Potential Proinvasive or Metastatic Effects of Preclinical Antiangiogenic Therapy Are Prevented by Concurrent Chemotherapy

Marta Paez-Ribes1, Shan Man1, Ping Xu1, and Robert S. Kerbel1,2

Abstract

Purpose: To resolve a controversy involving the therapeutic impact of antiangiogenic drugs and particularly antibodies targeting the VEGF pathway, namely, a body of preclinical mouse therapy studies showing such drugs can promote invasion and/or distant metastasis when used as monotherapies. In contrast, clinical studies have not shown such promalignancy effects. However, most such clinical studies have involved patients also treated with concurrent chemotherapy highlighting the possibility that chemotherapy may prevent any potential promalignancy effect caused by an antiangiogenic drug treatment.

Experimental Design: The impact of antiangiogenic therapy using DC101, an antibody targeting mouse VEGFR-2 with or without concurrent chemotherapy was assessed in multiple human breast cancer xenograft models, where impact on orthotopic primary tumors was evaluated. Metastasis was also assessed during adjuvant and neoadjuvant plus adjuvant therapy, after surgical resection of primary tumors, with the same combination therapies.

Results: Antiangiogenic therapy, while blunting tumor volume growth, was found to increase local invasion in multiple primary tumor models, including a patient-derived xenograft, but this effect was blocked by concurrent chemotherapy. Similarly, the combination of paclitaxel with DC101 caused a marked reduction of micro- or macrometastatic disease in contrast to DC101 monotherapy, which was associated with small increases in metastatic disease.

Conclusions: Conventional wisdom is that targeted biologic antiangiogenic agents such as bevacizumab when used with chemotherapy increase the efficacy of the chemotherapy treatment. Our results suggest the reverse may be true as well—chemotherapy may improve the impact of antiangiogenic drug treatment and, as a result, overall efficacy. Clin Cancer Res; 21(24): 5488–98. ©2015 AACR.

Introduction

A major development in medical oncology practice over the last decade has been the approval of numerous antiangiogenic drugs for the treatment of an extremely broad and diverse range of cancer types. The drugs primarily target the VEGF pathway and include the VEGF antibody bevacizumab, the VEGFR-2 antibody ramucirumab, and the soluble VEGF decoy receptor “trap” drug aflibercept (1–8), in addition to a number of tyrosine kinase inhibitors (TKI), including sunitinib, sorafenib, pazopanib, axitinib, regorafenib, and nintedanib (9–14). In the case of aflibercept, ramucirumab, and bevacizumab, these protein-based drugs are approved in combination with standard chemotherapy regimens, with the one exception of ramucirumab as a second-line monotherapy treatment for refractory gastric cancer (6). Bevacizumab is also used as a maintenance therapy after induction with chemotherapy in advanced ovarian cancer (3). As such, a question that remains under active investigation is the mechanism(s) by which drugs such as bevacizumab or ramucirumab improve the efficacy of chemotherapy. In contrast, there is only one approved combination therapy (in Europe currently) involving an antiangiogenic TKI with standard chemotherapy, namely nintedanib with docetaxel for second-line treatment of non–small cell lung cancer (14). The remaining numerous clinical trials testing such treatment combinations in a spectrum of malignancies, did not result in any marketed approvals (15–17).

Despite the aforementioned clinical successes with antiangiogenic drugs such as bevacizumab or ramucirumab, there are a number of concerns, the foremost of which is the generally modest clinical benefits in progression-free survival (PFS), and overall survival (OS), or lack of an OS benefit after a PFS benefit with these and other antiangiogenic therapies. In this regard, there are many initiatives underway to try and uncover the basis of both innate and acquired resistance to antiangiogenic drugs as an approach to improving their efficacy (18, 19). A second concern, one which may also account for some of the limited benefits of antiangiogenic drug based therapies, is the possibility that such drugs, at least in some circumstances, may promote local tumor invasion and distant metastases after causing an initial suppression of tumor growth, as reported in numerous preclinical studies (20–31). These promalignancy effects could occur as a result of several possible mechanisms; one is that the antiangiogenic treatment, by reducing blood flow and perfusion in tumors,
Translational Relevance

Numerous preclinical mouse studies have shown treatment with an antiangiogenic drug, including the VEGF antibody bevacizumab, or DC101, a VEGFR-2 antibody, may increase tumor invasion and/or metastasis. Such effects have not been observed clinically (with the exception of glioblastoma) in patients receiving bevacizumab or ramucirumab (a VEGFR-2 antibody), but who also received chemotherapy. We therefore undertook preclinical experiments in mice to assess the impact of concurrent chemotherapy in multiple primary tumor or post-surgical adjuvant therapy models of breast cancer. In all instances, any evidence of antiangiogenic drug increases in primary tumor local invasion or distant metastases were prevented by concurrent paclitaxel chemotherapy. The results, which may help resolve the preclinical/clinical discrepancies, also highlight the benefits of reproducing clinical treatment circumstances using appropriate translational preclinical models.

causes increases in tumor hypoxia and thus elevated HIF-1α or HIF-2α expression, leading to downstream induction or upregulation of genes involved in invasion or metastasis (as well as angiogenesis; refs. 22, 32). The increase in invasion may also foster a switch from tumors relying on angiogenesis to “vessel co-option” (33), thus serving as a possible mechanism responsible for resistance developing to the antiangiogenic treatment. One of the controversial aspects of the aforementioned preclinical studies concerns their clinical relevance. Thus, several groups have reported that increases in metastatic disease have not been generally noted in patients treated with antiangiogenic drugs, including bevacizumab (34, 35). However, as we first hypothesized in 2011 (25), a major difference between the preclinical and most clinical studies is that the latter involved patients treated with bevacizumab, ramucirumab, or alfibriccept almost always in combination with concurrent induction chemotherapy. In contrast, the preclinical studies showing antiangiogenic drug promotion of invasion or metastasis have all involved antiangiogenic drug monotherapy. Thus, a possible resolution to this preclinical/clinical discrepancy in results is the possibility that concurrent chemotherapy prevents or blocks the induction of any potential proinvasive or prometastatic effects induced by the antiangiogenic antibody therapy. In short, not only might such antiangiogenic therapy increase the efficacy of chemotherapy but the reverse may be true as well. There is some limited evidence for this concept with respect to distant metastases using sunitinib plus chemotherapy (36). However, there is no clinical approval of sunitinib plus chemotherapy in any cancer indication.

With this background in mind, the main purpose of this study was to evaluate the preclinical impact of antiangiogenic therapy using a VEGFR-2 antibody (DC101) for its potential proinvasive effects on established primary tumors when given alone or when administered with concurrent conventional chemotherapy. We also studied the impact of these therapies as adjuvant treatment on postsurgical development of metastatic disease and also as neoadjuvant therapy followed by adjuvant therapy. Using several models of orthotopic primary human breast cancers in mice, including patient-derived xenografts (PDX), DC101 was found to increase local tumor invasion, whereas this effect was virtually completely prevented by coadministration of a conventional chemotherapeutic drug, for example, paclitaxel or cyclophosphamide. We also found that the combination therapy of DC101 and paclitaxel also prevented the seeding of tumor cells in the lungs when used in the adjuvant setting, preventing metastasis. Taken together, the results raise the prospect of a new paradigm regarding the basis for the improved efficacy of certain targeted antiangiogenic antibody drugs when combined with chemotherapy.

Materials and Methods

Female yellow fluorescent protein (YFP) SCID mice (37) were bred in-house from breeding pairs generously provided by Dr. Janusz Rak (McGill University, Montreal, Quebec, Canada). Mice at 6 to 8 weeks of age were used. Human MDA-MB-231, MDA-MB-435, and MDA-MB-468 established breast cancer cell lines were grown in cell culture as previously described (38). The PDX tumor, HCl-002, was generously provided by Dr. Alana Welm (University of Utah, Salt Lake City, UT) and was propagated in SCID mice by serial passage: tumor tissue pieces 2 to 5 mm³ were implanted in the mammary fat pad of a new animal, as described previously for primary tumor therapy studies (39). Cell line authentication was carried out by genotyping using Illumina mouse linkage panel and confirmed to be human in origin. Routine mycoplasma screening, which is carried out in-house using commercial kits, which confirmed the cell lines were mycoplasma free. Mammary fat pad injections (2 × 10⁶ cells) and tissue fragment implantations were carried out as previously described (38, 39). Caliper measurements were carried out twice a week to determine tumor growth and tumor volume was calculated using the formula \(V = \frac{a \times b^2}{2}\) where \(a\) is the width and \(b\) is the length. Treatments of primary tumors were initiated when average volume was approximately 150 mm³. All mice were randomized just before initiation of treatment. DC101, the rat monoclonal antibody targeting mouse VEGF-2 was generously provided by Eli Lilly and Co. (through Dr Bronek Pytowski). Paclitaxel and cyclophosphamide were dispensed by Pharmacy Department, Odette Cancer Center, Sunnybrook Health Sciences Center (Toronto, Ontario, Canada). Control mice received normal saline. DC101 was administered intraperitoneally at 800 μg/mouse twice a week. Paclitaxel was administered intraperitoneally at 30 mg/kg every 2 weeks. Cyclophosphamide was scheduled as a 21-day cycle of 100 mg/kg, administrated intraperitoneally once every other day over 6 days followed by 2 weeks of rest (40).

Breast tumors were either embedded in Optimal Cutting Temperature (OCT) compound and frozen or fixed in 10% buffered formalin and embedded in paraffin. Detection of invasion was performed in at least 5 to 10 hematoxylin and eosin (H&E)-stained paraffin sections per animal, with a separation of 50 μm each. Antibodies used for specific tissue immunostaining included mouse monoclonal anti-human vimentin (clone 9, 1:100, Invitrogen), mouse monoclonal anti-HLA (1:200, Abcam) goat anti-human CAIX (1:100, R&D Systems), anti-Ki67 (1:1000, Vector), anti-caspase-3 (1:300, Cell Signaling Technology), and anti-ALDH1 (clone 44, 1:100 BD Biosciences). The LSAB + HRP-system from Dako was used as a secondary antibody.
All results were evaluated using the GraphPad Prism 4 software package. Due to the small sample size in each analysis and the fact that not all the data were normally distributed, nonparametric tests were used in each case (Kruskal–Wallis, Mann–Whitney, or χ²(test)). Survival curves were analyzed with the log-rank test. Comparisons were considered statistically significant if \( P < 0.05 \). Error bars represent SD.

**Results**

The addition of DC101 to chemotherapy increases tumor growth inhibition

Several different triple-negative breast cancer cell lines (MDA-MB-231, MDA-MB-468, and MDA-MB-435) and a triple-negative–derived PDX (HCI-002 PDX; ref. 39) were orthotopically implanted in the mammary fat pad of SCID mice. When primary tumors reached a volume of approximately 100 to 150 mm³, the DC101 antibody, paclitaxel or the combination of both drugs were administered over a period of 4 weeks or until tumors in control mice reached 1,700 mm³ (end point tumor volume). As shown in Fig. 1 (and Supplementary Fig. S1A and S1B) a robust growth delay was observed in all tumor models after therapy, especially when treated with the DC101–paclitaxel combination. While a growth delay was observed during therapy in the MDA-MB-231 tumor model (Fig. 1A), a more pronounced inhibition of growth in the PDX HCI-002 tumors was noted after therapy with DC101, or DC101 plus paclitaxel, given for 3 weeks (Fig. 1B). As shown in Fig. 1C and 1D this growth delay was likely the result of the inhibition of angiogenesis in these tumor models, as indicated by reduction of CD31 staining, detecting tumor-associated blood vessels.

![Figure 1](image_url)

**Figure 1.**

Tumor growth inhibition after therapy. A and B, primary tumor growth curves for the MDA-MB-231 established cell-line tumor model (A) and the PDX tumor HCI-002 (B). Tumor cells or tumor pieces were orthotopically implanted in the mammary fat pad of SCID mice. When tumor volumes reached 150 mm³, therapy was started with vehicle, DC101 (800 mg twice a week), paclitaxel (30 mg/kg once every 2 weeks) or the combination of DC101 and paclitaxel. Mice were sacrificed when control mice reached end point. Red arrows show when therapy was started. C and D, tumor vessel quantification for MDA-MB-231 (C) and HCI-002 (D) tumors. The Mann-Whitney test was used for statistics. Error bars, ±SD. In all groups, \( n \geq 6 \).
Treatment with DC101 monotherapy increases primary tumor invasion, which is blocked by concurrent chemotherapy. As summarized in the Introduction, a number of preclinical studies have shown an increase in tumor local invasion, albeit usually accompanied by tumor growth reduction and a subsequent survival benefit, as a result of antiangiogenic therapy (22, 23). To study the impact on local invasion of primary tumors during the different therapies tested, orthotopic primary tumors were fixed in formalin and paraffin embedded to preserve tissue architecture, and local invasion was assessed. As shown in Fig. 2A, a tumor was considered invasive when tumor cells were found to be clearly invading the

Figure 2.
Increase in local invasion after antiangiogenic therapy: blockade by concurrent chemotherapy. MDA-MB-231 tumor cells and the PDX tumor HCI-002 were orthotopically implanted in the mammary fat pad of SCID mice. When tumor volumes reached 150 mm^3, therapy was started with vehicle, DC101 (800 μg twice a week), paclitaxel (30 mg/kg once every 2 weeks), or the combination of DC101 and paclitaxel. Mice were sacrificed when control mice reached end point. A, tumors are considered invasive when tumor cells are encroaching into the adjacent muscular fibers of the abdominal wall. Left, a noninvasive MDA-MB-231 tumor stained for H&E (top) and anti-human vimentin (bottom, cells in brown). Right, invasive tumors stained for H&E (top) and anti-human vimentin (bottom). Ms, muscle cells of the abdominal wall; TC, tumor cells. Scale bars, 150 μm. B and C, the graphs show the percentage of invasive and noninvasive tumors in MDA-MB-231 (B) and PDX HCI-002 (C) breast tumors. The Mann-Whitney test was used for statistics. Error bars, ±SD. In all groups, n ≥ 6. In vivo experiment duplicated.
abdominal wall adjacent to the mammary fat pad. Serial sections of tumors were stained for HLA in the HCl-002 PDX tumor model and for human vimentin in the MDA-MB-231 tumors, to verify and detect human tumor cells encroaching the muscular fibers of the mouse abdominal wall. Detailed histologic analysis clearly showed an increase in local invasion after DC101 therapy in the different tumor models, but this effect was blocked when paclitaxel chemotherapy was administered with DC101 (Fig. 2B and C and Supplementary Fig. S1C and S1D). As shown in Fig. 2B and C, there was a decrease in the number of invasive tumors when mice were treated with paclitaxel compared with untreated controls despite the fact that paclitaxel was actually less efficacious in inhibiting primary tumor growth compared with DC101, as shown in Fig. 1A. This illustrates the divergent effects of the two types of drug treatment on growth versus invasion. Indeed, therapy with DC101 increased tumor invasiveness from 27% to 82% in MDA-MB-231 tumors, and from 37% to 71% in the HCl-002 PDX model. Addition of paclitaxel to DC101 drastically decreased the number of invasive tumors. Thus, no invasive lesions were observed in MDA-MB-231 tumors and only 8% in the HCl-002 tumors. Similar results were observed with the MDA-MB-435 and MDA-MB-468 cell lines (Supplementary Fig. S1C and S1D). In a separate experiment, we treated MDA-MB-231 tumor-bearing mice with cyclophosphamide, DC101, or the combination of both drugs, to determine whether a nontaxane chemotherapy drug had a similar effect in inhibiting local tumor invasion. The results (Supplementary Fig. S2) showed that chemotherapy-mediated inhibition of local tumor invasion was not restricted to paclitaxel.

A recent study using a lower dose of DC101 showed no increase in local invasion of primary tumors after 3 to 4 weeks of therapy in the genetically engineered spontaneous islet cell pancreatic mouse model, RIP-Tag2 (41). The effects of antiangiogenic drugs in modulating local tumor cell invasion may be dose-dependent. We therefore assessed the effects on tumor growth and invasion when using a lower dose of DC101 (reduced by half to 400 µg per injection). As shown in Supplementary Fig. S3, a lower dose of DC101 in the MDA-MB-231 tumor model still had a similar effect on tumor growth inhibition compared with the higher (800 µg) dose (Supplementary Fig. S3A), but when local invasion was assessed a dose-dependent increase in invasive tumors was observed. While the number of invasive tumors was doubled with respect to controls when using the high dose of 800 µg DC101, treatment with 400 µg dose of DC101, increased the amount of invasive tumors by 20% compared with controls. In the HCl-002 primary tumor model, the higher DC101 dose showed better inhibition of primary tumor growth compared with the lower dose (Supplementary Fig. S3B). In this model, although an increase in local tumor invasion was observed when treating with the higher dose of DC101, this was not statistically significant (Fig. 2C), and reducing the dose of DC101 caused similar effects on invasion (Supplementary Fig. S3D).

Antiangiogenic monotherapy increases proliferation, apoptosis, and expression of a stem cell marker, which are reversed by concurrent chemotherapy

Elevated hypoxia caused by antiangiogenic drug treatment has been postulated as a driving force responsible for causing changes that may lead to the increased invasion sometimes observed after such a therapy (20, 22). We therefore assessed a marker of hypoxia in treated tumors by evaluating expression of the hypoxia-inducible protein carbonic anhydrase IX (CAIX). As shown in Fig. 3A after DC101 therapy there was an increase in CAIX levels (4% of positive areas in control tumors vs. 12% in DC101-treated tumors, \( P = 0.037 \)), and this percentage remained the same after the addition of paclitaxel (10% of tumor area was CAIX-positive, \( P = 0.022 \)). This suggests that hypoxia may be a cause for tumors to switch to a more invasive phenotype, but is likely not the only factor, as the combination of DC101 with paclitaxel, although it induced similar changes in CAIX staining to DC101 monotherapy, resulted in decreased local invasion when compared with control-treated tumors.

We also assessed changes in proliferation in treated tumors, as shown in Fig. 3B. Therapy with DC101 monotherapy, unexpectedly, was associated with an increase in the percentage of proliferating cells from 6% in control tumors to 8% after 3 weeks of therapy (\( P = 0.016 \)). When tumors were treated with paclitaxel alone, a decrease in the proliferation rate was noticed, but when paclitaxel was combined with DC101 a more marked decrease in proliferation was observed, from 8% in tumors treated with DC101 to 3% in tumors treated with the drug combination (\( P < 0.001 \)). These results indicate that chemotherapy with a VEGFR-2 antibody can bring a change in the treated tumors that, counterintuitively, can actually lead to increased tumor cell proliferation as previously described (42), but this can be neutralized by concurrent chemotherapy. Interestingly, the number of apoptotic cells and the percentage of necrotic tissue were also increased in the DC101 monotherapy group (\( P \) values for apoptosis = 0.018; necrosis = 0.006, when comparing DC101 and control-treated tumors), which may help explain why DC101-treated tumors were growth inhibited while showing an increase in proliferation rate: the net balance is in favor of cell death (cell loss) over cell growth/renewal. In the paclitaxel-treated group or the combination treatment group, the number of apoptotic cells remained similar to that observed in the control tumors (Fig. 3C). The extent of necrosis was decreased when compared with control-treated tumors in the paclitaxel monotherapy group (\( P = 0.004 \)) or combined with DC101 (\( P = 0.025 \); ref. Fig. 3D).

The antiangiogenic TKI sunitinib has been reported to increase the population of aldehyde dehydrogenase 1 (ALDH1)-positive putative cancer stem cells in breast cancer xenografts (20). We evaluated whether this effect is also observed in our PDX HCl-002 tumor model when mice were treated with DC101 alone, or when treated with DC101 combined with chemotherapy. Paclitaxel alone showed a slight decrease in the percentage of ALDH1-positive cells, from 5% in controls to 3% in paclitaxel-treated tumors. DC101 monotherapy increased the amount of ALDH1-positive cells to 11% (\( P = 0.025 \)), but addition of paclitaxel to the antiangiogenic therapy decreased this number to 6%, similar to levels in control untreated tumors (Fig. 3E).

Taken together, these results show how the addition of chemotherapy to an antiangiogenic drug can reverse the proinvasive effects on local tumor invasion, and inhibit the increase in tumor cell proliferation as well as the ALDH1-positive population apparently promoted after antiangiogenic drug monotherapy.
Impact of DC101 combined with chemotherapy in the adjuvant setting decreased metastasis

As we reported in previous studies, improved therapeutic outcomes, such as increase in survival or inhibition of tumor growth, observed in primary tumor studies, do not necessarily predict or correlate with the efficacy of the same drug or therapy in postsurgical metastatic studies (43, 44). In this regard, the clinical analyses of the impact of antiangiogenic drugs have virtually all involved patients with early- or late-stage metastatic disease (34, 35). We therefore assessed the impact of DC101 plus paclitaxel in an adjuvant treatment model, where therapy was started soon after primary tumors were resected. To do so we used the highly metastatic variant LM2-4, selected in vivo from the commonly used MDA-MB-231 triple-negative human breast cancer cell line (38). At a tumor volume of 500 mm³, mammary fat pad primary tumors were resected, and 5 days later, therapy with vehicle, DC101, paclitaxel, or the combination of both drugs was initiated and continued until endpoint. As shown in Fig. 4A, therapy with paclitaxel or the combination of paclitaxel with DC101, prolonged survival of mice. When mice reached end point, they were sacrificed due to substantial loss of body weight, a toxicity symptom. The lungs were removed and paraffin embedded. Serial sections of lung tissue were stained for human vimentin, to detect micrometastases. This more detailed histopathologic study showed a significant difference between the control and the combination treatment mice (P = 0.0083; Fig. 4C and D), which indicated that the combination therapy used in this adjuvant treatment setting prevented the seeding and growth of tumor cells in the lungs. Mice that presented lung macrometastases at endpoint in the combination group had a lifespan similar to control mice, indicating that in those cases, therapy had no effect on their survival. These results appear consistent with those obtained in several phase III bevacizumab plus chemotherapy adjuvant clinical trials where the combination of bevacizumab and chemotherapy did not improve 3-year disease-free survival (DFS) when compared with chemotherapy, but also did not result in increased metastasis (34, 35). (45, 46). It is also consistent with the preclinical results of Rovida and colleagues (36) who reported that addition of chemotherapy to an antiangiogenic drug (sunitinib) counteracts the increase in metastatic dissemination promoted by sunitinib monotherapy in a kidney tumor model. In interpreting our results, and comparing them to the clinical

having a longer lifespan, the mice showed no sign of lung macrometastases at the time of necropsy. These mice nevertheless had to be sacrificed due to substantial loss of body weight, a toxicity symptom. The lungs were removed and paraffin embedded. Serial sections of lung tissue were stained for human vimentin, to detect micrometastases. This more detailed histopathologic study showed a significant difference between the control and the combination treatment mice (P = 0.0083; Fig. 4C and D), which indicated that the combination therapy used in this adjuvant treatment setting prevented the seeding and growth of tumor cells in the lungs. Mice that presented lung macrometastases at endpoint in the combination group had a lifespan similar to control mice, indicating that in those cases, therapy had no effect on their survival. These results appear consistent with those obtained in several phase III bevacizumab plus chemotherapy adjuvant clinical trials where the combination of bevacizumab and chemotherapy did not improve 3-year disease-free survival (DFS) when compared with chemotherapy, but also did not result in increased metastasis (34, 35). (45, 46). It is also consistent with the preclinical results of Rovida and colleagues (36) who reported that addition of chemotherapy to an antiangiogenic drug (sunitinib) counteracts the increase in metastatic dissemination promoted by sunitinib monotherapy in a kidney tumor model. In interpreting our results, and comparing them to the clinical
Figure 4.
Decrease in metastasis after DC101 and paclitaxel postsurgical adjuvant therapy. Mice were implanted with the metastatic variant LM2-4 and tumors were resected when they reached a volume of 500 mm$^3$. Therapy with DC101 (800 µg twice a week), paclitaxel (30 mg/kg once every 2 weeks) or the combination of DC101 and paclitaxel was started 5 days later. A, Kaplan–Meier survival curve (log-rank test used for statistics). B, percentage of mice with lung macrometastasis when they were sacrificed at endpoint. C, percentage of mice with lung micrometastasis at endpoint. The $\chi^2$ test was used for statistics. D, immunostaining for human vimentin in the lungs in a control (left) and DC101 and paclitaxel-treated mouse (right). Five serial sections for each lung, with a 50-µm separation in between, were stained to do the quantification. Scale bars, 150 µm. In all groups, n ≥ 7.
adjuvant trial studies, it should be noted that there was a clear trend of a benefit in patients receiving bevacizumab plus chemotherapy over chemotherapy plus placebo, but only while patients were on therapy and for a period after therapy was terminated; afterwards, this benefit was lost over time (35, 45, 46).

Impact of DC101 and chemotherapy in the neoadjuvant setting

In an attempt to minimize the toxic effects of the combination of DC101 and paclitaxel, we designed an experiment where we examined the effects of treatment in the neoadjuvant plus adjuvant setting. We implanted cells of the LM2-4 metastatic MDA-MB-231 variant into female SCID mice. When tumor volumes reached 150 mm³, mice were treated with saline, DC101 or the combination of DC101 and paclitaxel for 10 days and primary tumors were then resected. Five days after tumor resection, therapy combining paclitaxel and DC101 was restarted in all mice and maintained for 4 weeks. We wished to determine whether the combination of paclitaxel + DC101 administered as a neoadjuvant therapy, would improve the rate of macro and micrometastasis detected after adjuvant therapy. To our knowledge this type of preclinical experiment has not been undertaken previously, despite the protocol duplicating a common clinical treatment scenario (47–49). The experiment was also designed to reduce the level of toxicity observed in the adjuvant experiment shown in Fig. 4, as in this case we treated the mice after surgery for only 4 weeks.

Figure 5.
Neoadjuvant + adjuvant therapy with DC101 and paclitaxel increased survival and reduced toxicity. A, mice were implanted with the metastatic variant LM2-4; therapy was started when tumor volume reached 150 mm³ and maintained for 10 days (DC101 (800 mg twice a week) or the combination of DC101 and paclitaxel (30 mg/kg every other week)). Tumors were resected when they reached 500 mm³ and 5 days later therapy with DC101 and paclitaxel was started in all mice for 4 weeks. A, The Kaplan-Meier survival curve showing overall survival of mice after receiving neoadjuvant + adjuvant therapy. The log-rank test used for statistics. B, percentage of mice with lung macrometastasis when they were sacrificed at endpoint. The χ² test was used for statistics. In all groups, n ≥ 8.

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antiangiogenic drug was administered as monotherapy, an effect that was reversed/prevented as a result of concurrent chemotherapy.

Discussion
The results we have summarized herein provide a potential resolution, at least in part, to a fundamental discrepancy regarding the impact of antiangiogenic drugs on various aspects of tumor growth and progression in preclinical mouse therapy models on the one hand, with the results obtained in clinical trials on the other. This discrepancy concerns what now amounts to a large number of preclinical studies showing increases in local invasion of primary tumors, or exacerbation of distant metastatic disease during/after antiangiogenic therapy in mice (see Introduction), mostly involving treatment of mice with established primary tumors, in contrast to a lack of any such promalignancy effects observed in patients receiving antiangiogenic therapy (34, 35). However, as summarized in the Introduction, a major problem in trying to compare the existing published preclinical results with those obtained in patients is the fact that almost all of the clinical analyses undertaken involved patients who not only received an antiangiogenic drug, for example, bevacizumab, but also concurrent treatment with another therapeutic modality, which in the main has been standard-of-care cytotoxic chemotherapy (34, 35). We therefore undertook a series of experiments involving the impact of an antiangiogenic drug that targets the VEGF pathway, namely, DC101 (which blocks the function of VEGF-R2) either alone or when combined with a conventional chemotherapy regimen, using paclitaxel, in mice, in multiple breast cancer models in three different treatment circumstances. The first involves treatment of a number of different orthotopic human breast cancer xenograft models, either using cells from established cell lines, or tissue fragments of PDXs where the various therapies were assessed for their impact on local tumor invasiveness. The second model involved an analysis of the impact on metastatic disease progression using adjuvant treatments that were undertaken shortly after surgical resection of primary established tumors, and at a time when only microscopic early-stage disease would be present based on previous studies (21). The third model involved a combination of a targeted antiangiogenic agent with standard chemotherapy regimens involving neoadjuvant therapy prior to resection of primary tumors, followed by subsequent adjuvant therapy treatment, using the same drugs, a circumstance that has seldom (if at all) been modeled preclinically to the best of our knowledge.

The results of all three of these different cancer model settings, would indicate similar findings or trends, namely, that an antiangiogenic monotherapy can indeed have the potential to promote local tumor invasion (of established primary orthotopic tumors) and possibly also distant metastatic disease. In the case of the primary tumor therapy experiments, a robust antitumor effect was also caused by the treatment, in terms of growth delay. However, in all cases the use of concurrent chemotherapy not only blocked these undesirable effects on malignant tumor progression, but could also increase overall efficacy in some circumstances. Most definitive and clearest in this regard were the results obtained in the first model involving treatment of established primary orthotopic breast tumors. Thus, we found consistent morphologic evidence of increases in the local invasiveness of orthotopic primary breast tumors as a result of treatment with the antiangiogenic drug, DC101. However, in all cases we also found that this proinvasive effect was eliminated as a result of concurrent chemotherapy, in this case using paclitaxel. A similar blockade or prevention effect on metastasis was noted in the adjuvant and neoadjuvant/adjuvant therapy models.

Some comments about the results of our adjuvant model studies, and the extent to which they correlated with phase III clinical trial outcomes are in order. For example, we found that the DC101/paclitaxel combination caused a significant reduction in both microscopic and macroscopic metastases, which would seem at variance with phase III clinical trial results. Those trials were considered negative, that is, addition of bevacizumab to chemotherapy for the induction therapy, followed by prolonged (e.g., one year) maintenance therapy with bevacizumab did not result in an improvement in DFS at 3 years (35, 45, 46). However, a DFS benefit in favor of the combination treatment arm over chemotherapy alone was observed during therapy and for a period of time after termination of the maintenance phase of therapy. We continued therapy until endpoint in our studies and there was no maintenance phase of DC101 monotherapy. Moreover, there was no bevacizumab induction monotherapy arm in any of the phase III clinical trials. With these caveats in mind, our adjuvant results would appear to indicate the following findings: (i) DC101 monotherapy did not result in a decrease in metastasis—if anything, there was a trend to a small increase in both microscopic and macroscopic metastatic disease compared with untreated controls; (ii) the addition of DC101 to paclitaxel resulted in a reduction of both types of metastases compared with paclitaxel treatment alone; (iii) this reduction was associated with a prolongation of median survival (we did not assess ‘DFS’) from 73 days in the paclitaxel group to 82 days in the paclitaxel plus DC101 combination treatment group.

One limitation or weakness in our results concerns the experimental design of the neoadjuvant plus adjuvant therapy protocols shown in Fig. 5. Specifically, it would be informative including groups involving neoadjuvant paclitaxel alone, or paclitaxel plus DC101 as neoadjuvant therapy control (followed by surgery and no further therapy), to rule out the benefit of the paclitaxel + DC101 neoadjuvant therapy followed by the same combination therapy as adjuvant therapy, post-surgery, was not simply due to longer duration of the paclitaxel treatment. Previous studies we published indicated the paclitaxel protocol we used does not have any impact on primary tumor growth (43). Thus, it did not seem to us that there was a compelling rationale to use it as a neoadjuvant therapy control. Also, the primary purpose of undertaking the neoadjuvant + adjuvant experiment was to sustain the reduction in metastasis formation observed in the adjuvant therapy experiment. For both reasons, we decided not to include a group where only neoadjuvant therapy was administered followed by surgical resection of the treated tumor.

Taken together, there are several important translational implications of our results. First, they challenge conventional wisdom regarding the mechanism(s) by which the combination of a targeted antiangiogenic agent with standard chemotherapy causes increased overall efficacy. It is intuitive that if a targeted agent on its own has little or no discernible efficacy, in contrast to chemotherapy, that the overall increase in efficacy of the combination would be "unidirectional", that is, the targeted agent increases chemotherapy efficacy, not the reverse. However, our results, and those of Rovida and colleagues (36) suggest the benefits of the targeted agent, in our case an antiangiogenic VEGF pathway.
inhibiting antibody, can be improved by concurrent chemothera-
papy. In short, the benefits are mutual, not unidirectional. As such,
the results could have implications with other antibody-targeted
agents that are normally administered upfront as part of a com-
bination with chemotherapy, for example, EGF receptor or Her-2–
targeting antibodies such as cetuximab or trastuzumab. Similar to
bevacizumab, such agents are not used as standalone therapies
with the exception as maintenance after upfront/induction in
combination with chemotherapy (50).

Disclosure of Potential Conflicts of Interest

R.S. Kerbel reports receiving speakers bureau honoraria from Boehringer
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Angiocrine Biosciences, Cerulean Pharma, Eli Lilly (Ramlacimab Global
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Authors’ Contributions

Conception and design: M. Paz-Ribes, R.S. Kerbel
Development of methodology: M. Paz-Ribes, S. Man, R.S. Kerbel
Acquisition of data (provided animals, acquired and managed patients,
provided facilities, etc.): M. Paz-Ribes, S. Man, P. Xu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics,
computational analysis): M. Paz-Ribes, R.S. Kerbel

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Writing, review, and/or revision of the manuscript: M. Paz-Ribes, R.S. Kerbel
Administrative, technical, or material support (i.e., reporting or organizing
data, constructing databases): M. Paz-Ribes, S. Man, P. Xu

Study supervision: R.S. Kerbel

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Marta Paez-Ribes, Shan Man, Ping Xu, et al.


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