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SYNERGISTIC ACTIVITY OF LETROZOLE AND SORAFENIB ON BREAST CANCER CELLS

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ABSTRACT

Estrogens induce breast tumour cell proliferation by directly regulating gene expression via the estrogen receptor (ER) transcriptional activity and by affecting growth factor signalling pathways such as mitogen-activated protein kinase (MAPK) and AKT/mammalian target of rapamycin Complex1 (mTORC1) cascades. In this study we demonstrated the preclinical therapeutic efficacy of combining the aromatase inhibitor letrozole with the multi-kinase inhibitor sorafenib in aromatase-expressing breast cancer cell lines. Treatment with letrozole reduced testosterone-driven cell proliferation, by inhibiting the synthesis of estrogens. Sorafenib inhibited cell proliferation in a concentration-dependent manner; this effect was not dependent on sorafenib-mediated inhibition of Raf1, but involved the down-regulation of mTORC1 and its targets p70S6K and 4E-binding protein 1 (4E-BP1). At concentrations of 5-10 μ M the growth-inhibitory effect of sorafenib was associated with the induction of apoptosis, as indicated by release of cytochrome c and Apoptosis-Inducing Factor into the cytosol, activation of caspase-9 and caspase-7, and PARP-1 cleavage. Combination of letrozole and sorafenib produced a synergistic inhibition of cell proliferation associated with an enhanced accumulation of cells in the G₀/G₁ phase of the cell cycle and with a down-regulation of the cell cycle regulatory proteins c-myc, cyclin D1 and phospho-Rb. In addition, longer experiments (12 weeks) demonstrated that sorafenib may be effective in preventing the acquisition of resistance towards letrozole. Together, these results indicate that combination of letrozole and sorafenib might constitute a promising approach to the treatment of hormone-dependent breast cancer.

INTRODUCTION

Approximately 60% of primary breast cancers express the ER-alpha and are dependent on estrogens for their growth. ER is a member of the nuclear hormone receptor superfamily and 17 β -estradiol (E2) is the most potent ligand. Estrogens activate ER through genomic and non-genomic pathways, stimulating nuclear and cytoplasmatic processes that promote breast cancer cell proliferation. In the nucleus, ER regulates gene transcription either by direct binding to estrogen response elements (ERE) [1] or via protein-protein interactions with other transcription factors such as AP-1, SP-1, and NF-kB [2]. In addition, estrogens exert rapid non-genomic actions mediated by the interaction between membrane-bound ER and membrane kinase receptors such as IGF-IR, EGFR, and HER2 [1], which results in the activation of signalling cascades including the Ras/Raf/MAPK pathway [3] and the phosphatidylinositol-3-kinase (PI3K)/AKT/mTOR pathway [2]. Conversely, ER can be directly activated in the absence of estrogens by various kinases, including MAPK and PI3K/AKT [4, 5].

In postmenopausal women, estrogens are generated from androgens through aromatase-dependent enzymatic conversion [6]. Letrozole (Femara[®]) is a third-generation nonsteroidal aromatase inhibitor (AI) [7] approved for treatment of postmenopausal women with hormone-dependent breast cancer. By eradicating estrogens, AIs suppress both genomic and non-genomic action of ER and are proved to be effective as first-line therapy in advanced/metastatic breast cancer as well as in neoadjuvant, early adjuvant and extended adjuvant strategies [8]. However, either intrinsic or acquired resistance to AIs may occur, arising from activation of novel signalling mechanisms that involve crosstalk between ER and growth factor receptors [9]. This provides a strong rationale for combining endocrine therapies with signal transduction inhibitors to achieve a more potent anti-tumour effect or to bypass acquired resistance [10, 11].

Generali and co-workers demonstrated the role of mTOR, MAPK and HIF-1 α in endocrine resistance in patients receiving letrozole-based treatment supporting the development of new treatment strategies based on the combination of AIs with signal transduction inhibitors targeting mainly MAPK but also mTOR and HIF-1 α [12].

Sorafenib (Nexavar, BAY43-9006) is a multi-kinase inhibitor recently approved for treatment of advanced renal cell and hepatocellular carcinoma [13, 14]. It has been shown to block tumour cell proliferation and angiogenesis by inhibiting serine/threonine kinases (C-Raf, mutant and wild-type B-Raf) as well as vascular endothelial growth factor receptors (VEGFR2, VEGFR3) [15], and other tyrosine-kinase receptors such as PDGFR- β , FLT-3, Ret and c-KIT [15, 16]. In animal models, sorafenib effectively reduces melanoma, breast, colon and lung cancer growth [15, 17]. In addition, it has been reported that sorafenib induces apoptosis in a variety of tumour cell lines [18-22]. Recently, it was also shown that sorafenib modulates either mTOR signalling and its targets (p70S6K, S6R, 4EBP1) expression or HIF-1 α expression [23, 24].

The purpose of this study was to evaluate the potential for combining letrozole and sorafenib in breast cancer cell lines constitutively expressing the *aromatase* gene.

MATERIALS AND METHODS

Cell culture

The human breast cancer cell lines MCF-7/AROM-1 and T47D/AROM (clone 1 and 2), expressing high levels of aromatase, were generated by stable transfection of full-length human *aromatase* under control of the cytomegalovirus promoter. BT474/AROM were stably transduced with a retroviral construct pBabeAROM expressing *aromatase*. Cells were cultured as recommended [25, 26].

Before the experiments cells were steroid-deprived for 4 days using phenol red-free medium supplemented with 5% charcoal-stripped FCS (HyClone, Logan, UT) and then treated with 1nM E2 or stimulated with 100nM testosterone (T) in the absence or presence of the drugs. Media and drugs were changed every three days.

Compounds

Letrozole was provided by Novartis Institutes for BioMedical Research (Basel, Switzerland). Sorafenib was from Bayer HealthCare LLC (Tarrytown, NY). Drugs and hormones (E2 and T, Sigma-Aldrich, St. Louis, MO) were prepared in DMSO. The concentration of DMSO never exceeded 0.1% (v/v) and equal amounts of the solvent were added to control cells.

Antibodies and reagents

Media and FBS were from Euroclone (Devon, UK). Antibodies against cytochrome *c*, Apoptosis-Inducing Factor (AIF), p53, p21^{WAF1} were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP-1, caspase-7, caspase-8, caspase-9, phospho-ERK1/2(Thr202/Tyr204), ERK1/2, phospho-AKT(Ser473), AKT, phospho-mTOR(Ser2448), phospho-4E-BP1(Ser65), phospho-p70S6K(Thr389), p70S6K, c-myc,

cyclin D1, p27^{Kip1}, phospho-Rb(Ser780), and Rb were from Cell Signaling Technology (Beverly, MA). Anti-actin antibody was from Sigma-Aldrich. Horseradish peroxidase-conjugated secondary antibodies and chemiluminescence system were from Millipore (Millipore, MA). Reagents for electrophoresis and blotting analysis were from BIO-RAD Laboratories (Hercules, CA). Calcein-AM and propidium iodide (PI) were from Invitrogen (Milano, Italy), Draq5 was from Alexis Biochemicals (Lausen, Switzerland). Rapamycin and all other reagents were from Sigma-Aldrich.

Western blotting

Procedures for protein extraction and analysis by 1-D PAGE are described elsewhere [27]. Cytosolic and mitochondrial fractions were generated as previously described [28]. Protein levels were quantified by densitometric analysis (UN-SCAN-ITTMgel) and normalized to the actin levels; values are expressed as percent *versus* T-stimulated cells.

Analysis of cell proliferation, cell death and cell cycle

Cell proliferation was evaluated by cell counting and tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] (MTT) assay, as previously described [27]. Data are expressed as percent inhibition of cell proliferation *versus* T-stimulated cells, calculated by subtracting the amount of proliferation independent of hormone stimulation. A percent value of inhibition >100% indicates that hormone-independent cell proliferation was also inhibited.

Cell death was assessed as previously described [28]. In addition, real time analysis was performed using a Zeiss LSM510 Meta confocal microscope (CLSM) (Carl Zeiss, Jena, Germany) with a 63X NA 1.4 plan apo objective. Viability and morphology were assessed after cell loading with calcein-AM, PI and Draq5 excited with 488, 543, 633 laser lines

respectively. Acquisition was carried out in the multitrack mode, namely through consecutive and independent optical pathways.

Distribution of the cells in the cell cycle was determined by PI staining and flow cytometry as described elsewhere [27, 28].

Statistical Analysis

Statistical significance of differences among data was estimated by two-tailed Student's *t* test or one-way ANOVA (with Tukey test for pairwise comparisons). Differences were considered significant at $p < 0.05$.

IC₅₀ values, expressed as mean of three independent determinations ($\pm$ SD), were calculated by fitting the experimental data with a hyperbolic function and constraining $Y_{\max}$ to 100 (GraphPad-Prism 4.00).

The nature of the interaction between letrozole and sorafenib was calculated using the Bliss interaction model [29, 30]. A theoretical dose-response curve was calculated for combined inhibition using the equation $E_{\text{bliss}} = E_A + E_B - E_A \times E_B$, where E_A and E_B are the percent of inhibition *versus* testosterone-stimulated cells, obtained by drug A (sorafenib) and B (letrozole) alone and E_{bliss} is the percent of inhibition that would be expected if the combination was exactly additive. If the experimental percent of inhibition is $>E_{\text{bliss}}$ the combination is considered synergistic, if it is $<E_{\text{bliss}}$ the combination is antagonistic.

RESULTS

Letrozole and sorafenib inhibit estrogen-driven proliferation of MCF-7/AROM-1 cells.

MCF-7/AROM-1 cells were firstly analyzed for their estrogen sensitivity (fig.1a). Cell proliferation was slowed by steroid-deprivation and stimulated in a time-dependent manner by either E2 or testosterone, thus indicating that these cells mainly depend on estrogens for their growth. The effect of letrozole on cell proliferation was evaluated in testosterone-stimulated cells seeded at different densities. A clear concentration-dependent inhibition of testosterone-driven proliferation was observed in cells cultured at low density (fig.1b); in contrast, in cells cultured at higher density letrozole, even at 10 μ M, was unable to inhibit cell proliferation by more than 20%, indicating that the growth-inhibitory effect of the drug is affected by cell density. Indeed, in high density cell cultures a higher concentration of autocrine growth factors is presumably achieved [31], thus rendering cell proliferation less dependent on estrogen production. Treatments with letrozole up to 6 days, even at the highest concentrations, were never associated with induction of cell death (not shown).

Then we evaluated the effects of sorafenib on testosterone-driven proliferation. As demonstrated by cell counting (not shown) and MTT assay (fig.1c), sorafenib impaired cell proliferation in a concentration-dependent manner. In addition, sorafenib inhibited ERK1/2 phosphorylation only at concentrations higher than the IC₅₀ value (3 μ M) after 2h of treatment, whereas the activation status of the AKT/mTORC1 pathway was unaffected (fig.1d). It is noteworthy that the range of concentrations of sorafenib here used was below the plasma concentrations achieved *in vivo* at the clinically approved doses [32, 33].

Sorafenib at higher concentrations induces cell death by apoptosis.

Treatment with sorafenib at higher concentrations (5-10 μ M) was associated with the induction of cell death (fig.2a). Flow cytometry revealed the presence of Annexin V-FITC-

positive cells excluding PI at early time intervals (not shown), suggesting that sorafenib induced cell death by apoptosis. This result was confirmed by CSLM analysis (fig.2b). Sorafenib-treated cells exhibited some morphological hallmarks of apoptosis: rounded shape, nuclear condensation, blebbing and calcein-positivity until late stages [34]; the morphological changes associated with caspase-3 activity, such as fragmentation in apoptotic bodies, were not detected because MCF-7/AROM-1 cells are null for caspase-3 [35]. As shown in fig.2c sorafenib induced cleavage of the caspase substrate PARP-1 [36] and activation of the effector caspase-7. Also procaspase-9 was processed into active subunits, whereas procaspase-8 remained uncleaved, indicating that sorafenib-mediated apoptosis was associated with the activation of the mitochondrial intrinsic pathway. In line with this observation, cytochrome *c* was detected in the cytosol (fig.2d). Concomitantly, AIF was released from the mitochondria, suggesting that activation of both caspase-dependent and -independent pathways could be involved in the pro-apoptotic effects of sorafenib.

Combination of letrozole and sorafenib results in synergistic anti-proliferative activity in aromatase-expressing breast cancer cell lines.

Treatment of MCF-7/AROM-1 cells with 1 μ M letrozole and increasing concentrations of sorafenib (0.1-2.5 μ M) produced a synergistic inhibition of cell proliferation, as calculated by the Bliss interaction model (fig.3a). Combination with 5 μ M sorafenib failed to produce any enhancement of the growth-inhibitory effects, presumably because of the cytotoxic action of sorafenib at this concentration.

To evaluate whether the synergistic anti-proliferative action of letrozole and sorafenib required the simultaneous presence of both drugs from the start of treatment, we compared three different schedules: “simultaneous”, “sequential”, and “up-front” (letrozole before combination) treatment. As shown in fig.3b, the “sequential” treatment was not

effective in enhancing the anti-proliferative activity of the single agents. In contrast, the “up-front” schedule showed the same enhanced anti-proliferative effect as that produced by the “simultaneous” schedule.

We then examined the impact of the synergistic drug treatment on growth/survival signalling pathways. As shown in fig.3c, letrozole alone had no effect on the phosphorylation/expression of ERK1/2, AKT, mTORC1 and p70S6K. In contrast, sorafenib significantly decreased the phosphorylation of mTORC1 and its targets p70S6K and 4E-BP1 after 24-72h; concomitantly the phospho-AKT and phospho-ERK1/2 level increased, presumably as consequence of release of mTORC1-mediated inhibition on PI3K/AKT signalling [37]. The combination with letrozole did not produce any additional effect on these changes.

To evaluate whether the synergistic anti-proliferative effect of letrozole/sorafenib combination was not restricted to MCF7/AROM-1 cells, treatments with letrozole and sorafenib either alone or in combination were performed in other aromatase-expressing breast cancer cell lines, i.e. T47D/AROM cells (clone 1 and 2) and BT474/AROM cells. As demonstrated in MCF-7/AROM-1 cells, letrozole slowed cell proliferation (fig.4a). Treatment with sorafenib inhibited cell proliferation in a concentration-dependent manner (fig.4b) and was associated with inhibition of the mTORC1 pathway (not shown); in addition induction of cell death was detected on treatment with higher concentrations of the drug (fig.4c). Most importantly, the combined treatment produced a synergistic anti-proliferative effect, as demonstrated by the Bliss interaction model (fig.4d), thus strengthening the potential of combining letrozole and sorafenib for treatment of hormone-dependent breast cancer.

Letrozole and sorafenib cooperate to induce G₀/G₁ cell cycle arrest without inducing cell death.

Cell cycle analysis of MCF-7/AROM-1 cells (fig.5a) demonstrated that after 3 days of steroid-deprivation the cells were almost completely blocked in G₀/G₁ phase. Treatment with either letrozole or sorafenib also induced accumulation of cells in G₀/G₁ phase with a concomitant decrease of cells in S phase. The accumulation of cells in G₀/G₁ phase was further increased by the drug combination, thus confirming the synergistic growth-inhibitory effects. Western blot analysis demonstrated that neither the single drug treatment nor the combination affected the expression of p53 and p27 (fig.5b). Letrozole induced p21 expression; however, no further induction was observed in the presence of both drugs. In contrast, a combined effect was detectable on the expression of c-myc, cyclin D1 and phospho-Rb, whose levels were reduced after 3 days of treatment. Comparable effects were obtained combining letrozole with the mTORC1 inhibitor rapamycin (fig.5b), suggesting that the down-regulation of these cell cycle regulatory proteins required the simultaneous inhibition of the estrogen-dependent signalling and the mTORC1 pathway. Fluorescence microscopy demonstrated that letrozole 1 µM combined with sorafenib 1-2.5 µM up to 6 days failed to promote cell death (not shown), indicating that the drug combination had cytostatic and not cytotoxic effects.

Sorafenib treatment prevents the acquisition of resistance towards letrozole.

Acquisition of resistance to letrozole has been shown to eventually occur after prolonged treatments with the drug [38]. To evaluate whether sorafenib may prevent or delay the development of resistance to letrozole, long-lasting treatments with the single agents or the combination were performed in MCF-7/AROM-1 cells. As shown in fig.6, the proliferation index of cells continuously cultured in the presence of letrozole was lower than that observed in testosterone-stimulated cells. However, a more prolonged treatment with letrozole eventually promotes the acquisition of resistance to the drug. Also sorafenib treatment produced a decline in the proliferation index, however the cells continued to

proliferate, although at a very low rate. In contrast, continuous treatment with letrozole and sorafenib induced a rapid decline of the proliferation index followed by death of the entire cell population ($N/N_0 < 1$ after 9 weeks), indicating that MCF-7/AROM-1 cells were not able to adapt to the drug combination and therefore to acquire any form of resistance to letrozole.

DISCUSSION

In recent years, crosstalk between ER and growth factor receptors has been shown to contribute to both the failure of endocrine therapy as well as the development of resistance in breast cancer. Therefore, a clear rationale has been developed for combining endocrine therapies with signal transduction inhibitors to enhance endocrine responsiveness and to potentially circumvent or delay the onset of acquired resistance [10, 11].

The multi-kinase inhibitor sorafenib has been recently approved for treatment of advanced renal and hepatocellular carcinoma [13, 14] and has shown potent activity against a variety of tumour cell types [39]. In breast cancer cells sorafenib reduces cell proliferation, and sensitizes to chemotherapy via down-regulation of the Bcl-2-like anti-apoptotic protein Mcl-1 [22, 40]. Moreover, sorafenib combined with nanoliposomal ceramide has been shown to inhibit survival of MDA-MB-231 breast cancer cells and decrease tumour development *in vivo* [41].

Here we provide evidence that letrozole combined with sorafenib produces synergistic anti-proliferative effects in aromatase-expressing breast cancer cell lines (MCF-7/AROM-1, T47D/AROM and BT474/AROM cells).

Proliferation of these cells is affected by estrogen or testosterone stimulation and letrozole, by preventing testosterone aromatization, inhibited cell proliferation.

Also sorafenib inhibited cell proliferation in a concentration-dependent manner. It could be argued that sorafenib's anti-proliferative effect may result via direct inhibition of aromatase, however recent studies have shown this is not the case [26]. In addition, when sorafenib was used at higher concentrations (5-10 μ M) the inhibition of cell proliferation was associated with apoptosis induction, via the mitochondrial intrinsic pathway.

In testosterone-stimulated MCF-7/AROM-1 cells, sorafenib at 2.5 μ M significantly decreased the phosphorylation of mTORC1 and its targets p70S6K and 4E-BP1. A

sorafenib-mediated inhibition of the mTOR pathway has been described in melanoma cells as a consequence of MAPK signalling inhibition and ascribed to the existence of crosstalk between MAPK and mTOR signalling [42]. Under our experimental conditions, however, inhibition of the MAPK pathway was only observed with higher concentrations of sorafenib (5-10 μ M), suggesting that other mechanisms are involved in the down-regulation of the mTORC1 signalling. Sorafenib-mediated inhibition of mTORC1 signalling was downstream of AKT, being associated with AKT phosphorylation/activation. In this scenario, sorafenib seems to mimic a phenomenon that has been widely described in various cellular models, including MCF-7 cells, on treatment with rapamycin, i.e. the release of mTOR-dependent negative regulation of RTK/IRS-1/PI3K/AKT signalling [37]. In addition, the recent demonstration that mTORC1 inhibition increases RTK/IRS-1/PI3K activity towards Ras/MAPK [43] may provide a mechanistic explanation for how treatment with 2.5 μ M sorafenib also leads to ERK1/2 phosphorylation/activation. Together, these results demonstrate that the anti-proliferative effects of sorafenib were not dependent on its activity as a Raf1 inhibitor, but involved the inhibition of the mTORC1 pathway.

Combination of letrozole and sorafenib significantly enhanced the anti-proliferative activity compared with single agents alone in MCF7/AROM-1 cells as well as in T47D/AROM and BT474/AROM cells, with statistical analysis indicating synergistic interaction. The growth-inhibitory effects produced by a 6-days combined treatment were maintained by an “up-front” schedule (a 3-days treatment with letrozole followed by 3 days of exposure to both drugs). Considering the advantage of shortening drug treatments, this result suggests that the “up-front” schedule may provide the optimal strategy for combining letrozole with sorafenib.

In MCF7/AROM-1 cells, the synergistic inhibition of cell proliferation was associated with accumulation of cells in the G₀/G₁ phase of the cell cycle and with down-regulation of cyclin D1, c-myc and phospho-Rb expression. In the experimental conditions adopted in

the present study, both testosterone and serum growth factors contributed to sustain the expression of cyclin D1 and c-myc thus promoting cell proliferation. Estrogens induce the expression of these genes through a “non-classical” pathway in which ER binds to AP-1 proteins and functions as a coactivator to stimulate gene transcription [44]. Growth factor-mediated contribution to cyclin D1 and c-myc expression is quite complex and primarily involves the mTORC1 signalling cascade. In particular, mTORC1 can regulate the synthesis of both cyclin D1 and c-myc by controlling cap-dependent mRNA translation. However, when this pathway is inhibited, for example by rapamycin or sorafenib, translation can be initiated via IRES-mediated mechanisms [45]. Moreover, mTORC1 can induce *cyclin D1* and *c-myc* gene transcription by activating transcriptional factors such as STAT3 [46]. Since the regulation of these genes is governed by at least these two different mechanisms of control (ER-mediated genomic pathway and mTORC1 pathway), both have to be inhibited to reduce cyclin D1 and c-myc expression and to block cell cycle progression.

As previously mentioned, development of resistance to letrozole is a clinical issue. It has been reported that letrozole-resistant cell lines may be generated by prolonged culturing (more than 50 weeks) in the presence of the drug [38]. Research into the mechanism of endocrine responsiveness and resistance in breast cancer has revealed that various growth factor pathways, such as EGFR/HER2 or PI3K/AKT family, and oncogenes involved in the signal transduction cascades become activated and utilized by breast cancer cells to bypass the effects of endocrine treatments [47]. Such cascades would antagonize the anti-proliferative effect of anti-estrogens and thus represent attractive targets for pharmacologic intervention with signal transduction inhibitors that target aberrantly or excessively expressed oncogene products. Here we demonstrate that when letrozole is used in combination with sorafenib under long-term culture conditions (12

weeks), the cell proliferation rate rapidly drops as compared with letrozole-treated cells, and the cells ultimately die.

In conclusion, to our knowledge, this is the first *in vitro* study demonstrating the efficacy of combining letrozole with the multi-kinase inhibitor sorafenib for the treatment of breast cancer either to inhibit cell growth or to prevent the acquisition of resistance to Als therapy. To prove the clinical benefits of the association of letrozole with sorafenib a phase II clinical trial in breast cancer neo-adjuvant setting is on-going between our Institutions.

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FIGURE LEGENDS

Fig.1 MCF-7/AROM-1 cell proliferation is stimulated by estrogens and inhibited by letrozole and sorafenib

a, Steroid-deprived cells (10^4 cells/cm²) were treated with E2 or T up to 144h. Cell proliferation was assessed by MTT assay. Data are expressed as fold increase *versus* steroid-deprived cells.

b, Cells were seeded at the indicated density in 96-multiwell plates and 24h later were treated with T in the absence or presence of increasing concentrations of letrozole. After 6 days of incubation cell proliferation was assessed by MTT assay.

c, Steroid-deprived cells (10^4 cells/cm²) were treated with T in the absence or presence of increasing concentrations of sorafenib (0.1-5 μ M). After 6 days cell proliferation was assessed by MTT assay. The IC₅₀ value, calculated as reported in Material and Methods, was of 3 μ M.

Data in b and c are expressed as percent of inhibition of cell proliferation *versus* T-stimulated cells. *Columns*, means of three separate experiments; *bars*, SD. *p<0.05; **p<0.01.

d, Cells were pre-incubated for 30min with sorafenib (S, 1-10 μ M) and then stimulated with T for 2h in the absence or presence of the drug at the indicated concentrations. The cells were lysed and protein expression was assessed by Western blot analysis. Data are from a representative experiment. Each experiment, repeated three times, yielded similar results.

Fig. 2 Sorafenib at high concentrations induces cell death by apoptosis

Steroid-deprived MCF-7/AROM-1 cells were treated with T in the absence or presence of various concentrations of sorafenib (S, 1-10 μ M) up to 72h.

a, Cell death was quantitated at the indicated time intervals by fluorescence microscopy on Hoechst 33342/PI-stained cells. Data are expressed as percent values. *Columns*, means of three separate experiments; *bars*, SD. * $p < 0.05$; ** $p < 0.01$.

b, Morpho-functional analysis of cell death was evaluated by real time CLSM after 48h of treatment with sorafenib 10 μ M using the vital stain calcein-AM (green colour), and the nuclear dyes Draq5 (blue colour) and PI (red colour) to discriminate between viable/early apoptotic and dead cells. *Arrows*, blebs; *arrowheads*, calcein-AM positivity until late stages of cell death. *Bar*, 20 μ m.

c, Cleavage of PARP-1, procaspase-8, -9 and -7 were assessed at the indicated time by Western blot analysis.

d, Cytochrome c and AIF release from mitochondria was assessed after 24 and 48h of incubation with sorafenib 10 μ M by Western blot analysis of cell cytosolic and mitochondrial fractions.

Images shown in b and results presented in c and d are from a representative experiment. Each experiment, repeated three times, yielded similar results.

Fig. 3 Combination of letrozole and sorafenib potentiates the inhibition of cell proliferation

a, Steroid-deprived MCF-7/AROM-1 cells (10^4 cells/cm²) were treated with T in the absence or presence of 1 μ M letrozole combined with various concentrations of sorafenib (0.1-5 μ M). After 6 days cell proliferation was assessed by MTT assay and the effect of the drug combination was evaluated using the Bliss interaction model. Data are expressed as percent of inhibition of cell proliferation *versus* T-stimulated cells and are means of three separate experiments. *Bars*, SD. * $p < 0.05$; ** $p < 0.01$.

b, Cells (10^4 cells/cm²) were treated for 6 days with T in the absence or presence of 1 μ M letrozole (L1), 2.5 μ M sorafenib (S2.5) or three different combinations of the drugs: 6 days of continuous exposure to letrozole and sorafenib (L+S); a 3 days-treatment with letrozole followed by a 3 days-treatment with sorafenib (L before S); a 3 days-treatment with letrozole followed by 3 days of combined treatment (L before L+S). Cell proliferation was assessed by MTT assay. Data are expressed as percent of inhibition of cell proliferation *versus* T-stimulated cells. *Columns*, means of three separate experiments; *bars*, SD. *p<0.05; **p<0.01 (one-way ANOVA).

c, Cells were treated with T in the absence or presence of 1 μ M letrozole (L), 2.5 μ M sorafenib (S) or the combination of both. The cells were lysed at the indicated time and protein expression was assessed by Western blot analysis. The results are from a representative experiment. Each experiment, repeated three times, yielded similar results.

Fig. 4 Combination of letrozole and sorafenib produces a synergistic inhibition of cell proliferation in T47D/AROM and BT474/AROM cells

Steroid-deprived cells (10^4 cells/cm²) were treated with T in the absence or presence of increasing concentrations of letrozole (a) or sorafenib (b). After 6 days of incubation cell proliferation was assessed by MTT assay. Data are expressed as percent of inhibition of cell proliferation *versus* T-stimulated cells. *Columns*, means of three separate experiments; *bars*, SD. *p<0.05; **p<0.01.

c, Steroid-deprived cells were treated with T in the absence or presence of various concentrations of sorafenib up to 72h. Cell death was quantitated by fluorescence microscopy on Hoechst 33342/PI-stained cells. Data are expressed as percent values. *Columns*, means of three separate experiments; *bars*, SD. *p<0.05; **p<0.01.

d, Steroid-deprived cells (10^4 cells/cm²) were treated with T in the absence or presence of letrozole (1 μ M for T47D/AROM clone 1 and 2; 10^{-2} μ M for BT474/AROM cells) combined

with increasing concentrations of sorafenib. After 6 days cell proliferation was assessed by MTT assay and the effect of the drug combination was evaluated using the Bliss interaction model. Data are expressed as percent of inhibition of cell proliferation *versus* T-stimulated cells and are means of three separate experiments. Bars, SD. * $p < 0.05$; ** $p < 0.01$.

Fig. 5 Letrozole and sorafenib cooperate to inhibit cell cycle progression in G₁ phase
Steroid-deprived MCF-7/AROM-1 cells were untreated (without steroids) or treated with T in the absence or presence of 1 μ M letrozole (L1), 2.5 μ M sorafenib (S2.5) or a combination of both.

a, After 72h the cells were analyzed by flow cytometry for cell cycle-phase distribution. Mean percentages $\pm$ SD of cells residing in each cycle phase are reported in the table (* $p < 0.05$; ** $p < 0.01$, Student's *t* test *versus* T-stimulated cells; ### $p < 0.001$, one-way ANOVA *versus* single agent treatment).

b, After 72h of treatment the cells were lysed and protein expression was assessed by Western blot analysis. R: rapamycin. The results are from a representative experiment. Each experiment, repeated three times, yielded similar results.

Fig. 6 Effect of long-lasting treatments with letrozole and sorafenib on MCF-7/AROM-1 cell proliferation

MCF-7/AROM-1 T-stimulated cells were continuously cultured in the presence of 1 μ M letrozole (L1), 2.5 μ M sorafenib (S2.5), or the combination of both (L1+S2.5) up to 12 weeks. Once a week the cells were counted and reseeded at the density of 8×10^3 cells/cm². Data are expressed as proliferation index N/N_0 , where *N* is the number of cells counted after 7 days of culture and *N*₀ is the number of cells initially seeded.

Bonelli MA Fig.1

Bonelli MA Fig.2

Bonelli MA Fig.3

Bonelli MA Fig.4

Bonelli MA Fig.5

Bonelli MA Fig.6

Dean Evans is employed by Novartis Pharma AG.