Combination of Farnesyltransferase and Akt Inhibitors Is Synergistic in Breast Cancer Cells and Causes Significant Breast Tumor Regression in ErbB2 Transgenic Mice

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Abstract

The Akt activation inhibitor triciribine and the farnesyltransferase inhibitor tipifarnib have modest to little activity in clinical trials when used as single agents. In this article, preclinical data show that the combination is more effective than single agents both in cultured cells and in vivo. Combination index data analysis shows that this combination is highly synergistic at inhibiting anchorage-dependent growth of breast cancer cells. This synergistic interaction is also observed with structurally unrelated inhibitors of Akt (MK-2206) and farnesyltransferase (FTI-2153). The triciribine/tipifarnib synergistic effects are seen with several cancer cell lines including those from breast, leukemia, multiple myeloma and lung tumors with different genetic alterations such as K-Ras, B-Raf, PI3K (phosphoinositide 3-kinase), p53 and pRb mutations, PTEN, pRB and Ink4a deletions, and ErbB receptor overexpression. Furthermore, the combination is synergistic at inhibiting anchorage-independent growth and at inducing apoptosis in breast cancer cells. The combination is also more effective at inhibiting the Akt/mTOR/S6 kinase pathway. In an ErbB2-driven breast tumor transgenic mouse model, the combination, but not single agent, treatment with triciribine and tipifarnib induces significant breast tumor regression. Our findings warrant further investigation of the combination of farnesyltransferase and Akt inhibitors. Clin Cancer Res; 17(9): 2852–62. ©2011 AACR.

Introduction

Accumulation of genetic alterations during oncogenesis leads to deregulation of complex signal transduction pathways, which in turn results in several hallmarks of cancer such as uncontrolled cell division, resistance to apoptosis, angiogenesis, and metastasis (1, 2). Among the signal transduction pathways, most often found deregulated in cancer cells are those triggered by receptor tyrosine kinases (RTK) such as receptors for EGF (epidermal growth factor), PDGF (platelet-derived growth factor), IGF-I (insulin-like growth factor), and VEGF (3–5). These receptors activate complex and highly integrated signaling cascades that often culminate in the nucleus to regulate gene transcription. For example, binding of growth factors to their RTKs induces receptor dimerization and cross-autophosphorylation of receptor tyrosine, which leads to the recruitment of several key signaling proteins such as the GTP/GDP exchange factor m-SOS1 that activates the small GTPase Ras. Ras in turn can trigger a multitude of signaling cascades including the Raf/Mek/ERK1/2 (extracellular signal–regulated kinase) cascade and the Ras GDS/Ral A/B pathway (6–9). Activated RTKs can also recruit phosphoinositide 3-kinase (PI3K) which can activate several pathways including those mediated by Akt (see more later; ref. 10). Other pathways activated by RTKs involve non-RTKs such as JAK and Src which stimulate pathways mediated by signal transducers and activators of transcription such as STAT3 (11, 12). Although these RTK-driven pathways are highly regulated in normal cells, they are often found persistently hyperactivated in cancer cells. For example, aberrant activation, overexpression, and/or mutations in RTKs, Ras, PI3K, Akt, Ral GDS, and/or STAT3 are common occurrences in cancer cells, and consequently the pathways mediated by these proteins are found aberrantly activated (1). This prompted the development of inhibitors of these pathways as novel therapeutic agents, many of which are presently in clinical trials (6, 9, 13–20). Unfortunately, as single agents they often show little to no clinical efficacy, suggesting that inhibiting single targets in complex signal transduction pathways that contain multiple oncogenic lesions may not be sufficient, and that combination of
Antisignaling agents often show little to no clinical efficacy when used alone, suggesting that inhibiting single targets in complex signal transduction pathways with multiple oncogenic lesions may not be sufficient, and that combination of small molecules that target several pathways may be more effective at treating cancer. Our studies show that the combination of the Akt activation inhibitor triciribine and the farnesyltransferase inhibitor tipifarnib is highly effective at inhibiting tumor growth both in cultured cancer cells as well as in an ErbB2-driven breast cancer transgenic mouse model. This combination treatment may have widespread use because the synergistic effect is seen with human cancer cell lines of various lineage (breast, leukemia, multiple myeloma, and lung) and harbors a broad spectrum of genetic/oncogenic lesions. Thus, inhibition of farnesyltransferase coupled with inhibition of Akt activation is a novel drug combination treatment approach with a potential for broad applications for cancer therapy. Our findings warrant further translational studies with this combination.

Methods

Cell lines and small molecule inhibitors

Cell lines were purchased from American Type Culture Collection. TCN and TCN-P were obtained from National Cancer Institute (NCI; Bethesda, MD). FTI-2153 was synthesized as described previously (53). Tipifarnib was obtained from Johnson and Johnson Pharmaceutical. MK-2206 was purchased from Chemietek. AG1024 was purchased from EMD Chemicals. All drugs for cell culture were dissolved in dimethyl sulfoxide (DMSO; Sigma).

Western blot analyses

SDS-PAGE and Western blotting were carried out as previously described (39). Primary antibody sources were as follows: p-Akt (Ser473), p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-4EBP1 (Thr37/46), 4EBP1, p-p70S6K (Thr389), and p70S6K were from Cell Signaling Technology; HDJ-2 was from Labvision/Thermo Fisher Scientific; Rap1 and Akt1/2 were from Santa Cruz Biotechnology; β-actin and α-tubulin were from Sigma. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Translated Relevance

Antisignaling agents often show little to no clinical efficacy when used alone, suggesting that inhibiting single targets in complex signal transduction pathways with multiple oncogenic lesions may not be sufficient, and that combination of small molecules that target several pathways may be more effective at treating cancer (3–5, 21, 22).

One of the major pathways targeted for anticancer drug discovery is the PI3K/Akt pathway (10, 15–18). Stimulation of RTKs activates PI3K which converts phosphatidylinositol bisphosphate (PIP2) to PIP3. Akt and PDK1 are then recruited to the plasma membrane through binding of their pleckstrin homology (PH) domains to PIP3. PDK1 then phosphorylates Akt on Thr308, and full activation of Akt occurs when Ser473 is phosphorylated by kinases such as the TORC2 complex (23, 24). Activated Akt then phosphorylates a multitude of substrates involved in several hallmarks of cancer such as evasion of apoptosis, angiogenesis, and metastasis (10). We have identified triciribine (TCN/TCN-P), a small molecule inhibitor of the activation of all 3 isoforms of Akt: Akt1, Akt2, and Akt3 (25). TCN-P inhibits Akt activation by binding to the PH domain of Akt, blocking its membrane binding and subsequent phosphorylation (26). We and others have conducted clinical trials in both solid tumors (27) and leukemia (28) and found that TCN-P is able to inhibit p-Akt levels by comparing biopies before and during treatment (27). Unfortunately, no major clinical responses were observed, suggesting that inhibition of Akt activation alone is not sufficient and that combination therapy is warranted.

Many small G proteins that are involved in oncogenesis require posttranslational modifications with the lipids farnesyl or geranylgeranyl for their cancer-causing activity (29, 30). Farnesyltransferase (FTase) catalyzes the transfer of farnesyl from farnesylpyrophosphate to the thiol of cysteines at the carboxyl termini of proteins that end with the consensus sequence C-A-A-X where C = cysteine, A = any aliphatic amino acid, and X = any amino acid but preferably methionine or serine (30). Geranylgeranylationtransferase I (GGTase I) transfers geranylgeranyl from geranylgeranyl pyrophosphate to the thiol of C-A-A-X cysteine but prefers X to be leucine (30). Because farnesylated small G proteins, such as Ras and Rheb, and geranylgeranylated small G proteins, such as Rho and Ra1, require these posttranslational modifications for mediating malignant transformation, FTase and GGTase I inhibitors (FTIs and GGTIs) are being evaluated as potential anticancer drugs (29, 31–35). Preclinical studies showed that FTIs interfere with cell-cycle progression mainly during mitosis, whereas GGTIs induce cell-cycle arrest at the G1 phase. Furthermore, whereas FTIs have been shown to disrupt the Rheb/mTOR/S6Kinase pathway, GGTIs induce the accumulation of the CDK inhibitors p21waf1 and p27Kip1, inhibit CDK activity, and induce pRb hypophosphorylation (36–47). While several FTIs have been investigated in the clinic extensively, only 1 GGTI has entered phase I clinical trials recently (48, 49). Unfortunately, as single agents, FTIs, such as tipifarnib, have shown no clinical benefit in phase III clinical trials in metastatic colorectal and pancreatic cancer patients (50, 51). However, the combination of tipifarnib with doxorubicin and cyclophosphamide in phase II trials appears to benefit patients with locally advanced breast cancer, increasing the pathologic complete response rate (52). In this article, we provide preclinical data that show in cultured human breast cancer cells that the combination of the Akt activation inhibitor TCN and the FTI tipifarnib is synergistic at inhibiting anchorage-dependent and independent growth and inducing apoptosis. Furthermore, in an ErbB2-driven breast cancer transgenic animal model, the combination, but not single agent treatment, causes significant breast tumor regression.
**MTT assays**

Cells were cultured in 96-well plates and allowed to adhere overnight. The medium was then replaced with medium containing drugs as indicated for 72 hours before incubating with 1 mg/mL thiazolyl blue tetrazolium bromide (MTT; Sigma) for 3 hours. The medium was then removed and MTT crystals were solubilized in DMSO (Sigma). Plates were read on a μQuant microplate reader using KC4 data analysis software (Bio-Tek Instruments). The 540-nm absorbance of vehicle-treated wells was used to define 100% proliferation. Each condition was carried out in replicates of 8 wells.

**Synergy analysis**

The effects of drug combinations were evaluated with CalcuSyn software (Biosoft). This software uses the Chou–Talalay combination index method, which is based on the median–effect equation, itself a derivation from the mass–action law (54). For this analysis, Drug1 was combined with Drug2 at a constant ratio determined by IC50Drug1/IC50Drug2. We entered the resulting proliferation data, along with the data obtained from single drug treatments, into CalcuSyn to determine a combination index value (CI) for each combination point, which quantitatively defines additivity (CI = 1), synergy (CI < 1), and antagonism (CI > 1). The resulting values were used to construct a plot of CI values over a range of fractions affected (Fa-Ci plot; ref. 54).

**Active caspase-3 staining**

After drug treatment, cells were trypsinized, washed, and fixed using BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit from BD Biosciences. Cells were stained with FITC (fluorescein isothiocyanate)-conjugated antibody to the active form of caspase-3 (BD Biosciences) according to the manufacturer’s recommended protocol. Samples were analyzed using a FACSCalibur flow cytometer (BD).

**Soft agar assay**

Cells were seeded in regular growth medium containing 0.3% agar (Sigma) and drug was added at the indicated concentrations as previously described (39). Colonies were allowed to grow for 3 weeks before 1 mg/mL MTT (Sigma) solution was added overnight to facilitate counts. Colonies were scored and counted according to size as previously described (39).

**Mouse experimental procedures**

MMTV/neu transgenic mice [FVB/N-Tg(MMTVneu) 202Mul/J] were purchased from the Jackson Laboratory and bred to produce multiple litterers to maintain the colony. Female mice were palpated once each week for mammary gland tumor nodules. At the time of tumor onset, orthogonal measurements were taken 2 to 3 times per week and the tumor volume was calculated using the formula: \( V = \left( L^2W \right)/2 \), where \( L = \) length and \( W = \) width with width defined as the larger measurement. Drug treatment was initiated when the tumors grew to about 200 to 2,000 mm\(^3\) and treatment lasted up to 14 days. The treatment groups were as follows: (a) vehicle, (b) TCN-P, at a dose of 20 mg/kg/d in 1.5% sodium bicarbonate (Thermo Fisher) w/v in water, pH 8.0, administered in 100 μl intraperitoneal injections once daily, (c) tipifarnib, at a dose of 10 mg/kg/d in 20% 2-hydroxypropyl-β-cyclodextrin, was administered by a s.c. minipump (Alzet), releasing at a rate of 0.5 μL/h for 14 days, or (d) a combination of the 2 treatments. There was no evidence of gross toxicity in the drug-treated animals as measured by weight loss. All methods involving mice were approved by the Institutional Animal Care and Use Committee of the University of South Florida.

To analyze the effect of each treatment, we calculated the percent change in volume of each tumor during treatment. The percent change in volume was calculated on the basis of the tumor volume on the last day of treatment (\( V_t \)) relative to that on the day of initiation of treatment (\( V_i \)). The average percent change in tumor volume was then calculated for each treatment group.

**Statistical analysis**

For statistical analysis, percent volume change of 44 tumors was compared among the 4 treatment groups (vehicle, TCN-P, tipifarnib, and combination treatments) using a generalized linear model (Proc GLM in SAS V9.2). When differences were detected, Dunnett–Hsu test was conducted to adjust for multiple comparisons and to examine if the combination treatment is more effective than single agent treatment.

**Pre- and posttreatment tumor biopsies and Western blot analyses**

Incisional biopsies were taken from the same tumor before treatment and after 7 days of treatment with vehicle, tipifarnib, TCN-P, or the combination. Tumors were homogenized with a PowerGen 125 Tissue Homogenizer (Thermo Fisher) in T-PER Tissue Protein Extraction Reagent (Thermo Fisher) supplemented with 1× Complete Protease Inhibitor Cocktail (Roche Applied Science), 4 mmol/L sodium orthovanadate (Sigma), 10 mmol/L p-nitrophenyl phosphate (Sigma), and 0.5 mmol/L phenylmethylsulfonyl fluoride (Sigma). Homogenates were used in SDS-PAGE and Western blotting as described previously (39).

**Results**

**The Akt activation inhibitor TCN and the FTI tipifarnib synergize to inhibit the proliferation of human breast cancer cells**

To determine the effects of combination treatment of the Akt inhibitor TCN and the FTI tipifarnib on human breast tumor cell proliferation, we first treated MDA-MB-231 cells with various concentrations of TCN and tipifarnib alone and in combination and monitored cell proliferation by MTT assay as described under Methods. Figure 1A and B show that tipifarnib or TCN alone caused a concentration-dependent inhibition of proliferation with IC50 values of 18 μmol/L (tipifarnib) and greater than 30 μmol/L (TCN).
Furthermore, Figure 1B shows that treatment of MDA-MB-231 with fixed concentrations of tipifarnib decreased the IC$_{50}$ values of TCN from greater than 30 µmol/L (in the absence of tipifarnib) to 6 µmol/L (in the presence of 1 µmol/L tipifarnib) and 0.5 µmol/L (in the presence of 10 µmol/L tipifarnib). Therefore, tipifarnib sensitized MDA-MB-231 cells to TCN. Similarly, as shown in Figure 1A, TCN sensitized these cells to tipifarnib treatments, decreasing the IC$_{50}$ values of tipifarnib from 18 µmol/L in the absence of TCN, to 8 and 0.2 µmol/L in the presence of 1 and 10 µmol/L TCN, respectively. These data suggest that the 2 compounds, TCN and tipifarnib, may synergize to inhibit anchorage-dependent growth. To confirm this synergism, we treated cells with a combination of TCN and tipifarnib in a constant ratio to one another and used CalcuSyn software to generate Fa-CI plots as described under Methods. Figure 1C and Supplemental Table S3 show that all the experimental points have CI values of less than 1. The resulting Fa-CI plot curve falls well below 1 for the effect range, showing that the combination of TCN and tipifarnib is highly synergistic in MDA-MB-231 breast cancer cells.

We next determined whether this synergistic interaction between tipifarnib and TCN is unique to MDA-MB-231 cells or whether it can also occur in other human breast cancer cell lines as well as cell lines from other cancer types. To this end, we treated 2 additional breast cancer cell lines, (MCF-7 and MDA-MB-468), 1 lung (A549), 1 leukemia (MV4-11), and 1 multiple myeloma (U266) cancer cell lines, with TCN and tipifarnib, alone and in combination, monitored cell proliferation by MTT assay, and analyzed the data for synergy as described under Methods. Figure 1D and E show the resulting Fa-CI plots and show that in both MCF-7 (Fig. 1D) and MDA-MB-468 (Fig. 1E) cells, the combination treatment of TCN and tipifarnib resulted in synergistic inhibition of anchorage-dependent proliferation (also see Supplemental Table S3). A synergistic interaction was also observed with A-549, MV4-11, and U266 (Supplementary Fig. S1), suggesting that the combination is effective in a variety of human tumor types.

We next determined whether this synergistic interaction also occurs with inhibitors of Akt and farnesyltransferase that are structurally unrelated to TCN and tipifarnib. To this end, we first treated MDA-MB-231, MDA-MB-468, and MCF-7 cells, with the Akt inhibitor MK-2206 (55) or TCN in combination with tipifarnib as described under Methods. In all 3 cell lines, the MK-2206/tipifarnib combination was more effective than the single agent treatment (Supplementary Fig. S2). To determine if the combination is synergistic, we cotreated the cells with MK-2206 and tipifarnib in a constant ratio to one another and generated Fa-CI plots. Figure 2A and Supplemental

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Figure 1. TCN and tipifarnib synergize to inhibit anchorage-dependent growth of human breast cancer cells. A and B, MDA-MB-231 cells were plated in 96-well plates, treated for 72 hours with various concentrations of TCN and tipifarnib either alone or in combination, and processed for MTT staining as described under Methods. Each data point is the average of 8 wells each from 3 independent experiments. Error bars indicate standard error. C (MDA-MB-231), D (MCF-7), and E (MDA-MB-468) cells were plated, treated, and processed as in A and B, and CI analysis to determine synergy (defined as CI values < 1) was carried out using CalcuSyn software as described in Methods. x symbol designates the combination index value (CI) for each fraction affected (effect). The curves were generated by CalcuSyn software to fit the experimental points. The effect ranges from 0 (no inhibition) to 1 (complete inhibition). The data are representative of 3 independent experiments.
Table S3 show that in MDA-MB-231, MDA-MB-468, and MCF-7 cells, the combination of MK-2206 and tipifarnib was synergistic. We next treated MDA-MB-231, MDA-MB-468, and MCF-7 cells with the farnesyltransferase inhibitor FTI-2153 (53) in combination with the Akt activation inhibitor TCN and found this combination to also be more effective than single agent treatment (Supplementary Fig. S2) and synergistic (Fig. 2B) in all 3 cell lines. Thus, the synergistic interactions seen are not unique to TCN and tipifarnib and can also occur with other structurally unrelated inhibitors of Akt and farnesyltransferase.

TCN and tipifarnib combination treatment is more effective at inhibiting signal transduction pathways in human cancer cells

Several aberrant growth factor signal transduction pathways that rely on farnesylated small G proteins and Akt result in the persistent activation of the mTOR kinase (see Introduction). Therefore, we determined the effect of TCN and tipifarnib on the persistent activation of these pathways. To this end, we treated MDA-MB-231 cells with TCN and tipifarnib either alone or in combination and processed the cells for Western blotting as described under Methods. Figure 3A shows that treatment of the cells with the FTI tipifarnib alone inhibited the prenylation of the exclusively farnesylated HDJ2 protein without affecting the prenylation of the exclusively geranylgeranylated Rap1 protein, whereas treatment with the Akt activation inhibitor TCN alone resulted in decreased phosphorylation of Akt, showing that both drugs inhibited their targets. As single agents, TCN and tipifarnib partially decreased the phosphorylation levels of the mTOR kinase substrate p70S6K. When used in combination, the effects on the phosphorylation levels of p70S6K were more pronounced (Fig. 3A). Furthermore, the combination also decreased, but only slightly, the phosphorylation levels of 4EBP1.
(Fig. 3D). In contrast, the combination did not decrease the levels of p-ERK1/2 (Fig. 3D).

We next determined whether TCN and tipifarnib inhibit their targets and subsequent signaling in the other human cancer cell lines where they synergize to inhibit proliferation. To this end, MDA-MB-468, MCF-7, A-549, MV-4-11, and U266 cells were treated with TCN and tipifarnib alone and in combination and processed for Western blotting as described under Methods. D, MDA-MB-231 cells were treated and processed as in (A); in addition, the cells were treated with AG1024 either alone or in combination with tipifarnib (last 2 lanes). The data are representative of 2 independent experiments.

TCN and tipifarnib synergize to inhibit anchorage-independent tumor cell growth and to induce apoptosis

We next determined whether TCN and tipifarnib combination treatment is more effective than single agent treatment toward inhibiting the anchorage-independent growth of MDA-MB-231, MDA-MB-468, and MCF-7 breast cancer
Cells plated in soft agar were treated with TCN and tipifarnib alone or in combination. Colony numbers were counted after 3 weeks of treatment and analyzed for synergy using Calcusyn as described under Methods. Figure 4A and Supplemental Table S3 show that TCN and tipifarnib synergistically inhibited colony formation in all 3 breast cancer cell lines as shown by the fact that all experimental CI values are less than 1. We next determined whether the treatment with TCN and tipifarnib induces apoptosis and whether the combination is more effective than single agent treatment. To this end, MDA-MB-231 and MDA-MB-468 cells treated with TCN and tipifarnib alone and in combination were stained for active caspase-3 and analyzed with flow cytometry as described under Methods (MCF-7 cells do not express caspase-3; ref. 57). Figure 4B shows that the percentage of MDA-MB-231 cells with active caspase-3 increased from 5.4% in the vehicle-treated cells to 15.1% in the TCN-treated cells and 12.4% in the tipifarnib-treated cells. When the cells were treated with the combination, the percentage of cells with active caspase-3 increased to 44.9%, a 3- to 4-fold increase over either drug alone. Similarly, in MDA-MB-468 cells, the percentages of cells with active caspase-3 in the control, tipifarnib, TCN, and the combination treated cells were 4.1%, 9.3%, 22.1%, and 47.7%, respectively. These results suggest that TCN and tipifarnib also synergize to activate caspase-3 and induce apoptosis.

The combination of TCN-P and tipifarnib causes significant breast tumor regression in MMTV-ErbB2 transgenic mice

Figures 1 to 4 strongly suggest that the Akt activation inhibitor TCN and the FTI tipifarnib in combination are more effective at inhibiting signaling, anchorage-dependent and -independent tumor cell proliferation, as well as at inducing apoptosis in cultured breast cancer cells. We next determined whether this combination is also more efficacious against breast tumors in in vivo settings. To this end, we used an MMTV-Her2/neu/ErbB2 transgenic mouse model (58). In this model, mice spontaneously develop mammary gland tumors. As described in Methods, breast tumors were measured beginning at the time of tumor onset and treatment with vehicle, tipifarnib, TCN-P, or the TCN-P/tipifarnib combination began when tumor volumes reached about 200 to 2,000 mm³. A wide range of tumor volumes was used to ensure that responses were not volume dependent.

Figure 5A depicts representative tumor growth curves from animals treated either with vehicle, each drug alone, or in combination. The tumor from the vehicle-treated
mouse continued to grow and the tumors treated with either TCN-P or tipifarnib alone changed in size minimally, whereas the tumor from the mouse treated with the combination experienced significant regression as evident from a large decrease in tumor volume (Fig. 5A). Figure 5B shows the average percent change for each treatment group. Supplementary Table S1 shows the percent change in tumor volume of each tumor for a total of 44 tumors. The percent change was calculated from the tumor volume on the last day of treatment ($V_T$) relative to the volume on the day of initiation of treatment ($V_I$), as described in Methods. All tumors from mice treated with vehicle increased in size with an average percent change in tumor volume of 62.9% ± 18.8% (Fig. 5B and Supplementary Table S1). In contrast, tumors from mice treated with either TCN-P or tipifarnib as single agents had an average percent change in tumor volume of 3% ± 9.9% for TCN-P and 1.6% ± 9.2% for tipifarnib. There was a significant difference of percent volume change observed among treatment groups with statistical significance ($P < 10^{-4}$). To be conservative, even after adjusting for multiple comparisons using Dunnett–Hsu test, significant differences were still detected between the combination treatment group and TCN-P ($P = 0.03$), tipifarnib ($P = 0.004$), and the vehicle groups ($P < 10^{-4}$). Thus, the combination treatment of TCN-P and tipifarnib is significantly more effective than single agent treatment groups and causes breast tumor regression in vivo.

To determine whether tipifarnib and TCN-P reached their targets in vivo, we carried out incisional biopsies from the same tumor before treatment (pre) and after 7 days of treatment (post) with vehicle, tipifarnib, TCN-P, or the combination and processed the tumors for Western blotting as described under Methods. Figure 5C shows that neither HDJ-2 farnesylation nor Akt phosphorylation was inhibited in tumors from mice treated with vehicle control. In contrast, treatment with tipifarnib inhibited HDJ-2 farnesylation in tumors from all 4 mice treated (2 with tipifarnib as single agent and 2 with the combination of tipifarnib and TCN-P). Furthermore, TCN-P treatment resulted in decreased Akt phosphorylation levels in 3 of the 4 tumors from the mice treated (1 with TCN-P as a single agent and 2 with the combination of tipifarnib and TCN-P). In one of the mice treated with TCN-P alone, tumor levels of phosphorylated Akt increased but so did the tumor levels of total Akt (without effect on the loading control α-tubulin; Fig. 5C).
Discussion

The uncontrolled proliferation and resistance to apoptosis as well as the angiogenic and metastatic character of cancer cells are believed to be due to deregulated signal transduction pathways that are the results of multiple genetic lesions (see Introduction). The multitude of persistently activated signal transduction pathways in cancer cells allow them to survive under the high pressure of single agent treatments. Furthermore, interfering with persistently activated pathways to which tumor cells are not addicted may not be effective. Human clinical trials have taught us that single agent treatments rarely result in clinical benefits to cancer patients, suggesting that combination therapy may be necessary for effective treatment of tumors with multiple oncogenic lesions. In the present study, we have found that the Akt activation inhibitor TCN and the FTI tipifarnib synergize to inhibit proliferation of human breast cancer cells. Similar synergistic effects were also observed with leukemia (MV-4-11), multiple myeloma (U266), and lung (A-549) tumor cells. These synergistic effects are not unique to TCN and were also observed with the combination of tipifarnib with another structurally unrelated Akt inhibitor, MK-2206. Similarly, synergy was also seen with the combination of TCN with another structurally unrelated FTI, FF1-2153, suggesting that the synergistic effects are due, at least in part, to inhibition of Akt and farnesyltransferase. In addition to the effects on anchorage-dependent cell growth, the combination of TCN and tipifarnib was also synergistic at inhibiting anchorage-independent growth in soft agar. The combination was more effective at inducing apoptosis than the single agents alone. Finally, the combination was also much more effective than single agent treatment in vivo in the ErbB2-driven breast cancer transgenic mouse model. In this model, the combination of tipifarnib and TCN induced significant breast tumor regression.

Tumors from breast cancer patients often overexpress members of the ErbB family of RTKs such as EGFR and ErbB2, and this is associated with poor prognosis, resistance to chemotherapy, and shorter survival time (3-5, 52). Overexpression of ErbB family RTKs results in persistent activation of downstream signaling pathways such as those mediated by hyperphosphorylation of Akt, ERK1/2, and STAT3 (1, 2). We found that treatment with TCN alone completely inhibited the levels of p-Akt in MDA-MB-231 cells. However, in the other 2 breast cancer cell lines, MDA-MB-468 and MCF-7, TCN alone partially inhibited p-Akt levels. In these 3 cell lines, combination treatment with TCN and tipifarnib was more effective at inhibiting the levels of p-Akt, suggesting that farnesylated proteins need to be inhibited for efficient inhibition of p-Akt levels in MDA-MB-468 and in MCF-7, but not in MDA-MB-231. Considering that Akt phosphorylation is believed to be dependent on Akt recruitment to the membrane and that TCN inhibits such recruitment (26), these results also suggest that under the pressure of TCN treatment, some breast cancer cells may overcome the effects of TCN by harboring farnesylation-dependent pathways capable of phosphorylating Akt. However, the synergistic effects on tumor cell growth and apoptosis cannot be explained solely by this effect on p-Akt levels because, at least in MDA-MB-231, TCN by itself abolished p-Akt levels but synergy with tipifarnib was still seen. It is also important to point out that in MDA-MB-231 cells, tipifarnib treatment alone resulted in an increase in p-Akt levels. This is similar to the previously reported increase in p-Akt levels following treatment with the mTORC1 inhibitor rapamycin (56). A possible explanation is that inhibition of the farnesylated protein Rheb results in inhibition of mTORC1 which in turn inhibits the phosphorylation of IRS-1 by S6K, relieving the feedback loop previously proposed for rapamycin (56). However, the IGF-IR tyrosine kinase inhibitor AG1024 did not prevent tipifarnib from increasing the levels of p-Akt suggesting that this mechanism is not involved. Whether other feedback loops with other RTKs are involved is not known.

TCN inhibition of Akt activation (26) is anticipated to result in the activation of the Rheb GAP, TSC1/2, which in turn would inhibit Rheb activation, leading to the inhibition of mTORC1 phosphorylation of S6 Kinase (41–47). Furthermore, inhibition of Rheb farnesylation by tipifarnib is also anticipated to inhibit mTORC1-mediated phosphorylation of S6 Kinase (41–47). In all 3 breast cancer cell lines, the inhibition of p-S6 Kinase is only partial and requires combination treatment for a more complete inhibition. This suggests that neither inhibition of Rheb farnesylation nor prevention of the Akt-dependent inhibition of TSC1/2 is sufficient to fully inactivate mTORC1 from phosphorylating S6 Kinase. While these chemical biology studies are intriguing and suggest this combination approach is required to fully inactivate this pivotal signaling pathway, further studies are required to confirm that the synergistic effect of TCN and tipifarnib on tumor growth and survival is mediated, at least in part, by inhibition of mTORC1 phosphorylation of S6 Kinase.

An important finding of this study is that the combination of TCN and tipifarnib is synergistic in human cancer cell lines with a variety of genetic alterations. For example, the levels of expression of ErbB family members are different among these cell lines, with MDA-MB-468 and MDA-MB-231 cells expressing high levels of ErbB1 and ErbB3, and MCF-7 expressing high levels of ErbB3 and ErbB4 (Supplementary Table S2). In addition, while MDA-MB-231 and A-549 cells harbor K-Ras mutations, the other 4 cell lines have wild-type K-Ras. Furthermore, MDA-MB-468 has a deleted PTEN, whereas the other 5 cell lines have wild-type PTEN. While MDA-MB-231 and U266 cells have B-Raf mutations, the other 4 cell lines have wild-type B-Raf (for other mutations in PI3K, p53, pRb, and Ink4a, please refer to Supplementary Table S2). Despite these differences, the combination of TCN and tipifarnib was synergistic at inhibiting proliferation of all 6 cell lines evaluated. The fact that several tumor types with multiple genetic lesions are sensitive to this combination treatment suggests that the
dual targeting of the Akt/Rheb/mTOR pathway, a signaling node pivotal to tumor growth and survival, may be an effective approach to chemotherapy.

In summary, our studies have identified a combination treatment that appears to be highly effective at inhibiting tumor growth both in cultured cancer cells as well as in an ErbB2-driven breast cancer transgenic mouse model. This combination treatment may have widespread use because the synergistic effect does not depend on specific genetic/oncogenic lesions harbored by the tumor cell lines and was observed in human cancer cell lines of various lineage including breast, leukemia, multiple myeloma, and lung. Thus, inhibition of farnesyltransferase coupled with inhibition of Akt activation is a novel drug combination treatment approach with a broad application for cancer therapy. Further preclinical studies are warranted to confirm and validate this approach prior to clinical trials.

References


49. Rowlinsky EK. Lately, it occurs to me what a long, strange trip it’s been for the farnesyltransferase inhibitors. J Clin Oncol 2006;24:2881–4.


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