Bevacizumab-Induced Inhibition of Angiogenesis Promotes a More Homogeneous Intratumoral Distribution of Paclitaxel, Improving the Antitumor Response

Marta Cesca1, Lavinia Morosi1, Alexander Berndt2, Ilaria Fusco Nerini1, Roberta Frapolli1, Petra Richter2, Alessandra Decio1, Olaf Dirsch2, Edoardo Micotti3, Silvia Giordano4, Maurizio D’Incalci1, Enrico Davoli4, Massimo Zucchetti1, and Raffaella Giavazzi1

Abstract

The antitumor activity of angiogenesis inhibitors is reinforced in combination with chemotherapy. It is debated whether this potentiation is related to a better drug delivery to the tumor due to the antiangiogenic effects on tumor vessel phenotype and functionality. We addressed this question by combining bevacizumab with paclitaxel on A2780-1A9 ovarian carcinoma and HT-29 colon carcinoma transplanted ectopically in the subcutis of nude mice and on A2780-1A9 and IGROV1 ovarian carcinoma transplanted orthotopically in the bursa of the mouse ovary. Paclitaxel concentrations together with its distribution by MALDI mass spectrometry imaging (MALDI MSI) were measured to determine the drug in different areas of the tumor, which was immunostained to depict vessel morphology and tumor proliferation. Bevacizumab modified the vessel bed, assessed by CD31 staining and dynamic contrast enhanced MRI (DCE-MRI), and potentiated the antitumor activity of paclitaxel in all the models. Although tumor paclitaxel concentrations were lower after bevacizumab, the drug distributed more homogeneously, particularly in vascularized, non-necrotic areas, and was cleared more slowly than controls. This happened specifically in tumor tissue, as there was no change in paclitaxel pharmacokinetics or drug distribution in normal tissues. In addition, the drug concentration and distribution were not influenced by the site of tumor growth, as A2780-1A9 and IGROV1 growing in the ovary gave results similar to the tumor growing subcutaneously. We suggest that the changes in the tumor microenvironment architecture induced by bevacizumab, together with the better distribution of paclitaxel, may explain the significant antitumor potentiation by the combination.

Introduction

Angiogenesis-targeting agents are currently used in cancer treatment (1, 2). Targeting the VEGF–VEGFR signaling axis with ligand-binding drugs (i.e., with the antibody bevacizumab) or with low-molecular-weight VEGF receptor tyrosin kinase inhibitors (TKi) shows efficacy in different tumor types, though the survival benefit associated to these class of drugs is modest. In contrast with the TKi, which are in general given as single-agent therapy, bevacizumab, in most settings, requires the addition of cytotoxic therapy (2, 3). It has particularly beneficial effect when combined with chemotherapy, prolonging overall survival (OS) in patients with metastatic colorectal cancer (4), recurrent/advanced non–small cell lung cancer (NSCLC; ref. 5), and—more recently—with advanced cervical cancer (6). Bevacizumab, combined with chemotherapy, and in a maintenance regimen, extended progression-free survival in patients with advanced ovarian cancer (7, 8).

The mechanism by which antiangiogenic agents can boost the efficacy of chemotherapy is not completely understood, as these drugs can act on different tumor compartments, not necessarily linked (9–12). Several hypotheses have been proposed. Antiangiogenic drugs mainly have an antivascular effect, so that abnormal, inefficient vessels are destroyed and the remainder are remodeled toward a more “mature” or “normal” phenotype (13), which in turn can lead to better delivery of therapeutics. Additional mechanisms are related to their action as chemosensitizing agents by inhibiting chemotherapy-induced mobilization and tumor “homing” of proangiogenic bone marrow–derived circulating endothelial progenitors (14), or to chemotherapy-enhanced damage to endothelial cells when prosurvival signals of VEGF are blocked (15, 16). We showed induction of tumor necrosis by a vascular disrupting agent and increased proliferation in the remaining viable tumor tissue where chemotherapy may be more active (17).

In terms of drug delivery, giving chemotherapy after an antiangiogenic agent should not sound rational, since when the tumor vasculature is modified or even disrupted the delivery of...
cytotoxic drugs may be impaired and the response to therapy cannot improve. Normalization of the tumor vasculature (18, 19) offers a solution to this apparent paradox. An appropriate dose of antiangiogenic agents would transiently normalize the tumor vasculature, leading to better blood flow and lower interstitial fluid pressure (IFP), permitting an increase in drug delivery, thus improving the outcome of the therapy (13, 19, 20). Numerous preclinical studies, including ours, have shown the effect of antiangiogenic therapy on vessel morphology and function. However, experimental evidence of an enhanced antitumor effect of the combination due to better tumor drug uptake is scant and debated (21–23). There are few clinical studies reporting the effect of antiangiogenic therapy on tumor drug concentrations. Bevacizumab had avascular and normalizing action in patients with rectal carcinoma, indirectly indicating that blood vessels were more efficient after bevacizumab (24). On the other hand, it has also been reported that bevacizumab did not improve tumor drug delivery, and in fact had the opposite effect (25).

The scarcity of data on this issue prompted this study in which we investigated the relationship between the pharmacologic effects of bevacizumab and the tumor penetration of paclitaxel, combined. Using conventional analytic techniques based on liquid chromatography (LC) or LC coupled to mass spectrometry (LC/MS), which gives information on the total drug concentration inside the tumor, together with matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry imaging (MALDI MSI), which indicates the drug distribution in various compartments of the tissue (26, 27), we examined the intratumor distribution of paclitaxel, which is probably a key factor in the antitumor effect of the combination.

Our findings indicate that the greater antitumor activity of paclitaxel after bevacizumab is not necessarily due to overall increased drug delivery, but to the restoration of a more functional tumor microenvironment architecture that facilitates distribution of the cytotoxic drug in more vital, actively proliferating areas.

Materials and Methods

Drug preparation and administration

Bevacizumab (Avastin; Roche S.p.A.) was diluted in saline before use, and injected i.p. at the dose of 150 μg/mouse twice a week. For antitumor activity paclitaxel (Indena S.p.A.) was dissolved in 50% Cremophor EL (Sigma) and 50% ethanol, further diluted with saline immediately before use, and injected i.v. at a dose of 20 mg/kg weekly for three courses (Q7d), or 60 mg/kg (single bolus), as specified in the Results. Doxorubicin (Nerviano Medical Science) was dissolved in water and injected i.v. at the dose of 6 mg/kg Q7d × 3.

For pharmacokinetics and MALDI MSI, mice were given single bolus injections of chemotherapy, as detailed below. Control mice received the vehicles.

Animals

Six- to 8-week-old female NCr-nu/nu mice were obtained from Harlan Laboratories. They were maintained under specific pathogen-free conditions, housed in isolated vented cages, and handled using aseptic procedures. Procedures involving animals and their care were conducted in conformity with institutional guidelines that comply with national (Legislative Decree 26, March, 2014) and international (EEC Council Directive 2010/63, August, 2013) laws and policies, in line with guidelines for the welfare and use of animals in cancer research (28). Animal studies were approved by the Mario Negri Institute Animal Care and Use Committee and by Italian Ministerial decree no. 84-2013.

Tumor cell lines

The A2780-derived-1A9 (A2780-1A9) human ovarian cancer cell line (29) was kindly provided by Tito Fojo in 1994 (NCI-NIH) and the IGROV1 human ovarian cancer cell line (30) was obtained from the National Cancer Institute Tumor Repository in 1995. 1A9-luc and IGROV1-luc are their variants infected with lentiviral vector carrying the coding sequence of synthetic firefly luciferase gene luc2 (Photinus pyralis, refs. 31, 32). The HT-29 colon cancer line was obtained from the ATCC in the 1980s (33). Authentication of the cell lines was performed by comparing their DNA short tandem repeat profile (AmpFISTR identifier Plus PCR Amplification kit, Applied Biosystems) with a DNA fingerprinting database. Cell cultures were maintained in RPMI-1640 (BioWest) supplemented with 10% FBS (Euroclone). Master stocks of the cell lines were stored frozen in liquid nitrogen, new ampoules thawed at need and cultures maintained for no more than 6 weeks before use.

Xenograft tumor models and antitumor activity

Ectopic models. A2780-1A9 human ovarian carcinoma (10 × 10⁶ cells), or HT-29 human colon carcinoma (4 × 10⁶ cells), were implanted s.c. in the flank of nude mice (34, 35). Tumor growth was measured with a digital caliper two/three times a week and tumor volume (mm³) was calculated as [length (mm) × width² (mm²)]/2. We used the Study Director 2.1 software (Studylog System, Inc.) for animal management and data collection.

Results were plotted as relative tumor volume (RTV), calculated with the formula Vt/V₀, where Vt is the tumor volume on any day of measurement and V₀ is the tumor volume at the beginning of treatment. For A2780-1A9 the efficacy of the treatment was expressed as best tumor growth inhibition [%T/C = (median volume of treated tumors/median volume of control tumors)] × 100], or tumor log cell kill (LCK), with the formula GD/(3.32 ln 10), where GD (growth delay) is the difference in days required for the treated tumors to reach a target size (1,500 mm³) compared with the control group, and DT is the tumor doubling time. The combinations were considered additive, synergistic or antagonistic if the LCK (LCKobs) was, respectively, equal to, larger, or smaller than the expected LCK (LCKexp), calculated as the sum of the LCK of each single therapy. Animals were euthanized when mean primary tumor volume was ≤2,000 mm³.

Orthotopic models. A2780-1A9 (1A9-luc variant) and IGROV1-luc human ovarian carcinoma cells (1 × 10⁶) expressing the firefly luciferase gene luc-2 were injected orthotopically under the bursa of the mouse ovary, as previously reported (31, 32). Bioluminescence imaging (BLI) was used to confirm the presence of tumor in the ovary, to randomize mice at the beginning of treatment and to follow tumor progression as described in refs. (31, 32). Briefly, D-luciferin (150 mg/kg, i.p., Caliper Lifescience)–injected mice were scanned after 10 minutes with eXplore Optix MX2 (ART, Advanced Research Technologies Inc.) and images analyzed with Optiview software (ART, Advanced Research Technologies Inc.). Photon counts were indicative of tumor burden. For antitumor activity mice were killed at the first signs of discomfort (the day of death being considered the limit of survival) and results plotted as the percentage survival against days after tumor.
transplant. The increment of lifespan (ILS) was calculated as 100 × [(median survival day of treated group – median survival day of control group)/median survival day of control group].

Pharmacokinetic studies

Tumor-bearing mice (4/5 per time point) were given paclitaxel (20 or 60 mg/kg, day 0) or doxorubicin (6 mg/kg, day 0), alone or after bevacizumab (two injections, days −5 and −1). Biologic specimens were collected 0.25, 2, 6, 8, 24, 48, and 72 hours after 20 mg/kg paclitaxel or 6 hours after paclitaxel in the 60 mg/kg study, or 0.08, 0.25, 0.5, 1, 3, 6, 24, 48, 72 hours after doxorubicin. Blood was taken from the retro-orbital plexus under isoflurane anesthesia and collected in heparinized tubes. Animals were killed and tumors and liver were removed and immediately frozen at −80°C until analysis of the drug concentration. The plasma fraction was immediately separated by centrifugation (4,000 rpm, 15 minutes, 4°C) and stored at −20°C until analysis. Paclitaxel levels were determined by high-performance liquid chromatography (HPLC) with UV detection, modified for measurements in plasma and tissues (34). The total concentration of doxorubicin was determined by HPLC coupled to fluorescence detection (λex 475 nm; λem 550 nm) according to ref. 36.

Figure 1.

Antitumor activity and tumor uptake of chemotherapy in combination with bevacizumab in A2780-1A9 subcutaneous tumor-bearing mice. A, mice received bevacizumab (Bev, ▲, 150 μg/mouse, i.p., twice a week, from day −5, median tumor volume 150 mm³), alone or in combination with paclitaxel. Mice were randomized to start paclitaxel (PTX, ▲, 20 mg/kg, i.v) 24 hours after the second injection of bevacizumab (day 0) with a median tumor volume of 400 mm³. This schedule was repeated for 3 weeks, giving chemotherapy on days 0, 7, and 14 (Q7 × 3). Values are RTV (mean ± SEM, 10/group; ***, P < 0.0001 vs. chemotherapy alone). B, paclitaxel concentration (PTX, 20 mg/kg i.v., day 0) in tumors (left), livers and plasma (right), at 0.25, 2, 6, 8, 24, 48, and 72 hours, in mice pretreated with bevacizumab (Bev, 150 μg/mouse) or vehicle on days −5 and −1 (mean ± SEM, 4/group; ***, P < 0.01; ***, P < 0.0001). C, mice received bevacizumab (Bev, ▲, 150 μg/mouse, i.p., twice a week, from day −5, median tumor volume 150 mm³), alone or in combination with doxorubicin. Mice were randomized to start doxorubicin (Doxo, ▲, 6 mg/kg, i.v.) 24 hours after the second injection of bevacizumab (day 0) with a median tumor volume of 400 mm³. This schedule was repeated for 3 weeks, giving chemotherapy on days 0, 7, and 14 (Q7 × 3). Values are RTV (mean ± SEM, 10/group; ***, P < 0.0001 vs. chemotherapy alone). D, doxorubicin concentration (Doxo, 6 mg/kg i.v., day 0) in tumors (left), livers and plasma (right), at 0.08, 0.25, 0.5, 1, 3, 6, 24, 48, and 72 hours, in mice pretreated with bevacizumab or vehicle on days −5 and −1 (mean ± SEM, 4/group; ***, P < 0.05; ***, P < 0.001). E, DCE-MRI in tumor-bearing mice 24 hours after vehicle or bevacizumab (Bev, 150 μg/mouse, i.p., days −5 and −1). Left, tumor IAUC 5 is the initial AUC in the tumor region calculated from the first 5 minutes of the DCE-MRI signal time course (box plot, 6/group; *, P < 0.01). Right, representative images of the contrast agent enhancement superimposed over anatomical MRI images of tumors from a vehicle and a bevacizumab-treated mouse. F, vessel analysis from tumor harvested 24 hours after receiving vehicle or bevacizumab (Bev, 150 μg/mouse, i.p., days −5 and −1). From left to right, diameter, area, and number of vessels measured after immunostaining for CD31 (mean ± SEM, 5/group; ***, P < 0.001; ***, P < 0.0001); right, representative images of A2780-1A9 tumors stained for CD31 (scale bar, 200 μm).
Pharmacokinetic parameters were calculated using Phoenix WinNonlin 6.3 software (Pharsight).

MALDI mass spectrometry imaging analysis
Mice bearing different tumor models (12/group), pretreated or not with bevacizumab (two injections, days −5 and −1) were given 60 mg/kg of paclitaxel (day 0). After 6 hours, that is close to Cmax, tumors from five mice per group were collected, divided into two, and frozen in liquid nitrogen. The remaining 7 mice were followed for antitumor activity. For imaging analysis, half frozen tumor tissue was cut into 10-μm-thick sections (three from each tumor) at −20°C using a cryo-microtome (Leica Microsystems) and mounted on pre-cooled MALDI plates (Opti-TOF 384 Well insert) by standard thaw-mounting techniques. For each section, three adjacent slices (7-μm-thick) were cut for immunohistochemical analysis. Paclitaxel MALDI imaging analysis was done with a MALDI 4800 TOF-TOF (AB SCIEX Old Connecticut Path), as described (27), with details in Supplementary Methods. The distribution of the paclitaxel ion signal in the normalized images was analyzed by the software ImageJ (imagej.nih.gov/ji) as specified in Supplementary Methods. The other half of the frozen tumor tissue was analyzed for total paclitaxel content by HPLC, as described above.

Immunohistochemistry
To assess vessel density and size (CD31, MEC13.3 antibody, Beckton Dickinson GmbH), tumor proliferation (Ki-67, SP6 antibody, Thermo Fisher Scientific GmbH) and necrosis after treatments, tumors were excised, embedded in optimal cutting compound, snap-frozen, and stored at −80°C until immunohistochemical analysis (34). Tumor slices adjacent to MALDI images were analyzed for CD31 and Ki-67 to overlay vessel distribution/morphology and tumor cell proliferation/necrosis with MALDI-imaged paclitaxel distribution. Detailed procedures are described in Supplementary Methods. For documentation and comparison with MALDI MSI images, whole slides were scanned with the Hamamatsu NanoZoomer 2.0HT and processed with NDP.view2 Software (Hamamatsu Photonics). Image analyses were performed with computer-aided image analysis software (Axiovision Rel. 4.8.2; Zeiss).

In vivo magnetic resonance imaging
Dynamic contrast enhanced MRI (DCE-MRI) was done on A2780-1A9 tumor-bearing mice using a BioSpec AVIII system (Bruker BioSpin; ref. 35). Tumor-bearing mice (6/group, volume matched), were pretreated or not with bevacizumab (two injections, days −5 and −1); 24 hours after the last dose they were given gadopentetate dimeglumine Magnevist (Bayer, 0.125 mmol/kg, i.v.) and scanned, as detailed in Supplementary Methods. Image post-processing and analysis were done by in-house developed routines running under ImageJ (imagej.nih.gov/ji). IAUC was calculated as the initial area under the signal enhancement curve in the manually selected tumor region calculated from the first 5 minutes of the DCE-MRI signal time course.

Statistical analysis
Statistical analyses were done using Prism Software (GraphPad Prism 6 Software). Differences in tumor growth and pharmacokinetic profiles were analyzed by two-way ANOVA followed by the Tukey’s post-test. The Student unpaired t test or the nonparametric Mann–Whitney test was used for the other cases. Differences in survival were analyzed by the log-rank test. The P value <0.05 was considered significant.

Results and Discussion
Bevacizumab impaired the tumor uptake of chemotherapy drugs, despite slower efflux, but increased the antitumor activity of the combination
The main clinical use of bevacizumab in oncology is with chemotherapy (2, 3). Chemotherapy (including paclitaxel) combined with bevacizumab is becoming a standard of care for patients with ovarian cancer (37). We found that the combination of bevacizumab with paclitaxel had significantly greater effect on A2780-1A9 ovarian tumor xenografts than the single drugs (Fig. 1A). In the paclitaxel and bevacizumab groups, there was moderate antitumor activity (T/C% 51, 62; LCK 4.2, 3.3, respectively), whereas the combination gave a T/C% of 40, indicating a synergistic effect (LCKexp 7.5, LCKob 14.2).

To assess whether the antitumor activity of the combination was due to delivery of paclitaxel in the tumor, we measured its concentration in tumor-bearing animals pretreated with bevacizumab. The pharmacokinetics of paclitaxel in tumor, plasma and liver was studied at different points after a cycle of bevacizumab. Paclitaxel levels in tumor tissue were generally lower, particularly at early time points (30 minutes throughout 24 hours) in the bevacizumab-pretreated than in the vehicle-pretreated groups (Fig. 1B, left; Table 1). Bevacizumab pretreatment did not affect the systemic pharmacokinetics of paclitaxel, and plasma and liver

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Cmax (μg/g)</th>
<th>C48h (μg/g)</th>
<th>C72h (μg/g)</th>
<th>IAUC0–72h (μg/g × h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>Paclitaxel</td>
<td>6.30 ± 0.47</td>
<td>3.13 ± 0.50</td>
<td>2.59 ± 0.12</td>
<td>273.6</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab + paclitaxel</td>
<td>4.90 ± 0.24</td>
<td>2.70 ± 0.29</td>
<td>2.90 ± 0.21</td>
<td>205.2</td>
</tr>
<tr>
<td>Liver</td>
<td>Paclitaxel</td>
<td>95.70 ± 3.73</td>
<td>ND</td>
<td>ND</td>
<td>309.1</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab + paclitaxel</td>
<td>116.8 ± 3.31</td>
<td>ND</td>
<td>ND</td>
<td>347.0</td>
</tr>
<tr>
<td>Tumor</td>
<td>Doxorubicin</td>
<td>2.10 ± 0.22</td>
<td>1.46 ± 0.08</td>
<td>1.02 ± 0.06</td>
<td>111.8</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab + doxorubicin</td>
<td>1.60 ± 0.10</td>
<td>1.34 ± 0.06</td>
<td>1.24 ± 0.06</td>
<td>98.0</td>
</tr>
<tr>
<td>Liver</td>
<td>Doxorubicin</td>
<td>15.90 ± 0.86</td>
<td>1.50 ± 0.12</td>
<td>0.64 ± 0.01</td>
<td>290.2</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab + doxorubicin</td>
<td>17.30 ± 0.71</td>
<td>1.40 ± 0.04</td>
<td>0.75 ± 0.08</td>
<td>281.1</td>
</tr>
</tbody>
</table>

NOTE: n = 4 mice/group (mean ± SEM).
Abbreviations: AUC, area under the curve; Cmax, maximal drug concentration; C48h, and C72h, drug concentrations at 48 and 72 hours; ND, not detectable.

*Experiment was run as in Fig. 1B.
**Experiment was run as in Fig. 1D.

Table 1. Concentrations of paclitaxel and doxorubicin at different times and AUC in A2780-1A9 tumor and liver from mice pretreated or not with bevacizumab.
concentrations of paclitaxel were comparable in the two groups (Fig. 1B, right), thus not explaining the observations in tumor tissue.

In this preclinical setting, a similar pharmacokinetic profile was obtained with doxorubicin—another low-molecular-weight chemotherapy drug with different pharmacologic properties from...
paclitaxel—after bevacizumab. Doxorubicin levels in the A2780-1A9 tumor from mice receiving bevacizumab were lower than in control mice shortly after the injection, even though the combination had greater antitumor activity (Fig. 1C and D; left; Table 1). Again, liver and plasma levels of doxorubicin did not differ in the two groups (Fig. 1D, right). We infer that the lower uptake after bevacizumab is a common event for low-molecular-weight drugs.

A few preclinical studies have reported increased drug uptake in the tumor after antiangiogenic treatments (22). Among others, improved tumor perfusion mirrored by increased drug delivery after bevacizumab-induced vascular remodeling was described in a model of orthotopic neuroblastoma (38). In a colon carcinoma model, the CPT11 concentration tended to be higher after VEGF-blocking therapy, paralleling increased tumor perfusion (39). A reduction in vascular permeability after bevacizumab with increased paclitaxel concentration was described in a breast and a lung cancer xenograft models (40).

In our study though, drug concentrations were lower after bevacizumab. Interestingly, the pretreatment slowed the drug efflux from tumors, as evidenced by the paclitaxel or doxorubicin concentrations at 48 and 72 hours, which were comparable in the vehicle and bevacizumab-pretreated groups, in favor of antitumor activity (Fig. 1B and D). This agrees with our previous findings of reduced tumor uptake and slower efflux of paclitaxel, when combined with different tyrosine kinase receptor (TKR) inhibitors affecting angiogenesis (vandetanib-targeting EGFR and VEGFR in an ovarian cancer model, sunitinib targeting VEGFR and PDGFR and the dual inhibitors of VEGFR2 and FGFR2, brivanib and E-3810, in a mammary cancer model; refs. 34, 41). Therefore, this was true for different tumor models and with different angiogenesis inhibitors, where the outcome was always better with the combinations.

The most widely known theory to explain the antitumor activity of the combination is that vessel changes after antiangiogenesis treatment (i.e., normalization of the tumor microenvironment) can ultimately improve drug delivery, explaining the better response (13). Vessel normalization improves the supply of blood and oxygenation into the tumor, and reduces the IFP, so the delivery and penetration of therapeutics into tumor tissue improve (19). In our A2780-1A9 tumor model, 24 hours after bevacizumab DCE-MRI analysis showed a significant reduction in tumor IAUC (Fig. 1E) 5 minutes after injection of the contrast agent. Thus, bevacizumab reduced the overall delivery of a contrast agent to tumor tissue by altering tumor perfusion and/or permeability, a finding in line with a reduced uptake of chemotherapy (35). At this same time, CD31 staining of harvested tumors showed significant reductions in vessel diameter and area, but not in density, indicating a more regular vasculature (Fig. 1F).

Figure 3.
MALDI MSI quantification of paclitaxel distribution, tumor morphology assessment, paclitaxel tumor uptake, and antitumor activity. Mice bearing subcutaneous A2780-1A9 tumors were treated with paclitaxel (PTX) either alone or after bevacizumab (Bev) as per protocol in Fig. 2; controls were vehicles and bevacizumab alone. Tumors were excised 6 hours later (A–C and E), or mice were followed for antitumor activity (D). A, the percentage of paclitaxel pixels above the threshold calculated from MALDI MSI images as shown in Fig. 2 (sum of two independent experiments, B/group, mean ± SEM; ***P < 0.0001). B, the percentage of necrotic area (S/group, mean ± SEM; **P < 0.01). C, paclitaxel concentration in tumors measured by HPLC (sum of two independent experiments, B/group, mean ± SEM; ***P < 0.0001; △P < 0.001 vs. chemotherapy alone). D, antitumor activity in mice receiving bevacizumab (Bev, △), paclitaxel (PTX, △), or the combination; RTV (~/group, mean ± SEM; **P < 0.01; ***P < 0.0001). E, the percentage of Ki-67-positive proliferating tumor tissue (S/group, mean ± SEM; **P < 0.01; ***P < 0.0001).
Figure 4. MALDI MSI analysis, paclitaxel tumor uptake, and antitumor activity in orthotopic IGROV1-luc ovarian cancer from mice pretreated with bevacizumab. Mice bearing IGROV1-luc tumors in the ovary were randomized by photon count (day 14 after transplant) to receive bevacizumab or not (Bev, 150 µg/mouse, i.p., days 15 and 19), followed by paclitaxel (PTX, 60 mg/kg, i.v., day 20). Tumors were excised after 6 hours for MALDI MSI and histologic analysis (A–E), or mice were followed for antitumor activity (F and G). A and B, from top to bottom, overlapping whole-tumor MALDI MSI images of paclitaxel distribution, vessels (CD31) and proliferation/necrosis (Ki-67) in three adjacent slices of IGROV1-luc tumors, pretreated with vehicle (left), or bevacizumab (right) and followed by paclitaxel 24 hours later; representative images of two tumors/group; scale bar, 2.5 mm. B, enlargements as in A, images representative of one tumor/group; scale bar, 500 µm. C, the percentage of paclitaxel pixels above the threshold calculated from MALDI MSI images (5/group, mean ± SEM; ***, P < 0.0001). D, vessel analysis (number and diameter) from MALDI MSI overlapping images (5/group, mean ± SEM; ***, P < 0.0001). E, paclitaxel concentration in tumors measured by HPLC (5/group, mean ± SEM). F, mice were checked by BLI on day 18 (before receiving paclitaxel) and days 22, 35, and 42 (tumor progression); left, the time course of tumor growth in one representative mouse treated with paclitaxel (PTX) or bevacizumab plus paclitaxel (Bev + PTX); right, tumor burden is expressed as photon counts (7/group, mean ± SEM; **, P < 0.01; ***, P < 0.001). G, survival of mice receiving paclitaxel (PTX), alone or after bevacizumab (Bev + PTX; 7/group; **, P < 0.01).
Figure 5.
MALDI MSI analysis of paclitaxel, and antitumor activity in HT-29 tumors from mice pretreated with bevacizumab. Mice bearing subcutaneous HT-29 colon carcinoma were randomized at a median tumor volume of 400 mm$^3$ to receive a single dose of paclitaxel (PTX, 60 mg/kg, i.v., day 0) either alone or after bevacizumab (Bev, 150 μg/mouse, i.p., days −5 and −1). A, whole-tumor MALDI MSI images of paclitaxel distribution of HT-29 tumors, in mice pretreated with vehicle or bevacizumab and followed by paclitaxel 24 hours later. Tumors were excised 6 hours later; representative images of three tumors/group. (Continued on the following page.)
fact negatively affect the uptake of chemotherapy drug and ultimately the outcome of the combination therapy (13). Different tumor models may also account for this discrepancy. We found that at the same experimental conditions bevacizumab significantly decreased vessel density in the IGROV1-luc ovarian and in the HT-29 colon cancer models (see results in the paragraph below).

In line with our preclinical results, a human study in NSCLC, using positron emission tomography (PET), reported a rapid, significant reduction in perfusion and \(^{11}C\) docetaxel uptake after bevacizumab (25) and highlighted the importance of optimizing the scheduling of antiangiogenic drugs. Reduced vascular permeability and flow by DCE-MRI was described after bevacizumab in patients with advanced breast cancer (44).

These findings indicate that pretreatment with an antiangiogenic agent can lower the concentration of the anticancer drug used in combination. Nevertheless, here, the slower clearance—very likely due to vascular changes by bevacizumab—contributes to efficient tumor drug exposure.

**MALDI MSI visualization of paclitaxel shows more homogeneous intratumor distribution after bevacizumab**

The reduced tumor drug concentrations after bevacizumab is in apparent contrast with the better antitumor effect of the combination. This prompted us to investigate the spatial distribution of paclitaxel in tumors after bevacizumab, using a recent MALDI MSI technology (27, 45) with which drug localization in different areas of the tumor can be visualized. Visual inspection of the MALDI MSI images on A2780-1A9 tumor tissue sections from mice pretreated with bevacizumab showed more homogeneous paclitaxel distribution than in mice pretreated with vehicle, where the drug distribution was irregular (Fig. 2A and B, top). There was also a significantly larger percentage of pixels above the background noise threshold in sections from mice pretreated with bevacizumab than those from mice pretreated with vehicle, confirming the better paclitaxel tumor distribution (Fig. 3A).

Analysis of clusters of positive pixels in MALDI MSI tumor images showed that in the sections from mice pretreated with bevacizumab there were fewer aggregates of positive pixels and their mean size was bigger than in sections from mice pretreated with vehicle (Supplementary Fig. S1). Together with the different percentages of pixels above the background threshold these results highlight the better distribution of paclitaxel after bevacizumab pretreatment and particularly the presence of the drug in larger and more homogeneous areas of the tumor.

To find out whether the different distribution was linked to differences in tissue architecture and morphology, next we made a comparative histologic analysis on adjacent tumor sections from A2780-1A9, analyzed with MALDI MSI. Figure 2A (enlarged in Fig. 2B) shows the paclitaxel distribution overlapping optical images of adjacent tumor slices stained for CD31 (vessels) or Ki-67 (proliferation). Unlike with vehicle, bevacizumab-pretreated tumors had fewer necrotic areas (quantified in Fig. 3B), with vessels uniformly distributed throughout the tumor. This indicated that the tumor distribution of paclitaxel was associated with vasculature and proliferating, non-necrotic areas, with a more homogeneous pattern in the bevacizumab-treated tumors, where paclitaxel was regularly distributed, with no breaks in the tissue. This sheds some light on the apparent paradox of the reduced paclitaxel uptake after bevacizumab, providing evidence that the restoration of a functional tumor microenvironment architecture modifies the distribution and retention of the anticancer drug in the tumor.

Part of the same tumor analyzed with MALDI MSI was used to quantify paclitaxel content by HPLC, confirming that in this setting too total paclitaxel uptake in the tumors at 6 hours was lower after bevacizumab (Fig. 3C). Because we used 60 mg/kg paclitaxel for the MALDI MSI, in a parallel trial we tested the antitumor activity of this dose combined with bevacizumab. The combination was significantly more active than the single treatments (Fig. 3D), as illustrated by the significantly lower number of Ki-67-positive cells in tumor sections from mice receiving paclitaxel alone or the combination (Fig. 3E).

The site of tumor growth and the surrounding tumor environment can affect the biology of the tumor, influencing the delivery and activity of certain drugs (46). This prompted us to investigate paclitaxel distribution in orthotopically growing A2780-1A9. Again, the distribution of paclitaxel measured by MALDI MSI after bevacizumab in tumors growing under the bursa of the ovary was more homogeneous than in controls (Supplementary Fig. S2A and S2B) and was associated with a low paclitaxel concentration by HPLC analysis (Supplementary Fig. S2C). The comparable results on A2780-1A9 tumors growing ectopically in the subcutis or orthotopically in the ovary were consistent with our previous finding, showing that the histologic features (including vasculature) do not differ in the two sites (47).

To test the importance of these findings in another ovarian tumor model, mice bearing orthotopic IGROV1-luc ovarian tumor were pretreated or not with bevacizumab, before receiving paclitaxel, then randomized for MALDI MSI and HPLC analysis and treatment evaluation. MALDI MSI clearly showed a better, more uniform paclitaxel distribution in tumors from mice pretreated with bevacizumab than the vehicle-pretreated animals (Fig. 4A, top, enlarged in Fig. 4B); this was corroborated by the higher percentage of pixels above the threshold and the analysis of their cluster (Fig. 4C and Supplementary Fig. S3A).

The corresponding histologic images stained for CD31 and Ki-67 (Fig. 4A, bottom, enlarged in Fig. 4B) show solid micronodular growth of vehicle-treated tumors. Tumors are well vascularized with a characteristic continuous organization of the CD31-positive vessel structures encircling the tumor complexes. Bevacizumab-pretreated tumors show a more homogeneous growth pattern with a loss of “perinodular” vessel formation and a reduction in vessel number and diameter (quantified in Fig. 4D). Both groups show moderate proliferative activity with an accentuation of Ki-67-positive cells at the periphery of the microvessels (Fig. 4A and B, bottom).
At this time point, the paclitaxel concentration quantified by HPLC did not differ in the two groups, despite the significant histological changes (Fig. 4E). Bevacizumab combined with paclitaxel significantly delayed the growth of IGROV1-luc in the ovari (Fig. 4F), and increased survival (ILS 21%) compared with chemotherapy alone (Fig. 4G). These findings confirm the antiangiogenic effect of bevacizumab, though with a distinct pattern of vascular response in this model, and its ability to boost paclitaxel antitumor activity.

To test the significance of these findings in another tumor type for which bevacizumab is clinically used (4), we analyzed the MALDI MSI spatial distribution of paclitaxel after bevacizumab in the HT-29 colon cancer xenografts (Fig. 5). Again, paclitaxel ion distribution on HT-29 tumor sections from mice pretreated with bevacizumab appeared more homogeneous than in tumors from vehicle-pretreated mice, as confirmed by the significantly higher percentage of pixels above the 5th percentile threshold, and cluster size (Fig. 5A and B; Supplementary Fig. S3B). The corresponding histologic images show a reduction in CD31-stained vessels and Ki-67–positive cells in bevacizumab-pretreated tumors (Fig. 5C, quantified in Fig. 5D and E), which anticipated the antitumor effect of paclitaxel combined with bevacizumab evident from 6 days after treatment (Fig. 5F).

In conclusion, in bevacizumab-pretreated mice the tumor distribution of paclitaxel, shown by MALDI MSI (27), was more homogeneous than in mice pretreated with vehicle, with slower drug clearance from the tumor tissue. The discrete distribution of paclitaxel in tumors pretreated or not with bevacizumab is related to different morphologic features of the tissue, as we noted in microscopy IHC-stained tissue images superimposed on the MALDI MSI images. Drug penetration was inadequate in poorly vascularized or necrotic parts of the control tumors, and with the antiangiogenic pretreatment the anticancer drug was conveyed to proliferating areas of the tumor, leading to more even distribution. This was true for different tumor models independently of their origin or their site of implantation, and the different pattern of vascular response after antiangiogenic treatment.

References

These findings suggest that the better therapeutic activity of the combination is due not only to the sum of two drugs with different modes of action, but also to a more favorable distribution of the chemotherapy drug in the tumor. The better tumor distribution of paclitaxel is in keeping with its good antitumor activity when it is combined with bevacizumab.

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

Authors’ Contributions
Conception and design: M. Cesca, R. Frapoll, M. D’Incalci, M. Zucchetti, R. Giavazzi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Cesca, L. Morosi, R. Frapoll, O. Dirsch, E. Micotti, S. Giordano, R. Giavazzi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Cesca, L. Morosi, A. Berndt, R. Frapoll, P. Richter, A. Decio, S. Giordano, M. Zucchetti, R. Giavazzi
Writing, review, and/or revision of the manuscript: M. Cesca, A. Berndt, O. Dirsch, M. D’Incalci, M. Zucchetti, R. Giavazzi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I.F. Nerini, E. Davoli
Study supervision: R. Giavazzi, M. Cesca

Acknowledgments
The authors thank Katja Grun for assistance in histologic analysis and I.D. Baggett for editing the article. I. Fuson Nerini and A. Decio are the recipients of a fellowship from the Italian Foundation for Cancer Research (IFRC).

Grant Support
This study was supported by grants from the Italian Association for Cancer Research (IG14532 and 12182; to R. Giavazzi) and the Fondazione CARIPO (no. 2011-0614; to M. Cesca).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 22, 2015; revised October 5, 2015; accepted October 9, 2015; published OnlineFirst October 22, 2015.
Molecular Cancer Therapeutics

Bevacizumab-Induced Inhibition of Angiogenesis Promotes a More Homogeneous Intratumoral Distribution of Paclitaxel, Improving the Antitumor Response

Marta Cesca, Lavinia Morosi, Alexander Berndt, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0063

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2015/10/21/1535-7163.MCT-15-0063.DC1

Cited articles
This article cites 47 articles, 16 of which you can access for free at:
http://mct.aacrjournals.org/content/15/1/125.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/15/1/125.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.