

# Preclinical evaluation of the tyrosine kinase inhibitor SU11248 as a single agent and in combination with "standard of care" therapeutic agents for the treatment of breast cancer

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## Abstract

SU11248 is an oral multitargeted tyrosine kinase inhibitor with antitumor and antiangiogenic activities through targeting platelet-derived growth factor receptor, vascular endothelial growth factor receptor, KIT, and FLT3, the first three of which are expressed in human breast cancer and/or its supporting tissues. The purpose of the present studies was to demonstrate the potent anticancer activity of SU11248 alone or in combination with conventional cytotoxic agents against several distinct preclinical models of breast cancer. SU11248 was administered as a monotherapy to (1) mouse mammary tumor virus-*v-Ha-ras* mice and 7,12-dimethylbenz(a)anthracene-treated rats bearing mammary tumors and (2) mice bearing human breast cancer xenografts of s.c. MX-1 tumors and osseous metastasis of a MDA-MB-435-derived cell line (435/HAL-Luc). SU11248 was also administered in combination with docetaxel both in xenograft models and in combination with 5-fluorouracil and doxorubicin in the MX-1 model. SU11248 treatment potently regressed growth of mammary cancers in mouse mammary tumor virus-*v-Ha-ras* transgenic mice (82% regression) and 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats (99% regression at the highest dose;  $P < 0.05$  for both). This agent also inhibited MX-1 tumor growth by 52%, with markedly enhanced anticancer effects when administered in combination with docetaxel, 5-fluorouracil, or doxorubicin compared with either agent alone ( $P < 0.05$ ). SU11248 treatment in combination with docetaxel effectively prolonged survival of mice with 435/HAL-Luc cancer xeno-

grafts established in bone compared with either agent alone ( $P < 0.05$ ). These results demonstrate that SU11248 is effective in preclinical breast cancer models and suggest that it may be useful in the treatment of breast cancer in the clinic. (Mol Cancer Ther. 2003;2:1011–1021)

## Introduction

Breast cancer is the most common malignancy in women in the United States, with an estimated 212,600 new invasive cases expected to be diagnosed among women during 2003 and an estimated 40,200 deaths (American Cancer Society, *Cancer Facts & Figures 2003*), largely due to the poor long-term clinical response of breast tumors to conventional chemotherapeutics. Docetaxel (Taxotere), doxorubicin (Adriamycin), and 5-fluorouracil (5-FU), commonly administered in prodrug form as capecitabine (Xeloda), are among the most active agents against human breast cancer. Despite clinical improvements attributed to treatment with such agents, a significant number of patients will eventually become refractory to these therapies, and many of the current drugs have significant side effects at the therapeutic dose. New treatment options are urgently needed to improve survival of breast cancer patients.

The first molecular targeted therapy for the treatment of metastatic breast cancer, Herceptin (trastuzumab), has substantial clinical benefit and serves as proof of principle for oncogene-targeted therapy. Based on this same rationale that targeting key molecules or combinations of molecules in signal transduction pathways can achieve clinical responses in breast cancer, SU11248 was developed as an oral multitargeted receptor tyrosine kinase (RTK) inhibitor with antitumor and antiangiogenic activities through targeting platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), KIT, and FLT3, the first three of which are found to be expressed in human breast cancer and/or its supporting tissues. SU11248 has shown antitumor activity by inhibiting both RTKs expressed by cancer cells and directly involved in cancer proliferation and survival and RTKs expressed on endothelial or stromal cells supporting the cancer (1). As examples of the former, SU11248 blocks the activity of wild-type and activated FLT3 expressed by acute myelogenous leukemia-derived cell lines and KIT expressed by small cell lung cancer-derived cell lines, resulting in tumor regression and improved survival for acute myelogenous leukemia preclinical models and tumor growth inhibition in small cell lung cancer tumor models, respectively (2, 3). The latter antitumor mechanism is supported by SU11248 inhibition of VEGFR2 (FLK-1/KDR) and PDGFR- $\beta$ , both of which play a prominent role in

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angiogenesis (4). Recent studies in preclinical tumor models have demonstrated inhibition of target RTKs and concurrent tumor growth effects with SU11248 in a variety of cancer types (1–3).

The targets of SU11248 are believed to play important roles in growth and survival of human breast cancers. VEGFR2 is expressed in the endothelium of blood vessels (5), and PDGFR- $\beta$  is expressed in breast tumor stroma (6), including the pericytes that support blood vessels. Their ligands VEGF- $\alpha$ , VEGF- $\beta$ , and PDGF- $\beta$  are expressed by breast cancer epithelia (7, 8), suggesting the possibilities of paracrine signaling by these receptor-ligand complexes. Consistent with the proposed role of VEGF and PDGF in this disease, elevated plasma VEGF levels and PDGF- $\beta$  protein expression have been reported as negative prognostic indicators in breast carcinoma (9, 10). The role of the third SU11248 target, KIT, in breast cancer is not well understood, with evidence of KIT expression in most breast cancer specimens (11, 12), counteracted by several reports indicating that KIT protein levels negatively correlate with advancement of breast tumors (13–15).

Because several SU11248 target RTKs may be involved in breast cancer growth, we evaluated the potential therapeutic utility of SU11248 in preclinical models of breast cancer as a single agent and in combination with established therapies for the disease. The therapeutic effect of SU11248 was evaluated against mammary cancers in oncogene-driven mouse mammary tumor virus (MMTV)-*v-Ha-ras* transgenic mice (16) and in 7,12-dimethylbenz(*a*)anthracene (DMBA)-treated rats (17). SU11248 activity was also examined in two human breast cancer xenograft models: s.c. MX-1 tumor growth and site-specific bone metastasis of a MDA-MB-435-derived cell line (435/HAL-Luc) (18). In addition to single-agent treatment, anticancer activity of SU11248 was evaluated in combination with three common chemotherapeutic agents for breast cancer: the microtubule stabilizer, docetaxel, the pyrimidine antagonist, 5-FU, and the topoisomerase II inhibitor, doxorubicin, in the s.c. MX-1 tumor growth model. SU11248 and docetaxel were also combined in the 435/HAL-Luc experimental model of osseous metastasis. These cytotoxic agents act via different mechanisms from each other and from SU11248 and have different tolerability profiles. We hypothesize that combination therapy using the antitumor and antiangiogenic properties of SU11248 together with the cytotoxic properties of these chemotherapeutic agents will result in the most beneficial activity against primary and metastatic growth of breast cancer through targeting multiple mechanisms.

## Materials and Methods

### Animals

The MX-1 human breast cancer xenograft studies were performed at the preclinical contract research organization Piedmont Research Center, Inc. (Morrisville, NC). Thirteen-week-old female athymic *nu/nu* mice purchased from Harlan (Indianapolis, IN) were used. For xenograft studies at SUGEN, Inc. examining inhibition of growth in bone of

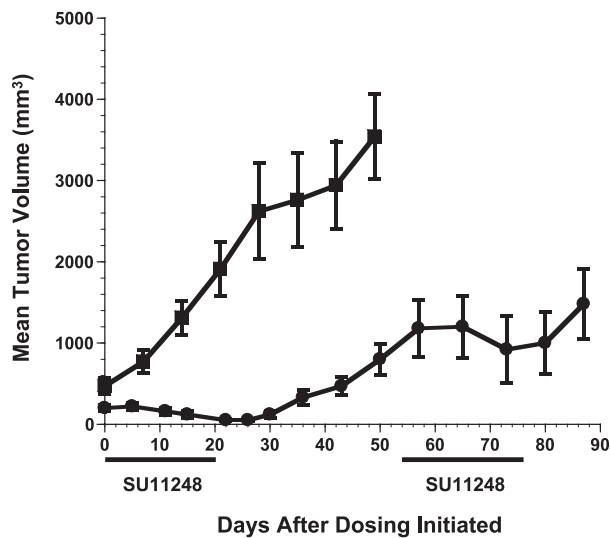
435/HAL-Luc, a line derived from the human breast cancer MDA-MB-435 (18), 7–8-week-old female athymic *nu/nu* mice purchased from Charles River Breeding Laboratories (Wilmington, MA) were used. Mice were housed under pathogen-free conditions in microisolator cages, with irradiated rodent chow and water available *ad libitum*. All xenograft animal studies were carried out with the approval of either the Piedmont Research Center or the SUGEN Institutional Animal Care and Use Committee in an AAALAC International accredited vivarium and in accordance with the Institute of Laboratory Animal Research (NIH, Bethesda, MD) *Guide for the Care and Use of Laboratory Animals*. MMTV-*v-Ha-ras* transgenic mice were originally obtained from Charles River Breeding Laboratories and maintained in a FVB background. Female Sprague-Dawley rats (IOPS-OFA) 7 weeks of age were supplied by Iffa-Credo (France). The rats were housed two per cage in cages, with rodent chow and water available *ad libitum*. The MMTV-*v-Ha-ras* transgenic mouse and DMBA-treated rat studies were performed using procedures in compliance with Italian Legislative Decree N.116 dated January 27, 1992, enforcing the European Communities Council Directive N.86/609/EEC concerning the protection of animals used for experimental or other scientific purposes and according to Institutional Policy Regarding the Care and Use of Laboratory Animals.

### Treatment of Mouse Mammary Tumor Virus-*v-Ha-ras* Transgenic Mice with Established Mammary Tumors

Compound administration began when mammary tumors in MMTV-*v-Ha-ras* mice were established to an average volume in the range of 300–500 mm<sup>3</sup>. Mice were treated p.o. by gavage once daily with either a suspension of 40 mg/kg/day of SU11248 (established as the efficacious dose in previous tumor xenograft models (1) or its vehicle, 0.5% carboxymethylcellulose, for the first 20 days, followed by cessation of dosing to monitor tumor regrowth. Treatment resumed on day 54 until dosing stopped on day 74. Tumor growth was measured approximately once weekly for 8 weeks using Vernier calipers for the duration of the treatment. The two perpendicular tumor axes were measured and tumor volume was calculated according to the formula  $d^2 \times D/2$ , where  $d$  is the minimum and  $D$  the maximum diameter. *P*-values were calculated using the two-tailed Student's *t* test to assess differences in tumor volumes between treated and control groups ( $P \leq 0.05$  was considered significant).

### Treatment of Rats with Established 7,12-Dimethylbenz(*a*)anthracene-Induced Mammary Tumors

DMBA and its vehicle sesame oil were purchased from Sigma-Aldrich (St. Louis, MO). Female 50-day-old Sprague-Dawley rats were intubated with a single intragastric dose of 20 mg of DMBA in 1.0 ml of sesame oil. After ~40 days, animals were examined weekly by palpation. When at least one mammary tumor measuring 1 cm in diameter was identified, the rats were placed sequentially into two groups and treated p.o. once daily with 5, 10, or 20 mg/kg/day of SU11248 or its vehicle. The 20-mg/kg dose of SU11248 in these rats is approximately equivalent to the



**Figure 1.** Antitumor activity of SU11248 on spontaneous mammary carcinomas in MMTV-v-Ha-ras transgenic mice. Oral administration of SU11248 at 40 mg/kg/day (●) or vehicle (■) was initiated when tumors reached an average of  $\sim 300$  mm<sup>3</sup> and continued to day 20. At that time, dosing was ceased to monitor tumor regrowth. Compound administration was reinitiated on day 54, with cessation of dosing on day 74 to monitor regrowth. Tumor volume was measured on the indicated days. Lines, mean tumor volume for each group; bars, SEM.

efficacious dose of 40 mg/kg in the mouse based on allometric scaling. The tumor volume was measured weekly using Vernier calipers for the duration of the treatment. *P*-values were calculated using the two-tailed Student's *t* test to assess differences in tumor volumes between treated and control groups ( $P \leq 0.05$  was considered significant).

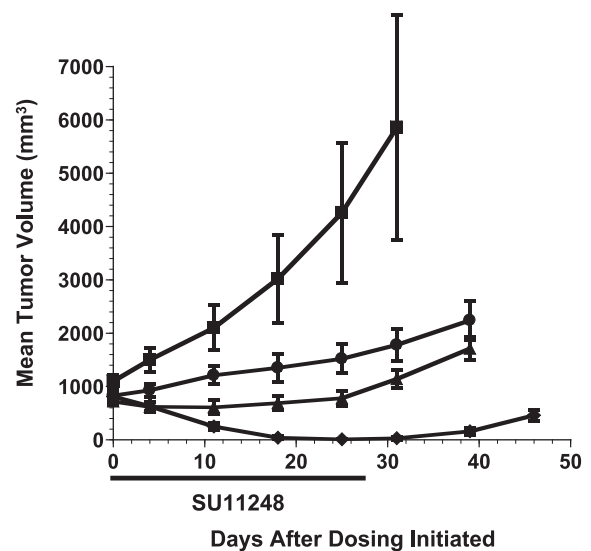
#### Combination Treatment of SU11248 and Docetaxel, Doxorubicin, or 5-Fluorouracil against s.c. MX-1 Human Breast Cancer Xenografts

Athymic mice implanted s.c. with 1-mm<sup>3</sup> MX-1 human breast carcinoma tumor fragments in the hind flank were randomized into treatment groups of 9–10 mice each when tumors reached  $\sim 100$  mm<sup>3</sup> in volume. Tumor-bearing mice were treated p.o. once daily with SU11248 to the end of the study, with a chemotherapeutic agent, or a combination of the two or their vehicles, as indicated in figure legends and tables. SU11248 was delivered p.o. in a carboxymethylcellulose suspension by gavage to the end of the study. Docetaxel (Taxotere) was obtained as the pharmaceutical drug (Aventis, Bridgewater, NJ) and was diluted for i.v. dosing in a vehicle containing 7.5% ethanol and 7.5% Tween 80 in 5% dextrose in water. It was dosed once a week for 3 weeks at 5, 10, or 15 mg/kg concentrations. Clinical 5-FU (American Pharmaceutical Partners, Inc., Schaumburg, IL), diluted for i.p. dosing to an appropriate concentration with 5% dextrose in water, was administered once a week for 3 weeks at a 100-mg/kg concentration. Clinical doxorubicin (Adriamycin; Pharmacia Corp., Peapack, NJ), diluted for i.v. dosing to an appropriate concentration for dosing with 0.9% saline, was adminis-

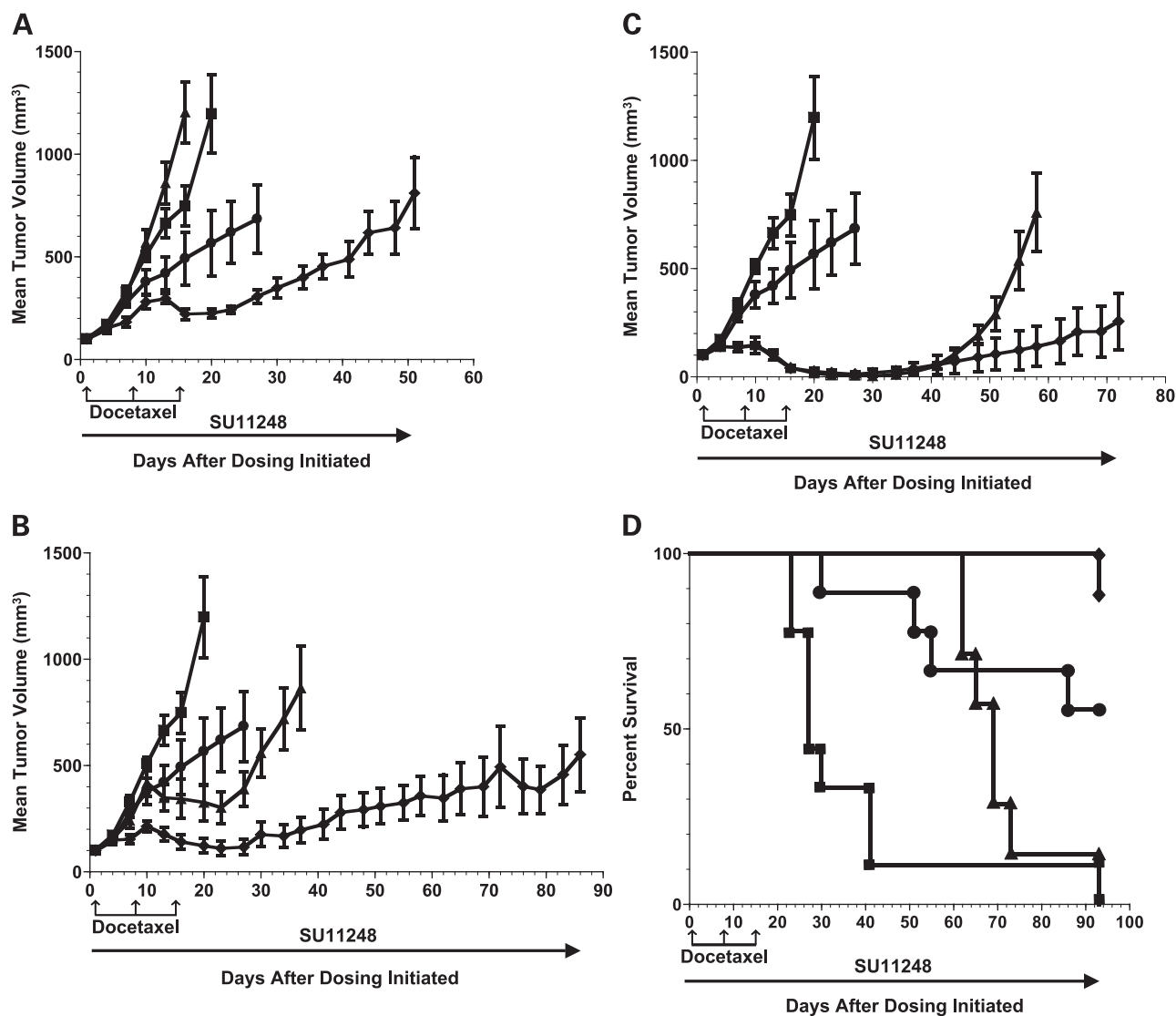
tered every other day for three doses at a 4-mg/kg concentration. Tumor growth was measured twice weekly using Vernier calipers for the duration of the treatment. Tumor growth inhibition of treated mice was compared with control mice. Tumor growth delay was calculated as the time taken for tumors to reach 500 mm<sup>3</sup> in treated versus control groups. The survival data were based on the day mice were euthanized as a cancer death when tumors reach the 1500-mm<sup>3</sup> cutoff. For all studies, *P*-values were calculated using the two-tailed Student's *t* test to assess differences between treated and control groups ( $P \leq 0.05$  was considered significant).

#### Combination Treatment of SU11248 and Docetaxel against 435/HAL-Luc Human Breast Cancer Colonized in Bone

The 435/HAL-Luc line is derived from the human breast cancer line MDA-MB-435 and selected for enhanced growth *in vivo* after multiple passage of tumors in mice (kindly provided by Dr. David Griggs, Pharmacia Corp.; 18) followed by introduction of stable luciferase expression by transfection of the pGL3 luciferase reporter vector (Promega, Madison, WI). Luciferase reporter activity was used to group mice with tumor in bone before initiation of treatment using the Xenogen IVIS Imaging System (Xenogen Corp., Alameda, CA). These cells were cultured using standard culture technique in RPMI 1640 supplemented with 10% fetal bovine serum, 2-mM glutamine, and 1-mM sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD) and maintained routinely in a humidified chamber at 37°C and 5% carbon dioxide. Cells were harvested from cell culture flasks during exponential growth, washed once with sterile PBS, counted, and resuspended in PBS to a



**Figure 2.** Antitumor activity of SU11248 on DMBA-induced mammary carcinomas in Sprague-Dawley rats. Oral administration of SU11248 at 5 (●), 10 (▲), or 20 (◆) mg/kg/day or vehicle (■) was initiated when tumors reached an average of between 700 and 1100 mm<sup>3</sup> and continued to day 28. Tumor volume was measured on the indicated days. Lines, mean tumor volume for each group of 12 animals; bars, SEM.



**Figure 3.** Combination of SU11248 with docetaxel significantly improved growth inhibition of MX-1 human breast cancer xenografts in athymic mice. Oral administration of SU11248 (●) at 40 mg/kg/day to end of study, i.v. administration of docetaxel (▲) at 5 (A), 10 (B), or 15 (C) mg/kg once weekly for 3 weeks, combination of the two agents (◆), or vehicle (■) were initiated when tumors reached an average of ~100 mm<sup>3</sup>. Tumor volume was measured on the indicated days. Lines, mean tumor volume for each group of nine animals; bars, SEM. The time for tumors treated with SU11248, docetaxel at 15 mg/kg, or their combination to reach 1500 mm<sup>3</sup> is represented as a Kaplan-Meier survival graph (D).

suitable concentration prior to implantation. Athymic mice received injections into the left ventricle of the heart (19) with  $2 \times 10^6$  cells. Subsequently, cells colonize and continue to grow in bone, often resulting in spinal cord compression, with secondary vertebral collapse leading to hind limb paralysis.

When the first mouse began to demonstrate signs of hind limb paralysis 4 weeks after cell implantation, indicating tumor growth in bone, the remaining mice with no initial appearance of paralysis or weight loss were randomized into treatment groups of 15 mice each for efficacy studies. Athymic mice bearing 435/HAL-Luc human breast cancer growing in bone were administered 40 mg/kg/day of SU11248 p.o. once daily to the end of the study, 5 mg/kg/

day of docetaxel i.v. once a week for 3 weeks as described above, or a combination of the two or their vehicles, as indicated in the figure legend and Table 4. At experimental end points exhibited by hind limb paralysis or 20% weight loss, mice were humanely euthanized, with the remaining percentage of mice surviving plotted over time. *P*-values were calculated using the two-tailed Student's *t* test and  $\chi^2$  test to assess differences between treated and control groups ( $P \leq 0.05$  was considered significant).

#### Immunoprecipitation and Western Blot Analysis *in Vivo*

MX-1, MMTV-*v-Ha-ras*, or 435/HAL-Luc mammary tumors from mice were pulverized in liquid nitrogen into a powder, which was then lysed, and protein concentration



**Table 1. Evaluation of SU11248 combined with docetaxel compared with monotherapies against 100-mm<sup>3</sup> established human breast cancer MX-1 in athymic mice**

Agent <sup>a</sup>	Dose (mg/kg)	Mean tumor volume (mm <sup>3</sup> ± SEM) (day)	%T/C <sup>b</sup> (day)	P-value	Mean day for tumor to reach 500 mm <sup>3</sup>	T – C <sup>b</sup> (days)
Control	N/A	748 ± 97 (16)	N/A	N/A	10	–
SU11248	40	492 ± 129 (16)	34 (16)	NS	18	8
		566 ± 156 (20)	53 (20)	0.02		
Docetaxel	5	1204 ± 148 (16)	0 (16)	NS	9	0
Docetaxel	10	343 ± 91 (16)	60 (16)	0.005	29	19
Docetaxel	15	41 ± 12 (16)	95 (16)	<0.0001	54	44
		759 ± 181 (58)				
SU11248 + Docetaxel	40 + 5	221 ± 26 (16)	70 (16)	<0.0001	42	32
			vs. SU11248: 55 (16)	0.06 (NS)		
			vs. Docetaxel: 82 (16)	<0.0001		
			vs. SU11248: 55 (27)	0.04		
SU11248 + Docetaxel	40 + 10	141 ± 34 (16)	81 (16)	<0.0001	85	75
		122 ± 34 (20)				
			vs. SU11248: 71 (16)	0.02		
			vs. Docetaxel: 59 (16)	0.05		
			vs. SU11248: 78 (20)	0.01		
			vs. Docetaxel: 62 (20)	0.04		
			vs. Docetaxel: 77 (37)	0.005		
SU11248 + Docetaxel	40 + 15	140 ± 92 (58)	vs. Docetaxel: 82 (58)	0.008	91	81

<sup>a</sup>SU11248 given p.o. once daily to study end; docetaxel given i.v. once weekly for 3 weeks.

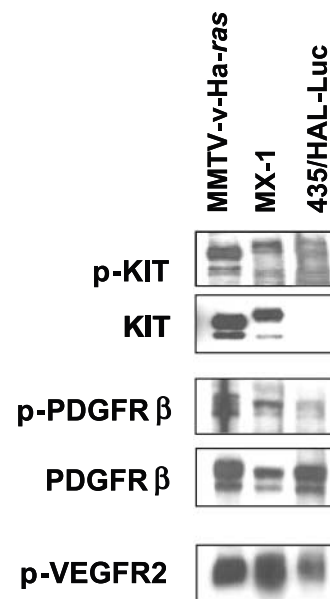
<sup>b</sup>T/C, tumor growth inhibition, where T is the mean tumor volume of the treated group and C is the mean tumor volume of the control group on the designated day. T – C, tumor growth delay, median time (days) for the treated tumors to reach 500-mm<sup>3</sup> volume minus the median time for control group to reach 500 mm<sup>3</sup> in volume.

was determined as previously described (3). One milligram of protein from each sample was immunoprecipitated overnight at 4 °C with an anti-KIT (SC-1493-AC, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-PDGFR (06-498, Upstate Biotechnology, Lake Placid, NY) antibody and protein A agarose beads (16-125, Upstate Biotechnology). Immune complexes were washed with HNTG lysis buffer containing inhibitors. Proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were probed with an anti-phospho-KIT antibody (epitope Y719; 3391, Cell Signaling Technology, Beverly, MA) or, to identify phospho-PDGFR, probed with antiphosphotyrosine antibody (SC-7020, Santa Cruz Biotechnology) then stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL). To detect total KIT and PDGFR levels, membranes were reprobed with the same anti-KIT and anti-PDGFR antibodies, respectively, which were used for the immunoprecipitation. For detection of KDR, tumor lysates were immunoprecipitated with an antibody to total phosphotyrosine (4G10-AC, Upstate Biotechnology) followed by immunoblot with an anti-KDR antibody (SC-504, Santa Cruz Biotechnology) using techniques described above.

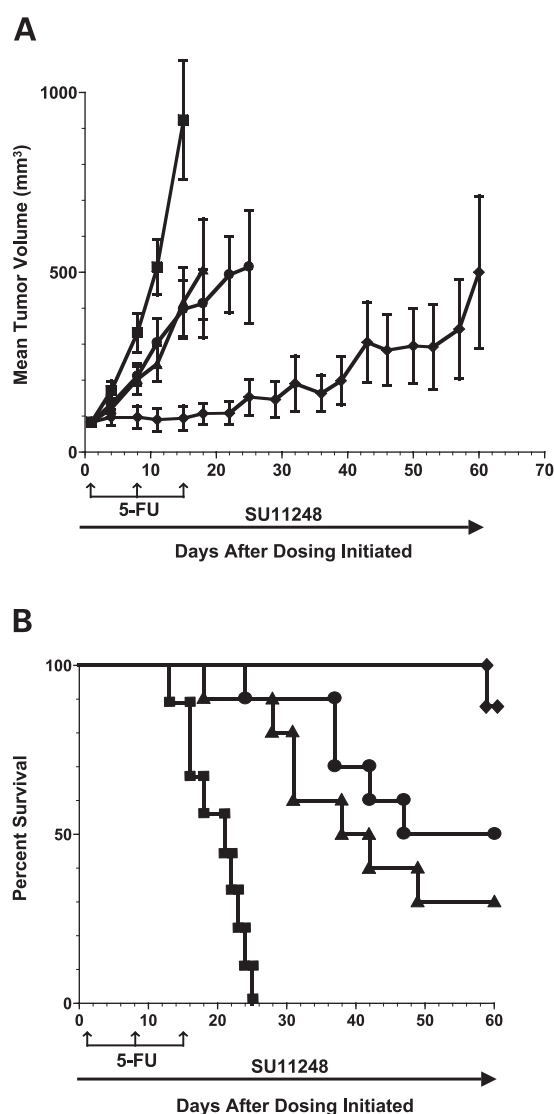
## Results

### SU11248 Was Efficacious in Diverse Preclinical Models of Breast Cancer

The effect of SU11248 on tumor growth was evaluated in two different models of primary mammary cancers that



**Figure 4.** Expression and phosphorylation levels of KIT, PDGFR- $\beta$ , and VEGFR2 in MMTV-v-Ha-ras, MX-1, and 435/HAL-Luc mammary tumors from mice. Tumor lysates were prepared as described in "Materials and Methods." Protein was immunoprecipitated with antibodies to KIT, PDGFR- $\beta$ , or, for the case of VEGFR2 analysis, total phosphotyrosine. Protein levels of KIT and PDGFR- $\beta$  were determined by immunoblotting using antibodies for total KIT and PDGFR- $\beta$ . Phosphotyrosine levels of all three receptors were determined by immunoblotting using antibodies for phospho-KIT (Y719), total phosphotyrosine for PDGFR- $\beta$ , or VEGFR2.



**Figure 5.** Combination of SU11248 with 5-FU improved growth inhibition of MX-1 human breast cancer xenografts in athymic mice. Oral administration of SU11248 (●) at 40 mg/kg/day to end of study, i.p. administration of 5-FU (▲) at 100 mg/kg once weekly for 3 weeks, combination of the two agents (◆), or vehicle (■) were initiated when tumors reached an average volume of ~100 mm<sup>3</sup>. Tumor volume was measured on the indicated days. Lines, mean tumor volume for each group of 10 animals; bars, SEM (A). The time for tumors treated with SU11248, 5-FU, or their combination to reach 1500 mm<sup>3</sup> is represented as a Kaplan-Meier survival graph (B).

develop *de novo*. MMTV-*v-Ha-ras* transgenic mouse mammary carcinomas and DMBA-induced rat mammary carcinomas model human cancer in their stochastic development in their natural tissue of origin.

The effect of SU11248 on the growth of MMTV-*v-Ha-ras* mouse mammary tumors was evaluated using the established efficacious dose of 40 mg/kg/day of SU11248 (1). This agent administered daily for the first 20 days resulted in marked tumor regression (82%,  $P = 0.0002$ ) (Fig. 1), indicating that SU11248 was active in this model. After 20 days of dosing, SU11248 treatment was

stopped, resulting in tumor regrowth. When tumors again grew to a large size, SU11248 treatment was reinitiated, resulting in tumor stasis (Fig. 1). After 17 days of the second round of dosing, compound administration ceased a second time, and tumors again began to grow. In a second study, SU11248 was administered daily for 4 weeks to rats with established DMBA-induced mammary tumors, resulting in marked tumor regression at the 20 mg/kg/day dose (99% regression;  $P < 0.0001$ ) and tumor growth inhibition by 5 and 10 mg/kg/day (64%,  $P = 0.04$  and 82%,  $P = 0.006$ , respectively) (Fig. 2). After dosing was ceased, tumors regrew in all groups.

Anticipating combination studies of SU11248 with chemotherapeutic agents, SU11248 was administered p.o. at 40 mg/kg/day to mice bearing MX-1 human breast cancer-derived xenografts. Significant tumor growth inhibition was observed, as shown in Fig. 3 and Table 1, and this dose was used for all combination studies. Three of nine mice survived for 53 days after dosing stopped. The SU11248 target KIT is expressed and phosphorylated in these tumors in mice (Fig. 4), as are PDGFR- $\beta$  and VEGFR2 (Fig. 4), with KIT localized to the epithelium and PDGFR- $\beta$  to the stroma (data not shown). VEGFR2 localization was not determined.

#### SU11248 in Combination with Docetaxel, 5-Fluorouracil, or Doxorubicin Significantly Enhanced Growth Inhibition of s.c. MX-1 Tumors

Because taxane, fluoropyrimidine, and anthracycline drugs are three major therapeutic approaches for advanced breast cancer in patients (20), the *in vivo* effects of SU11248 in combination with an agent from each of these classes (docetaxel, 5-FU, or doxorubicin, respectively) were examined in the MX-1 tumor model. Each chemotherapy was first administered as a single agent at several doses in regimens modeled on the clinical treatment regimen, and a subefficacious dose was selected for combination with SU11248. In combination studies, docetaxel (5, 10, or 15 mg/kg) and 5-FU (100 mg/kg) were administered i.v. and i.p., respectively, once weekly for 3 weeks. Doxorubicin was administered i.v. at 4 mg/kg every other day for three doses. In each combination, SU11248 was administered p.o. at a daily dose of 40 mg/kg to the end of the study. Tumor volumes were compared with those in animals receiving each agent alone.

Docetaxel treatment of MX-1 tumors resulted in a dose-dependent effect, with no tumor growth inhibition at 5 mg/kg, 60% inhibition at 10 mg/kg, and 95% inhibition at 15 mg/kg after 16 days of dosing. SU11248 combined with the ineffective dose of 5 mg/kg of docetaxel demonstrated greater than additive antitumor activity of SU11248, resulting in 55% inhibition on day 27 of dosing and a 24-day tumor growth delay as compared with SU11248 alone (Fig. 3A; Table 1). The antitumor activity of either SU11248 or 10 mg/kg docetaxel alone was enhanced by coadministration of the two compounds, resulting in a 78% inhibition *versus* SU11248 alone and 62% inhibition *versus* docetaxel alone after 20 days of dosing (Fig. 3B; Table 1). This combination resulted in a 67- and 56-day tumor growth delay as compared with single administration of

**Table 2. Evaluation of SU11248 combined with 5-FU compared with monotherapies against 100-mm<sup>3</sup> established human breast cancer MX-1 in athymic mice**

Agent <sup>a</sup>	Dose (mg/kg)	Mean tumor volume (mm <sup>3</sup> ± SEM) (day)	%T/C <sup>b</sup> (day)	P-value	Mean day for tumor to reach 500 mm <sup>3</sup>	T - C <sup>b</sup> (days)
Control	N/A	1071 ± 195 (15)	N/A	N/A	11	-
SU11248	40	397 ± 81 (15)	57 (15)	0.01	25	14
5-FU	100	417 ± 96 (15)	55 (15)	0.02	18	7
SU11248 + 5-FU	40 + 100	94 ± 34 (15)	90 (15)	0.0001	60	49
			<i>vs.</i> SU11248: 76 (15)	0.003		
			<i>vs.</i> 5-FU: 77 (15)	0.005		

<sup>a</sup>SU11248 given p.o. once daily to study end; 5-FU given i.p. once weekly for 3 weeks.

<sup>b</sup>T/C, tumor growth inhibition, where T is the mean tumor volume of the treated group and C is the mean tumor volume of the control group on the designated day. T - C, tumor growth delay, median time (days) for the treated tumors to reach 500-mm<sup>3</sup> volume minus the median time for control group to reach 500 mm<sup>3</sup> in volume.

SU11248 or docetaxel, respectively. Administration at 15 mg/kg almost fully regressed tumors, with tumor regrowth observed after cessation of treatment. In combination with SU11248, this regrowth was greatly delayed (Fig. 3C; Table 1), with 82% tumor growth inhibition after 58 days of dosing and a 73- and 37-day tumor growth delay as compared with SU11248 or docetaxel alone, respectively, conferring a survival benefit as shown in Fig. 3D. With this combination, three of nine (33%) mice were tumor free 3 months after dosing ceased, while no mice were tumor free with docetaxel alone and only one of nine (11%) was with SU11248 treatment. The combination of SU11248 and docetaxel was generally well tolerated, and although there were no deaths in the group receiving SU11248 in combination with the highest dose of docetaxel (15 mg/kg), there were 4 of 10 unexplained deaths in the group receiving 10 mg/kg of docetaxel in combination with SU11248. In a repeat study yielding similar efficacy results, no treatment-related deaths were observed in mice treated with SU11248 in combination with docetaxel at the 5, 10, or 15 mg/kg dose.

SU11248 was also combined with two other chemotherapeutic agents used to treat breast cancer. The combination of SU11248 and 5-FU resulted in 74% inhibition *versus* SU11248 and 79% inhibition *versus* 5-FU (Fig. 5A; Table 2). The combination resulted in greater than additive tumor growth delays of 35 and 42 days to reach 500 mm<sup>3</sup> in volume as compared with SU11248 and 5-FU, respectively, conferring a survival benefit as shown in Fig. 5B. The combination was generally well tolerated; however, 2 of 10 mice died of unknown cause in the group receiving the combination treatment, while no weight loss was observed in any mice other than these two.

The combination of SU11248 with doxorubicin was well tolerated, inducing no mortality or weight loss, and resulted in 60% and 81% inhibition 31 days after dosing, as compared with SU11248 and doxorubicin alone, respectively (Fig. 6A; Table 3). The combination resulted in greater than additive tumor growth delays of 32 and 46 days to reach 400 mm<sup>3</sup> in volume as compared with SU11248 and doxorubicin, respectively, conferring a survival benefit as shown in Fig. 6B.

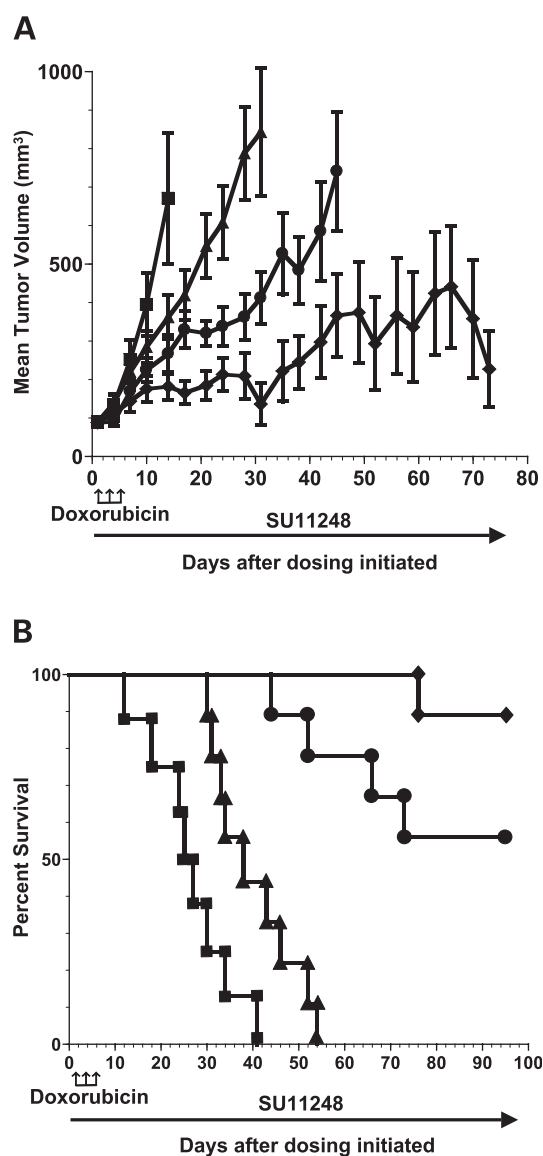
### SU11248 Enhanced Docetaxel-Induced Tumor Growth Delay in 435/HAL-Luc Breast Carcinoma Growth in Bone

To model the aggressive metastatic nature of advanced human breast cancer, the faster-growing MDA-MB-435-derived cell line 435/HAL (18) was transfected to stably express luciferase and used in bioluminescent imaging to initially identify colonization to bone. Cell inoculation via the left ventricle resulted in bone colonization (19), providing a model to investigate the effects of SU11248 on growth of breast cancer in the bone. As shown in Fig. 7 and Table 4, daily treatment with 40 mg/kg/day of SU11248 or once weekly treatment with 5 mg/kg of docetaxel for 3 weeks resulted in prolongation of the mean day of survival (MDS) from 46 days after cell implantation in the vehicle-treated group to 52 days in the two monotherapy groups ( $P = 0.02$  for each). In combination, the MDS was prolonged further to 60 days ( $P = 0.0001$  *versus* vehicle treatment,  $P = 0.005$  *versus* SU11248 treatment,  $P = 0.0007$  *versus* docetaxel treatment). On day 61, the last mouse succumbed to disease in the SU11248-treated group, while 8 of 13 mice (62%,  $P = 0.0002$ ) still remained healthy in the group receiving combination therapy.

### Discussion

SU11248 offers the potential for novel therapeutic intervention in breast cancer by inhibiting the signaling of RTKs contributing to both tumor proliferation and stromal and blood vessel support of tumor growth. SU11248 has been shown to exhibit potent antitumor activity in many s.c. tumor xenograft models derived from human cancers of diverse tissue origin (1–3). In the current studies, SU11248 has now demonstrated effective single-agent anticancer activity in all the human breast cancer models evaluated: oncogene-driven mammary cancers in transgenic mice, carcinogen-induced mammary cancers in rats, and human breast cancer xenografts in mice.

The relationship between SU11248 pharmacokinetics and inhibition of its targets PDGFR- $\beta$ , VEGFR2, and KIT has been previously characterized in a number of preclinical



**Figure 6.** Combination of SU11248 with doxorubicin improved inhibition of MX-1 human breast cancer xenografts in athymic mice. Oral administration of SU11248 (●) at 40 mg/kg/day to end of study, i.v. administration of doxorubicin (▲) at 4 mg/kg every other day for three doses, combination of the two agents (◆), or vehicle (■) were initiated when tumors reached an average volume of ~100 mm<sup>3</sup>. Tumor volume was measured on the indicated days. Lines, mean tumor volume for each group of nine animals; bars, SEM. (A). The time for tumors treated with SU11248, doxorubicin, or their combination to reach 1500 mm<sup>3</sup> is represented as a Kaplan-Meier survival graph (B).

models (1–3). A consistent finding has been that administration of a single dose of SU11248 at the fully efficacious dose of 40 mg/kg results in a marked reduction of phosphotyrosine levels of VEGFR2, PDGFR- $\beta$ , and KIT for up to 12 h, correlating with tumor growth inhibition on daily administration. The predictable dose-dependent pharmacokinetics and reproducible extent and duration of SU11248 target inhibition support the likelihood that similar pharmacodynamics of target inhibition occurred in the breast cancer models used in the current report.

At least one target of SU11248 was expressed in each of the tumor models studied. However, we have not addressed the relative contribution of each target to the antitumor effect. A common approach to evaluate the role of targets directly expressed by the tumor cells is to examine the effect of drug treatment on tumor cells in a controlled cell culture setting. Of the four models reported in this manuscript, only the 435/HAL-Luc exists as a cell line. A related manuscript currently under review<sup>1</sup> reports that anchorage-independent growth of 435/HAL-Luc cells in soft agar was not inhibited by SU11248 at the submicromolar, target-selective concentrations that reduce phosphotyrosine levels of PDGFR, VEGFR, and KIT. Thus, the inhibitory effects of SU11248 on 435/HAL-Luc tumors are likely acting through effects on the microenvironment and vasculature of the tumor.

The microenvironment for tumor xenograft growth is quite different from the cancer's original milieu and may explain some of the differences in tumor biology seen between primary human tumors and tumor xenografts in athymic mice, such as different responses to drug treatment (21). Therefore, SU11248 efficacy was evaluated in two breast cancer models where tumors arose either through transgenic oncogene activation or through chemical carcinogenesis. SU11248 inhibited growth of activated RAS-driven mammary tumors in MMTV-*v-Ha-ras* transgenic mice, which exhibit many characteristics of human breast tumors, including spontaneous development and local invasiveness. SU11248 treatment of mice bearing established tumors resulted in dramatic tumor regression followed by regrowth when SU11248 dosing ceased. When the resulting large tumors were subsequently redosed with SU11248, stasis or a modest regression was observed. These results demonstrated that mammary tumors in these transgenic mice respond to multiple rounds of SU11248 treatment and that even advanced tumors responded to therapy. In this model, SU11248 activity may be via several mechanisms, such as inhibition of KIT signaling via a non-RAS pathway and/or through inhibition of VEGFR2 or PDGFR- $\beta$ , both of which regulate the supporting tumor stroma (22, 23). In addition, SU11248 administration to rats harboring DMBA-induced mammary tumors also resulted in marked inhibition of the malignant tumors, demonstrating the effectiveness of this agent against mammary cancers arising from genetic insult by carcinogen exposure in rodents.

The anticancer activity of SU11248 against oncogene-driven and carcinogen-induced rodent mammary cancers was reproduced in the MX-1 human breast cancer s.c. tumor xenograft model. When SU11248 was administered at the efficacious dose, three of nine mice were long-term survivors, surviving 53 days after dosing ceased on day 93. The tumor epithelium of MX-1 xenografts expresses KIT; thus, the contribution of compound activity may be

<sup>1</sup> L. J. Murray, T. J. Abrams, K. R. Long, T. J. Ngai, L. M. Olson, M. D. Pagel, W. Hong, P. K. Keast, J. A. Brassard, A. M. O'Farrell, J. M. Cherrington, and N. K. Pryer. SU11248 inhibits tumor growth and CSF-1R-dependent osteolysis in an experimental breast cancer bone metastasis model, submitted for publication.



**Table 3. Evaluation of SU11248 combined with doxorubicin compared with monotherapies against 100-mm<sup>3</sup> established human breast cancer MX-1 in athymic mice**

Agent <sup>a</sup>	Dose (mg/kg)	Mean tumor volume (mm <sup>3</sup> ± SEM) (day)	%T/C <sup>b</sup> (day)	P-value	Mean day for tumor to reach 400 mm <sup>3</sup>	T – C <sup>b</sup> (days)
Control	N/A	700 ± 70 (14)	N/A	N/A	11	–
SU11248	40	267 ± 48 (14) 412 ± 68 (31)	62 (14)	0.02	31	20
Doxorubicin	4	362 ± 57 (14) 843 ± 167 (31)	48 (14)	0.07 (NS)	17	6
SU11248 + Doxorubicin	40 + 4	182 ± 34 (14) 163 ± 55(31)	74 (14) vs. SU11248: 60 (31) vs. Doxorubicin: 81 (31)	0.009 0.01 0.001	63	52

<sup>a</sup>SU11248 given p.o. once daily to study end; doxorubicin given i.v. every other day for three doses.

<sup>b</sup>T/C, tumor growth inhibition, where T is the mean tumor volume of the treated group and C is the mean tumor volume of the control group on the designated day. T – C, tumor growth delay, median time (days) for the treated tumors to reach 400-mm<sup>3</sup> volume minus the median time for control group to reach 400 mm<sup>3</sup> in volume.

through direct inhibition of the tumor proliferation and survival as well as through targeting the supporting tumor stroma. SU11248 anticancer activity was somewhat less effective against these highly aggressive estrogen-independent MX-1 tumors than in many previous xenograft models (1, 2), providing an attractive opportunity to examine combined activity of SU11248 with traditional chemotherapeutic agents.

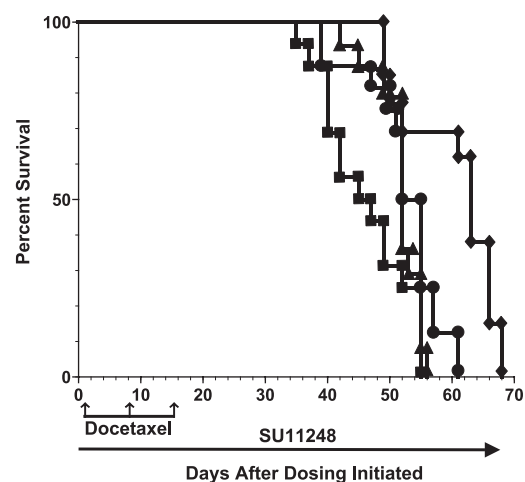
The combination of conventional cytotoxic drugs with novel molecularly targeted agents that specifically interfere with key pathways controlling cancer cell survival, proliferation, invasion, and/or metastatic spread has generated wide interest in recent years. This is likely to be a promising therapeutic approach for several reasons: (a) the cellular targets and mechanism(s) of action are different in that combinations may prevent or prolong initiation of drug resistance in tumors and (b) changes in the expression and/or the activity of genes that regulate mitogenic signals directly inhibit cell growth and in addition may affect the sensitivity of cancer cells to conventional chemotherapy (24).

Enhanced antitumor activity was observed in mice bearing established MX-1 human breast cancer xenografts treated with SU11248 in combination with the antineoplastic drugs docetaxel, 5-FU, or doxorubicin. This effect was accompanied by significantly increased survival in the combined therapy groups as compared with the groups treated with a single agent. Treatment with each of the cytotoxic agents alone only transiently inhibited tumor growth, and the tumors resumed the growth rate of untreated controls after cessation of therapy. In contrast, when combined with SU11248 administered to the end of the study, tumor growth delays were observed after the end of treatment with the cytotoxic agents. The extended maintenance of tumor growth inhibition after chemotherapy by continuous treatment with SU11248 supports investigation of these combinations in the clinic.

The combined treatment with SU11248 and each of the three cytotoxic agents was well tolerated by the mice, without overt signs of weight loss or toxicity. Doses of the

chemotherapeutic agents in mice were below the maximum tolerated dose and/or most efficacious dose to study combined effects with SU11248. SU11248 significantly enhanced tumor growth inhibition at these lower, well-tolerated doses with all three agents tested, suggesting that the standard doses of these agents, which confer significant side effects, may potentially be reduced by coadministration with SU11248.

The presence of disseminated disease is a hallmark of advanced breast cancer. To study the effects of SU11248 on breast cancer-derived cells growing in bone, a model recapitulating aggressive breast cancer growth in the bone of athymic mice was used, leading to eventual paralysis and



**Figure 7.** Combination of SU11248 with docetaxel prolonged survival of athymic mice with human breast cancer xenografts 435/HAL-Luc growing in bone. Oral administration of SU11248 (●) at 40 mg/kg/day to end of study, i.v. administration of docetaxel (▲) at 5 mg/kg once weekly for 3 weeks, combination of the two agents (◆), or vehicle (■) were initiated 4 weeks after implantation of tumor cells. Survival was measured as the time for animals to exhibit the disease phenotypes of hind limb paralysis or 20% weight loss on the indicated days. Each group consisted of 15 mice.

**Table 4.** Evaluation of SU11248 combined with docetaxel compared with monotherapies against established human breast cancer 435/ HAL-Luc growth in bone of athymic mice

Agent <sup>a</sup>	Dose (mg/kg)	n	MDS ± SEM (days)	Difference between means (P-value)	χ <sup>2</sup> test: difference in survival on designated day (P-value)
Control	N/A	16	46 ± 2	N/A	–
SU11248	40	15	52 ± 2	0.02	–
Docetaxel	5	13	52 ± 1	0.02	–
SU11248 + Docetaxel	40 + 5	15	60 ± 2	0.0001 vs. SU11248: 0.005 vs. Docetaxel: 0.0007	Day 61 vs. SU11248: 0.0002

<sup>a</sup>SU11248 given p.o. once daily to study end; docetaxel given i.v. once weekly for 3 weeks.

cachexia (18, 25). This reflects more advanced metastatic breast cancer cases, which ultimately metastasize to bone in ~70% of patients (26) and is typically associated with pain, pathologic fractures, spinal cord compression, and hypercalcemia (27–29). SU11248 was administered in combination with docetaxel, beginning at an advanced point of disease where the first mice began to show signs of paralysis. A statistically significant increase in survival was observed with the combination of SU11248 and docetaxel in mice with established breast cancer bone lesions.

The combination of SU11248 and each of the three cytotoxic agents evaluated in these studies produced significantly longer survival of tumor-bearing mice than that of either agent alone, suggesting that the combination effect may be independent of the precise mechanism(s) of action of three distinct drugs with highly diverse mechanisms of action. While both 5-FU and doxorubicin induce DNA damage, they achieve this by very different processes, and docetaxel is an antimetabolic drug that targets microtubule dynamics. We hypothesize that inhibition of mitogenic signaling by SU11248, in combination with cytotoxic drugs, could cause irreparable tumor cell damage, leading to apoptotic cell death, as postulated for epidermal growth factor receptor inhibitors in combination with cytotoxic drugs (24). In addition, cytotoxic agent damage of tumor cells may be exacerbated by inhibition of tumor-supporting stroma and blood vessels by SU11248. Clearly, there are multiple points in tumor biology where SU11248 could potentiate the effect of the chemotherapeutic agents and vice versa. Further mechanistic analyses would be required to elucidate the nature of the interaction between SU11248 and these cytotoxic agents.

The multiplicity of aberrant processes and highly mutagenic capability of malignant cells require a therapeutic arsenal capable of targeting multiple mechanisms of tumorigenesis individually and in combination. Tumor resistance to both traditional chemotherapy and novel molecular targeted therapies is in many cases inevitable. Multitargeted inhibitors such as SU11248 provide the opportunity to attack tumor growth and survival by multiple mechanisms. The combination of SU11248 with chemotherapeutic agents in use in the clinic provides

further opportunities to enhance or extend the activity of approved therapies and to administer better-tolerated doses of approved agents with comparable or enhanced activity.

In summary, our experiments demonstrate that SU11248 as a single agent exhibits potent anticancer activity in preclinical breast cancer models and further enhances the effects of docetaxel, 5-FU, and doxorubicin in human breast cancer xenografts, including a model of disseminated disease in mice, demonstrating the benefit of combining two therapeutic modalities that have entirely different mechanisms of activity. The enhanced activity of SU11248 in combination with “standard of care” chemotherapeutic agents for breast cancer activity presents a compelling rationale for comparable clinical studies in this disease. SU11248 is currently in Phase 1/2 clinical trials in patients with advanced cancers.

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#### References

- Mendel, D. B., Laird, A. D., Xin, X., Louie, S. G., Christensen, J. G., Li, G., Schreck, R. E., Abrams, T. J., Ngai, T. J., Lee, L. B., Murray, L. J., Carver, J., Chan, E., Moss, K. G., Haznedar, J. O., Sukbuntherng, J., Blake, R. A., Sun, C., Tang, C., Miller, T., Shirazian, S., McMahon, G., and Cherrington, J. M. *In vivo* antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin. Cancer Res.*, 9: 327–337, 2003.
- O’Farrell, A. M., Abrams, T. J., Yuen, H. A., Ngai, T. J., Louie, S. G., Yee, K. W. H., Wong, L. M., Hong, W. M., Lee, L. B., Town, A., Smolich, B. D., Manning, W. C., Murray, L. J., Heinrich, M. C., and Cherrington, J. M. SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity *in vitro* and *in vivo*. *Blood*, 101: 3597–3605, 2003.
- Abrams, T., Lee, L. B., Murray, L. J., Pryer, N. K., and Cherrington, J. M. SU11248 inhibits KIT and platelet derived growth factor receptor  $\beta$  in preclinical models of human small cell lung cancer. *Mol. Cancer Ther.*, 2: 471–478, 2003.
- Cherrington, J. M., Strawn, L. M., and Shawver, L. K. New paradigms for the treatment of cancer; the role of anti-angiogenesis agents. *In: G. F. Vande Woude (ed.), Advances in Cancer Res.*, 1st edition, vol. 79, pp. 1–38. San Diego, CA: Academic Press, 2000.
- Nakopoulou, L., Stefanaki, K., Panayotopoulou, E., Giannopoulou, I., Athanassiadou, P., Gakiopoulou-Givalou, H., and Louvrou, A. Expression

- of the vascular endothelial growth factor receptor-2/Flk-1 in breast carcinomas: correlation with proliferation (Comment). *Hum. Pathol.*, **33**: 863–870, 2002.
6. de Jong, J. S., van Diest, P. J., van der Valk, P., and Baak, J. P. Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I. An inventory in search of autocrine and paracrine loops. *J. Pathol.*, **184**: 44–52, 1998.
  7. Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Guidi, A. J., Dvorak, H. F., Senger, D. R., Connolly, J. L., and Schnitt, S. J. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum. Pathol.*, **26**: 86–91, 1995.
  8. Kranz, A., Mattfeldt, T., and Waltenberger, J. Molecular mediators of tumor angiogenesis: enhanced expression and activation of vascular endothelial growth factor receptor KDR in primary breast cancer. *Int. J. Cancer*, **84**: 293–298, 1999.
  9. Manders, P., Beex, L. V., Tjan-Heijnen, V. C., Geurts-Moespot, J., Van Tienoven, T. H., Foekens, J. A., and Sweep, C. G. The prognostic value of vascular endothelial growth factor in 574 node-negative breast cancer patients who did not receive adjuvant systemic therapy. *Br. J. Cancer*, **87**: 772–778, 2002.
  10. Coltrera, M. D., Wang, J., Porter, P. L., and Gown, A. M. Expression of platelet-derived growth factor B-chain and the platelet-derived growth factor receptor  $\beta$  subunit in human breast tissue and breast carcinoma. *Cancer Res.*, **55**: 2703–2708, 1995.
  11. Hines, S. J., Organ, C., Kornstein, M. J., and Krystal, G. W. Coexpression of the *c-kit* and stem cell factor genes in breast carcinomas. *Cell Growth & Differ.*, **6**: 769–779, 1995.
  12. Palmu, S., Soderstrom, K. O., Quazi, K., Isola, J., and Salminen, E. Expression of C-KIT and HER-2 tyrosine kinase receptors in poor-prognosis breast cancer. *Anticancer Res.*, **22**: 411–414, 2002.
  13. Natali, P. G., Nicotra, M. R., Sures, I., Mottolese, M., Botti, C., and Ullrich, A. Breast cancer is associated with loss of the *c-kit* oncogene product. *Int. J. Cancer*, **52**: 713–717, 1992.
  14. Chui, X., Egami, H., Yamashita, J., Kurizaki, T., Ohmachi, H., Yamamoto, S., and Ogawa, M. Immunohistochemical expression of the *c-kit* proto-oncogene product in human malignant and non-malignant breast tissues. *Br. J. Cancer*, **73**: 1233–1236, 1996.
  15. Tsuura, Y., Suzuki, T., Honma, K., and Sano, M. Expression of *c-kit* protein in proliferative lesions of human breast: sexual difference and close association with phosphotyrosine status. *J. Cancer Res. Clin. Oncol.*, **128**: 239–246, 2002.
  16. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. Coexpression of MMTV/*v-Ha-ras* and MMTV/*c-myc* genes in transgenic mice: synergistic action of oncogenes *in vivo*. *Cell*, **49**: 465–475, 1987.
  17. Welsch, C. W. Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. *Cancer Res.*, **45**: 3415–3443, 1985.
  18. Schmidt, C. M., Settle, S. L., Keene, J. L., Westlin, W. F., Nickols, G. A., and Griggs, D. W. Characterization of spontaneous metastasis in an aggressive breast carcinoma model using flow cytometry. *Clin. & Exp. Metastasis*, **17**: 537–544, 1999.
  19. Sasaki, A., Boyce, B. F., Story, B., Wright, K. R., Chapman, M., Boyce, R., Mundy, G. R., and Yoneda, T. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. *Cancer Res.*, **55**: 3551–3557, 1995.
  20. Crown, J., Dieras, V., Kaufmann, M., von Minckwitz, G., Kaye, S., Leonard, R., Marty, M., Misset, J. L., Osterwalder, B., and Piccart, M. Chemotherapy for metastatic breast cancer—report of a European expert panel. *Lancet Oncol.*, **3**: 719–727, 2002.
  21. Fidler, I. J. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Res.*, **50**: 6130–6138, 1990.
  22. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.*, **13**: 9–22, 1999.
  23. Sundberg, C., Ljungstrom, M., Lindmark, G., Gerdin, B., and Rubin, K. Microvascular pericytes express platelet-derived growth factor- $\beta$  receptors in human healing wounds and colorectal adenocarcinoma. *Am. J. Pathol.*, **143**: 1377–1388, 1993.
  24. Mendelsohn, J. and Fan, Z. Epidermal growth factor receptor family and chemosensitization (Comment) *J. Natl. Cancer Inst.*, **89**: 341–343, 1997.
  25. Price, J. E. Metastasis from human breast cancer cell lines. *Breast Cancer Res. Treat.*, **39**: 93–102, 1996.
  26. Hortobagyi, G. N. Bone metastases in breast cancer patients. *Semin. Oncol.*, **18**: 11–15, 1991.
  27. Poitout, D. G. (ed.). Bone metastases: diagnosis and treatment, pp. 5–15. New York: Springer-Verlag, 1991.
  28. Berenson, J. R., Lichtenstein, A., Porter, L., Dimopoulos, M. A., Bordoni, R., George, S., Lipton, A., Keller, A., Ballester, O., Kovacs, M. J., Blacklock, H. A., Bell, R., Simeone, J., Reitsma, D. J., Heffernan, M., Seaman, J., and Knight, R. D. Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma. Myeloma Aredia Study Group (Comment). *N. Engl. J. Med.*, **334**: 488–493, 1996.
  29. Mundy, G. R. and Bertolini, D. R. Bone destruction and hypercalcemia in plasma cell myeloma (Erratum appears in *Semin. Oncol.* **13** (4): Ixiii, 1986). *Semin. Oncol.*, **13**: 291–299, 1986.