Low-dose metronomic cyclophosphamide combined with vascular disrupting therapy induces potent antitumor activity in preclinical human tumor xenograft models

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Abstract

Vascular disrupting agents preferentially target the established but abnormal tumor vasculature, resulting in extensive intratumoral hypoxia and cell death. However, a rim of viable tumor tissue remains from which angiogenesis-dependent regrowth can occur, in part through the mobilization and tumor colonization of circulating endothelial progenitor cells (CEP). Cotreatment with an agent that blocks CEPs, such as a vascular endothelial growth factor pathway–targeting biological antiangiogenic drug, results in enhanced antitumor efficacy. We asked whether an alternative therapeutic modality, low-dose metronomic chemotherapy, could achieve the same result given its CEP-targeting effects. We studied the combination of the vascular disrupting agent OXi4503 with daily administration of CEP-inhibiting, low-dose metronomic cyclophosphamide to treat primary orthotopic tumors with the use of the 231/LM2-4 breast cancer cell line and MeWo melanoma cell line. In addition, CEP mobilization and various tumor characteristics were assessed. We found that daily p.o. low-dose metronomic cyclophosphamide was capable of preventing the CEP spike and tumor colonization induced by OXi4503. This was associated with a decrease in the tumor rim and marked suppression of primary 231/LM2-4 growth in nude as well as severe combined immunodeficient mice. Similar results were found in MeWo-bearing nude mice. The delay in tumor growth was accompanied by significant decreases in microvessel density, perfusion, and proliferation, and a significant increase in tumor cell apoptosis. No overt toxicity was observed. The combination of OXi4503 and metronomic chemotherapy results in prolonged tumor control, thereby expanding the list of therapeutic agents that can be successfully integrated with metronomic low-dose chemotherapy. [Mol Cancer Ther 2009;8(10):2872–81]

Introduction

Angiogenesis, the growth of new blood vessels from existing mature vasculature, has been shown to be an important functional target in experimental and clinical oncology. After the 2004 Food and Drug Administration approval of bevacizumab, a humanized monoclonal antibody (mAb) directed against vascular endothelial growth factor (VEGF), for the treatment of metastatic colorectal cancer (1) and, subsequently, non–small cell lung cancer and breast cancer, many other antiangiogenic drugs have been studied in phase I/II/III trials, some of which have been approved for clinical practice (2). Two small-molecule receptor tyrosine kinase inhibitors, sunitinib and sorafenib, are used as single agents in the treatment of advanced renal cell carcinoma (2–4). Sorafenib monotherapy has shown benefit in hepatocellular carcinoma as well (2, 5). Both drugs target VEGF receptors (VEGFR) and platelet-derived growth factor receptors, among other receptor tyrosine kinases.

Vascular disrupting agents (VDA) represent a relatively novel class of vascular targeting drugs that specifically target the established but abnormal tumor vasculature. A subset of these drugs, the combretastatin family, including combretastatin A-4 phosphate and its second-generation prodrug derivative OXi4503 (combretastatin A-1 phosphate), bind preferentially to endothelial cell–associated tubulin, inducing rapid microtubular depolymerization and vascular shutdown in solid tumors. Severe tumor hypoxia subsequently ensues, followed by extensive intratumoral necrosis. However, rapid tumor regrowth occurs from a rim of remaining viable tissue at the leading edge of the tumor (6). Considerable effort has therefore been made to interfere with this particular tumor repopulation phenomenon by combining VDAs with other anticancer agents that preferentially target the well-oxygenated, angiogenic, and proliferative tumor cell rim. Multiple strategies have been tested preclinically, such as VDAs combined with radiation

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therapy (7) or conventional, maximum tolerated dose chemotherapy (8).

A prime example of a strategy that effectively enhances the antitumor activity of a VDA in a complementary manner is combination with an antiangiogenic agent. The addition of a potent inhibitor of VEGFR-2–associated tyrosine kinase, ZD6474, to the VDA ZD6126 resulted in a significantly enhanced tumor growth delay and tumor-free survival in mouse models of renal cell carcinoma and Kaposi sarcoma (9). Combining bevacizumab, the anti-VEGF antibody, with combretastatin A-4 phosphate showed similar effects (10). A mechanistic rationale for the prolonged suppression of tumor growth with the use of such drug combinations was recently provided by the results of studies from our laboratory. We have shown that mobilization into the bloodstream of bone marrow–derived circulating endothelial progenitor cells (CEP), and possibly other types of bone marrow–derived cells, takes place rapidly (within 4 hours) after treatment with OXi4503 or combretastatin A-4 phosphate. These cells subsequently invade and colonize the viable tumor rim, where they are incorporated into growing vessels and thus contribute to tumor regrowth. The administration of the antiangiogenic drug DC101, a rat mAb blocking the mouse VEGFR-2, just before OXi4503 can inhibit the acute elevation of CEP levels, thereby blunting regrowth from the viable tumor rim and even causing tumor shrinkage (11). Also of interest, we have recently found that CEP mobilization and the subsequent antitumor benefit gained by cotreatment with DC101 is not limited to VDAs, but is also observed when certain chemotherapeutics (e.g., taxanes) are given at their maximum tolerated dose, implicating a clear possibility that this phenomenon might be more widely applicable (12).

Preliminary clinical studies have revealed results that seem to support, at least tentatively, our preclinical results with OXi4503. Elevated levels of circulating bone marrow–derived CD133+ cells and CD34+ cells were found in cancer patients within 4 hours to days after treatment with a VDA (13), implying that there might be a clinical rationale for the combination of a VDA with an agent that targets bone marrow cell-mediated vasculogenesis/angiogenesis, such as bevacizumab.

The concept of 'metronomic' chemotherapy (14), that is, the frequent administration of chemotherapeutic agents at doses well below the maximum tolerated dose with no prolonged drug-free breaks, has been shown to induce antiangiogenic effects (15). Moreover, such drugs, including cyclophosphamide, are able to suppress circulating endothelial (progenitor) cell levels when given metronomically (16). This suggests metronomic chemotherapy could be a rational treatment for suppressing the regrowth of the viable tumor rim that remains after VDA treatment. There are several circumstances in which low-dose metronomic (LDM) chemotherapy could have an advantage over the use of drugs such as bevacizumab when combined with VDA therapy (e.g., when patients may be intrinsically resistant to the antiangiogenic agent or acquire resistance to it). Reduced costs when using a drug such as cyclophosphamide may be another advantage (17), as is the safety profile of LDM cyclophosphamide (18, 19).

Here we show that the combination of OXi4503 with LDM cyclophosphamide is highly effective and safe in the treatment of primary orthotopic human breast carcinoma and melanoma transplanted xenografts. Robust increases in tumor necrosis and apoptosis were observed, which were accompanied by decreases in microvessel density, perfusion, and proliferation. The viable rim that normally remains after VDA monotherapy was found to be smaller, which was accompanied by a decrease in bone marrow–derived cells homing to the tumor.

Materials and Methods

Primary Tumor and Animal Models

An aggressive variant of the human MDA-MB-231 breast cancer cell line called 231/LM2-4 was isolated as previously described (20). This line was previously selected for its high-grade metastatic ability and was used for the present primary tumor therapy studies rather than the parental MDA-MB-231 line because this would allow us to compare the results with those obtained in future studies involving the treatment of established metastatic disease. Cells (2×10^6 231/LM2-4) were injected orthotopically into the right inguinal mammary fat pads of 6-week-old female immunodeficient athymic nude mice (Harlan Laboratories Inc., Indianapolis, IN), or into athymic nude mice that were previously lethally irradiated (900 rad) and subsequently transplanted with 10^7 green fluorescent protein (GFP)+ bone marrow cells from syngeneic nude GFP+ donors (21). In other experiments, 2×10^6 human melanoma MeWo cells (ATCC, Manassas, VA) were injected orthotopically (subdermally) into adult 6-week-old female immunodeficient athymic nude mice, or 2×10^6 231/LM2-4 cells were implanted orthotopically into the right inguinal mammary fat pads of 6-week-old female CB-17 severe combined immunodeficient mice. Tumor volume was assessed regularly with Vernier calipers, with the use of the formula length $\times$ width$^2 \times 0.5$. When tumor size reached 400 to 500 mm^3, treatment was initiated with either LDM cyclophosphamide, OXi4503, or a combination of the two drugs. Biweekly weight assessment was used as a surrogate marker for toxicity. Mice were sacrificed when tumor sizes reached 1700 mm^3 and in accordance with the guidelines of Sunnybrook Health Sciences Centre.

Drugs and Schedule

Cyclophosphamide (Baxter Oncology GmbH, Mississauga, Ontario, Canada) was purchased from the institutional pharmacy. It was reconstituted according to the instructions of the manufacturer to a stock concentration of 20 mg/mL and given through the drinking water to provide a dose of 20 mg/kg/d, based on the estimated daily consumption of 3 mL for a 20-g mouse, as previously described (22). OXi4503, a vascular microtubule disrupting agent, was given i.p. at a dose of 50 mg/kg, as described

previously (23). For the combination therapy, OXi4503 was given 6 d after the start of LDM cyclophosphamide. For long-term treatment, 50 mg/kg OXi4503 injection was repeated every 2 wk.

Bone Marrow Transplantation

GFP+ bone marrow cells (10^7) isolated from femurs of GFP+ nude mice (21) were injected into the tail veins of 6-to-8-week-old lethally irradiated (900 rad) female athymic nude mice. Four to six weeks later, recipient mice were implanted with 231/LM2-4.

Cells and Culture Conditions

231/LM2-4 and MeWo cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Hyclone, South Logan, UT).

Evaluation of Viable CEPs by Flow Cytometry

Blood was drawn from the retro-orbital sinus of anesthetized mice. Viable CEPs were counted with the use of five-color flow cytometry. Briefly, mAbs specific for CD45 were used to exclude CD45+ hematopoietic cells, and CEPs were detected as being positive for the murine endothelial markers fetal liver kinase 1/VEGFR-2, CD13 (aminopeptidase N), and CD117 (c-kit; BD Biosciences, Mississauga, ON, Canada; ref. 24). After red cell lysis, cell suspensions were analyzed on an LSR II (BD Biosciences). After acquisition of at least 100,000 cells per sample, analyses were considered informative when an adequate number of events (i.e., >25, typically 50-150) were collected in the CEP enumeration gate in untreated control animals. Percentages of stained cells were determined and compared with appropriate negative controls. Positive staining was defined as being greater than nonspecific background staining, and 7-aminoactinomycin D was used to distinguish apoptotic and dead cells from viable cells (25).

Evaluation of a Marker for Perfusion

For blood vessel perfusion analysis, mice were injected i.v. with the fluorescent, DNA-binding dye Hoechst 33342 (40 mg/kg; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) 1 min before euthanasia (26). After euthanasia, tumors were removed and either fixed in 10% buffered formalin for 24 h, followed by 70% ethanol, or tumors and organs were frozen on dry ice in Tissue-Tek OCT Compound (Miles Inc., Elkhart, IN) and kept in the dark at −70°C.

Tissue Processing and Immunohistochemistry

Tissue processing and immunohistochemistry were done as described (26). Briefly, formalin-fixed, paraffin-embedded tumors were sectioned (5 μm thick) and stained with H&E. Necrosis was detected as autofluorescence in the FITC channel. Tumor tissues were quantified for perfusion by analysis of Hoechst 33342 staining on cryosections. The microvessel density was analyzed by immunostaining with an anti-CD31 antibody (1:200; BD Biosciences) and secondary Cy3-conjugated donkey anti-rat antibody (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Proliferation was determined by immunostaining with a rabbit polyclonal Ki-67 antibody (Vector Laboratories Inc., Burlington, Ontario, Canada) and secondary Texas Red–conjugated goat anti-rabbit antibody (1:200; Jackson ImmunoResearch Laboratories Inc.). Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (Roche Diagnostics, Indianapolis, IN). Controls were immunostained with a secondary antibody alone.

Image Acquisition and Analysis

Image acquisition and analysis were carried out as previously described (11). Tumor sections were visualized under a Carl Zeiss Axioplan 2 microscope (Carl Zeiss Canada Inc., Toronto, Ontario, Canada) with a bright field and the following fluorescence filters: 4′, 6-diamidino-2-phenylindole (350 nm excitation) for Hoechst 33342, Cy3 (540 nm excitation) for CD31 staining, and GFP (470 nm excitation) for pimonidazole, GFP+ bone marrow positive cell staining, or autofluorescence of necrotic tissue. Images were captured with a Zeiss Axiocam digital camera connected to the microscope with the use of AxioVision 3.0 software. The number of fields per tumor sample varied from 5 to 15 depending on the tumor size. Perfusion was assessed by systematically analyzing ×200 magnified images of tumor tissue sections stained with Hoechst 33342 and calculating the areas that are positively stained (blue) as a fraction of the total tumor area. Similarly, necrosis was assessed by analyzing ×25 magnified images for tissue autofluorescence (green). Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA) was used to quantify perfused and necrotic fractions, which are then expressed as percentages of the total tumor area. For necrosis and perfusion, a total of at least 15 fields per group were analyzed. Longitudinal cross-sections of the tumors were made to allow all the tumor areas to be represented in the sample. For the analysis of GFP+ and CD31+ cells, a Zeiss Axiovert 100 M confocal laser scanning microscope was used at ×200 magnification, and analysis was done with the Zeiss LSM Image Browser software version 4,2,0,121. The number of vascular structures (CD31+) and cells (GFP+) per field was counted.

Analysis of Tumors by Flow Cytometry

A representative longitudinal section of tumor tissue was prepared as a single-cell suspension by digestion with an enzyme cocktail made up of collagenase 3 (4 mg/mL; Worthington Biochemical Corporation, Lakewood, NJ), hyaluronidase (2 mg/mL; Sigma), and collagenase IV (2 mg/mL; Sigma). Subsequently, the cells in suspension were immunostained with mAbs against CD45, CD31, and VEGFR-2 (fetal liver kinase 1) markers (BD Biosciences). Evaluation of positive cells was conducted with the use of flow cytometry, and >150,000 events were collected for each sample tested.

Statistical Analysis

The SPSS statistical package version 12.0.1 was used to assess the statistical significance of the differences in mean values. For each value, Levene's test was used to determine the equality of variances. Depending on the outcome of the Levene's test, two-tailed Student's *t* test or Wilcoxon test was used to assess the significance of the mean difference. Differences between designated groups compared with the control-untreated group (unless indicated otherwise) were considered significant at

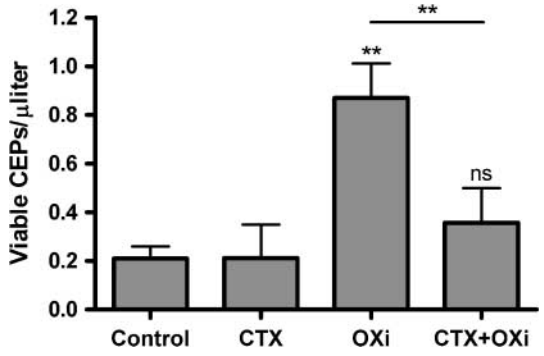


Figure 1. Evaluation of viable CEP levels in mice treated with metronomic CTX, OXi, or a combination of both drugs. Non–tumor-bearing nude mice ($n = 5$ per group) were treated with LDM CTX and OXi. Untreated mice and mice treated with either of the drugs alone were used as controls. Four hours after OXi administration, blood was drawn for evaluation of viable CEPs through five-color flow cytometry. *Error bars,* + SD; *, $0.01 < P < 0.05$; **, $P < 0.01$ with the use of unpaired Student's t test. Values are compared with control mice unless indicated otherwise. CTX, cyclophosphamide; OXi, OXi4503.

values of $0.05 > P > 0.01$ (*) or $P < 0.01$ (**). Data are expressed as mean ± SD.

Results

The Combination of OXi4503 with Metronomic Cyclophosphamide Inhibits the CEP Spike Induced by OXi4503

The administration of LDM cyclophosphamide suppresses the levels of CEPs in peripheral blood of human tumor–bearing mice [i.e., lymphoma-bearing mice (ref. 16) and melanoma-bearing mice (ref. 27)] even within 1 week of daily treatment (16). We asked whether the acute elevation in CEPs found 4 hours after administration of OXi4503 can be inhibited by prior treatment with LDM cyclophosphamide. We reasoned that to prevent this rapidly induced endothelial progenitor cell spike, CEP levels had to be suppressed by LDM cyclophosphamide at the time of administration of OXi4503. Therefore, we evaluated whether 6 days of daily metronomic cyclophosphamide given continuously through the drinking water (22) was sufficient to suppress CEP levels. Consistent with previously published results, we found that it did so (data not shown).

Next, nude mice were first treated with daily low-dose cyclophosphamide for 6 days through the drinking water at an initial dose of 20 mg/kg/d, at which time OXi4503 was given i.p. in a nontoxic dose of 50 mg/kg. Analysis of viable CEPs after 4 hours showed that treatment with OXi4503 alone increases CEP levels in the peripheral blood ($P < 0.001$; Fig. 1) in accordance with previous studies (11). However, pretreatment with metronomic cyclophosphamide is capable of significantly lowering the number of viable CEPs ($P = 0.002$) to a level approaching that in the cyclophosphamide monotherapy control group ($P > 0.05$ compared with control mice). In non–tumor-bearing nude mice, however, cyclophosphamide alone did not significantly suppress CEP levels compared with control, which can be attributed to the extremely low CEP levels in untreated nude mice (ref. 28; Fig. 1). A similar inhibiting effect of the combination treatment on CEPs was found in different mouse strains, such as C57Bl/6J, treated with LDM cyclophosphamide and OXi4503 (data not shown).

The Combination of OXi4503 with Metronomic Cyclophosphamide Delays Primary Tumor Growth without Overt Toxicity

Next, we asked whether the CEP suppression observed in the combination treatment is associated with a delay in primary tumor growth. To this end, nude mice were orthotopically implanted with a previously selected aggressive variant of the MDA-MB-231 human breast cancer cell line called 231/LM2-4 (20). When the primary tumors had reached an average size of 400 mm^3, treatment with low-dose cyclophosphamide was started, 6 days after which biweekly injections with OXi-4503 were initiated. In untreated control mice, rapid tumor growth was observed, reaching the tumor end point as early as 34 days after tumor cell implantation (Fig. 2A). Metronomic cyclophosphamide alone only resulted in a small delay of tumor growth in this model. OXi4503 monotherapy showed considerable benefit in suppressing tumor growth, but the initial reduction in tumor volume was followed by significant regrowth within 4 weeks of treatment with OXi4503 when compared with the tumor size at the time of initiation of therapy ($P = 0.037$ from day 55 onward, paired t test). In contrast, the combination of cyclical OXi4503 and continuous daily metronomic cyclophosphamide showed a striking antitumor activity with no significant signs of regrowth during the first 4 weeks of therapy ($P > 0.1$ not significant, paired t test), resulting in a significant benefit over OXi4503 monotherapy after 34 days ($P = 0.028$, unpaired t test), 55 days ($P = 0.030$, unpaired t test), and 62 days ($P = 0.025$, unpaired t test). Moreover, when combination treatment was given, tumor control was achieved during a prolonged period of time (data not shown). No overt toxicity was observed compared with OXi4503 alone, as measured by regular assessments of body weight (Fig. 2B).

Comparable Effects of the Combination of OXi4503 and Cyclophosphamide in Different Tumor and Mouse Models

The effect of our treatment on tumor growth was subsequently analyzed in CD17 severe combined immunodeficient mice. Similar treatment effects were seen. LDM cyclophosphamide monotherapy only delayed tumor growth by a few days (Fig. 2C), whereas OXi4503 monotherapy was again associated with initial tumor control followed by potent regrowth within two treatment cycles ($P < 0.04$ from day 39 onward, paired t test). OXi4503-treated mice had to be sacrificed 23 days after tumor implantation because the tumor endpoint was reached. When OXi4503 was combined with metronomic cyclophosphamide, a prolonged antitumor effect was seen, even resulting in significant tumor size reduction (Fig. 2C; $P < 0.05$ on days 29, 34, 39, 47, and 61; paired t test). From day 39 onward, there was a

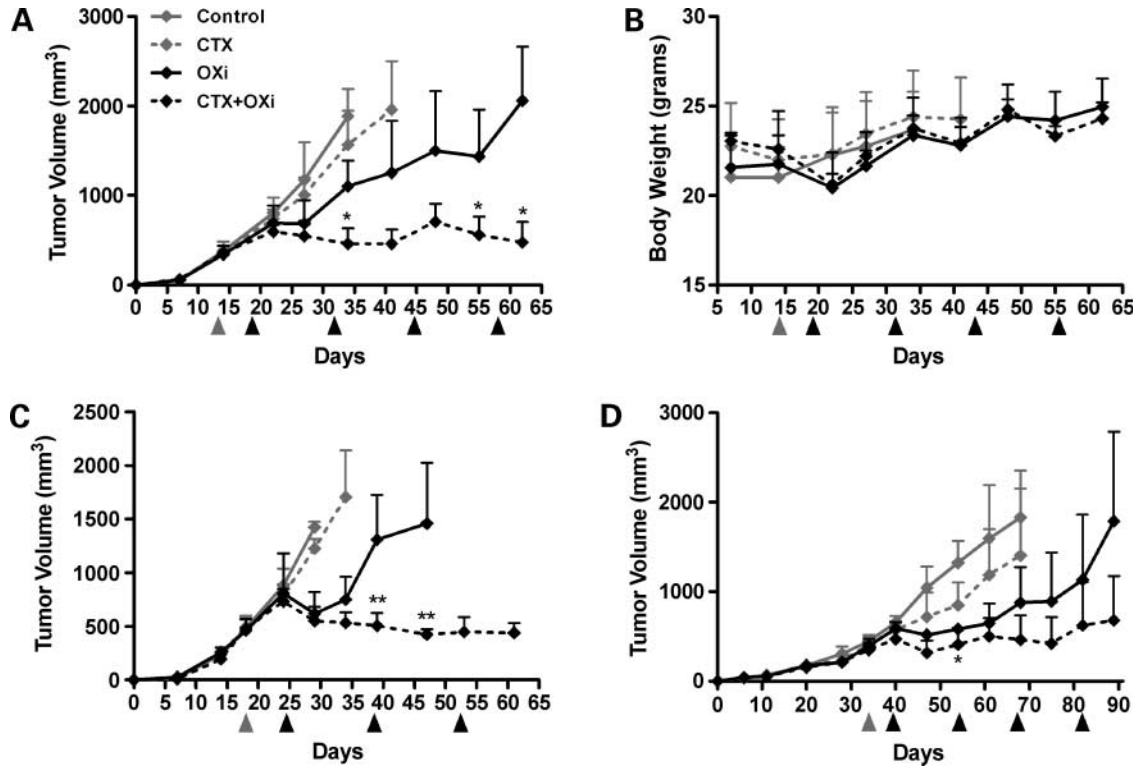


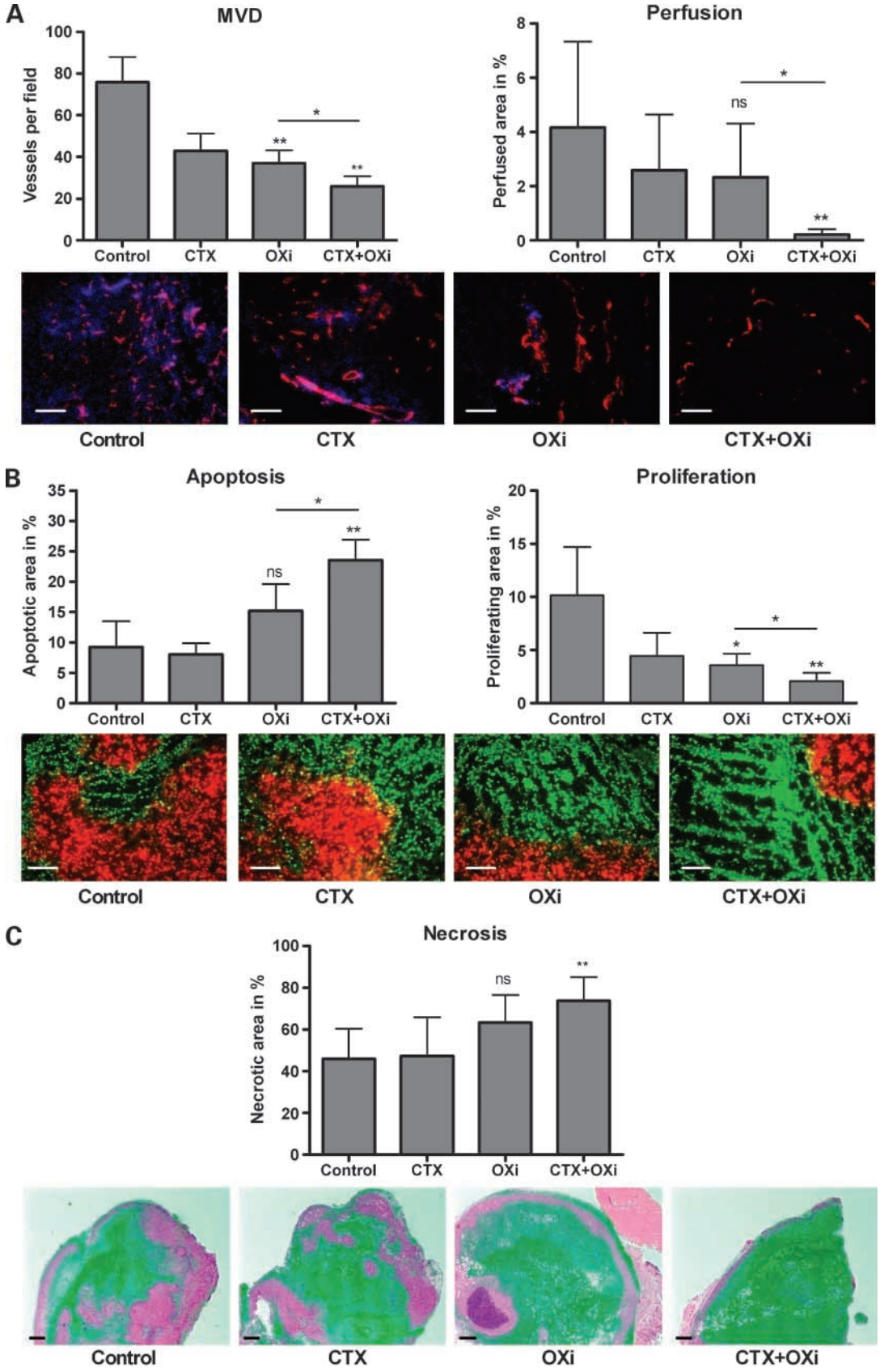
Figure 2. Evaluation of antitumor effect and body weight in mice treated with metronomic CTX, OXi4503, or a combination of both drugs. **A,** tumor volume of nude mice ($n = 5$ per group) orthotopically implanted with 231/LM2-4 cells, and treated with CTX and OXi4503. **B,** body weight of nude mice bearing 231/LM2-4 tumors treated with CTX and OXi4503 ($n = 5$ per group). **C,** tumor volume of CB-17 SCID mice ($n = 5$ per group) orthotopically implanted with 231/LM2-4 cells, and treated with CTX and OXi4503. **D,** nude mice ($n = 4$ per group) s.c. implanted with MeWo cells, and treated with CTX and OXi4503. *Gray arrows,* start of continuous CTX administration; *black arrows,* OXi4503 administration. *Error bars,* +SD. CTX + OXi values are compared with OXi4503 alone. *, $0.01 < P < 0.05$; **, $P < 0.01$ with the use of two-tailed unpaired Student's *t* test. SCID, severe combined immunodeficient.

significant difference between the two treatments ($P < 0.01$). A similar trend, albeit less significant, was found when another tumor cell line (MeWo human melanoma) was implanted orthotopically in nude mice and treated according to the same schedule (Fig. 2D).

Combination of OXi4503 and Metronomic Cyclophosphamide Increases Tumor Hypoxia, Necrosis, and Apoptosis while Decreasing Microvessel Density and Proliferation

To further characterize the antitumor effect of the combination treatment, 231/LM2-4 tumors were grown in nude mice and treated when tumor size reached 400 mm^3. Three days after administration of OXi4503, tumors were removed for immunohistochemistry to assess microvessel density, perfusion, apoptosis, proliferation, and necrosis. As shown in Fig. 3A, both LDM cyclophosphamide and OXi4503 monotherapy diminished microvessel density (both $P = 0.001$), an effect that could be significantly enhanced by combining OXi4503 with LDM cyclophosphamide ($P = 0.01$). Perfusion was unchanged in mice treated with OXi4503 alone ($P = 0.26$), whereas the addition of LDM cyclophosphamide significantly lowered tumor perfusion (Fig. 3A, *right*; $P = 0.046$). This was accompanied by significant increases in tumor cell apoptosis, as measured by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (Fig. 3B, *left*; $P = 0.043$), and decreases in proliferation rates, as measured by Ki-67 staining (Fig. 3B, *right*; $P = 0.047$). Because the 231/LM2-4 tumors show some necrosis even when untreated, the increases in necrosis after OXi4503 treatment were modest compared with previously studied tumor models (e.g., Lewis lung carcinoma in C57Bl/6 mice or MeWo human melanoma in athymic nude mice; ref. 11). However, a clear trend toward increased level of necrosis was nevertheless observed when the two treatments were combined (Fig. 3C; $P = 0.002$ compared with control). As shown in Fig. 3C, the viable rim, although present, was considerably reduced after the addition of cyclophosphamide to OXi4503.

Figure 3. Analysis of MVD, perfusion, necrosis, tumor cell proliferation, and apoptosis in tumors grown in nude mice after treatment with CTX and OXi4503. 231/LM2-4–bearing nude mice (400 mm^3) were treated with LDM CTX and OXi4503. Three days after OXi4503 administration, tumors were harvested and sections were prepared for assessment of (**A**) MVD (*red*) and perfusion (*blue*; *bar,* 50 μm); (**B**) tumor cell proliferation (*red*) and apoptosis (*green*; *bar,* 50 μm); and (**C**) necrosis (*green*) on H&E staining (*bar,* 100 μm). *Error bars,* +SD. Values are compared with untreated mice unless indicated otherwise. *, $0.01 < P < 0.05$; **, $P < 0.01$ with the use of two-tailed unpaired Student's *t* test. MVD, microvessel density.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Combination of OXi4503 and Metronomic Cyclophosphamide Is Capable of Diminishing Homing of GFP+ Bone Marrow Cells to Tumors

Because we have previously reported that the administration of the mouse anti–VEGFR-2 mAb DC101 24 hours before OXi4503 improves antitumor activity, at least in part, by blocking the mobilization and subsequent homing of CEPs and possibly other bone marrow–derived cells to the viable tumor rim (11), we analyzed whether pretreatment with LDM cyclophosphamide also causes these same effects. Hence, 231/LM2-4 tumors were implanted into nude mice that had previously been lethally irradiated and transplanted with GFP-tagged bone marrow from GFP+ nude donor mice (21). Once again, tumors were allowed to reach 400 mm^3 before treatment was initiated. Analysis of the tumors by immunohistochemistry revealed a significant increase in the number of bone marrow cells homing to the viable rim in mice treated with OXi4503 alone (Fig. 4A; $P < 0.001$). Cotreatment with LDM cyclophosphamide resulted in a significant decrease of bone marrow–derived cells homing to the tumor ($P < 0.001$ compared with OXi4503 alone). Furthermore, to confirm these results, fluorescence-activated cell sorting analysis was done on single-cell suspensions prepared by enzymatic digestion of portions of the tumors (Fig. 4B, *left*). Staining of these cells with antibodies against CD45, CD31, and VEGFR-2 showed a decrease in GFP+ endothelial cells ('CEPs') homing to the tumor (Fig. 4B, *right*). However, analysis of both immunohistochemistry and fluorescence-activated cell sorting data revealed that the incorporation of GFP+ cells, including CEPs, was not completely blocked in the combination group, which corresponds with the small remaining viable tumor rim (Fig. 3C). One explanation for this is our finding that LDM cyclophosphamide is unable to completely block CEP mobilization (Fig. 1). Post-treatment levels in peripheral blood and tumors are comparable with control levels, unlike DC101, which almost completely inhibits CEP mobilization, thereby causing the viable tumor rim to disappear.

Our results suggest that the effects of the combination of OXi4503 and cyclophosphamide can inhibit systemic angiogenesis/vasculogenesis to an extent sufficient to significantly improve the effect of OXi4503 when treating primary tumors.

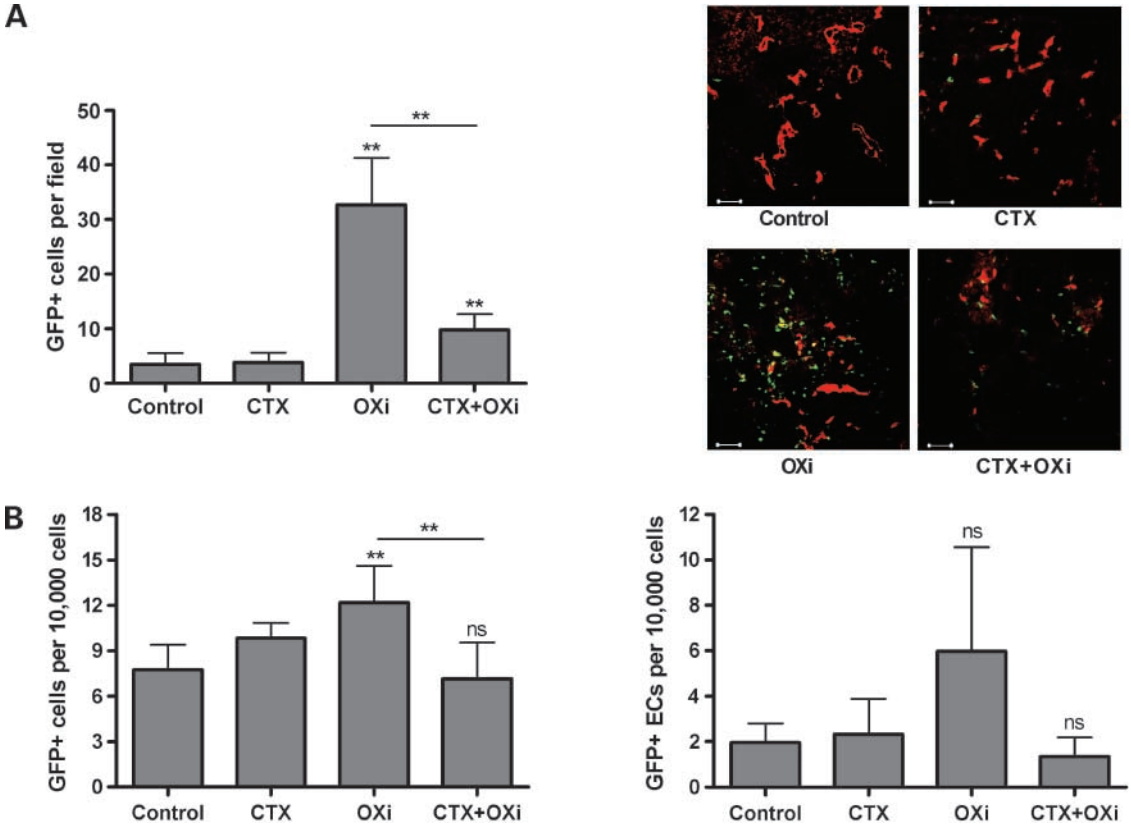

Figure 4. Homing of GFP+ bone marrow–derived cells to tumors. Lethally irradiated nude mice were reconstituted with GFP+ bone marrow, and 4 wk later 231/LM2-4 cells were orthotopically implanted. CTX and OXi4503 therapy was initiated when tumor volumes reached 400 mm^3. Three days after OXi4503 administration, tumors were removed and GFP+ cells homing to the tumors were analyzed on prepared sections (**A**); *bars,* 50 μm. Parts of the tumors were analyzed for the presence of GFP+ cells by flow cytometry (**B**, *left*). These tumor cells were stained with CD45, CD31, and VEGFR-2 to analyze bone marrow–derived GFP+ ECs by flow cytometry ('CEPs'; **B**, *right*). *Error bars,* +SD. Values are compared with untreated mice unless indicated otherwise. *, $0.01 < P < 0.05$; **, $P < 0.01$ with the use of two-tailed unpaired Student's *t* test. EC, endothelial cell.

Discussion

In this study we analyzed a new combination treatment strategy with the use of two mouse strains and two different tumor models. We report that combining a potent VDA (OXi4503) with metronomic chemotherapy with the use of cyclophosphamide led to inhibition of the OXi4503-induced CEP spike, which was accompanied by a marked growth inhibition of primary orthotopically transplanted 231/LM2-4 and MeWo tumors in nude mice, compared with OXi4503 treatment alone. Comparable results were obtained in 231/LM2-4–bearing severe combined immunodeficient mice and in MeWo-bearing nude mice. Our results thus expand the list of biological therapeutic agents that can be combined successfully with LDM chemotherapy based on both preclinical studies, and recent or ongoing phase II clinical trials. With respect to preclinical studies, some notable examples include anti–VEGFR-2 mAbs (29), TNP-470 (30), sunitinib (31), tumor vaccines/immunotherapy (32), and trastuzumab (33), among others (15, 34). With respect to clinical trials, various LDM chemotherapy regimens have been evaluated in phase II clinical trials in combination with biological agents, such as bevacizumab (18, 19), aromatase inhibitors (e.g., letrozole; ref. 35), and cyclooxygenase-2 inhibitors (e.g., celecoxib; ref. 36). Over 40 ongoing or completed trials currently listed[6] also show the diversity of biological agents being tested in a variety of indications in combination with a number of different LDM chemotherapy drugs and protocols. Many of these trials involve LDM cyclophosphamide or LDM cyclophosphamide with methotrexate (37, 38).

Because our results suggest a potential new role for LDM chemotherapy (i.e., as part of a combination therapy with a VDA), they also indicate an alternative for the combination of a VDA with an antiangiogenic drug, such as bevacizumab. Combining VDAs with drugs targeting angiogenesis is a rational step because the regrowth from the viable tumor rim that remains after VDA therapy is driven by angiogenesis. Indeed, preclinical studies combining a VDA with a drug targeting the VEGF pathway have shown that the viable rim almost entirely disappears when the antiangiogenic drug is added, resulting in more potent antitumor effects (11). Furthermore, VDAs have been shown to cause a direct upregulation of VEGF (39), which could be host-derived as well as tumor-dependent (i.e., a consequence of the marked increase in intratumoral hypoxia induced by VDA treatment). This increased level of VEGF would be rendered ineffective as a proangiogenic effect by treatments that specifically block the VEGF pathway function.

However, there are some potential concerns about the use of a VDA with such a VEGF(R)-targeting agent. One is the high, if not excessive, potential costs that would be associated with a treatment involving two such biological anticancer agents (40). In addition, such combinations may exacerbate vascular-associated toxicities that are common to both drugs, such as hypertension or adverse cardiovascular events (41, 42). Although the ongoing phase II trial evaluating the VDA combretastatin A-4 phosphate with bevacizumab has thus far shown surprisingly mild side effects (13), only a limited amount of patients have been treated, and it remains to be determined whether larger randomized phase II or III trials will confirm this result. Finally, a third concern is that resistance may develop rapidly to an antiangiogenic drug targeting a single proangiogenic pathway, because tumors can produce multiple compensatory proangiogenic growth factors (43). In this regard, when combined with a VDA, metronomic chemotherapy could conceivably have several benefits over targeted antiangiogenic agents, at least in theory. Importantly, the use of off-patent drugs, such as cyclophosphamide, would reduce costs significantly. LDM cyclophosphamide at a dose of 50 mg/d costs about 10 U.S. dollars per month (17). Furthermore, it has the benefit of p.o. administration. Because of its favorable toxicity profile, it is less likely to cause potential synergistic serious toxicities when combined with a VDA. Finally, with respect to the issue of acquired resistance to targeted antiangiogenic drugs as discussed above, including bevacizumab (43), there will be a need for second-line drugs to be used in combination with a VDA. Metronomic chemotherapy could potentially fulfill such a role.

CEP levels can be used as a surrogate marker for angiogenic activity in mice (28). In a clinical setting, the levels of both CEPs and mature circulating endothelial cells are increased in the blood of cancer patients, and correlate with angiogenesis and tumor volume, therefore potentially serving as a biomarker to determine progressive disease, prognosis, and response to therapy (44). In addition to mobilizing CEPs, other bone marrow–derived cells might be induced by VDAs, such as Tie-2–expressing monocytes, CD11b+Gr1+ myeloid cells, mesenchymal stem cells, and VEGFR-1+ hematopoietic progenitor cells (45). Corecruitment of VEGFR-1+ hematopoietic progenitor cells, together with CEPs, has been shown to stabilize tumor vasculature and facilitate CEP incorporation (46). Interestingly, in this regard we have recently found that OXi4503 not only mobilizes VEGFR-2+ CEPs but also leads to an increase in circulating bone marrow–derived VEGFR-1+ hematopoietic progenitor cells.[7] Prolonged repetitive exposure to a low dose of cyclophosphamide seems to specifically target (VEGFR-2+) CEPs. Thus, VEGFR-1+ cells that are mobilized by OXi4503 may not be inhibited by metronomic cyclophosphamide. It would be of interest to assess whether adding antibodies against VEGFR-1+ hematopoietic progenitor cells to our combination treatment could improve tumor response. Additionally, VEGFR-1+ cells have been suggested to play a role in the first steps of the development of metastasis by creating a premetastatic niche in distant organs where tumor cells can home (47).

Gao et al. have shown that after these initiating steps, VEGFR-2+ CEPs control the angiogenic switch mediating

[6] www.clinicaltrials.gov

[7] Y. Shaked, unpublished observation.

the progression of lung micrometastasis into macrometastasis (48). These results suggest differential roles for VEGFR-1+ hematopoietic progenitor cells and VEGFR-2+ CEPs in metastasis, and a need to block both VEGFR-1 and VEGFR-2 signaling. As reported in another study, only concurrent treatment with neutralizing antibodies against both VEGFR-1 and VEGFR-2 substantially suppressed the formation and growth of lung metastasis in a B16 melanoma model (49). It would be of interest to investigate the effects of our therapy in a metastatic model and compare this with a situation in which both VEGFR-1 and VEGFR-2 are blocked.

Although our results are consistent with the possibility that the improved tumor control achieved by combining metronomic cyclophosphamide with a VDA is due to the blockade of the acute CEP host response induced by the VDA, there may be other or additional mechanisms involved. These include a greater degree of direct killing of either vascular endothelial cells present in the tumor vasculature (because both types of therapy can cause endothelial cell death) or of the tumor cells themselves. Both types of killing therapy, which include VDAs, may cause some direct cytotoxic cell effects.

Finally, consideration should be given to the possibility that VDAs themselves may be given in a low(er)-dose metronomic fashion. The availability of p.o. VDAs, such as CYT997, makes this a potentially feasible prospect (50). One advantage of such an approach would be the reduced possibilities of acute cardiovascular toxicities induced by the VDA. In this regard, other p.o. microtubule inhibiting drugs, such as vinorelbine, are now being tested in LDM chemotherapy clinical trials.[8]

In summary, our results suggest that integration of metronomic chemotherapy with VDA treatment caused potently enhanced VDA-mediated antitumor efficacy when using various primary tumor models for therapy testing. These findings support further preclinical testing of the combination of a VDA with metronomic chemotherapy, such as through an approach that could possibly serve as a first-line or second-line alternative to targeted drugs, such as bevacizumab.

[8] www.clinicaltrials.gov

Disclosure of Potential Conflicts of Interest

R.S. Kerbel: consultant, Oxigene, Inc. and Taiho Pharmaceuticals, Inc. D.J. Chaplin: employee of OXiGENE Inc.; R.M. Hoffman: Chief Executive Officer of AntiCancer, Inc. No other potential conflicts of interest were disclosed.

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References

1. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 2004;3:391–400.

2. Kerbel RS. Tumor angiogenesis. N Engl J Med 2008;358:2039–49.

3. Motzer RJ, Michaelson MD, Redman BG, et al. Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 2006;24:16–24.

4. Escudier B, Eisen T, Stadler WM, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med 2007;356:125–34.

5. Llovet J, Ricci S, Mazzaferro V, et al. Sorafenib improves survival in advanced hepatocellular carcinoma (HCC): results of a phase III randomized placebo-controlled trial (SHARP trial). J Clin Oncol ASCO Annual Meeting Proceedings 2007;25;LBA1.

6. Tozer GM, Kanthou C, Baguley BC. Disrupting tumour blood vessels. Nat Rev Cancer 2005;5:423–35.

7. Ng QS, Goh V, Carnell D, et al. Tumor antivascular effects of radiotherapy combined with combretastatin a4 phosphate in human non-small-cell lung cancer. Int J Radiat Oncol Biol Phys 2007;67:1375–80.

8. Horsman MR, Siemann DW. Pathophysiologic effects of vascular-targeting agents and the implications for combination with conventional therapies. Cancer Res 2006;66:11520–39.

9. Siemann DW, Shi W. Efficacy of combined antiangiogenic and vascular disrupting agents in treatment of solid tumors. Int J Radiat Oncol Biol Phys 2004;60:1233–40.

10. Siemann DW, Shi W. Dual targeting of tumor vasculature: combining Avastin and vascular disrupting agents (CA4P or OXi4503). Anticancer Res 2008;28:2027–31.

11. Shaked Y, Ciarrocchi A, Franco M, et al. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 2006;313:1785–7.

12. Shaked Y, Henke E, Roodhart JM, et al. Rapid chemotherapy-induced acute endothelial progenitor cell mobilization: implications for antiangiogenic drugs as chemosensitizing agents. Cancer Cell 2008;14:263–73.

13. Nathan PD, Judson I, Padhani A, et al. A phase I study of combretastatin A4 phosphate (CA4P) and bevacizumab in subjects with advanced solid tumors. J Clin Oncol 2008;26 suppl:ASCO:Abstract 3550.

14. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 2000;105:1045–7.

15. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 2004;4:423–36.

16. Bertolini F, Paul S, Mancuso P, et al. Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. Cancer Res 2003;63:4342–6.

17. Bocci G, Tuccori M, Emmenegger U, et al. Cyclophosphamide-methotrexate 'metronomic' chemotherapy for the palliative treatment of metastatic breast cancer. A comparative pharmacoeconomic evaluation. Ann Oncol 2005;16:1243–52.

18. Dellapasqua S, Bertolini F, Bagnardi V, et al. Metronomic cyclophosphamide and capecitabine combined with bevacizumab in advanced breast cancer. J Clin Oncol 2008;26:4899–905.

19. Garcia AA, Hirte H, Fleming G, et al. Phase II clinical trial of bevacizumab and low-dose metronomic oral cyclophosphamide in recurrent ovarian cancer: a trial of the California, Chicago, and Princess Margaret Hospital phase II consortia. J Clin Oncol 2008;26:76–82.

20. Munoz R, Man S, Shaked Y, et al. Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. Cancer Res 2006;66:3386–91.

21. Yang M, Reynoso J, Jiang P, Li L, Moossa AR, Hoffman RM. Transgenic nude mouse with ubiquitous green fluorescent protein expression as a host for human tumors. Cancer Res 2004;64:8651–6.

22. Man S, Bocci G, Francia G, et al. Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. Cancer Res 2002;62:2731–5.

23. Hill SA, Toze GM, Pettit GR, Chaplin DJ. Preclinical evaluation of the antitumour activity of the novel vascular targeting agent Oxi 4503. Anticancer Res 2002;22:1453–8.

24. Monestiroli S, Mancuso P, Burlini A, et al. Kinetics and viability of

circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. Cancer Res 2001;61:4341–4.

25. Philpott NJ, Turner AJ, Scopes J, et al. The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. Blood 1996;87:2244–51.

26. Rijken PF, Bernsen HJ, Peters JP, Hodgkiss RJ, Raleigh JA, Van Der Kogel AJ. Spatial relationship between hypoxia and the (perfused) vascular network in a human glioma xenograft: a quantitative multi-parameter analysis. Int J Radiat Oncol Biol Phys 2000;48:571–82.

27. Shaked Y, Emmenegger U, Man S, et al. Optimal biologic dose of metronomic chemotherapy regimens is associated with maximum antiangiogenic activity. Blood 2005;106:3058–61.

28. Shaked Y, Bertolini F, Man S, et al. Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis; implications for cellular surrogate marker analysis of antiangiogenesis. Cancer Cell 2005;7:101–11.

29. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 2000;105:R15–24.

30. Browder T, Butterfield CE, Kraling BM, et al. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 2000;60:1878–86.

31. Pietras K, Hanahan D. A multitargeted, metronomic, and maximum-tolerated dose "chemo-switch" regimen is antiangiogenic, producing objective responses and survival benefit in a mouse model of cancer. J Clin Oncol 2005;23:939–52.

32. Hermans IF, Chong TW, Palmowski MJ, Harris AL, Cerundolo V. Synergistic effect of metronomic dosing of cyclophosphamide combined with specific antitumor immunotherapy in a murine melanoma model. Cancer Res 2003;63:8408–13.

33. Francia G, Emmenegger U, Lee CR, et al. Long-term progression and therapeutic response of visceral metastatic disease non-invasively monitored in mouse urine using β-human choriogonadotropin secreting tumor cell lines. Mol Cancer Ther 2008;7:3452–9.

34. Scharovsky OG, Mainetti LE, Rozados VR. Metronomic chemotherapy: changing the paradigm that more is better. Curr Oncol 2009;16:7–15.

35. Bottini A, Generali D, Brizzi MP, et al. Randomized phase II trial of letrozole and letrozole plus low-dose metronomic oral cyclophosphamide as primary systemic treatment in elderly breast cancer patients. J Clin Oncol 2006;24:3623–8.

36. Buckstein R, Kerbel RS, Shaked Y, et al. High-dose celecoxib and metronomic "low-dose" cyclophosphamide is an effective and safe therapy in patients with relapsed and refractory aggressive histology non-Hodgkin's lymphoma. Clin Cancer Res 2006;12:5190–8.

37. Colleoni M, Orlando L, Sanna G, et al. Metronomic low-dose oral cyclophosphamide and methotrexate plus or minus thalidomide in metastatic breast cancer: antitumor activity and biological effects. Ann Oncol 2006; 17:232–8.

38. Orlando L, Cardillo A, Ghisini R, et al. Trastuzumab in combination with metronomic cyclophosphamide and methotrexate in patients with HER-2 positive metastatic breast cancer. BMC Cancer 2006;6:225.

39. Shaked Y, Tang T, Woloszynek J, et al. Contribution of granulocyte colony-stimulating factor to the acute mobilization of endothelial precursor cells by vascular disrupting agents. Cancer Res 2009;69:7524–8.

40. Kolata G, Pollack A. Costly cancer drug offers hope, but also a dilemma. The New York Times 2008;Jul 6;1.

41. Beerepoot LV, Radema SA, Witteveen EO, et al. Phase I clinical evaluation of weekly administration of the novel vascular-targeting agent, ZD6126, in patients with solid tumors. J Clin Oncol 2006;24: 1491–8.

42. Cooney MM, Radivoyevitch T, Dowlati A, et al. Cardiovascular safety profile of combretastatin a4 phosphate in a single-dose phase I study in patients with advanced cancer. Clin Cancer Res 2004;10:96–100.

43. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 2008;8:592–603.

44. Roodhart JM, Langenberg MH, Daenen LG, Voest EE. Translating preclinical findings of (endothelial) progenitor cell mobilization into the clinic; from bedside to bench and back. Biochim Biophys Acta 2009;1796: 41–9.

45. Rafii S, Lyden D, Benezra R, Hattori K, Heissig B. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat Rev Cancer 2002;2:826–35.

46. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194–201.

47. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 2005;438:820–7.

48. Gao D, Nolan DJ, Mellick AS, Bambino K, McDonnell K, Mittal V. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. Science 2008;319:195–8.

49. Gille J, Heidenreich R, Pinter A, et al. Simultaneous blockade of VEGFR-1 and VEGFR-2 activation is necessary to efficiently inhibit experimental melanoma growth and metastasis formation. Int J Cancer 2007; 120:1899–908.

50. Francesconi A, Kotasek D, Burge M, Smith G, Lickliter J. Phase I evaluation of orally-administered CYT997, a novel cytotoxic vascular-disrupting agent, in patients with advanced cancer. J Clin Oncol ASCO Annual Meeting Proceedings 2009;27:3568.