Cancer Therapeutics Insights

Chemotherapy Counteracts Metastatic Dissemination Induced by Antiangiogenic Treatment in Mice

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Abstract

The development of resistance and progressive disease after treatment with angiogenesis inhibitors is becoming a controversial issue. We investigated the experimental conditions that cause multireceptor tyrosine kinase inhibitors (RTKI) to augment metastasis and whether opportune combinations with chemotherapy could counteract this effect. The renal Renca-luc tumor was transplanted orthotopically in the kidney of Balb/c mice, which then were or were not nephrectomized. The Lewis Lung carcinoma (LLC) was transplanted in the tibial muscle of C57/B6 mice. Treatment with the RTKI sunitinib started at different stages of tumor progression, mimicking neoadjuvant or adjuvant settings. Combination studies with paclitaxel, doxorubicin, cisplatin, gemcitabine, and topotecan were done on the LLC model, using opportune regimens. In a neoadjuvant setting, sunitinib inhibited Renca-luc tumor growth, prolonging survival despite an increase in lung metastasis; treatment after primary tumor surgery (adjuvant setting) or on established metastasis prolonged survival and decreased metastasis. Sunitinib increased lung metastasis from mice bearing early-stage LLC, but did not affect established metastases (no acceleration) from advanced tumors. Combinations with doxorubicin, topotecan, gemcitabine, but not cisplatin and paclitaxel, counteracted the increase in metastasis from LLC, partly reflecting their antitumor activity. Histology analysis after sunitinib confirmed tumor vascular changes and increased hypoxia. Topotecan at suboptimal daily doses reduced sunitinib-related metastasis, reducing tumor hypoxia. Tyrosine kinase inhibitors, as sunitinib, can have adverse malignant effects mainly in the neoadjuvant setting. The addition of chemotherapy might influence metastasis, depending on each drug mechanism of action and its regimen of administration. Mol Cancer Ther; 12(10); 2237–47. ©2013 AACR.

Introduction

Angiogenesis is required for tumor growth, invasion, and metastatic dissemination, hence the strong rationale for an antiangiogenic therapy. Numerous angiogenesis-targeting agents have been admitted to the ranks of cancer therapeutics (1); most of them are used in polytherapy regimens (2). The most validated antiangiogenic strategy targets the VEGF axis. VEGF can be blocked directly, as with the antibody bevacizumab (Avastin), or indirectly by inhibiting the receptor activity with small molecules such as multiple tyrosine kinase receptor inhibitors (RTKI).

Among these, sunitinib (Sutent), sorafenib (Nexavar), and pazopanib (Votrient) have been approved by the U.S. Food and Drug Administration for a number of malignancies (3). The approval of inhibitors of angiogenesis has been limited because after an initial prolongation of progression-free survival and improved patient response rates, they do not always translate into better overall survival, thus casting doubt on the overall efficacy of antiangiogenic therapy (4–6). Resistance to antiangiogenic therapy has also been reported both in preclinical and clinical studies often associated with the activation of alternative proangiogenic pathways (7, 8).

The progression of a growing tumor to distant metastases involves a number of steps. There is the loss of cell-to-cell adhesion, increased motility/invasion, intravasation in the bloodstream, extravasation, and homing in a different site. All require permissive angiogenesis, which is also vital for the survival and proliferation of micrometastases (9–12). The majority of preclinical studies have focused on the effect of antiangiogenic therapy on primary tumor growth, with less attention to metastasis. The results are poor and controversial. In some studies, antiangiogenic therapy was extremely effective on the primary tumor and metastasis, improving survival (13–15). However, surprisingly, recent studies reported that treatment of tumor-bearing mice,
mainly with anti-VEGF/VEGFR-related compounds, increased tumor invasiveness and metastasis (16–22). Clinical data on the effect of antiangiogenesis in general, or anti-VEGF therapy, on malignant progression are lacking and widely debated. With certain tumors, such as glioblastoma, there was an increase in the volume of infiltrative tumor after bevacizumab, or a switch to more infiltrating growth after cediranib (a pan-VEGF-RTKI; refs. 23, 24). A retrospective analysis of five placebo-controlled phase III clinical trials in patients with breast, colorectal, renal, and pancreatic cancer did not support worse clinical outcome or altered disease progression after cessation of bevacizumab (25). Results that sunitinib did not accelerate tumor growth in patients with metastatic renal cell carcinoma have been recently published (26).

Treatment with bevacizumab generally consists of combinations with standard-of-care chemotherapy, which might help reduce the unwanted effects of the angiogenesis inhibitors (2). Unlike bevacizumab, the RTKI have antitumor activity in monotherapy, but there appears to be no survival benefit from the combination with conventional chemotherapy (27).

In this study, we hypothesized that chemotherapy combined with angiogenesis inhibitors might influence tumor dissemination and metastasis. We therefore investigated the effect of the RTKI, sunitinib, alone or with chemotherapy on relevant murine metastatic tumor models. While sunitinib alone, in the neoadjuvant setting, increased metastasis, the cytotoxic chemotherapy combination counteracted this unwanted tumor dissemination and overcame the resistance to angiogenesis inhibitors.

**Materials and Methods**

**Animals**

Six- to 8-week-old female Balb/c and C57/Bl6 mice were obtained from Harlan Laboratories and maintained under specific pathogen-free conditions.

Procedures involving animals and their care were conducted in conformity with institutional guidelines that comply with National (Legislative Decree 116 of January 27, 1992, Authorization n.19/2008-A issued March 6, 2008, by the Italian Ministry of Health) and international laws and policies (EEC Council Directive 86/609, Of L 358, 1, December 12, 1987; standards for the Care and Use of Laboratory Animals, United States National Research Council, Statement of Compliance A5023-01, October 28, 2008), and in line with Guidelines for the welfare and use of animals in cancer research (28).

**Tumor lines**

The renal adenocarcinoma cell line Renca was purchased from American Type Culture Collection (ATCC) and used within 6 months after receipt; the Renca-Luc variant was obtained by infecting Renca cells with lentiviral vector carrying the coding sequence of synthetic firefly luciferase gene, luc2 (*Photinus pyralis*; ref. 22). Tumor cells were maintained in RPMI-1640 (Voden Medical Instrument), supplemented with 10% fetal calf serum, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 2 mmol/L L-glutamine (Lonza Sales). Stocks of the cell line were stored frozen in liquid nitrogen and kept in culture for no more than 3 to 4 weeks before injection in mice.

The Lewis Lung carcinoma cell line (LLC), obtained from DTP, DCTD Tumor Repository (www.dtp.nci.nih.gov), was used within 6 months after receipt, stocked frozen as *in vivo* derived tumor fragments and maintained subcutaneously in C57/BL6 mice for no more than two generations before testing.

**Renca-luc metastatic tumor model**

For spontaneous metastasis studies, Renca-luc cell suspensions were inoculated orthotopically (5 × 10⁵) under the right renal capsule of BALB/c mice, as previously described (29), and the primary tumor was surgically removed (nephrectomy) or not. Artificial metastases were obtained by injecting Renca-luc cells (1 × 10⁵) into the tail vein (30).

*In vivo* bioluminescence imaging (BLI) was used to confirm the presence of tumors, to randomize mice to start therapy or for nephrectomy, and to follow tumor growth and metastatic progression (22). Results are plotted as photon count (PC). When specified, *ex vivo* BLI of the lung was used to quantify metastatic spread. See Supplementary Methods for details.

Metastases in the lung were evaluated at the end of treatment (day 19, interim), or when primary tumors reached the same size (PC = 2 × 10⁵, ~1 g).

For survival studies, mice were constantly monitored and killed at the first signs of discomfort (the day of death being considered the limit of survival). Results are plotted as the percentage survival against days after tumor transplant. The increment of lifespan (ILS) was calculated as [median survival day of treated group – median survival day of control group] × 100.

**Lewis lung metastatic tumor model**

Tumor cells (5 × 10⁵) from enzymatically digested subcutaneous tumors were injected into the right tibial muscle of C57/Bl6, as described (30). Primary tumor growth was measured with a digital caliper three times a week and tumor volume (mm³) was calculated as: [length (mm) × width² (mm²)]/2.

Animal management and data collection were carried out with the help of the Study Director 1.8 software (Studylog System, Inc.). Results were plotted as the mean tumor volume against days after transplantation. Efficacy of the treatment was expressed as best tumor growth inhibition [%T/C = (median weight of treated tumors/median weight of control tumors) × 100].

Animals were euthanized at specific points after treatment started (same day, interim), or when mean primary tumor volume was ≤2,000 mm³ (different days, terminal). Primary tumors were fixed in formalin and embedded in paraffin (FFPE), or frozen on liquid nitrogen, for further analysis. Lungs were excised and fixed in Bouin’s...
solution (Bio-Optica) and superficial metastatic nodules were counted and measured using a dissecting microscope, as described in the Supplementary Methods (31).

**Drugs and treatments**

Sunitinib (Chemietek; 30 mg/kg) and sorafenib (Chemietek; 40 mg/kg) were dissolved in methocell 0.5% and administered daily orally. Both compounds were used at or near clinical doses (32). Paclitaxel (Indena S.p.A., 20 mg/kg, i.v.) was dissolved in 50% Cremophor EL (Sigma-Aldrich) and 50% ethanol and further diluted with saline before use. Cisplatin (Sigma-Aldrich; 5 mg/kg, i.v.) was dissolved in 0.9% NaCl. Gemcitabine (Eli Lilly and Company; 100 mg/kg, i.p.) was diluted in sterile water. Doxorubicin (Nerviano Medical Science; 6 mg/kg i.v.) was dissolved in saline. Topotecan (NCI/DTP Repositories, 10 mg/kg, 1 mg/kg or 0.2 mg/kg, i.p.) was dissolved in PBS. The cytotoxic compounds were administered every 4 days for three cycles (Q4 × 3), except for 1 and 0.2 mg/kg topotecan, which were given daily for 10 days.

**Histopathological examination**

Primary tumors and lungs from euthanized mice were FFPE for histologic analysis. Micrometastases in the lung were evaluated by hematoxylin–eosin, as specified in the Supplementary Methods. Microvessel density and maturation in the primary tumor (CD31 and CD31/JC–2 staining), and intratumor hypoxia were evaluated by immunohistochemistry as described in the Supplementary Methods.

**Western blotting**

For immunoblotting, total proteins were extracted from frozen tumor samples. Blots were probed with goat anti HIF-1α antibody, 1:300 (R&D System) or mouse anti β-tubulin antibody (1:1000, Sigma Aldrich). See Supplementary Methods for details.

**Statistical analyses**

Statistical analyses were done using Prism Software (GraphPad Prism 6 Software). Differences in tumor growth were evaluated by two-way ANOVA followed by Bonferroni’s post-test. Differences in survival were analyzed by the log-rank test. The unpaired Student’s t test or the nonparametric Mann–Whitney test was used to compare two groups. For more than two groups, one-way ANOVA followed by Bonferroni’s post-test or the nonparametric Kruskal–Wallis test followed by Dunn’s post-test was used. P < 0.05 was considered significant.

**Results**

**Sunitinib in the neoadjuvant setting increases pulmonary metastases from the renal orthotopic model Renca-luc**

We tested the effect of sunitinib on tumor metastasis in an orthotopic model of murine renal carcinoma, as the drug is currently clinical used for this kind of tumor. Mice bearing Renca-luc tumors were randomized by their photon count (PC) to receive either sunitinib or vehicle (Fig. 1A and B, neoadjuvant setting). Sunitinib delayed primary tumor growth (Fig. 1A, left) and significantly increased survival (ILS 37%, Fig. 1A, right). Mice killed at the same primary tumor burden (PC = 2 × 106, ~1 g, Fig. 1B, left), had significantly more lung metastases with sunitinib than with vehicle (Fig. 1B, right). It is worth noting that at the end of treatment (day 19, interim), at a smaller primary tumor by sunitinib treatment did not correspond less metastases, compared with vehicle (Supplementary Fig. S1). These findings indicate that the increase in metastases is not dependent on the effect of sunitinib on the primary tumor. To determine whether the sunitinib-related increase also affected established metastases, in a second experiment, the primary tumor was surgically removed before treatment (i.e. adjuvant setting; Fig. 1C). Two days after removal of the primary tumor, sunitinib treatment started and metastasis progression was followed. As shown in Fig. 1C (left), sunitinib did not influence metastasis progression, and improved overall survival, though not significantly (ILS 50%, Fig. 1C, right).

To confirm that sunitinib had no adverse effect on implanted metastases, mice were injected intravenously with Renca-luc cells and sunitinib treatment started as soon as lung micrometastases were detectable by BLI (Fig. 1D, left and middle). Sunitinib significantly reduced metastatic growth in the lung, with consequent prolongation of survival (ILS 19%, Fig. 1D, right). At death, all mice presented a similar metastatic burden (number of nodules >300, not shown). Pretreatment with sunitinib for 1 week before intravenous injection of Renca-luc cells did not affect metastatic growth in the lung or survival (data not shown).

**The increase in metastases due to sunitinib depends on tumor stage**

We used the murine LLC model, which spontaneously metastasizes to the lung, to confirm the above findings and to study the effects of sunitinib on metastasis formation at different tumor stages. Mice bearing LLC were treated with sunitinib, starting when tumors were palpable (Fig. 2A), but no metastasis to the lung was detectable by histology (Fig. 2A, inset) or when tumors were more advanced (Fig. 2B) and metastases were already established (Fig. 2B, inset). When started at an early stage, the drug had a moderate antitumor effect (T/C 77%; Fig. 2A, left), but it significantly increased the number of lung metastases compared with controls (median 123 and 53, respectively; Fig. 2A, middle), counted at a comparable primary tumor dimension (different days, terminal). Sunitinib increased the number of medium and large nodules (diameter > 1 mm), whereas small metastases (diameter < 1 mm) were not affected by the treatment. A similar increase was observed when metastases were evaluated at the end of treatment (same day, interim) despite sunitinib-treated

![Image](https://www.aacrjournals.org/molcanther/article-pdf/12/10/2239/4541968/mct-13-0244.pdf)
mice had a smaller primary tumor (Supplementary Fig. S2A and S2B), again suggesting that the increase in metastasis was not directly related to the size of primary tumor.

At a more advanced tumor stage, with established lung metastases, sunitinib had a similarly modest effect on primary tumor growth (T/C 72%; Fig. 2B, left), but there was no significant difference in the number of metastases (Fig. 2B, middle), with a trend, not significant, in more small nodules (Fig. 2B, right). Similar results were obtained with sorafenib, another RTKI with a similar target receptor profile (Supplementary Fig. S2).

These findings show for both models that sunitinib augments metastasis in neoadjuvant-like setting and has no adverse effect on established metastases, suggesting that the increase in metastasis is related to the presence of the primary tumor, and this set the basis for our further experiments.
The combination with chemotherapy curbed the increase in metastasis driven by sunitinib

Angiogenesis inhibitors are generally used in combination with chemotherapy, which ultimately might limit or mask the negative effect of these drugs on metastasis formation.

We investigated this possibility in experiments combining sunitinib and chemotherapy drugs with different mechanisms of action and efficacy on the LLC model: paclitaxel, cisplatin, doxorubicin, gemcitabine and topotecan. The antitumor and antimetastatic activities of these treatments are summarized in Fig. 3 and detailed in Supplementary Table S1. Sunitinib caused a significant increase in metastases to the lung, with a modest effect on the growth of the primary tumor. The effect of the combination followed three patterns: (i) Drugs (i.e., paclitaxel and cisplatin) which as single agents only minimally affected metastasis and primary tumor growth (T/C 76% and 83%), in combination with sunitinib moderately improved antitumor activity but did not counteract the augmentation of metastasis by sunitinib (Fig. 3A and D); (ii) doxorubicin, modestly active on primary tumor growth (T/C 53%), was significantly active on metastasis formation; in combination with sunitinib, its antitumor activity increased and it prevented the sunitinib-driven metastasis augmentation (Fig. 3B and D); (iii) drugs like gemcitabine and topotecan, which are highly active on the primary tumor (T/C 5% and 18%) and on metastasis as single agents, when combined with sunitinib did not further increase the antitumor activity, but counteracted the augmentation of metastasis (Fig. 3C and D).

Sunitinib reduced tumor vasculature, improved pericyte coverage, and increased intratumor hypoxia in LLC

To elucidate the vascular alterations within the primary tumor that may enhance tumor invasiveness (33), microvessel density, area, and maturation were measured in tumor samples collected after 7 days of treatment with sunitinib and from time-matched controls (interim). Tumor vessel density and dimension were significantly lower in sunitinib than vehicle-treated animals. As expected, the
percentage of double-positive CD31/α-SMA vessels was higher in sunitinib-treated tumors, an indicator of mature vessels (Fig. 4A and C). The reduction of vessel area induced by sunitinib was accompanied by a significantly larger percentage of hypoxic areas in tumors treated with sunitinib than in time-matched controls (Fig. 4B and C).

Metronomic low doses of topotecan counteracted the metastatic spread elicited by sunitinib, acting on tumor hypoxia

The findings set out above illustrate the possibility, reported in several studies, that under certain experimental conditions, angiogenesis inhibitors can promote a metastatic phenotype, partly by creating an increasingly hypoxic tumor microenvironment. We investigated the effect on metastasis formation of sunitinib with topotecan administered daily at suboptimal doses, previously reported to affect the hypoxic tumor microenvironment (34, 35).

The combination of topotecan at the metronomic dose of 1 mg/kg (TPT-1) with sunitinib gave significantly better antitumor activity than the single agents alone (T/C 65%, 32%, 3% for sunitinib, TPT-1, and Sun-TPT-1) and reduced the number of metastases, even below those of untreated mice (Fig 5A–C and Supplementary Table S2). The decrease in metastases was observed at the terminal analysis, therefore at a later time, due to the slowing of the primary tumor growth, once again suggesting that the effect on metastasis is independent from the size of the primary tumor and persisted after treatment suspension. Interestingly, topotecan at the metronomic dose of 0.2 mg/kg (TPT-0.2), had almost no effect on tumor growth.

Figure 3. Antitumor and antimetastatic activity on LLC of sunitinib in combination with different chemotherapeutic agents. LLC cells were injected in the tibial muscle of C57BL6 mice, which were randomized on day 7 (8–10/group, palpable tumors) to receive either vehicle, sunitinib, or one of the chemotherapeutic agents alone or in combination: A, paclitaxel (PTX, 20 mg/kg) and cisplatin (DDP, 5 mg/kg); B, doxorubicin (Doxo, 6 mg/kg); C, gemcitabine (Gem, 100 mg/kg) and topotecan (TPT, 10 mg/kg). All drugs were given Q4×3, singly (▲) or with sunitinib (Sun, 30 mg/kg, ▼), daily for 12 days. A to C, antitumor activity of chemotherapy together with sunitinib (mean tumor volume ± SEM); D, number of metastatic nodules at scheduled death (i.e. primary tumor volume of 2,000 mm³). For vehicle and sunitinib groups, values in the figure are representative of three independent experiments (▲, P < 0.05; ▼, P < 0.01; ▲▲▲, P < 0.001 vs. vehicle, ▼▼▼, P < 0.001 vs. sunitinib). Statistical analysis was done on the corresponding vehicle and sunitinib groups.
(Fig. 5B), but combined with sunitinib it reduced metastases too, and significantly counteracted their increase due to sunitinib (Fig. 5C and Supplementary Table S2).

To examine the mechanism by which topotecan contrasted sunitinib-induced metastatic spread, we analyzed intratumor hypoxia by pimonidazole staining after metronomic TPT-1 treatment (Fig. 5D, top). TPT-1 alone or with sunitinib significantly reduced intratumor hypoxia compared with the sunitinib and vehicle groups. Thus, hypoxia-induced HIF1-α protein accumulation in sunitinib-treated tumors was lowered by the combination with TPT-1 (Fig. 5D, bottom). Intratumor hypoxia and HIF1-α protein levels were not significantly affected in the TPT-0.2-treated tumors, probably because of the slight, not experimentally appreciable, effect of this low dose (data not shown).

Discussion

The discovery of VEGF as a major regulator of endothelial cell growth and survival paved the way for translating these concepts into clinical practice, by targeting the VEGF signaling pathway as a way to block angiogenesis and inhibit tumor growth (1). However, the overall survival benefits of those antiangiogenic drugs have been modest: often the response to therapy is short lasting, and patients become refractory or escape treatment (8, 36). Recent preclinical studies have even shown increased invasion and metastasis in mouse tumor models treated with certain angiogenesis inhibitors (16–22). Less clear is whether the resistance/escape to therapy using angiogenesis inhibitors, with accelerated disease progression or increased mortality, also happens in patients. Noteworthy, preclinical studies were obtained with single-agent treatment, although most clinical trials with angiogenesis inhibitors are in patients already treated with- or added to- chemotherapy, which could well be responsible for the final outcome.

In this study, first we reexamined the experimental conditions in which small-molecule RTKI (e.g., sunitinib) boost metastasis, and we found that sunitinib promoted metastasis only in a neoadjuvant setting. We also confirmed that sunitinib can suppress metastatic growth, leading to an overall survival benefit for mice. Then, in the LLC model, we assessed whether chemotherapy could counteract the proinvasive/prometastatic effects of sunitinib, and found that these effects were chemotherapysensitive and dose schedule-dependent. It is known that...
the host plays a role in the response/resistance to anti-angiogenic therapy, but also to chemotherapy (37). Therefore, we chose to work with murine tumor models that allow us to study metastatic spread in immunocompetent hosts. Similar results were obtained with immunodeficient nude mice bearing LLC and treated with sunitinib (data not shown).

When used as a single agent, sunitinib increased spontaneous metastasis to the lung in two murine metastatic models, the Renca renal carcinoma and the LLC. Increased invasion and accelerated metastasis with inhibitors of tumor angiogenesis, including sunitinib, were originally shown by Ebos and colleagues and Paez-Ribes and colleagues (16, 17). More recent studies have reported that the adverse effects of angiogenesis inhibitors on metastasis are dose-, drug class-, and tumor model–dependent (18, 19, 22).

Sunitinib is approved for the treatment of metastatic renal carcinoma (38). However, we found that in Renca model transplanted orthotopically in the kidney of mice, sunitinib, at clinically relevant doses (32) promoted metastasis in a tumor stage–dependent fashion; it had negative effects on metastasis dissemination only in the neoadjuvant setting when metastases were not yet implanted at the beginning of treatment. Conversely, in the adjuvant setting, when mice had been nephrectomized and metastases were established, sunitinib did not exacerbate metastatic progression. This is in contrast with previous reports of increased metastasis formation after surgery of the primary tumor (16, 19). However, those studies used sunitinib at high doses for a short period (a schedule not reflecting clinical practice), recently reported to be the only conditions in which metastasis is facilitated (19, 39).

Interestingly, while leading to an increase in metastasis, sunitinib per se inhibited the growth of the primary tumor, mirrored by increased mouse survival. Sunitinib can extend progression free survival and overall survival in patients with metastatic renal carcinoma (38). Therefore, we believe that in this model and at these experimental conditions, the survival gain is due to sunitinib’s strong effect on the primary tumor and on established metastases, hiding the negative influence on metastasis formation. In light of the above observation, our findings might explain why an increase in metastasis has never been observed in the clinical practice and sunitinib therapy is beneficial for patients with metastatic renal cell carcinoma. In fact, patients do not receive sunitinib in the neoadjuvant setting, but it is often given later after the removal of the primary tumor (26, 38).
Contrary to previous reports in which treatment with sunitinib before intravenous injection of tumor cells led to a greater metastatic take \( (16, 19) \), in our experimental conditions, we did not find any conditioning effect to promote the growth of metastasis. Rather, as recently reported \( (39) \), we can confirm that sunitinib significantly affects the growth of Renca established lung nodules (experimental metastasis). Our results are important, given patients with metastatic RCC continuously treated with these drugs.

The LLC model enabled us to strengthen the results, showing that sunitinib (and sorafenib), which per se only moderately affected primary tumor growth, caused a dramatic and reproducible increment of lung metastases; again this was true only when metastases were not yet implanted at the start of treatment, and not when they were fully established (late-stage treatment), thus mimicking an adjuvant setting. The increase in metastasis is evident independently of the day of scheduled death (different times but same tumor size) or the size of the tumor (end of treatment, different tumor size).

Sunitinib thus seems to exert its main effect on the initial escape of metastatic cells from the primary tumor, providing a possible explanation of the discrepancy between preclinical data (augmentation of metastasis) and clinical observation (in general no worsen outcome; ref. 26). Whether augmentation of metastasis was a consequence of the escape from the sunitinib-treated primary tumor, one would expect an increased number of micrometastases in the lung. Surprisingly, the distribution of small metastases was not influenced by the treatment, ruling out an antiangiogenic effect at the secondary site. The second question is whether concomitant chemotherapy in some tumor types and with certain drug indications modifies or overcomes this aggressive metastatic behavior. The best combination regimens of antiangiogenic compounds and conventional chemotherapy or novel biologicals must be optimized to obtain better therapeutic responses \( (2) \). It is worth noting that no change in disease progression patterns was observed in a retrospective analysis of a number of clinical trials, with several thousand patients treated with bevacizumab plus chemotherapy \( (25) \).

Having set the appropriate experimental conditions, we found that an opportune combination therapy can curb sunitinib’s adverse effects on spontaneous metastasis from the LLC. However, different cytotoxic drugs affected metastasis to different degrees, partially reflecting their intrinsic antitumor activity. Only the combination of sunitinib with gemcitabine or topotecan (but not paclitaxel and cisplatin) reduced the metastatic burden compared with untreated controls, and there was a strong effect on the primary tumor as well. This prompts us to hypothesize that the combination’s final outcome might be due to the antitumor effect of the chemotherapy. With doxorubicin, however, there was strong abrogation of the sunitinib-augmented metastasis, but only a moderate effect on the primary tumor. Cytotoxic drugs have different activity on the primary tumor and metastatic sites and this was particularly evident for doxorubicin \( (40, 41) \). However, it is very possible that the outcome of chemotherapy is influenced by the tumor microenvironment, such as vascular cells that can be susceptible to angiogenesis inhibitors in combination with chemotherapy \( (42) \). Furthermore, the host immune system can influence the response to chemotherapy at multiple levels and ultimately influence metastasis \( (31, 43) \). Sunitinib is rarely used in combination with chemotherapy in clinical practice, as no clear benefit has been reported combining multitargeted antiangiogenic RTKI with chemotherapy \( (27, 44) \). The limited advantage we observed combining sunitinib with chemotherapy raises the question whether these RTKI differ for example from the anti-VEGF antibody bevacizumab in their antiangiogenic actions or in their ability to facilitate chemotherapy drug delivery and activity. Mechanism-based combination therapies aimed at impeding the “bad” consequences of antiangiogenic therapy and improving the overall therapeutic response are needed \( (45) \).

Several mechanisms have been proposed to explain the increase in metastasis by angiogenesis inhibitors observed in preclinical studies. They relate to the acquisition of a more malignant tumor phenotype, or to changes of the tumor vasculature, or to abnormal recruitment of proinflammatory cells and production of cytokines \( (4) \). Hypoxia is a key mediator of tumor progression, contributing to poor patient survival \( (46) \). After anti-VEGF-based treatment, tumor hypoxia increases, and HIF-1α is activated, so the tumor responds by adapting or evading it \( (46, 47) \). Pharmacodynamic analyses on sunitinib-treated LLC did in fact show an impairment of vascularization with an increase in intratumor hypoxia and upregulation of HIF-1α. Rapisarda and colleagues reported that topotecan, at low daily doses (metronomic regimen), inhibited the accumulation of the α subunit of HIF-1 by a mechanism independent of DNA replication-mediated DNA damage \( (34) \), then confirmed in a multi-histology target-driven pilot trial on tumor biopsies from patients receiving topotecan \( (48) \).

We found that (i) metronomic topotecan counteracted the sunitinib-increased metastasis more than a standard dose; (ii) even at the suboptimal dose of 0.2 mg/kg, which does not affect tumor growth, topotecan contained the increment of metastasis; (iii) metronomic topotecan added to sunitinib-reduced intratumor hypoxia and inhibited HIF-1α protein accumulation. Increased activity of bevacizumab in combination with daily low doses of topotecan was reported to be associated with inhibition of HIF-1α transcription \( (35) \); however, tumor invasiveness was not evaluated in that study. Although these findings suffer from the limited specificity of topotecan as an HIF-1α-targeting drug, we feel the effect of metronomic topotecan on metastasis is hypoxia-mediated rather than a consequence of a direct effect on tumor cells, because suboptimal dose of topotecan \( (0.2 \text{ mg/kg}) \) counteracted sunitinib-due increase in metastases without affecting primary tumor growth. The conclusion that in our model, hypoxia...
in the primary tumor is what accelerates metastases, and appropriate drugs in combination regimens can impair this, is in line with two recent reports pointed to HIF-1α as a major mediator of metastasis dissemination induced by antiangiogenic treatment (20, 21).

Our preclinical findings indicate that the combination of RTKI with chemotherapy can influence metastasis and this might depend on the mechanism of action of the drug and its regimen. Our study used only two murine tumors and few angiogenesis inhibitors, so extrapolation to humans and other angiogenesis inhibitors calls for caution. Recently, different outcomes in the promotion of metastasis have been shown, depending on the angiogenesis inhibitor, but also on the preclinical tumor model (19).

Angiogenesis therapies targeting VEGF/VEGFR pathways are giving some benefits to patients with cancer. However, the often modest effects might depend not only on the lack of response of the tumor, but also on our inability to use those drugs appropriately. Combining antiangiogenic compounds with other treatment modalities is a reasonable strategy for the best therapeutic results. Selecting the right combination and the best administration regimen could help avoid unwanted effects and potentiate the final outcome.

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

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Conception and design: A. Rovida, R. Giavazzi, M. Cesca
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References
Chemotherapy Counteracts Angiogenic Metastasis


Correction: Chemotherapy Counteracts Metastatic Dissemination Induced by Antiangiogenic Treatment in Mice

In this article (Mol Cancer Ther 2013;12:2237–47), which appeared in the October 2013 issue of Molecular Cancer Therapeutics (1), the authors regret that in some instances the term “neoadjuvant” is used incorrectly. The term “neoadjuvant” refers to treatment prior to surgical resection of a primary tumor. In this case, however, the term “neoadjuvant” is used to describe experiments where treatment is given to animals with primary tumors, and then the experiment is terminated.

In the interest of clarification, the authors note that the intent, as specified in the Discussion section, was to distinguish between treatment in the presence of a primary tumor and the likely absence of established metastases (mimicking a neoadjuvant setting) from postsurgical treatment without a primary tumor, but eventually with established metastases (adjuvant). Accordingly, in some instances they use the descriptions “mimicking neoadjuvant,” and a “neoadjuvant-like setting.”

Reference


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