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 multikinase receptor 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 were then or not nephrectomized. The Lewis Lung carcinoma (LLC) was transplanted in the tibial muscle of C57/Bl6 mice. Treatment with the RTKI sunitinib started at different stages of tumor progression, mimicking neo-adjuvant or adjuvant settings. Combination studies with paclitaxel, doxorubicin, cisplatin, gemcitabine and topotecan were done on the LLC model, using opportune regimens.

In a neo-adjuvant 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.

Tyrosin kinase inhibitors, as sunitinib, can have adverse malignant effects mainly in the neo-adjuvant setting. The addition of chemotherapy might influence metastasis, depending on each drug mechanism of action and its regimen of administration.
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 vascular endothelial growth factor (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 (PFS) and improved patient response rates, they do not always translated into better overall survival (OS), 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, 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) (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 shave 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 neo-adjuvant 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 eight-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, OJ 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) (22). Tumor cells were maintained in RPMI 1640 (Voden Medical Instrument), supplemented with 10% FCS, 0.1 mM NEAA, 1 mM sodium pyruvate and 2 mM L-glutamine (Lonza Sales). Stocks of the cell line were stored frozen in liquid nitrogen, and kept in culture for no more than 3-4 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 (5x10^5) into 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 (1x10^5) 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.
Metastasis in the lung were evaluated at the end of treatment (day 19, interim), or when primary tumor reached the same size (PC = 2x10^5, approx. 1g).

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) / median survival day of control group] x 100.

**Lewis lung metastatic tumor model**

Tumor cells (5x10^4) 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 (mm^3) was calculated as: (length [mm] × width^2 [mm^2])/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 ≤2000 mm^3 (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 Buin’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 (PTX, Indena S.p.A., 20 mg/kg, i.v.) was dissolved in 50% CremophorEL (Sigma-Aldrich) and 50% ethanol and further diluted with saline before use. Cisplatin (DDP, Sigma-Aldrich; 5 mg/kg, i.v.) was dissolved in 0.9% NaCl. Gemcitabine (GEM, Eli Lilly and Company; 100 mg/kg, i.p.) was diluted in sterile water. Doxorubicin (Doxo, Nerviano Medical Science; 6 mg/kg i.v.) was dissolved in saline. Topotecan (TPT, 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 four days for three cycles (Q4x3), except for 1-0.2 mg/kg topotecan, which were given daily for ten days.
**Histopathological examination**

Primary tumors and lungs from euthanized mice were FFPE for histological 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/α-SMA double staining), and intra-tumor 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 non-parametric 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 neo-adjuvant 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 (Figure 1A, B, neoadjuvant setting). Sunitinib delayed primary tumor growth (Figure 1A, left) and significantly increased survival (ILS 37%, Figure 1A, right). Mice killed at the same primary tumor burden (PC = 2x10^5, approx. 1g, Figure 1B, left), had significantly more lung metastases with sunitinib than with vehicle (Figure 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 to vehicle (Supplementary Figure 1). These findings indicate that sunitinib-due increase in metastases is not dependent on the effect 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), (Figure 1C). Two days after removal of the primary tumor sunitinib treatment started and metastasis progression was followed. As shown in Figure 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 i.v. with Renca-luc cells and sunitinib treatment started as soon as lung micro-metastases were detectable by BLI (Figure 1D, left and middle). Sunitinib significantly reduced metastatic growth in the lung, with consequent prolongation of survival (ILS 19%, Figure 1D, right). At death all mice presented a similar metastatic burden (number of nodules >300, not shown). Pre-treatment with sunitinib for one week before i.v. 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 Lewis Lung carcinoma (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 (Figure 2A), but no metastasis to the lung was detectable by histology (Figure 2A, inset) or when tumors were more advanced (Figure 2B) and metastases were already established (Figure 2B, inset). When started at an early stage the drug had a moderate antitumor effect (T/C 77%, Figure 2A, left), but it significantly increased the number of lung metastases compared to controls (median
123 and 53 respectively, Figure 2A, middle), counted at a comparable primary tumor dimension (different days, terminal). Sunitinib increased the number of medium and large nodules (diameter > 1mm), while small metastases (diameter < 1mm) 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 mice had a smaller primary tumor (Supplementary Figure 2A, B), 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%, Figure 2B, left), but there was no significant difference in the number of metastases (Figure 2B, middle), with a trend, not significant, in more small nodules. (Figure 2B, right). Similar results were obtained with sorafenib, another RTKI with a similar target receptor profile (Supplementary Figure 2).

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 (PTX), cisplatin (DDP), doxorubicin (Doxo), gemcitabine (Gem) and topotecan (TPT). The antitumor and anti-metastatic activities of these treatments are summarized in Figure 3 and detailed in Supplementary Table 1.

Sunitinib caused a significant increase in metastases to the lung, with a modest effect on the growth of the primary tumor. These effects followed three patterns: 1) 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 (Figure 3A, D). 2) 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 (Figure 3B, D). 3) 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 (Figure 3C, D).
Sunitinib reduced tumor vasculature, improved pericyte coverage and increased intra-tumor 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 seven 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 (Figure 4A, 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 (Figure 4B, 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 TPT 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 5, A-C and Supplementary Table 2). 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, TPT at the metronomic dose of 0.2 mg/kg (TPT 0.2), had almost no effect on tumor growth (Figure 5B), but combined with sunitinib it reduced metastases too, and significantly counteracted their increase due to sunitinib (Figure 5C and Supplementary Table 2).

To examine the mechanism by which topotecan contrasted sunitinib-induced metastatic spread, we analyzed intra-tumor hypoxia by pimonidazole staining after metronomic TPT 1 treatment (Figure 5D, top). TPT 1 alone or with sunitinib significantly reduced intra-tumor hypoxia compared to the sunitinib and vehicle groups. Thus hypoxia-induced HIF1-α protein accumulation in sunitinib-treated tumors was lowered by the combination with TPT 1 (Figure 5D, bottom). Intra-tumor 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 the 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 re-examined the experimental conditions in which small-molecule RTKI (e.g. sunitinib) boost metastasis, and we found that sunitinib promoted metastasis only in a neo-adjuvant 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 pro-invasive/pro-metastatic effects of sunitinib, and found that these effects were chemotherapy-sensitive and dose-schedule-dependent. It is known that the host response plays a role in the response/resistance to antiangiogenic 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 Lewis Lung carcinoma. Increased invasion and accelerated metastasis with inhibitors of tumor angiogenesis, including sunitinib, were originally shown by Ebos et al. and Paez-Ribes et al. (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 neo-adjuvant setting when metastases are 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 metastatic renal carcinoma patients (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 have never been observed in the clinical practice and sunitinib therapy is beneficial for patients with mRCC. In fact, patients do not receive sunitinib in the neo-adjuvant 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 prior to 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 the adjuvant studies in patients with metastatic RCC continuously treated with these drugs.

The Lewis Lung 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) (26). Whether augmentation of metastasis was a consequence of the escape from the sunitinib-treated primary tumor, one would expect an increased number of micro-metastases 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 to 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 clinical 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 pro-inflammatory 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 intra-tumor hypoxia and upregulation of HIF-1α. Melillo and coworkers 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 intra-tumor 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 sub-optimal dose of topotecan (0.2 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 cancer patients. 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.
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Figure Legends

**Figure 1: Effect of sunitinib on metastatic Renca-luc tumors**

**(A-B) Orthotopic injection, neoadjuvant setting.** Renca-luc cells were injected under the renal capsule (i.k.) in Balb/c mice and on day 5 tumor take was assessed by bioluminescence (BLI) and animals were randomized by photon count (PC) to treatment (T↑) with either vehicle (n=20) or sunitinib (n=20, 30 mg/kg) for 12 days. Primary tumor growth and metastatic burden were followed by BLI and expressed as PC. **(A)** Effect of sunitinib on the lifespan of mice (10/group). Left: primary tumor growth (PC) along the test period (mean ± s.e.m., **p<0.01, ***p<0.001). Right: effect of sunitinib on survival (**p<0.001). **(B)** Animals (10/group) were killed with a median primary tumor of 2x10^5 PC (tumor weight ~1g) on different days (median vehicle 19 (16-26); sunitinib 32 (19-34), and lung metastases were assessed by BLI (mean ± s.e.m., *p<0.05). Left: primary tumor (PC); right: lung metastatic burden (PC) at scheduled death (mean ± s.e.m., *p<0.05).

**(C) Orthotopic injection, adjuvant setting.** Renca-luc cells were injected i.k. in Balb/c mice, which were nephrectomized (primary tumor resection) on day 6, one day after the BLI assessment of tumor take and lung metastases implantation. Two days after surgery (day 8) mice (10/group) were treated (T↑) with either vehicle or sunitinib (30 mg/kg) for 12 days; metastatic spread was followed by BLI. Left: effect of sunitinib on the metastatic spread (PC) in mice after surgery (mean ± s.e.m.); right: effect of sunitinib on lifespan (**p<0.001).

**(D) Intravenous injection, artificial metastases.** Renca-luc cells were injected into the tail vein (i.v.) of Balb/c mice, which were treated (T↑) with either vehicle or sunitinib (30 mg/kg) until scheduled death, starting from day 7 after tumor injection (10/group). From left to right: effect of sunitinib on metastatic spread along the test period, followed by BLI (PC), (mean ± s.e.m., ***p<0.001); one representative mouse of the vehicle and sunitinib-treated groups imaged on day 17; effect of sunitinib on lifespan ( ***p<0.001).

**Figure 2: Effect of sunitinib on metastatic Lewis Lung carcinoma**

LLC cells were injected in the tibial muscle of C57/BL6 mice, which then received vehicle or sunitinib (30 mg/kg, ↑, 10/group) for 12 days starting on day 7 (A), when tumors were palpable (60-80 mm³) and there were no lung metastases (A, inset), or from day 11 (B), when the mean tumor volume was 300 mm³ and lung metastases were established (B, inset). **A-B,** left: antitumor activity of sunitinib (mean tumor volume ± s.e.m, ** p<0.01, ***p<0.001); the inset reports the metastatic foci (histological score) at the beginning of treatment. A-B, middle: total number of metastatic...
nODULES AT DEATH (I.E. PRIMARY TUMOR VOLUME 2000 MM³) (** P<0.001). A-B, RIGHT: NUMBER OF METASTATIC NODULES DIVIDED PER SIZE, *P<0.05, ** P<0.01.

**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 C57/BL6 mice, which were randomized on day 7 (8-10/group, palpable tumors) to receive either vehicle, or one of the chemotherapeutic agents (▲): (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 Q4x3, singly or with sunitinib (30 mg/kg, †, daily for 12 days). (A-B-C) Antitumor activity of chemotherapy together with sunitinib (mean tumor volume ± s.e.m); (D) Number of metastatic nodules at scheduled death (i.e. primary tumor volume of 2000 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).

**Figure 4: Pharmacodynamic analyses on LLC from sunitinib treated and time-matched control tumors**

LLC tumor-bearing mice were treated as in Figure 2A for 7 days, and vessel morphology was analyzed (5/group). (A) From left to right: vessel number per field and vessel area, by CD31 immunostaining; vessel maturation index by CD31/α-SMA double staining (% α-SMA/CD31 double-positive vessels/total vessels). (B) Percentage intra-tumor hypoxia area (pimonidazole adduct) per field; (mean ± s.e.m.*P<0.05,**P<0.01). (C) Representative images of vessel morphology; from left to right: CD31, α-SMA/CD31 (scale bar 100 µm), pimonidazole (scale bar 400 µm).

**Figure 5: Antitumor and antimetastatic activity of sunitinib in combination with metronomic topotecan on LLC**

LLC cells were injected in the tibial muscle of C57/BL6 mice, which were treated daily with sunitinib (30 mg/kg, p.o.†), starting from day 7 (palpable tumors), alone or in combination with metronomic, low-dose, topotecan (TPT 1, 1 mg/kg or TPT 0.2, 0.2 mg/kg, ▲, 8-10/group), daily for 10 days. (A, B) Antitumor activity of sunitinib in combination with TPT 1 (A) or TPT 0.2 (B) (mean tumor volume ± s.e.m); (C) Number of lung metastases at scheduled death (i.e. primary tumor volume of 2000 mm³). (***P<0.001 vs. vehicle, ^P<0.05, ^^^P<0.001 vs sunitinib). (D) Top:
percentage intra-tumor hypoxic area (pimonidazole adduct) per field as from (A), (mean ± s.e.m., 5/group, *p<0.05, ***p<0.001 vs. vehicle, ^^^p<0.001 vs. sunitinib); bottom: Western Blot analysis for HIF-1α, on parallel tissue samples, (2 mice/group).
Figure 1

A. Orthotopic neoadjuvant

B. Orthotopic neoadjuvant

C. Orthotopic adjuvant

D. Artificial metastasis

Graphs showing the effects of Sunitinib and Vehicle on primary tumor and lung metastases over time in different experimental conditions.
Figure 2

A

Tumor volume (mm³)

Lung metastases (score)

Vehicle

Sunitinib

day 7

Lung metastases (no.)

Vehicle

Sunitinib

Lung metastases (no. per size)

Vehicle

Sunitinib

B

Tumor volume (mm³)

Lung metastases (score)

Vehicle

Sunitinib

day 7

Lung metastases (no.)

Vehicle

Sunitinib

Lung metastases (no. per size)

Vehicle

Sunitinib
Figure 3

A

Tumor volume (mm³)

Vehicle
Sunitinib
PTX
Sun+PTX

Days

B

Tumor volume (mm³)

Vehicle
Sunitinib
DDP
Sun+DDP

Days

C

Tumor volume (mm³)

Vehicle
Sunitinib
Gem
Sun+Gem

Days

D

Lung metastases (no.)

Vehicle
Sunitinib
PTX
Sun+PTX
DDP
Sun+DDP
Doxo
Sun+Doxo
Gem
Sun+Gem
TPT
Sun+TPT

Legend:

* p < 0.05
** p < 0.01
*** p < 0.001

Note: The images depict the effect of different treatments on tumor volume and lung metastases over time.
Figure 5

A

Tumor volume (mm$^3$)

0 1000 2000 3000

0 10 20 30 40 Days

- Vehicle
- Sunitinib
- TPT 1
- Sun+TPT 1

B

Tumor volume (mm$^3$)

0 1000 2000 3000

0 10 20 30 40 Days

- Vehicle
- Sunitinib
- TPT 0.2
- Sun+TPT 0.2

C

Lung metastases (no.)

0 50 100 150

- Vehicle
- Sunitinib
- TPT 1
- TPT 0.2
- Sun+TPT 1

D

% Hypoxic area

0 1 2 3 4 5

- Vehicle
- Sunitinib
- TPT 1
- Sun+TPT 1

HIF-1α

β-Tubulin
Chemotherapy counteracts metastatic dissemination induced by antiangiogenic treatment in mice

Alessandra Rovida, Vittoria Castiglioni, Alessandra Decio, et al.

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