Chemical Therapeutics

The Tyrosine Kinase Inhibitor E-3810 Combined with Paclitaxel Inhibits the Growth of Advanced-Stage Triple-Negative Breast Cancer Xenografts

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Abstract

E-3810 is a novel small molecule that inhibits VEGF receptor-1, -2, and -3 and fibroblast growth factor receptor-1 tyrosine kinases at nmol/L concentrations currently in phase clinical II. In preclinical studies, it had a broad spectrum of antitumor activity when used as monotherapy in a variety of human xenografts. We here investigated the activity of E-3810 combined with different cytotoxic agents in a MDA-MB-231 triple-negative breast cancer xenograft model. The molecule could be safely administered with 5-fluorouracil, cisplatin, and paclitaxel. The E-3810–paclitaxel combination showed a striking activity with complete, lasting tumor regressions; the antitumor activity of the combination was also confirmed in another triple-negative breast xenograft, MX-1. The activity was superior to that of the combinations paclitaxel + brivanib and paclitaxel + sunitinib. Pharmacokinetics studies suggest that the extra antitumor activity of the combination is not due to higher paclitaxel tumor levels, which in fact were lower in mice pretreated with all three kinase inhibitors, and the paclitaxel plasma levels excluded reduced drug availability. Pharmacodynamic studies showed that E-3810, brivanib, and sunitinib given as single agents or in combination with paclitaxel reduced the number of vessels, but did not modify vessel maturation. Reduced tumor collagen IV and increased plasma collagen IV, associated with increased matrix metalloproteinases (MMP), particularly host MMP-9, indicate a proteolytic remodeling of the extracellular matrix caused by E-3810 that in conjunction with the cytotoxic effect of paclitaxel on the tumor cells (caspase-3/7 activity) may contribute to the striking activity of their combination. These data support the therapeutic potential of combining E-3810 with conventional chemotherapy. Mol Cancer Ther; 12(2); 1–10. ©2012 AACR.

Introduction

Treatment of cancer is by definition multimodal, including surgery, chemotherapy, and radiotherapy (1). The underlying rationale is to improve the therapeutic index, resulting from the summing of anticancer effects and the nonoverlapping side effects of the different treatment modalities. This implies that all the new active drugs will at some points in their clinical development be used in combination with standard care (radio- and chemotherapy). This is also true for targeted therapy. Antiangiogenic treatment is the most obvious example: angiogenesis, the growth of new vessels from existing ones, has been defined as a hallmark for the growth and maintenance of tumors (2), and inhibition of angiogenesis is a recognized mechanism of various clinically effective anticancer therapies (3, 4). These include bevacizumab, a monoclonal antibody to VEGF-A, as well as sunitinib, sorafenib, and other small molecules with inhibitory activity against the receptors involved in angiogenesis [i.e., VEGF receptor (VEGFR), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR); ref. 5].

Pure antiangiogenic drugs are typically cytostatic and even though are active as single agents in preclinical and some clinical studies, their primary use is in combination with chemotherapy. Bevacizumab as monotherapy causes a tumor response in glioblastoma (6), ovarian carcinoma (7), and colorectal liver metastases (8), but only when combined with chemotherapy it significantly prolonged overall survival in patients with metastatic colorectal cancer, in metastatic nonsquamous non–small cell lung carcinoma (NSCLC; ref. 9). Moreover, because of the multiplicity and complexity of pathways that can be involved in tumor inception and growth, inhibiting multiple targets and/or developing rational combinations

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with other treatment modalities particularly with cytotoxic, seems a reasonable goal.

Triple-negative breast cancer (TNBC) comprises a heterogeneous group of tumors, accounting for 15% of breast tumors, with a very poor prognosis, with no or minimal expression of estrogen/progesterone receptors and EGF receptor (EGFR)-2 (10). Therefore, it is not amenable to hormone therapy or the anti-HER2 monoclonal antibody trastuzumab, and systemic treatment options are currently limited to cytotoxic therapy, including paclitaxel. A number of target approaches are under clinical evaluation: PARP inhibitors, antiangiogenic drugs, and anti-EGFR agents (11, 12). FGFR signaling may be important for the growth of a certain proportion of TNBC, providing a rationale for assessing FGFR inhibitors, or therapies targeting FGF2 ligand in TNBC and basal-like breast cancer (13).

E-3810 is a new, orally active tyrosine kinase inhibitor with antitumor activities attributable to the inhibition of VEGFR-1, -2, -3, fibroblast growth factor receptor-1 (FGFR-1), and colony-stimulating factor receptor 1 (CSF-1R; Fig. 1). In preclinical studies, E-3810 has shown a broad spectrum of antitumor activity when used as monotherapy in a variety of human xenografts (14). We investigated the activity of E-3810 combined with different cytotoxic agents in TNBC xenograft models. The molecule could be safely administered with these drugs and the E-3810–paclitaxel combination showed a striking activity. Pharmacokinetics and pharmacodynamic studies were carried out aimed at clarifying the observed antitumor effect. These findings support the therapeutic potential of combining E-3810 with conventional chemotherapy.

Materials and Methods

Cell cultures

MDA-MB-231 (human TNBC) cells were obtained from the American Type Culture Collection and maintained in RPMI medium supplemented with 10% (v/v) FBS and 1% (v/v) L-glutamine. Cell line authentication was not carried out by the authors within the last 6 months.

Drugs

E-3810, sunitinib, and brivanib were synthesized by ChemPartner Co. Ltd.. All drugs were dissolved in 100% dimethyl sulfoxide (DMSO) at a final concentration of 10 mmol/L for in vitro treatment. For in vivo experiments, these drugs were dissolved in 0.5% Methocel. Drug solutions were made every 5 days and kept at 4°C, as these working solutions are stable for at least 7 days, with the exception of brivanib, which was dissolved immediately before use. The drugs were administered orally by gavage, daily at the doses and schedules detailed in Results. Paclitaxel (kindly provided by Indena S.p.A.) was dissolved in 50% Cremophor EL (Sigma-Aldrich) and 50% ethanol, further diluted with saline immediately before use and injected intravenously at a dose of 20 mg/kg; both cisplatin (DDP) and 5-fluorouracil (5-FU) were supplied by Sigma-Aldrich and dissolved in NaCl 0.9%. Control mice received the corresponding vehicle.

Antiproliferative assays

Exponentially growing MDA-MB-231 cells were seeded in 96-well plates at a density of 3 to 6 × 10³ cells/100 μL/well in complete medium. After 24 hours, cells were
treated with both different paclitaxel concentrations and 2 fixed doses (0.15 and 3 μmol/L) of E-3810, sunitinib, and brivanib for 72 hours. The antiproliferative effect was evaluated by MTS at the end of treatment time.

**Xenograft models**

Six- to 8-week-old female NCr-nu/nu mice were obtained from Harlan S.p.A. Mice were maintained under specific pathogen-free conditions, housed in isolated vented cages, and handled using aseptic procedures. Procedures involving animals and their care were conducted in conformity with institutional guidelines in compliance with national and international laws and policies.

MDA-MB-231 exponentially growing cells (3–5 × 10³ cells) were detached from flask, resuspended in PBS, and implanted subcutaneously into the right flank region of athymic mice. MX-1 human breast tumor was subcutaneously transplanted as fragments. Unless otherwise specified, mice were randomized when the average tumor size was 350 to 400 mm² (advanced stage: 8–10 per group).

Tumor growth was measured twice weekly with a Vernier caliper, and the tumor weights (mg = mm³) were calculated as follows: [length (mm) × width (mm)²]/2. Efficacy was expressed as best tumor growth inhibition [TGI%; 1 − (median tumor weight of treated tumors/median tumor weight of control tumors) × 100] or tumor growth delay (T–C = median time to reach 500 or 1,000 mg in treated tumor − median time to reach 500 or 1,000 mg in control tumor). Survival curves were drawn as a percentage of survival animals in all the experimental groups.

**Paclitaxel pharmacokinetics**

MDA-MB-231 tumor-bearing mice were randomized when their tumor masses were about 350 to 400 mg to receive E-3810, brivanib, and sunitinib at the doses used for the antitumor activity trial, for 10 days. Four hours after the antiangiogenic dose of day 7, paclitaxel was injected intravenously at the dose of 20 mg/kg and tumor and plasma samples were collected after 1, 4, and 24 hours in all the groups (each group consisting of 3 animals). At the indicated sampling time, mice were anesthetized, blood was collected from the retro-orbital plexus into heparinized tubes, and the plasma fraction was separated.

Mice were killed by cervical dislocation, and tumors excised and snap-frozen. The samples were analyzed by high-performance liquid chromatography (HPLC) with UV detection at 230 nm as described in the Supplementary Information.

**Pharmacodynamic studies**

MDA-MB-231 tumor-bearing mice were treated daily with E-3810, brivanib, and sunitinib as described in the Paclitaxel pharmacokinetics section. At 24 and 72 hours after paclitaxel treatment, blood from control and treated mice was collected from the retro-orbital plexus into heparinized tubes, and the plasma fraction was separated. Mice were killed by cervical dislocation, and tumors were excised, and directly snap-frozen or embedded in optimal cutting compound (OCT), snap-frozen, and stored at −80°C. Cryosections (4 μm) were fixed in methanol/acetic acid at room temperature.

A number of pharmacodynamic markers were examined on tumor samples. Vessel density and morphology were assessed by immunostaining with anti-CD31 antibody MEC13.3 (Beckton Dickinson GmbH), followed by a biotin-conjugated mouse anti-rat immunoglobulin G (IgG)1/2a monoclonal antibody (clone C28-5, Beckton Dickinson GmbH), and a streptavidin-alkaline phosphatase (AP) conjugate (Biozol Diagnostika GmbH). For chromogenic detection, the chromogenic solution of the Dako REAL Detection System AP/RED (Dako Deutschland GmbH) was applied. Three randomly selected measurement fields (0.57 mm²) from each tumor with representative vessel density were scanned and all CD31-stained vascular structures were marked by the examiner. The vessel density, mean vessel area, and mean vessel diameter (represented by the shortest Feret’s diameter) were determined using computer-aided image analysis software (AxioVision Rel. 4.6.2; Zeiss).

For assessment of collagen type IV and proliferative activity, cryosections were fixed with 4% paraformaldehyde for 15 minutes. Then the rabbit-anti-murine collagen type IV antibody AB756 (Millipore/Chemicon) or the rabbit-anti-human Ki67 antibody SP6 (Thermo Fisher Scientific GmbH) were applied followed by biotin-SP–conjugated AffiniPure goat anti-rabbit IgG (H+L; Diannova GmbH) and the detection system as described earlier. To assess the extent of collagen type IV or Ki67 staining, 3 randomly selected areas (0.57 mm²) of each tumor were photographically captured. The red stained area (representing collagen IV deposition or proliferative cells) was recorded by computer-aided image analysis and given as percentage of the whole area (Axio Vision Release 4.6).

The necrotic tumor area was measured after H&E staining. Whole tumor sections were scanned at low power (×4 objective = 3.58 mm²). The total tumor area and the necrotic tumor area were marked by the examiner on the scanned microscopic images. The areas were calculated by computer-aided image analysis and the tumor necrosis area was given as a percentage of the total tumor area.

Caspase-3/7 activity was assessed in snap-frozen tumor tissue samples from whole protein extraction by homogenization in radioimmunoprecipitation assay (RIPA) buffer. Total proteins were quantified by Bradford assay and the caspase-3/7 activity was measured by a fluorimetric assay in a white 96-well plate. Briefly, in each well, 5 μL of protein lysates were mixed with 45 μL of apoptosis activity buffer (20 mmol/L HEPES pH 7.5, 10% glycerol, 10 mmol/L dithiothreitol) and Ac-DEVD-AMc substrate (final concentration: 12.5 μmol/L). The Ac-DEVD-AMc cleavage reaction was monitored by a kinetic approach and the data were used to determine the slope (relative fluorescence...
unit/min) of the reaction curves, normalizing the slope of each sample for its protein concentration and calculating the ratios of the apoptotic signal from treated to control samples.

Extracellular matrix-degrading proteolytic activity was assessed by gelatin zymography. Tumor lysates (25 μg) were separated on SDS-PAGE (8%) copolymerized with 1 mg/mL gelatin (Sigma), as described (15). Gels were washed twice in 2.5% Triton X-100, incubated overnight in 50 mmol/L Tris–HCl, pH 7.6, 100 mmol/L NaCl, 0.02% Brij 35, and 5 mmol/L CaCl₂, at 37°C, and stained with Coomassie Blue. Gelatinases appear as white bands on a dark background, indicating proteolysis of the substrate. Supernatants of HT1080 fibrosarcoma and NIH-3T3 cells were used as a reference standard for respectively human and murine matrix metalloproteinase (MMP)-2 and -9.

Circulating biomarkers in plasma samples of treated and control mice were measured by ELISA (Murine Collagen IV ELISA Kit, Exocell; mouse soluble VEGFR-2, mouse FGF-21, and mouse VEGF immunoassays; R&D Systems) according to vendor instructions.

Results

Antitumor activity of E-3810 in triple-negative human breast cancer xenografts as single agent or with chemotherapeutic agents

The activity of E-3810 given at the doses of 15 mg/kg was tested on MDA-MB-231 breast cancer transplanted subcutaneously, at a late stage, when tumor masses reached 350 to 400 mg. This tumor xenograft was very sensitive to E-3810 (Fig. 2A), with complete tumor stabilization lasting throughout the 30-day treatment. As in other tumor models, tumors regrew after withdrawal of E-3810 at a rate similar to control tumors. 5-FU and DDP treatments had little activity in inhibiting MDA-MB-231 growth; when the drugs were combined with E-3810, growth inhibition was slightly better only in the mice treated with 5-FU and E-3810 but increased no further in T–C and TGI% over E-3810 singly (Fig. 2B; data not shown). 5-FU and DDP could be safely combined with E-3810, as suggested by body weight loss, which never exceeds 15% (Supplementary Fig. S1A and S1B).

In the same experimental setting, we also explored the activity of E-3810 given for 30 days starting 35 days from inoculum, and paclitaxel at the dose of 20 mg/kg weekly for 3 weeks. The combination was well tolerated; although weight loss was greater in the mice treated with both drugs than with each one singly agent, it never exceeded 15% (Supplementary Fig. S1C). One death occurred with E-3810 and 1 in the E-3810–paclitaxel combination group on day 62 and 55, respectively, in mice whose tumor was regressing (data not shown). E-3810 and paclitaxel had a similar growth inhibitory activity (Fig. 3A and Table 1); with the 2 drugs combined, all tumors regressed and by day 83 all mice were tumor free although by day 93, 1 tumor was regrowing with a rate similar to controls. The tumor growth delay and the survival curves clearly indicate a more than additive effect of the combination over the 2 drugs singly (Supplementary Fig. S2A and Supplementary Table S1).

A subsequent experiment confirmed the striking activity of E-3810–paclitaxel (at the lower E-3810 dose of 15 mg/kg) and compared this activity with the combinations of paclitaxel with sunitinib and brivanib, 2 targeted agents inhibiting kinase receptors for VEGF and FGF among others. Sunitinib and brivanib singly, at optimal dosing schedules (14, 16, 17), reduced MDA-MB-231 tumor growth but with a lower activity than E-3810 and paclitaxel (Fig. 3B and Table 1). All the mice treated with paclitaxel and E-3810, paclitaxel and sunitinib, and paclitaxel and brivanib experienced tumor regression (Fig. 3B); however, 10 days after withdrawing the drugs, tumors resumed their growth in all the mice treated with paclitaxel and brivanib and in almost all those treated with paclitaxel and sunitinib (8 of 10 mice). Tumors regrew much more slowly in mice treated with E-3810–paclitaxel (Fig. 3B). This latter combination was more...
20 mg/kg every 7 days for 3 times (Fig. 3C and Table 1). When the experiment was stopped, 5 of 9 mice were tumor-free, corroborating the data obtained on MBA-MD-231 tumor xenograft.

**Pharmacokinetics and pharmacodynamics**

We did *in vitro* and *in vivo* pharmacokinetics/pharmacodynamics studies to clarify the synergy of E-3810 and paclitaxel, also in comparison with the other combinations tested.

*In vitro* combination treatment of E-3810, sunitinib, or brivanib and different doses of paclitaxel in MDA-MB-231 cells never had any synergistic effect (Supplementary Fig. S3). This suggests that the enhanced antitumor activity observed *in vivo* is likely to be related to the antiangiogenic effects of these agents or possibly to more complex interactions between host factors and tumor cells.

To assess whether pretreatment with E-3810, sunitinib, and brivanib affected paclitaxel pharmacokinetics, we studied paclitaxel plasma and tumor levels. We selected the 3 time points (1, 4, and 24 hours) after an intravenous dose of 20 mg/kg paclitaxel, as previous data from our laboratory indicated that these times are adequate for assessing paclitaxel plasma and tumor exposure. As shown in Fig. 4A, plasma paclitaxel levels were similar in mice pretreated with vehicle and with the active agents, indicating that pretreatment does not alter paclitaxel plasma kinetics. However, paclitaxel tumor levels were significantly lower in the 3 groups that had been pretreated with the tyrosine kinase inhibitors, with a decrease in tumor paclitaxel uptake (compared with vehicle-pretreated mice) from 72% to 75%, from 60% to 70%, and from 40% to 72%, respectively, in E-3810, sunitinib, and brivanib pretreated mice (Fig. 4B) at all 3 time points. Paclitaxel tumor levels were similar in E-3810, sunitinib, and sunitinib pretreated tumors.

We also examined the effects on tumor cells and tumor vasculature induced by the different treatments. We selected 2 time points, 24 and 72 hours after paclitaxel as representative of early and late biologic effects. Immunohistochemical staining for Ki67, a marker of active cell proliferation, showed that Ki67-positive cell population was around 30% in the control group, with no major differences in all the experimental groups at 24 and 72 hours (Fig. 5A). However, the proportion of necrosis was 2 to 20 times higher than in controls in all groups that received active treatments, except for paclitaxel single-agent, with the largest increase in the groups treated with E-3810 alone or in combination (Fig. 5B). The pattern was similar for caspase-3/7 activity, indicative of apoptosis (Fig. 5C). Maximum induction of caspase-3/7 was detected in tumors 72 hours after treatment with the

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**Figure 3.** Inhibition of tumor growth after treatment with E-3810, brivanib, sunitinib, paclitaxel (PTX), and their combinations. A, the antitumor activity of E-3810, paclitaxel, and their combinations in MDA-MB-231 subcutaneously transplanted tumors. When tumor masses reached 300 to 350 mg, mice were randomized to receive vehicle (○); E-3810 20 mg/kg (■) every day for 30 days, paclitaxel 20 mg/kg every 7 days for 3 times (△) and their combination (▲). B, the antitumor activity of E-3810, brivanib, sunitinib, paclitaxel, and their combinations in MDA-MB-231 subcutaneously transplanted tumors. When tumor masses reached 300 to 350 mg, mice were randomized to receive vehicle (○); E-3810 15 mg/kg (■) every day for 30 days, brivanib 100 mg/kg (□) every day for 30 days, sunitinib 40 mg/kg (●) every day for 30 days; paclitaxel 20 mg/kg every 7 days for 3 times (△); E-3810-paclitaxel (▲); brivanib-paclitaxel (■) and sunitinib-paclitaxel (●). C, the antitumor activity of E-3810, paclitaxel, and their combinations in MX-1 subcutaneously transplanted tumors. When tumor masses reached 300 to 350 mg, mice were randomized to receive vehicle (○); E-3810 20 mg/kg (■) every day for 30 days and paclitaxel 20 mg/kg every 7 days for 3 times (△) and their combination (▲).

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active than the other 2 in term of T–C (respectively 87, 46, and 42 days), survival curves and long-term survivors (Table 1 and Supplementary Fig. S2A). All the combinations seemed well tolerated, with no clinical signs or significant weight loss (Supplementary Fig. S1D).

To strengthen the evidence of the synergy of E-3810 plus paclitaxel, this combination was tested in another xenograft model: the MX-1 triple-negative breast xenograft.
E-3810–paclitaxel combination, indicating that increased apoptosis might contribute to the activity of the combination.

Then, we examined different morphologic parameters of the tumor vasculature: mean vessel number, mean vessel area, and mean vessel diameter. As expected, all 3 antiangiogenic inhibitors clearly reduced the mean vessel number, but had no effect on mean vessel area or mean vessel diameter (Fig. 5D and E; data not shown); paclitaxel caused no modification in the CD31 count compared with control tumors and no further decrease was found in tumors from mice treated with E-3810, sunitinib, or brivanib combined with paclitaxel. We were unable to identify any differences in tumor vasculature in the 3 combination treatment groups that could even partially explain the better antitumor activity of the combination E-3810 and paclitaxel.

A reduction in collagen IV immunoreactivity was observed in tumors of mice treated with E-3810 (not statistically significant) and E-3810–paclitaxel (72 hours) although not in the other experimental groups (Fig. 5F). To analyze if the decrease in collagen IV might depend on increased tumor proteolytic activity, we analyzed the pattern of collagenase/gelatinase expression (MMP-2 and MMP-9). Zymographic analysis of the tumors revealed a marked increase in collagenases, particularly murine MMP-9, following treatment with E-3810 alone and in combination with paclitaxel (Fig. 5G). The relevant increase in murine (host) MMP-9 points to activation of the proteolytic activity of the stroma cells as a major mechanism of the observed matrix remodeling.

E-3810, either as single agent or in combination with paclitaxel, did not increase the tumor content of natural killer cells (assessed by immunohistochemistry with anti- asialo GM1 and F4-80, respectively; data not shown) as compared with control tumors (data not shown), indicating that recruitment of infiltrating immune cells do not apparently contribute to matrix degradation induced by E-3810 or to the antineoplastic activity of the E-3810–paclitaxel combination.

Circulating factors (mVEGF, mFGF-21, msVEGFR-2, and soluble mCollagen IV) were measured. E-3810 raised the concentration of mVEGF (Supplementary Fig. S4A), but no changes in its plasma levels were detected in mice treated continuously for 10 days with brivanib or sunitinib and at 24 and 72 hours after paclitaxel. The mVEGF levels in brivanib and sunitinib + paclitaxel combination groups paralleled the findings in the single agent groups. Plasma levels of msVEGFR-2 in mice treated with E-3810, brivanib, and paclitaxel (alone or with paclitaxel) were lower than in untreated mice, but there was no change in msVEGFR-2 in mice treated with sunitinib and the combination paclitaxel + sunitinib were the same as in untreated mice. In agreement with the finding of decreased collagen IV levels in the tumor tissue, soluble collagen IV plasma levels were raised in mice treated with E-3810 alone or with paclitaxel; whereas the opposite pattern was found in mice treated with sunitinib, paclitaxel, and their combination (Supplementary Fig. S4D).

Discussion

As new-targeted agents for cancer treatment enter clinical use, it is important to understand their potential interactions with traditional chemotherapy, ideally to...
incorporate them in multiple radio/chemotherapeutic regimens. The rationale of combining antiangiogenic agents with chemotherapy is to target 2 different tumor hallmarks: angiogenesis and cell proliferation (2). Inhibition of angiogenesis would deprive tumor cells of nutrients and oxygen, whereas direct killing of tumor cells would clearly reduce tumor burden. Even if action on the tumor vasculature could potentially impair the delivery of cytotoxic drugs and paradoxically limit their activity, these combinations have been shown to be additive/synergic in different preclinical (18–22) and, for bevacicubumab, clinical settings (23–25).

E-3810 is a novel small molecule that inhibits VEGFR-1, -2, and -3 and FGF-1 tyrosine kinases with IC50 < 30 nmol/L; at higher concentrations, it also inhibits FGFR-2. We have previously shown that the compound has a strong antiangiogenic effect in vivo and potent antitumor activity as single agent in the different human xenograft models in which it has been tested (14). The compound is active than brivanib + paclitaxel and sunitinib + paclitaxel; in fact, even though the T/C ratios were similar, they were reached at quite different times in the combination groups (75, 64, and 57, respectively, for E-3810 + paclitaxel, brivanib + paclitaxel, and sunitinib + paclitaxel), and were higher for the E-3810 pretreated mice.

The mechanisms of the additive/synergistic activity of antiangiogenic and chemotherapy are not clear although several have been proposed: enhanced delivery of the chemotherapeutic drug due to vascular normalization induced by the antiangiogenic therapy (33); a direct effect on tumor cells whose growth depends on the VEGF/VEGFR or FGF/FGFR pathways (34–36); sensitization of endothelial and/or circulating tumor cells to the cytotoxic damage after VEGF depletion (37); and additive/synergic effects through the concomitant destruction of tumor and endothelial cells (38). These mechanisms have been shown in experimental settings where cytotoxic agents were combined with monoclonal antibody and pan-tyrosine kinase inhibitors (39).

Normalization of the abnormal tumor vasculature with an increased delivery of chemotherapy during the "normalization window" has been suggested to occur during antiangiogenic treatment (33, 40) helping improved delivery of chemotherapy and enhancing its efficacy. However, the finding of lower paclitaxel levels in tumors pretreated with all 3 antiangiogenic drugs suggest that these tumors are much less perfused, as the paclitaxel plasma levels exclude reduced drug availability. Even though in these experiments we have not assessed the perfusion status of the tumors pretreated with the active drugs, E-3810, sunitinib, and brivanib
has been shown to decrease perfusion in other experimental settings and in clinical trials (14, 41, 42). The lower paclitaxel tumor levels and the higher antitumor activity of the combination than single-agent paclitaxel corroborate the findings in tumor xenografts with other antiangiogenic and cytotoxic agents. For example, axitinib combined with cyclophosphamide has greater antitumor activity in rat 9L gliosarcoma despite a substantial decrease in tumor uptake of the active metabolite (43). In A2780-1A9 ovarian tumor xenograft, pretreatment with vandetanib lowered tumor paclitaxel levels, despite the fact that the antitumor activity was better in the combination group than with vandetanib or paclitaxel alone (20). In addition, the infusion of bevacizumab in patients with NSCLC was recently shown to reduce tumor perfusion and the tumor net influx rate of [11C] docetaxel within 5 hours (44).

Our data indicate that all 3 targeted agents clearly reduced the number of vessels, with no apparent effects on their morphology. Detailed analysis of tumor vessel and drug distribution might disclose a more complex heterogeneity with some tumor regions better perfused than others; conceivably tyrosine kinase inhibitors may have different overall activity depending on their affinity profile.

In this model, a direct effect of the drugs on tumor cells can be excluded by the results in vitro, including the lack of cytotoxic activity at concentrations in line with those observed in vivo (14) and the lack of additive/synergistic effect when combined with paclitaxel. Induction of necrosis emerged as a distinctive feature of E-3810 activity, although the effect was not significantly increased by paclitaxel. Increase in apoptosis markers was highest in tumors treated with the E-3810–paclitaxel combination,

Figure 5. Immunohistochemistry and pharmacodynamics of MDA-MB-231 tumors not treated (C) or treated with E-3810 (E), brivanib (B), sunitinib (S), paclitaxel and their combinations. A, analysis of tumor cell proliferation by Ki67 immunohistochemistry results are given as Ki67-positive area in percentage of the whole examined area. B, necrosis area given in percentage of the whole tumor area. C, caspase-3/7 activity expressed as RLU/min/mg. D, vessel density (CD31) given as mean vessel number per scanned tumor area. E, vessel area given as mean vessel area in μm². F, collagen IV deposition: stained area given as percentage of the whole examined area. G, zymographic analysis of tumor lysates. Conditioned medium of HT1080 fibrosarcoma cells (1) and NIH3T3 fibroblasts (2) were used as reference for human and murine MMP9 and MMP2, respectively. Experimental groups are indicated: controls (C), E-3810 (E), sunitinib (S), brivanib (B), paclitaxel (P); E-3810–paclitaxel (E+P); E-sunitinib–paclitaxel (S+P), brivanib–paclitaxel (E+P). Data are expressed as the mean ± SE at 2 different time points after paclitaxel treatment 24 (□) and 72 hours (■), *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired t test with Welch correction).
suggesting the involvement of this process in the activity of the combination.

The effect on the vascular compartments of E-3810, given singly or with paclitaxel, was not dissimilar to that of brivanib and sunitinib, as it reduced the vessel counts without modifying vessel diameters. At variance, we found that E-3810 alone and in combination with paclitaxel caused a remodeling of the extracellular matrix, documented by reduced collagen IV in the tumor and increased levels of plasma collagen IV. This is in agreement with our previous study in which E-3810 lowered collagen IV expression in A498 xenografts (14, 45), although in this case the effect of E-3810 on tumor collagen IV did not reach statistical significance probably because of differences in treatment schedules and the experimental model used. E-3810-induced matrix remodeling was associated with an increased level of MMPs in the tumors following treatment with E-3810, alone or in combination with paclitaxel. The remarkable induction of murine (host-derived) MMP-9 indicates that E-3810 potentiates the proteolytic activity of the stroma cells, suggesting that the concomitant remodeling activity of E-3810 on the tumor stroma along with the direct cytotoxic effect of paclitaxel on the tumor cells contribute to the remarkable effect of the E-3810–paclitaxel combination. Our results show that E-3810 as a single agent has a potent effect on the MDA-MB-231 chemoresistant model and can synergize with paclitaxel in 2 late-stage triple-negative breast xenografts. Two heavily pretreated patients with TNBC and altered FGF pathway who received single-agent E-3810 in the ongoing clinical trial showed a significant objective response (27). Further exploration of the combination of E-3810 and paclitaxel is therefore warranted and may, in time, address a highly unmet need in this challenging population of patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: E. Cavalletti, R. Giavazzi, G. Camboni, G. Damia
Development of methodology: G. Damia
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Bello, G. Tarabottini, S.A. Licandro
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