is mediated by in part by Ras [9,10]. In order for Ras to localize to the membrane either a farnesyl or a geranylgeranyl lipid moiety must be attached to its C-terminus [11–13]. Lonafarnib and other farnesyltransferase inhibitors (FTIs) were developed to inhibit the farnesylation of Ras and prevent membrane localization [12]. This strategy was anticipated to inhibit growth factor-mediated signal transduction pathways. In FTI-treated cells, however, two of the Ras isoforms (K-Ras and N-Ras) undergo alternative geranylgeranylation and continue to localize to the plasma membrane [14,15]. Only the membrane localization of H-Ras is inhibited by FTIs. Therefore, in tumor cells with mutated or over-expressed K-Ras or N-Ras, FTIs do not inhibit Ras-mediated activation of the ERK and AKT signal transduction pathways.

Despite the alternative prenylation of K-Ras and N-Ras, lonafarnib is effective at inhibiting the growth of a number of cancer cell lines in culture and tumor xenografts *in vivo* [16,17]. This indicates that the ability of lonafarnib to inhibit tumor growth is most likely due to the inhibition of farnesylation and functional activity of additional proteins. Potential candidate proteins include the mitotic proteins CENP-E and CENP-F, the PRL family of nuclear phosphatases, the small GTPases RhoB and Rheb, and HDJ-2 (reviewed in [12]).

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Breast tumors have a low incidence of activating Ras mutations [18]. In both preclinical and clinical studies, however, several FTIs are effective at inhibiting breast cancer cell and tumor growth. Preclinically, MCF-7 human breast cancer cells are sensitive to the antiproliferative effects of the FTIs L-744, 832 [19], tipifarnib [20–22] and FTI-277 [23,24]. In addition, when FTIs are combined preclinically with the antiestrogen tamoxifen, breast cancer cell and tumor growth is inhibited in either an additive [21,23] or synergistic manner [24]. Clinically, the FTI tipifarnib is effective as a single agent [25] and when combined with chemotherapy [26].

This report describes the effects of combining lonafarnib with endocrine agents in hormone-dependent breast cancer models. Lonafarnib combined with the antiestrogen 4-OH tamoxifen (4-OH Tam) inhibits the growth of a panel of hormone-dependent breast cancer cells *in vitro*. In MCF-7 cells this combination prevents transition from G_1 through S phase of the cell cycle and enhances apoptosis. In addition, when combined with either the antiestrogen tamoxifen or the aromatase inhibitor anastrozole, lonafarnib enhances the antitumor activity of these agents on the growth of parental MCF-7 or aromatase-transfected MCF-7*Ca* human breast tumor xenografts *in vivo*.

Materials and methods

Materials

Lonafarnib (SCH66336) is synthesized by Schering-Plough (Kenilworth, New Jersey, USA) [27]. Tissue culture media were from Invitrogen (Carlsbad, California, USA). Phenol-red free trypsin/versene was from Biosource International (Rockville, Maryland, USA). Heat-inactivated fetal bovine serum (FBS) and heat-inactivated dextran-coated charcoal-stripped fetal bovine serum (CSS) were from Hyclone (Logan, UTAH, USA). E2, tamoxifen, 4-OH Tam, propidium iodide, Triton X-100, RNase A, dimethyl sulfoxide and paraformaldehyde were from Sigma (St Louis, Missouri, USA). Anastrozole was from Sequoia Research Products (Oxford, UK). Hydroxypropyl-β-cyclodextrin (HPBCD) was from Cargill Food and Pharma Specialities (Cedar Rapids, Iowa, USA).

Cell culture

Aromatase-transfected MCF-7*Ca* cells obtained from Dr S. Chen (City of Hope, Duarte, California, USA) [28] were grown in Dulbecco's modified Eagle's medium (DMEM), containing 5% FBS, 1% penicillin/streptomycin, and supplemented with 600 μg/ml G418. MCF-7 cells from Dr. A. Brodie (University of Maryland School of Medicine, Baltimore, Maryland, USA) were maintained in DMEM with 5% FBS and 1% penicillin/streptomycin. BT-474, T-47D and ZR-75-1 cells were from the American Type Culture Collection (Manassas, Virginia, USA), and were maintained in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin.

For in-vitro cell culture assays, cells in log-phase growth were first synchronized by changing the media to a steroid-free medium that consisted of phenol red-free DMEM/F12 supplemented with 10% CSS and 1% penicillin/streptomycin solution [steroid-free media (SFM)]. Cells were cultured in this SFM for 6 days before plating for all experiments [29]. Cells were then treated with SFM supplemented with E2 (1 nmol/l) and containing lonafarnib, 4-OH Tam or the combination of both agents. Cells were treated for 6 days and the medium was refreshed on day 3.

Growth assays were performed by plating synchronized cells into 96-well plates (2×10^3 cells/well). Cells in quadruplet wells were treated with compounds. Cell viability was determined using the CellTitre-Glo luminescent assay (Promega, Madison, Wisconsin, USA). Luminescent signal intensity was determined using a Molecular Analyst (Molecular Devices, Sunnyvale, California). All growth assays were performed in triplicate.

Cell cycle analyses were performed by plating synchronized MCF-7 cells into 10-cm dishes (5×10^5 cells/plate) in SFM. Cells were collected, and after fixing with methanol and staining with propidium iodide, analysis was performed as described previously [30]. All cell cycle assays were performed with cells treated in triplicate plates. The assays were repeated three times.

TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling) detection of apoptosis was performed by plating synchronized MCF-7 cells into 10-cm Petri dishes (5×10^5 cells/plate) in SFM. Cells were collected and fixed in 1% paraformaldehyde in phosphate-buffered saline. After fixing the cells in 70% ice-cold ethanol, the percentage of apoptotic cells was determined using APO-BRDU kits (BD Biosciences, San Diego, California, USA). Apoptotic cells were then detected by flow cytometry analysis. All TUNEL assays were repeated in duplicate.

Western blotting analyses were performed on synchronized MCF-7 cells after seeding into 10 cm Petri dishes (1×10^6 cells/plate) in SFM. Cells were lysed in RIPA buffer and Western blotting was performed as previously described [31]. Antibodies used were against E2F-1, retinoblastoma protein (Rb), the cleaved p85 fragment of poly(ADP-ribose) polymerase (PARP) (BD Pharmingen, San Diego, California), ERK, phospho-ERK (Thr^{202}/Tyr^{204}), S6 ribosomal protein, phospho-S6 ribosomal protein ($Ser^{235/236}$), eukaryotic initiation factor 4E-binding protein-1 (4EBP-1), phospho-4EBP-1 ($Thr^{37/46}$) and phospho-4EBP-1 (Ser^{65}) (Cell Signaling Technology,

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Beverly, Massachusetts, USA), and β-actin (Sigma). Densitometric quantification of each band was determined using Quantity One software (PDI, Huntington Station, New York, USA). Densitometric quantification of each band was normalized in comparison with the relevant expression of β-actin in the reprobed blot.

Animal studies

Female ovariectomized athymic nude mice (4–6 weeks of age) from Charles River Laboratories (Wilmington, Massachusetts, USA) were maintained in a VAF-barrier facility. Animal procedures were performed in accordance with the rules set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Schering-Plough Animal Care and Use Committee. All animals received food and water *ad libitum*.

MCF-7 breast tumor fragments from rapidly growing tumors were cut into small pieces and transplanted into the left flank of mice in Matrigel. MCF-7 tumor growth was maintained by weekly subcutaneous administration of estradiol cypionate [3 mg/kg body weight (mpk), Pfizer, Ann Arbor, Michigan, USA; adapted from [32]]. When tumor volumes were palpable, animals were grouped ($n = 10$) for treatment twice daily with vehicle or lonafarnib (40 mpk [16,17]), once daily (q.d.) with tamoxifen (20 mpk [33]) or the combination of both agents. Tumor volumes were measured twice weekly using calipers and calculated by the formulae (width × length × height)/2.

MCF-7*Ca* tumors were grown as described above except animals were implanted subcutaneously with a 15-mg, 90-day release androstenedione pellet before the inoculation of 5×10^6 MCF-7*Ca* human breast cancer cells in Matrigel (200 µl). In the first experiment, when tumors were palpable, animals were grouped ($n = 10$) for treatment with vehicle, lonafarnib (40 mpk, twice daily), anastrozole (5 mpk, twice daily) or the combination of both agents. Tumor volumes were measured twice weekly as described above. In the second experiment, when tumors were palpable, animals were grouped ($n = 10$) for treatment with vehicle, lonafarnib (40 mpk, twice daily), a higher dose of anastrozole (25 mpk, twice daily) or the combination of both agents.

Statistical analysis

Statistically significant differences were determined by the Student's *t*-test at the 95% confidence level using GraphPad Prism.

Results

Effect of lonafarnib in combination with 4-hydroxy tamoxifen on hormone-dependent breast cancer cells *in vitro*

The concentration of 4-OH Tam required to inhibit the growth of a panel of five hormone-dependent breast cancer cells by 50% (IC_{50}) ranged from 0.45 to 3.33 µmol/l (Table 1). The IC_{50} concentrations of lonafarnib ranged from 0.04 to 6.9 µmol/l (Table 1). As shown in Fig. 1a, lonafarnib (0.1–1.0 µmol/l) significantly enhanced the growth inhibitory effects of 4-OH Tam on MCF-7 cells ($P < 0.05$). Lonafarnib also significantly enhanced the growth inhibitory effects of 4-OH Tam on MCF-7*Ca*, ZR-75-1, and BT-474 cells (Fig. 1b–d).These cell lines were, however, not as sensitive to single-agent lonafarnib as parental MCF-7 cells and with these cells the enhanced growth inhibitory effects of lonafarnib were only consistently observed at the 1.0 µmol/l concentration. With the T-47D cell line, lonafarnib (0.1–1.0 µmol/l) did not enhance the growth inhibitory effects of 4-OH Tam (Fig. 1e).

Table 1 Sensitivity of hormone-dependent human breast cancer cell lines to the antiproliferative effects of lonafarnib and 4-hydroxy tamoxifen

Cell line	Lonafarnib IC_{50} (µmol/l)	4-hydroxy Tamoxifen IC_{50} (µmol/l)
MCF-7	0.04 ± 0.01	0.45 ± 0.05
BT-474	1.23 ± 0.06	0.47 ± 0.05
MCF-7Ca	0.44 ± 0.04	3.33 ± 0.83
ZR-75-1	3.66 ± 0.18	3.20 ± 1.15
T-47D	6.90 ± 0.90	2.05 ± 0.52

Results show the mean ± the standard error from three independent experiments.

Cell cycle analyses showed that both single-agent 4-OH Tam ($P < 0.01$) and single-agent lonafarnib significantly reduced ($P < 0.01$) the percentage of MCF-7 cells in S phase of the cell cycle (Fig. 2a). Both of these single-agent treatments caused MCF-7 cells to accumulate in the G_1 phase of the cell cycle. Accumulation in G_1 was associated with reduced levels of E2F-1 and the hyperphosphorylated form of Rb (Fig. 2b). Both of these proteins are involved in transitioning cells though the $G_1 \rightarrow$ S phase of the cell cycle [34,35]. Levels of both E2F-1 and hyperphosphorylated Rb were further reduced when MCF-7 cells were treated with the combination of lonafarnib plus 4-OH Tam.

The combination of lonafarnib plus 4-OH Tam was associated with the appearance of a significant ($P < 0.001$) population of MCF-7 cells in the sub-G_1 phase of the cell cycle indicating the induction of apoptosis (Fig. 2a). Apoptosis induced by this combination was further confirmed using TUNEL analysis as well as Western blotting for the cleaved fragment of PARP (Fig. 3a and b). TUNEL analysis showed that the combination of both agents significantly enhanced the percentage of cells undergoing apoptosis compared with treatment with single-agent 4-OH Tam ($P < 0.05$). Densitometric quantification of the cleaved p85 PARP band showed increased band intensity when MCF-7 cells were treated with the combination of both agents.

In MCF-7 cells the combination of 4-OH Tam plus lonafarnib did not inhibit either the ERK or AKT signal

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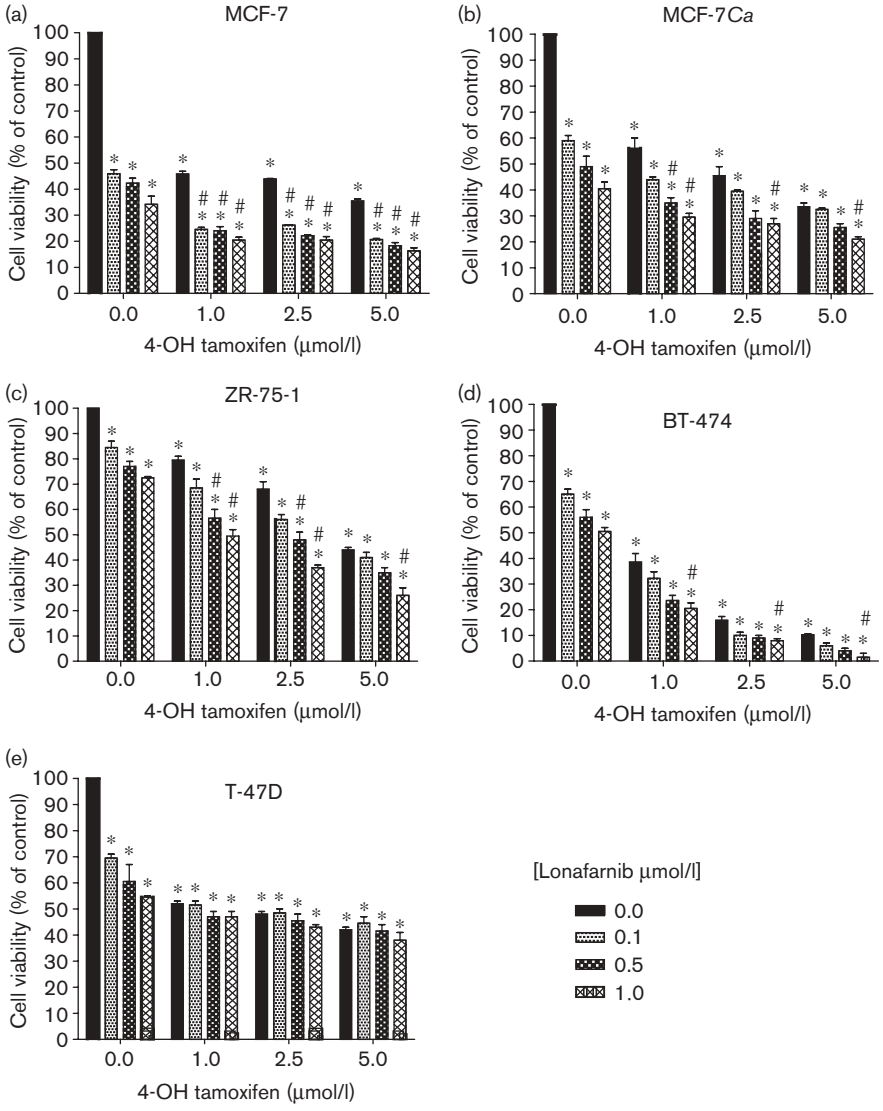
Fig. 1



Lonafarnib enhances the antiproliferative effects of 4-OH Tam on (a) MCF-7, (b) MCF-7Ca, (c) ZR-75-1 and (d) BT-474 human breast cancer cells *in vitro*. Lonafarnib does not enhance the antiproliferative effects of 4-OH Tam on T-47D human breast cancer cells *in vitro* (e). Cell viability is expressed as a percentage of the control E2-treated cells. *$P<0.01$ versus control cells. #$P<0.05$ versus single-agent 4-OH Tam. E2, 17β-estradiol; 4-OH Tam, 4-hydroxy tamoxifen.

transduction pathways as assessed by the phosphorylation status of these proteins (Fig. 4a). To assess effects on the mammalian target of rapamycin (mTOR) signaling pathway, we examined the phosphorylation status of the mTOR downstream targets, S6 ribosomal protein and 4EBP-1 (Fig. 4b). The combination of 4-OH Tam and lonafarnib reduced levels of phospho-S6 as well as phospho-4E-BP-1. Single-agent lonafarnib downregulated phosphorylation of both of these proteins. A more complete downregulation was, however, accomplished by the combination of both agents (Fig. 4b).

Effect of lonafarnib in combination with endocrine therapies on the growth of hormone-dependent breast cancer xenografts *in vivo*

The effects of combining lonafarnib plus tamoxifen on the growth of MCF-7 tumor xenografts and with anastrozole on the growth of MCF-Ca tumor xenografts were determined.

Single-agent tamoxifen and single-agent lonafarnib significantly inhibited the growth of MCF-7 breast tumors. After 21 days of treatment, final tumor volumes in the

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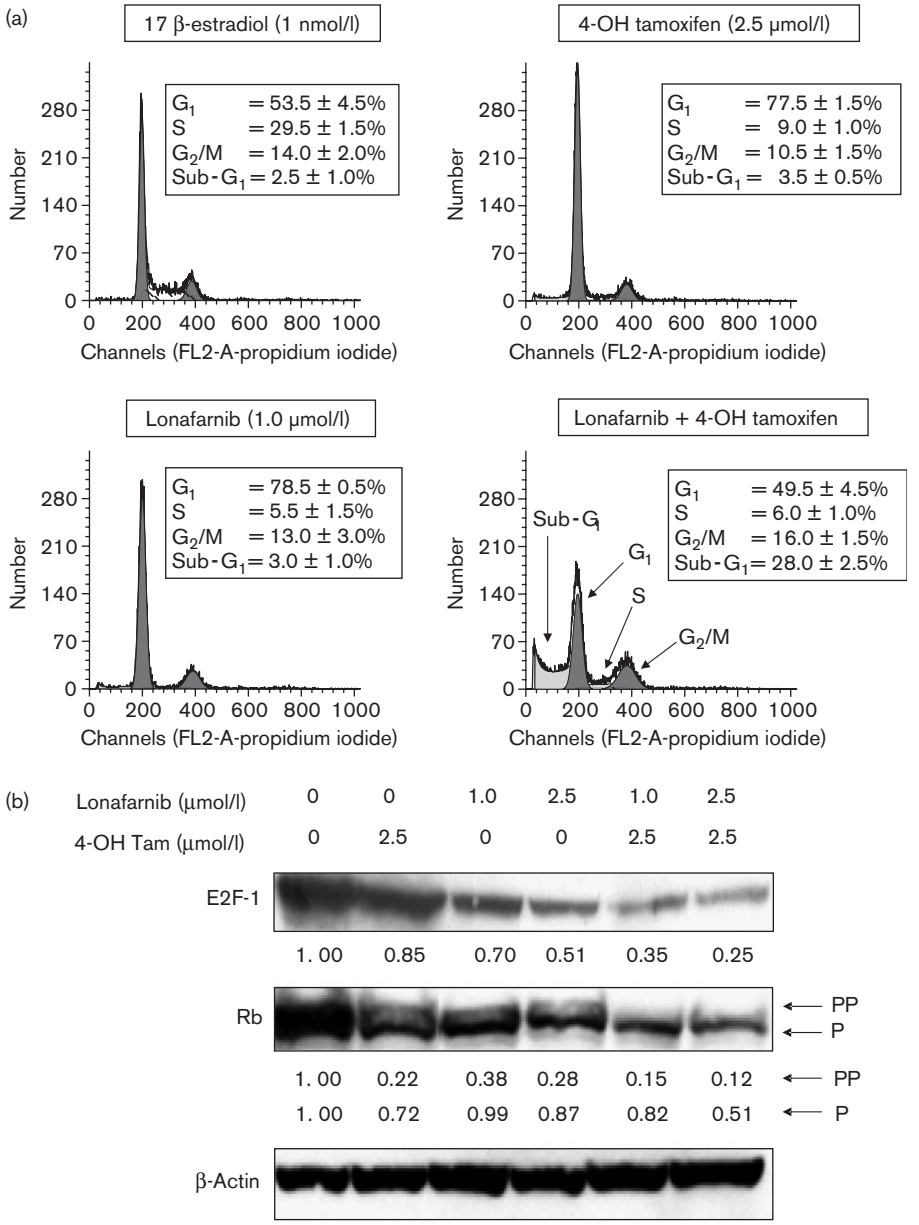
Fig. 2



(a) The combination of lonafarnib plus 4-OH Tam prevents MCF-7 cells from transitioning into S phase of the cell cycle. Single-agent lonafarnib and single-agent 4-OH Tam induce G_1 arrest in MCF-7 cells and this is associated with significantly less cells in S phase of the cell cycle ($P<0.01$). The combination of lonafarnib plus 4-OH Tam induces significant accumulation of MCF-7 cells in sub-G_1 ($P<0.0001$). (b) Effect of the combination of lonafarnib plus 4-OH Tam on expression of E2F-1 and hyperphosphorylated Rb in MCF-7 cells. Levels of E2F-1 and hyperphosphorylated Rb are reduced after treatment with the combination. Densitometric quantitation of each band was normalized in comparison with the relative expression of β-actin and the values are indicated at the bottom. 4-OH Tam, 4-hydroxy tamoxifen; P, phosphorylated; PP, hyperphosphorylated, Rb, retinoblastoma protein. β-Actin served as the loading control.

single-agent therapy arms were significantly smaller compared with the control, vehicle-treated arms ($P < 0.001$ for both arms. When combined with tamoxifen, lonafarnib caused MCF-7 tumors to regress by 31% such that final tumor volumes were 69% of their initial volume (Fig. 5a). Tumor volumes in the combination arm were significantly smaller compared with single-agent tamoxifen from day 8 of treatment ($P < 0.05$). This statistically significant difference was maintained for the duration of the experiment. At the end of treatment, final tumor volumes in the combination arm were significantly smaller than tumors in the animals that received single-agent tamoxifen or single-agent lonafarnib ($P < 0.01$ versus both single-agent arms). Moreover, in this model,

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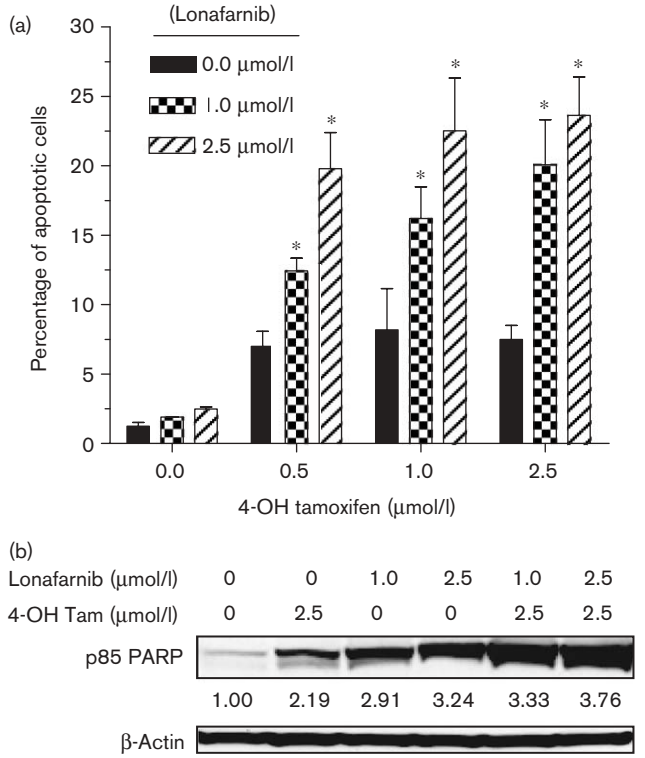
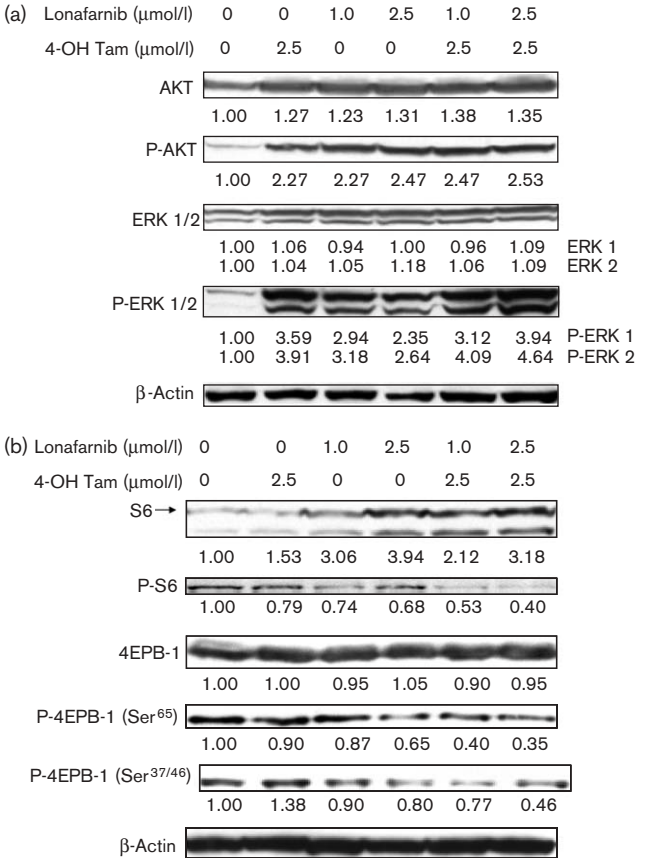
Fig. 3



The combination of lonafarnib plus 4-OH Tam induces apoptosis in MCF-7 cells. (a) Apoptosis was determined by TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling) analyses using APO-BRDU kits. *$P<0.05$ versus single-agent 4-OH Tam. (b) Levels of the p85 cleaved fragment of PARP in MCF-7 cells are increased after treatment with the combination of lonafarnib plus 4-OH Tam. Densitometric quantitation of each band was normalized in comparison with the relative expression of β-actin and the values are indicated at the bottom. β-Actin served as the loading control. 4-OH Tam, 4-hydroxy tamoxifen; PARP, poly(ADP-ribose) polymerase.

Fig. 4



(a) Effect of the combination of lonafarnib plus 4-OH Tam on expression levels of AKT phospho-AKT, ERK and phospho-ERK in MCF-7 cells. Levels of phospho-AKT and phospho-ERK were not reduced after treatment with the combination. (b) Effect of the combination of lonafarnib plus 4-OH Tam on the phosphorylation of S6 and 4EBP-1 in MCF-7 cells. Levels of phospho-S6 and phospho-4EBP-1 were reduced after treatment with the combination. Densitometric quantitation of each band was normalized in comparison with the relative expression of β-actin and the values are indicated at the bottom. β-Actin served as the loading control. 4EBP-1, 4E-binding protein-1; ERK, extracellular-signal related kinase; 4-OH, Tam, 4-hydroxy tamoxifen.

neither single-agent tamoxifen nor single-agent lonafarnib caused MCF-7 tumors to regress.

Aromatase inhibitors provide superior clinical benefit than the antiestrogen tamoxifen in hormone-dependent breast cancer [35]. We also determined the effects of combining lonafarnib with the aromatase inhibitor anastrozole. In the first experiment, anastrozole was administered at a dose of 5 mpk, twice daily. After 28 days of treatment, final tumor volumes in the single-agent arms were significantly smaller than tumors in the vehicle-treated arms ($P < 0.05$ versus anastrozole and $P < 0.01$ versus lonafarnib). When combined with anastrozole (5 mpk), lonafarnib caused MCF-7*Ca* tumors to regress by 33% such that final tumor volumes were 67% of their initial volume (Fig. 5b). Tumor volumes in the combination arm were significantly smaller compared with single-agent anastrozole (5 mpk) from day 8 of treatment ($P < 0.01$). This statistically significant difference was then maintained for the duration of the experiment. At the end of treatment, final tumor volumes in the combination arm were significantly smaller than tumors in the animals that received single-agent anastrozole (5 mpk) or single-agent lonafarnib ($P < 0.01$ versus both single-agent arms). In this experiment, single-agent anastrozole did not cause MCF-7*Ca* tumors to regress, whereas single-agent lonafanib caused minor regressions of 16%.

In the second MCF-7*Ca* experiment, lonafarnib was combined with a higher dose of anastrozole (25 mpk, Fig. 5c). After 28 days of treatment with this dose of

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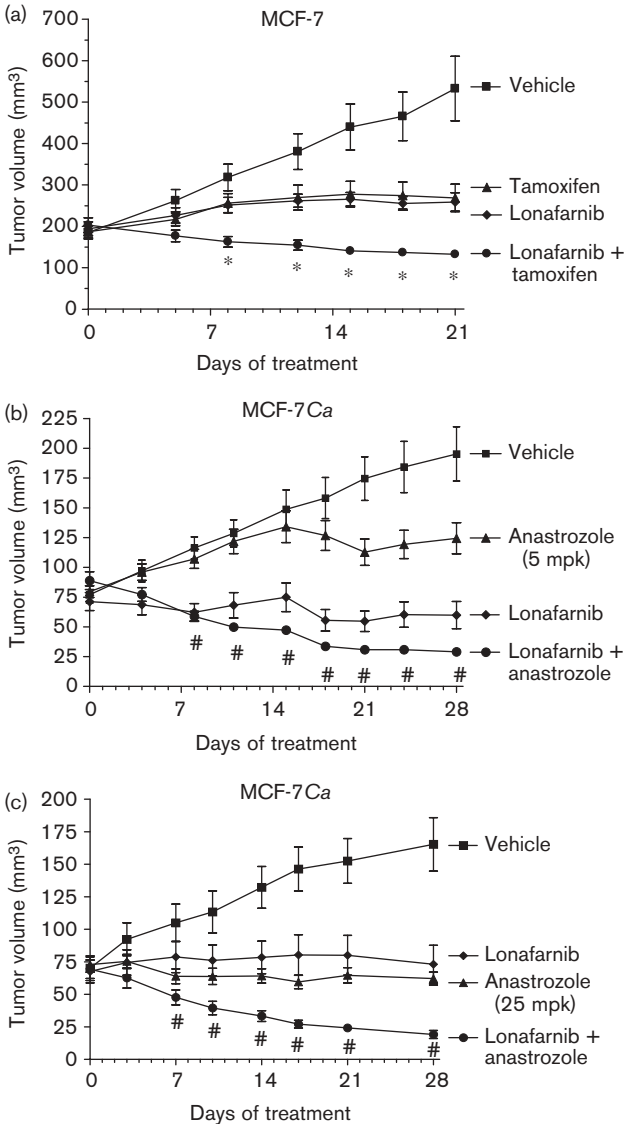
anastrozole, MCF-7*Ca* tumors regressed by 14%. Final MCF-7*Ca* tumor volumes in the anastrozole (25 mpk) arm were significantly smaller compared with the vehicle-treated arm ($P < 0.01$). Final tumor volumes in the single-agent lonafarnib arm were also significantly smaller than the control arm ($P < 0.01$). The combination of lonafarnib plus anastrozole (25 mpk) again induced marked MCF-7*Ca* tumor regressions (72%). Tumor volumes in the combination arm were significantly smaller compared with single-agent anastrozole (25 mpk) from day 7 of treatment ($P < 0.01$). This statistically significant difference was then maintained for the duration of the experiment. At the end of treatment, final tumor volumes in the combination arm were significantly smaller than tumors in the animals that received single-agent anastrozole (25 mpk) or single-agent lonafarnib ($P < 0.01$ versus both single-agent arms).

Fig. 5



(a) Effect of combined lonafarnib (40 mpk, twice daily) plus tamoxifen (20 mpk, q.d.) on the growth of parental MCF-7 breast tumor xenografts in female ovariectomized athymic nude mice *in vivo*. After 21 days of treatment, final tumor volumes in the single-agent arms were significantly smaller compared with the tumor volumes in the vehicle-treated arm ($P < 0.001$ versus both agents). *Tumor volumes in the combination arm were significantly smaller ($P < 0.05$) versus single-agent tamoxifen from day 8 of treatment and remained significantly smaller for the duration of the experiment. (b) Effect of combining lonafarnib (40 mpk, twice daily) plus anastrozole (5 mpk, twice daily) on the growth of aromatase-transfected MCF-7*Ca* breast tumor xenografts in female ovariectomized athymic nude mice *in vivo*. After 28 days of treatment, final tumor volumes in the single-agent arms were significantly smaller compared with the tumor volumes in the vehicle-treated arm ($P < 0.05$ versus anastrozole and $P < 0.01$ versus lonafarnib). #Tumor volumes in the combination arm were significantly smaller ($P < 0.01$) versus single-agent anastrozole from day 8 of treatment and remained significantly smaller for the duration of the experiment. (c) Effect of combining lonafarnib (40 mpk, twice daily) plus anastrozole (25 mpk, twicw daily) on the growth of aromatase-transfected MCF-7*Ca* breast tumor xenografts in female ovariectomized athymic nude mice *in vivo*. After 28 days of treatment, final tumor volumes in the single-agent arms were significantly smaller compared with the tumor volumes in the vehicle-treated arm ($P < 0.01$ versus both agents). #Tumor volumes in the combination arm were significantly smaller ($P < 0.01$) versus single-agent anastrozole from day 7 of treatment and remained significantly smaller for the duration of the experiment. mpk, mg/kg body weight; q.d., once daily.

Discussion

This is the first report describing the ability of lonafarnib to enhance the growth inhibitory effects of the anti-estrogen tamoxifen as well as the aromatase inhibitor anastrozole on hormone-dependent breast cancer cells and breast tumor xenografts *in vitro* and *in vivo*.

In four of five hormone-dependent breast cancer cell lines lonafarnib enhanced the growth inhibitory effects of 4-OH Tam. T-47D cells did not respond to the combination of lonafanib plus 4-OH Tam with enhanced growth inhibition. This is most likely because T-47D cells are relatively resistant to the antiproliferative effects of single-agent lonafanib. The reasons underlying this resistance are under investigation and, to date, no explanation has been found. It has been shown previously [19] that the ability of the FTI L-744,832 to inhibit the growth of a panel of breast cancer cell lines *in vitro* does not correlate with expression of total Ras, differential expression of the Ras isoforms, or Ras mutational status. This suggests that the molecular mechanisms contributing to the resistance of T-47D cells to FTIs may be associated with the prenylation status and functional activity of proteins other than Ras. The differential sensitivity of MCF-7 and T-47D cells to lonafarnib is intriguing and warrants further investigation. A molecular understanding of this differential sensitivity could assist in selecting breast cancer patients who would be expected to have a therapeutic benefit from lonafarnib.

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The ability of lonafanib to enhance the antiproliferative effects of 4-OH Tam on MCF-7 cells is associated with the ability of the combination to prevent $G_1 \rightarrow S$ transition through the cell cycle and to induce apoptosis. Similar effects have been reported when the farnesyltransferase inhibitors FTI-277 [23] or tipifarnib [21] are combined with tamoxifen. Doisneau-Sixou *et al.* [23] showed that in MCF-7 cells the combination of FTI-277 plus tamoxifen caused accumulation of p21 that led to inactivation of cyclin E/cyclin-dependent kinase-2 complexes and reduced levels of phospho-Rb. This resulted in an enhanced effect on MCF-7 cell cycle arrest. We show here that the combination of lonafarnib plus 4-OH Tam reduced levels of the hyperphosphorylated form of Rb. In addition, we show that this combination also reduced levels of E2F-1. Both of these proteins play important roles in transitioning cells from G_1 through S phase of the cell cycle [34,35] and the depletion in their levels correlates well with the observed cell cycle arrest.

In MCF-7 breast cancer cells treated with the combination of 4-OH Tam plus lonafarnib the classical Ras-mediated ERK and AKT signal transduction pathways are not inhibited, indicating that these pathways are not mediating the antiproliferative activity of this combination. Therefore, we determined whether this combination might be inhibiting the mTOR signal transduction pathway. Single-agent lonafarnib had an inhibitory effect on the phosphorylation status of S6 ribosomal protein and 4EBP-1. It was, however, only after treatment with both agents that marked inhibition of phospho-S6 ribosomal protein and phospho-4EBP-1 was observed. The ability of other FTIs [31,37] as well as the Ras antagonist farnesylthiosalicylic acid [38,39] to inhibit mTOR signaling has been previously documented. We have recently reported that lonafarnib inhibits mTOR signaling by blocking the farnesylation of Rheb, a small GTPase that is an upstream regulator of mTOR [31].

When mice bearing MCF-7 human breast tumor xenografts were treated with the combination of lonafarnib plus tamoxifen, marked tumor regressions were observed. Similar results were reported previously when established MCF-7 breast tumor xenografts were treated with the combination of the FTI tipifarnib plus tamoxifen [33]. Our data further show that when combined with the aromatase inhibitor anastrozole, lonafarnib also induced marked tumor regressions in the MCF-7*Ca* postmenopausal breast cancer model. These regressions were observed when anastrozole was administered to the animals at a relatively low dose (5 mpk, twice daily) and at a much higher dose (25 mpk, twice daily). The reason for using two different doses of anastrozole in this report is because anastrozole has a poor pharmacokinetic profile in mice and is quickly metabolized (data not shown). That the MCF-7*Ca* tumors regressed even when lonafarnib was combined with a low dose of anastrozole suggests that this combination may be effective in an ongoing clinical trial. The MCF-7*Ca* postmenopausal breast cancer model has been used previously to show that aromatase inhibitors are more effective than antiestrogens at inhibiting the growth of hormone-dependent breast cancer [40,41]. This is the first report documenting the use of this model to determine the effects of combining an aromatase inhibitor with an FTI.

In summary, we have shown that lonafarnib enhances the growth inhibitory effects of the antiestrogen tamoxifen as well as the aromatase inhibitor anastrozole *in vitro* and *in vivo*. The combination of lonafarnib plus 4-OH Tam caused MCF-7 cell cycle arrest and enhanced apoptosis. Marked tumor regressions were observed *in vivo* when lonafarnib was combined with either tamoxifen or anastrozole. Taken together, these data provide preclinical rationale for the clinical evaluation of the combination of lonafarnib with an endocrine therapy in postmenopausal patients with hormone-dependent breast cancer.

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