The tyrosine kinase inhibitor E-3810 combined with paclitaxel inhibits the growth of advanced-stage triple-negative breast cancer xenografts

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Running title: E-3810 and anticancer drugs in triple negative breast tumors

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CONFLICT OF INTEREST STATEMENT
None declared

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ABSTRACT

E-3810 is a novel small molecule that inhibits VEGFR-1, -2 and -3 and FGFR-1 tyrosine kinases at nM 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 pre-treated 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, 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) might contribute to the striking activity of their combination. These data support the therapeutic potential of combining E-3810 with conventional chemotherapy.
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 non-overlapping 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. VEGFR, FGFR, PDGFR) (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 mono-therapy 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 non-squamous non small cell lung cancer (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 with other treatment modalities particularly with cytotoxics, appears 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 epidermal growth factor receptor-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: poly(ADP-ribose)polymerase inhibitors, antiangiogenic drugs and anti-epidermal growth factor receptor agents (11, 12). FGFR signalling 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 triple negative and basal-like breast cancer (13).

E-3810 is a new, orally active tyrosin kinase inhibitor with antitumor activities attributable to the inhibition of vascular endothelial growth factor receptors (VEGFR-1, -2, -3), fibroblast growth factor receptors (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 triple negative breast carcinoma) cells were obtained from the American Type Culture Collection and maintained in RPMI medium supplemented with 10% (v/v) Foetal Bovine Serum (FBS), 1% (v/v) L-Glutammine. 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 (China). All drugs were dissolved in 100% DMSO at a final concentration of 10 mM 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 (PTX; kindly provided by Indena S.p.A., Milan, Italy) was dissolved in 50% Cremophor EL (Sigma-Aldrich) and 50% ethanol, further diluted with saline immediately before use and injected intravenously (i.v.) at a dose of 20 mg/kg; both cisplatin (DDP) and 5-fluorouracil (5FU) 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 3-6 x 10^3 cells/100 μL/well in complete medium. After 24 h cells were treated with both different PTX concentrations and two fixed doses (0.15 μM and 3 μM) of E-3810, sunitinib and brivanib for 72 h. The anti-proliferative effect was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) at the end of treatment time.

Xenograft models
Six- to eight-week-old female NCr-nu/nu mice were obtained from Harlan S.p.A, Italy. 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 x 10^6 cells) were detached from flask, resuspended in PBS and implanted s.c. into the right flank region of athymic mice. MX-1 human breast tumor was s.c transplanted as fragments. Unless otherwise specified, mice were randomized when the average tumor size was 350-400 mm^3 (advanced stage) (8-10 per group). Tumor growth was measured twice weekly
with a Vernier caliper, and the tumor weights (mg = mm$^3$) were calculated as follows: (length [mm] × width [mm$^2$])/2. Efficacy was expressed as best tumor growth inhibition (TGI%) \[1 - \frac{\text{median tumor weight of treated tumors}}{\text{median tumor weight of control tumors}} \times 100\] or tumor growth delay (T−C = median time to reach 500 or 1000 mg in treated tumor − median time to reach 500 or 1000 mg in control tumor). Survival curves were drawn as a percentage of survival animals in all the experimental groups.

**PTX pharmacokinetics**

MDA-MB-231 tumor bearing mice were randomized when their tumor masses were about 350-400 mg to receive E-3810, brivanib and sunitinib at the doses used for the antitumor activity trial, for ten days. Four hrs after the anti-angiogenic dose of day 7, PTX was injected iv at the dose of 20 mg/kg and tumor and plasma samples were collected after 1, 4, and 24 h in all the groups (each group consisting of three 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 HPLC with UV detection at 230 nm as described in Supplementary Information. Noncompartmental pharmacokinetic parameters were calculated by WinNonlin Pro Node 4.1 pharmacokinetic software (Pharsight Co. Mountain View, CA).

**Pharmacodynamic studies**

MDA-MB-231 tumor bearing mice were treated daily with E-3810, brivanib and sunitinib as described in the PTX pharmacokinetics section. At 24 and 72 h after PTX 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/acetone at room temperature.

A number of pharmacodynamic (PD) markers were examined on tumor samples. Vessel density and morphology were assessed by immunostaining with anti-CD31 antibody MEC13.3 (Beckton Dickinson GmbH, Heidelberg, Germany), followed by a biotin-conjugated mouse anti-rat IgG1/2a monoclonal antibody (clone G28-5, Beckton Dickinson GmbH), and a streptavidin-alkaline phosphatase (AP) conjugate (Biozol Diagnostika GmbH, Eching, Germany). For chromogenic detection, the chromogen solution of the Dako REAL™ Detection System AP/RED (Dako Deutschland GmbH, Hamburg, Germany) was applied. Three randomly selected measurement fields (0.57 mm$^2$) 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, Oberkochen, Germany).

For assessment of collagen type IV and proliferative activity, cryosections were fixed with 4% paraformaldehyde for 15 min. Then the rabbit-anti-murine collagen type IV antibody AB756 (Millipore/Chemicon, Schwalbach/Ts., Germany) or the rabbit-anti-human Ki67 antibody SP6 (Thermo Fisher Scientific GmbH, Schwerte, Germany) were applied followed by Biotin-SP-conjugated AffiniPure Goat anti Rabbit IgG(H+L) (Dianova GmbH, Hamburg, Germany) and the detection system as described above. To assess the extent of collagen type IV or Ki67 staining, three 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 (4X 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 percent of the total tumor area.

Caspase 3/7 activity was assessed in snap frozen tumor tissue samples from whole protein extraction by homogenization in 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 mM Hepes pH 7.5, 10% Glycerol, 10 mM DTT) and Ac-DEVD-Amc substrate (final concentration: 12.5 μM). The Ac-DEVD-Amc cleavage reaction was monitored by a kinetic approach and the data were used to determine the slope (RFU/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 (25μg) were separated on SDS-polyacrylamide gels (8%) copolymerized with 1mg/ml gelatin (Sigma), as described (15). Gels were washed twice in 2.5% Triton X-100, incubated overnight in 50mM Tris–HCl, pH 7.6, 100mM NaCl, 0.02% Brij 35, and 5 mM 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 MMP-2 and MMP-9.

Circulating biomarkers in plasma samples of treated and control mice were measured by ELISA (Murine Collagen IV ELISA Kit, Exocell, Philadelphia; mouse soluble VEGFR-2, mouse FGF-21 and mouse VEGF immunoassays; R&D Systems, Minneapolis, MN) 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 350-400 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 re-grew after withdrawal of E-3810 at a rate similar to control tumors. 5FU 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 5FU and E-3810, but increased no further in T-C and TGI% over E-3810 singly (Fig. 2B and data not shown). 5FU and DDP could be safely combined with E-3810, as suggested by body weight loss which never exceed 15% (Supplementary Fig. 1A and B).

In the same experimental setting, we also explored the activity of E-3810 given for 30 days starting 35 day from inoculum, and PTX at the dose of 20 mg/kg weekly for three weeks. The combination was well tolerated; although weight loss was greater in the mice treated with both drugs than with each one singly, it never exceeded 15% (Supplementary Fig. 1C). One death occurred with E-3810 and one in the E-3810+PTX combination group on day 62 and 55 respectively in mice whose tumor was regressing (data not shown). E-3810 and PTX had a similar growth inhibitory activity (Fig. 3A and Table 1); with the two drugs combined, all tumors regressed and by day 83 all mice were tumor free though by day 93 one tumor was re-growing 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 two drugs singly (Supplementary Fig. 2A and Table 1).

A subsequent experiment confirmed the striking activity of E-3810 plus PTX (at the lower E-3810 dose of 15 mg/kg) and compared this activity with the combinations of PTX with sunitinib and brivanib, two 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 PTX (Fig. 3B and Table 1). All the mice treated with PTX and E-3810, PTX and sunitinib and PTX and brivanib experienced tumor regression (Fig. 3B); however, 10 days after withdrawing the drugs, tumors resumed their growth in all the mice treated with PTX and brivanib and in almost all those treated with PTX and sunitinib (8 out of 10 mice). Tumors re-grew much more slowly in mice treated with E-3810+PTX (Fig. 3B). This latter combination was more active than the other two in term of T-C (respectively 87, 46 and 42 days), survival curves and long-term survivors (Table 1 and Supplementary Fig. 2A). All the combinations appeared well tolerated, with no clinical signs or significant weight loss (Supplementary Fig. 1D).
In order to strengthen the evidence of the synergy of E-3810 plus PTX, this combination was tested in another xenograft model: the MX-1 triple negative breast xenograft. As shown in Fig. 3C and Table 1, E-3810 at the dose of 20 mg/kg for 30 days was active and able to induce tumor stabilization, PTX given once a week for three weeks reduced tumor growth and when the two drugs were given in combination again a striking activity was observed with all the mice experiencing tumor regression that lasted for a long time (Fig. 3C and Table 1). When the experiment was stopped, 5 out 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 in order 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 PTX in MDA-MB-231 cells never had any synergistic effect (Supplementary Fig. 3). 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 pre-treatment with E-3810, sunitinib and brivanib affected PTX pharmacokinetics, we studied PTX plasma and tumor levels. We selected the three time points (1, 4 and 24 h) after an i.v. dose of 20 mg/kg PTX, as previous data from our laboratory indicated that these times are adequate for assessing PTX plasma and tumor exposure. As shown in Fig. 4A, plasma PTX levels were similar in mice pre-treated with vehicle and with the active agents, indicating that pre-treatment does not alter PTX plasma kinetics. However, PTX tumor levels were significantly lower in the three groups that had been pre-treated with the tyrosine kinase inhibitors, with a decrease in tumor PTX uptake (compared to vehicle-pre-treated mice) from 72 to 75%, from 60 to 70% and from 40 to 72% respectively in E-3810, sunitinib and brivanib pre-treated mice (panel B) at all three time points. PTX tumor levels were similar in E-3810, brivanib and sunitinib pre-treated tumors.

We also examined the effects on tumor cells and tumor vasculature induced by the different treatments. We selected two time points, 24 and 72 h after PTX as representative of early and late biological effects. Immuno-histochemical 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 h (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 PTX 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 72h after treatment with the E-3810-PTX combination, indicating that increased apoptosis might contribute to the activity of the combination.

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

A reduction in collagen IV immunoreactivity was observed in tumors of mice treated with E-3810 (not statistically significant) and E-3810+PTX (72 h) 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 MMP9, following treatment with E-3810 alone and in combination with PTX. The relevant increase in murine (host) MMP9 points to activation of the proteolytic activity of the stroma cells as a major mechanism of the observed matrix remodelling.

E-3810, either as single agent or in combination with PTX, did not increase the tumor content of NK cells (assessed by IHC with anti-asialo GM1 and F4-80, respectively-data not shown) as compared to controls tumors (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/PTX combination.

Circulating factors (mVEGF, mFGF-21, msVEGFR-2 and soluble mCollagen IV) were measured. E-3810 raised the concentration of mVEGF (Supplementary Fig. 4A), 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 h after PTX. The mVEGF levels in brivanib and sunitinib+PTX combination groups paralleled the findings in the single agent groups. Plasma levels of msVEGFR-2 in mice treated with E-3810, brivanib and PTX (alone or with PTX) were lower than in untreated mice but there was no change in msVEGFR-2 in mice treated with sunitinib and the combination PTX+sunitinib (Supplementary Fig. 4B) or in the levels of mFGF-21 in any of the experimental groups (Supplementary Fig. 4C). 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 PTX; whereas the opposite pattern was found in mice treated with sunitinib, PTX and their combination (Supplementary Fig. 4D).
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 two different tumor hallmarks: angiogenesis and cell proliferation (1). Inhibition of angiogenesis would deprive tumor cells of nutrients and oxygen, while 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 bevacizumab, clinical settings (23-25).

E-3810 is a novel small molecule that inhibits VEGFR-1, -2 and -3 and FGFR-1 tyrosine kinases with IC₅₀ < 30 nM; at higher concentrations it also inhibits FGFR-2. We have previously shown that the compound has a strong antiangiogenic effects 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 now undergoing a Phase I-II clinical trial in Europe (26, 27), with very promising results.

We did a number of experiments to see whether E-3810 could be safely combined with chemotherapeutic agents and if these combinations gave better antitumor activity.

We selected MDA-MB-231 and MX-1, two triple-negative human breast cancer xenografts, and a late stage tumor setting, randomizing to treatment mice with high tumor burden. MDA-MB-231 model showed minimal sensitivity to 5FU and DDP as already reported (28), but good sensitivity to PTX and even more to E-3810, suggesting that antiangiogenic therapy and/or inhibition of the FGF pathway might be effective in triple-negative breast cancer and in tumors resistant to chemotherapy, as already shown in cell lines and tumor models for other VEGFR tyrosine kinase inhibitors (13, 16, 29, 30). The combinations of E-3810 plus 5FU and DDP show similar activity to single-agent E-3810. It seems reasonable to hypothesize that in this model the lack of activity of DDP and 5FU is due to biological characteristics of the tumor cell (such as a high level of DNA repair, increased GSH levels, high level of TS) (31, 32) that cannot be overcome by co-administering of E-3810. However, the combinations of 5FU and DDP with E-3810 were well tolerated with no clinical signs or significant weight loss, suggesting that the spectrum of toxicities is not overlapping.

In contrast, there was striking enhancement of the antitumor effect, with long-term tumor-free survivors, when E-3810 was combined with PTX. These data have also been corroborated in MX-1, another triple negative breast human xenograft. In both experimental settings, the combination induced lasting tumor regressions with half of the mice free of tumors and high T/C ratios. In the MDA-MB-231 xenograft, the combination was more active than brivanib+PTX and sunitinib+PTX; 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+PTX, brivanib+PTX and sunitinib+PTX), and were higher for the E-3810 pre-
treated mice.

The mechanisms of the additive/synergistic activity of antiangiogenic and chemo-therapy are not clear
though 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
demonstrated 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 PTX levels in tumors pretreated with all three antiangiogenic
drugs suggest that these tumors are much less perfused, as the PTX 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 PTX tumor levels and the higher
antitumor activity of the combination than single-agent PTX 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 PTX levels, despite the fact that the antitumor activity was better in the
combination group than with vandetanib or PTX alone (20). In addition, the infusion of bevacizumab in
NSCLC patients was recently shown to reduce tumor perfusion and the tumor net influx rate of $[11C]$ 
docetaxel within 5 h (44).

Our data indicate that all three 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 PTX. Induction of necrosis emerged as a distinctive
feature of E-3810 activity, though the effect was not significantly increased by PTX. Raise in apoptosis
markers was highest in tumors treated with the E-3810+PTX combination 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 PTX, 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 PTX 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 remodelling was associated with an increased level of MMPs in the tumors following treatment with E-3810, alone or in combination with PTX. 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 remodelling activity of E-3810 on the tumor stroma along with the direct cytotoxic effect of PTX on the tumor cells contribute to the remarkable effect of the E-3810+PTX combination. Our results show that E-3810 as a single agent has a potent effect on the MDA-MB-231 chemo-resistant model and can synergize with PTX in two late-stage triple negative breast xenografts. Two heavily pre-treated patients with triple negative breast cancer 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 PTX is therefore warranted and may, in time, address a highly unmet need in this challenging population of patients.
REFERENCES


Table 1. Antitumor activity of E-3810 lone or in combination with PTX in MDA-MB-231 and MX-1 tumor bearing mice

<table>
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<th>Schedule (days)</th>
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<th>T-C (days)</th>
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<td></td>
<td>PTX+sunitinib</td>
<td>95 (57)</td>
<td>42</td>
<td>2/9 (104)</td>
</tr>
<tr>
<td>MX-1</td>
<td>PTX 20 mg/kg</td>
<td>63 (24)</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E-3810 20 mg/kg</td>
<td>79 (24)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTX+E-3810 20 mg/kg</td>
<td>99 (55)</td>
<td>na*</td>
<td>5/9 (96)</td>
</tr>
</tbody>
</table>

% TGI: tumor growth inhibition
T-C (days): difference between treated and control mice in time to reach 1,000 mg.
* not yet reach at day 96, day of study termination
LEGENDS TO THE FIGURES

Figure 1. Chemical structure of E-3810, sunitinib, brivanib, and paclitaxel.

Figure 2. Inhibition of tumor growth after treatment with E-3810, 5FU, DDP and their combinations.
The antitumor activity of E-3810, 5FU and DDP was tested in MDA-MB-231 subcutaneously transplanted tumors. When tumor masses reached 300-350 mg, mice were randomized to receive: A: vehicle (---○---); E-3810 15 mg/kg (---●---) qdx30, DDP 4 mg/kg q7x3 (---□---), E-3810+DDP (---■---). B: vehicle (---○---); E-3810 15 mg/kg (---●---) qdx30, 5FU 75 mg/kg q7x3 (---△---), E-3810+5FU (---▲---);

Figure 3. Inhibition of tumor growth after treatment with E-3810, brivanib, sunitinib, PTX and their combinations.
A. The antitumor activity of E-3810, PTX and their combinations in MDA-MB-231 subcutaneously transplanted tumors. When tumor masses reached 300-350 mg, mice were randomized to receive vehicle (---○---); E-3810 20 mg/kg (---●---) qdx30 and PTX 20 mg/kg q7x3 (---△---) and their combination (---▲---).
B. The antitumor activity of E-3810, brivanib, sunitinib, PTX and their combinations in MDA-MB-231 subcutaneously transplanted tumors. When tumor masses reached 300-350 mg, mice were randomized to receive vehicle (---○---), E-3810 20 mg/kg (---●---) qdx30, brivanib 100 mg/kg (---□---) qdx30, sunitinib 40 mg/kg (---■---) qdx30; PTX 20 mg/kg q7x3 (---△---), E-3810+PTX (---▲---); brivanib+PTX (---■---) and sunitinib+PTX (---■---).
C. The antitumor activity of E-3810, PTX and their combinations in MX-1 subcutaneously transplanted tumors. When tumor masses reached 300-350 mg, mice were randomized to receive vehicle (---○---); E-3810 20 mg/kg (---●---) qdx30 and PTX 20 mg/kg q7x3 (---△---) and their combination (---▲---).

Figure 4. PTX plasma and tumor levels in mice bearing MDA-MB-231 xenograft. Tumor bearing mice were treated for 10 days with vehicle, E-3810 20 mg/kg, sunitinib 40 mg/kg and brivanib 100 mg/kg. On day 7, PTX was given i.v. at the dose of 20 mg/kg and its levels were measured in plasma (A) and tumor (B) 1, 4 and 24 h after the injection. Vehicle pre-treated mice (□); E-3810 pre-treated mice (■); brivanib pre-treated mice (○); sunitinib pre-treated mouse (△).
* p < 0.05; ** p < 0.01; *** p < 0.001 (Dunnet’s Multiple Comparison Test).

Figure 5. Immunohistochemistry and pharmacodynamics of MDA-MB-231 tumors not treated (C) or treated with E-3810 (E), brivanib (B), sunitinib (S), paclitaxel (PTX) and their combinations.
A. Analysis of tumor cell proliferation by Ki67 immunohistochemistry results are given as Ki67 positive area in % of the whole examined area. B. Necrosis area given in % of the whole tumor area. C. Caspase 3/7 activity expressed as RLU/min.µg 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 % of the whole examined area. G. Zymografic analysis of tumor lysates. Conditioned medium of HT1080 fibrosarcoma cells (1) and NIH3T3 fibroblasts (2) were used as reference for human and murine MMP9 andMMP2, respectively. Experimental groups are indicated: controls (C), E-3810 (E), sunitinib (S), brivanib (B), PTX (P); E-3810+PTX (E+P), E-sunitinib+PTX (S+P), brivanib+PTX (E+P). Data are expressed as the mean±SE at two different time points after PTX treatment 24h ( ■ ) and 72h ( □ ). * p < 0.05; ** p < 0.01; *** p < 0.001 (Unpaired t test with Welch's correction).

ACKNOWLEDGMENTS
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CONFLICT OF INTEREST STATEMENT
None declared
Figure # 1
Figure #3
Figure # 4
Figure 5

A

% Ki67 positive cells

B

% of necrosis

C

Caspase 3/7 activity (RFU/min/mg)

D

Vessel density

E

Vessel area (µm²)

F

% of collagen IV positive area

G

MMP-2

MMP-9
The tyrosine kinase inhibitor E-3810 combined with paclitaxel inhibits the growth of advanced-stage triple-negative breast cancer xenografts

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