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Low-Dose Metronomic Cyclophosphamide Combined with Vascular Disrupting Therapy Induces Potent Anti-Tumor Activity in Preclinical Human Tumor Xenograft Models

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Abstract

Purpose—Vascular disrupting agents (VDAs) 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 via mobilization and tumor colonization of circulating endothelial progenitor cells (CEPs). Co-treatment with an agent that blocks CEPs, such as VEGF-pathway targeting biologic antiangiogenic drugs, results in enhanced anti-tumor efficacy. We asked whether an alternative therapeutic modality – low-dose metronomic (LDM) chemotherapy could achieve the same result, given its CEP targeting effects.

Experimental Design—We studied the combination of the VDA OXi-4503 with daily administration of CEP-inhibiting, low-dose metronomic (LDM) cyclophosphamide to treat primary orthotopic tumors using the 231/LM2-4 breast cancer cell line and MeWo melanoma cell line. In addition, CEP mobilization and various tumor characteristics were assessed.

Results—We found that daily oral LDM cyclophosphamide was capable of preventing the CEP spike and tumor colonization induced by OXi-4503; this was associated with a decrease in the tumor rim and marked suppression of primary 231/LM2-4 growth in nude as well as SCID mice. Similar results were found in MeWo bearing nude mice. The delay in tumor growth was accompanied by significant decreases in micro-vessel density, perfusion and proliferation, and a significant increase in tumor cell apoptosis. No overt toxicity was observed.

Conclusions—The combination of OXi-4503 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.

Keywords

OXi-4503; low-dose cyclophosphamide; breast cancer; melanoma; endothelial progenitor cells

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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. Following the 2004 FDA approval of bevacizumab, a humanized monoclonal antibody 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 (RTKIs), sunitinib and sorafenib, are used as single agents in the treatment of advanced renal cell carcinoma (2,3,4). Sorafenib monotherapy has shown benefit in hepatocellular carcinoma as well (2,5). Both drugs target VEGF receptors and PDGF receptors, among other receptor tyrosine kinases.

Vascular disrupting agents (VDAs) 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 (CA4P) and its second generation prodrug derivative OXi-4503 (CA1P), 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 intra-tumoral 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, e.g. VDAs combined with radiation therapy (7) or maximum tolerated dose (MTD), conventional chemotherapy (8).

A prime example of a strategy that effectively enhances the anti-tumor activity of a VDA in a complementary manner is through combination with an antiangiogenic agent. Addition of a potent inhibitor of VEGF receptor 2-associated tyrosine kinase, ZD6474, to vascular disrupting agent 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 CA4P showed similar effects (10). A mechanistic rationale for the prolonged suppression of tumor growth using such drug combinations was recently provided by the results of studies from our lab. We have shown that mobilization into the bloodstream of bone marrow (BM)-derived CEPs, and possibly other types of BM-derived cells (BMDCs), takes place rapidly, within 4 hours, after treatment with OXi-4503 or CA4P. These cells subsequently invade and colonize the viable tumor rim, where they are incorporated into growing vessels and thus contribute to tumor regrowth. Administration of the antiangiogenic drug DC101, a rat monoclonal antibody blocking the mouse VEGF receptor 2, just prior to OXi-4503 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 EPC mobilization and the subsequent anti-tumor benefit gained by co-treatment with DC101 is not limited to VDAs, but is also observed when certain chemotherapeutics (eg taxanes) are administered at their MTD, implicating the clear possibility that this phenomenon might be more widely applicable (12).

Preliminary clinical studies have revealed results that appear to support, at least tentatively, our preclinical results with OXi-4503. 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 systemic BMDC-mediated vasculogenesis/angiogenesis, such as bevacizumab.

The concept of ‘metronomic’ chemotherapy (14), i.e. the frequent administration of chemotherapeutic agents at doses well below the maximum tolerated dose (MTD) 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 administered 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 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 (17), as is the safety profile of LDM cyclophosphamide (18,19).

Here we show that the combination of OXi-4503 with low-dose metronomic 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 (MVD), perfusion and proliferation. The viable rim that normally remains after VDA monotherapy was found to be smaller, which was accompanied by a decrease of BMDCs 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 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 treatment of established metastatic disease. 2×10^6 231/LM2-4 cells were injected orthotopically into the right inguinal mammary fat pads (MFP) of 6-week-old female immunodeficient athymic nude mice (Harlan Biotech Industries, USA), 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. 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 MFP of 6-week-old female CB-17 severe combined immunodeficient (SCID) mice. Tumor size was assessed regularly with Vernier calipers, using the formula $\text{length} \times \text{width}^2 \times 0.5$. When tumor size reached 400–500 mm³, treatment was initiated with either LDM cyclophosphamide, OXi-4503 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 mm³, and in accordance to 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 as per instructions of the manufacturer to a stock concentration of 20 mg/mL and administered via drinking water to provide a dose of 20 mg/kg/day, based on the estimated daily consumption of 3 mL for a 20-g mouse, as previously described (21). OXi-4503, a vascular microtubule disrupting agent, was administered intraperitoneally at a dose of 50 mg/kg, as described previously (22). For the

combination therapy, OXi-4503 was given 6 days after the start of LDM cyclophosphamide. For long-term treatment, 50 mg/kg OXi-4503 injection was repeated every two weeks.

Bone Marrow Transplantation

GFP+ bone marrow cells (10^7) isolated from femurs of GFP+ nude mice (23) were injected into the tail veins of 6–8 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, Utah).

Evaluation of Viable CEPs by Flow Cytometry

Blood was drawn from the retro-orbital sinus of anaesthetized mice. Viable CEPs were counted using five color flow cytometry. Briefly, monoclonal antibodies 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/VEGF receptor 2 (*flk-1/VEGFR-2*), CD13 (aminopeptidase N) and CD117 (c-kit) (BD Biosciences, San Diego, CA) (24). After red cell lysis, cell suspensions were analyzed on a LSR II (BD). 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 non-specific background staining, and 7-aminoactinomycin D (7AAD) was used to distinguish apoptotic and dead cells from viable cells (25).

Evaluation of a marker for Perfusion

For blood vessel perfusion analysis, 1 min before euthanasia mice were injected intravenously with the fluorescent, DNA-binding dye, Hoechst 33342 (40 mg/kg) (Sigma-Aldrich Canada Ltd., Oakville, ON Canada) (26). After euthanasia, tumors were removed and either fixed in 10% buffered formalin for 24 hours, 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 performed as described (26). Briefly, formalin-fixed, paraffin-embedded tumors were sectioned (5 μm thick) and stained with hematoxylin and eosin (H&E). Necrosis was detected as autofluorescence in the fluorescein isothiocyanate (FITC) channel. Tumor tissues were quantified for perfusion by analysis of Hoechst 33342 staining on cryosections. The microvessel density (MVD) was analyzed by immunostaining with an anti-CD31 antibody (1:200, BD Biosciences, San Diego, CA) and secondary Cy3-conjugated donkey anti-rat (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, ON, Canada), and secondary Texas-Red conjugated goat anti-rabbit (1:200, Jackson ImmunoResearch Laboratories Inc.). Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (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, ON, Canada), using bright field and the following fluorescence filters: DAPI (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 using 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 200x magnified images of tumor tissue sections stained with Hoechst 33342 and calculating the areas which are positively stained (blue) as a fraction of the total tumor area. Similarly, necrosis was assessed by analyzing 25x 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 magnification of 200x times and analysis was performed with Zeiss LSM Image Browser software, version 4,2,0,121. The number of vascular structures (CD31-positive) and cells (GFP-positive) per field were counted. The total number of positive cells or structures per field for each tumor sample was counted.

Analysis of tumors using 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 monoclonal antibodies against CD45, CD31 and VEGFR-2 (flk-1) markers (BD Pharmingen). Evaluation of positive cells was conducted using flow cytometry, and more than 150,000 events were collected for each sample tested.

Statistical Analysis

SPSS statistical packages version 12.0.1 were used to assess the statistical significance of differences in mean values. For each value, Levene's test was used to determine the equality of variances. Depending on the outcome of Levene's test, the two-tailed student's *t*-test or Wilcoxon test was used to assess the significance of the mean difference. Differences between designated groups compared to control-untreated group (unless indicated otherwise) were considered significant at values of $0.05 > P > 0.01$ (*) or $P < 0.01$ (**). Data are expressed as mean $\pm$ S.D.

Results

The combination of OXi-4503 with metronomic cyclophosphamide inhibits the CEP spike induced by OXi-4503

The administration of LDM cyclophosphamide suppresses levels of CEPs in peripheral blood of human tumor-bearing mice, i.e. lymphoma-bearing mice (16) and melanoma-bearing mice (27) even within one week of daily treatment (16). We asked whether the acute elevation in CEPs found 4 hours after administration of OXi-4503 can be inhibited by prior treatment with LDM cyclophosphamide. We reasoned that in order to prevent this rapidly-induced EPC spike, CEP levels had to be suppressed by LDM cyclophosphamide at the time of administration of OXi-4503. Therefore, we evaluated whether 6 days of daily metronomic cyclophosphamide

administered continuously through the drinking water (21) was sufficient to suppress CEP levels. Consistent with previously published results we found that it did so (data not shown). Next, nude mice were treated with daily low-dose cyclophosphamide for 6 days via the drinking water at an initial dose of 20 mg/kg/day, at which time OXi-4503 was administered intraperitoneally in a non-toxic dose of 50 mg/kg. Analysis of viable CEPs after 4 hours showed that treatment with OXi-4503 increases CEP levels in the peripheral blood ($p < 0.001$; Fig 1), in accordance with previous studies (11). However, pre-treatment 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 to control mice). In non-tumor bearing nude mice, however, cyclophosphamide alone did not significantly suppress CEP levels compared to control, which can be attributed to the extremely low CEP levels in untreated nude mice (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 OXi-4503 (data not shown).

The combination of OXi-4503 with metronomic cyclophosphamide delays primary tumor growth without overt toxicity

Next, we asked whether the CEP suppression that is 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 mm³, 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 endpoint 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. OXi-4503 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 OXi-4503, when compared to the tumor size at time of initiation of therapy ($p = 0.037$ from day 55 on, paired t-test). In contrast, the combination of cyclical OXi-4503 and continuous daily metronomic cyclophosphamide showed a striking anti-tumor activity with no significant signs of regrowth during the first four weeks of therapy ($p > 0.1$ n.s., paired t-test), resulting in a significant benefit over OXi-4503 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 administered, tumor control was achieved during a prolonged period of time (data not shown). No overt toxicity was observed in comparison to OXi-4503 alone, as measured by regular assessments of body weight (Fig 2B).

Comparable effects of the combination of OXi-4503 and cyclophosphamide in different tumor and mice models

The effect of our treatment on tumor growth was subsequently analyzed in CD17 SCID mice. Similar treatment effects were seen. LDM cyclophosphamide monotherapy only delayed tumor growth by a few days (fig 2C), whereas OXi-4503 monotherapy was again associated with initial tumor control followed by potent regrowth within two treatment cycles ($p < 0.04$ from day 39 on, paired t-test). OXi-4503 treated mice had to be sacrificed 23 days after tumor implantation, because the tumor endpoint was reached. When OXi-4503 was combined with metronomic cyclophosphamide, a prolonged anti-tumor effect was seen, even resulting in significant tumor size reduction (fig 2C; $p < 0.05$ on day 29, 34, 39, 47 and 61, paired t-test). From day 39 on, there is a significant difference between both 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 OXi-4503 and metronomic cyclophosphamide increases tumor hypoxia, necrosis and apoptosis, while decreasing microvessel density and proliferation

To further characterize the anti-tumor effect of the combination treatment, 231/LM2-4 tumors were grown in nude mice and treated when tumor size reached 400 mm³. Three days after administration of OXi-4503, tumors were removed for immunohistochemistry to assess microvessel density (MVD), perfusion, apoptosis, proliferation and necrosis. As shown in figure 3A, both LDM cyclophosphamide and OXi-4503 monotherapy diminished MVD (both $p=0.001$), an effect that could be significantly enhanced by combining OXi-4503 with LDM cyclophosphamide ($p=0.01$). Perfusion was unchanged in mice treated with OXi-4503 alone ($p=0.26$), whereas the addition of LDM cyclophosphamide significantly lowered tumor perfusion (Fig 3A right panel, $p=0.046$). This was accompanied by significant increases in tumor cell apoptosis as measured by TUNEL assay (Fig 3B, left panel, $p=0.043$), and decreases in proliferation rates as measured by Ki-67 staining (Fig 3B, right panel, $p=0.047$). Since the 231/LM2-4 tumors show some necrosis even when untreated, the increases in necrosis after OXi-4503 treatment were modest compared to previously studied tumor models (e.g. Lewis Lung Carcinoma in C57Bl/6 mice or MeWo human melanoma model in athymic nude mice (11)), but nevertheless a clear trend towards increased level of necrosis was observed when the two treatments were combined (Fig 3C; $p=0.002$ as compared to control). As shown in Figure 3C, the viable rim, even though present, was considerably reduced after addition of cyclophosphamide to OXi-4503.

Combination of OXi-4503 and metronomic cyclophosphamide is capable of diminishing homing of GFP+ bone marrow cells to tumors

Since we have previously reported that the administration of the mouse anti-VEGFR-2 monoclonal antibody DC101 24 hours prior to OXi-4503 improves anti-tumor activity, at least in part, by blocking the mobilization and subsequent homing of CEPs and possibly other BMDCs to the viable tumor rim (11), we analyzed whether pre-treatment 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-positive nude donor mice (23). Once again, tumors were allowed to reach 400 mm³ before treatment was initiated. Analysis of the tumors by immunohistochemistry indeed revealed a significant increase in the number of bone marrow cells homing to the viable rim in mice treated with OXi-4503 alone (Fig 4A, $p<0.001$). The co-treatment with LDM cyclophosphamide resulted in a significant decrease of BMDCs homing to the tumor ($p<0.001$ compared to OXi-4503 alone). Furthermore, to confirm these results, FACS analysis was performed on single cell suspensions prepared by enzymatic digestion of portions of the tumors (Fig 4B, left panel). Staining of these cells with antibodies against CD45, CD31 and VEGFR-2 showed a decrease in GFP+ ECs ('CEPs') homing to the tumor (Fig 4B, right panel). However, analysis of both immunohistochemistry and FACS 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 rim (Fig 3C). One explanation for this is our finding that LDM cyclophosphamide is unable to completely block CEP mobilization (fig 1A). Post-treatment levels in peripheral blood and tumors are comparable to 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 OXi-4503 and cyclophosphamide can inhibit systemic angiogenesis/vasculogenesis to an extent sufficient to significantly improve the effect of OXi-4503 when treating primary tumors.

Discussion

In this study we analyzed a new combinatorial treatment strategy using two mouse strains and two different tumor models. We report that combining a potent VDA (OXi-4503) with metronomic chemotherapy using cyclophosphamide led to an inhibition of the OXi-4503 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 to OXi-4503 treatment alone. Comparable results were obtained in 231/LM2-4 bearing SCID mice and in MeWo bearing nude mice. Our results thus expand the list of biologic therapeutic agents which 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 monoclonal antibodies (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 biologic agents such as bevacizumab (18,19), aromatase inhibitors, e.g. letrozole (35) and COX-2 inhibitors, e.g. celecoxib (36). Almost 40 ongoing or completed trials currently listed in the website www.clinicaltrials.gov also show the diversity of biologic 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 low-dose metronomic chemotherapy, i.e., as part of a combination therapy with a VDA, they also implicate an alternative for the combination of a VDA with an anti-angiogenic drug, such as bevacizumab. Combining VDAs with drugs targeting angiogenesis is a rational step, since the regrowth from the viable 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 anti-tumor 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 pro-angiogenic effect by treatments which specifically block VEGF pathway function.

However, there are some potential concerns regarding 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 anti-cancer 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). Even though the ongoing phase II trial evaluating the VDA CA4P 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 a targeted antiangiogenic drug targeting a single pro-angiogenic pathway, as a result of tumors producing multiple compensatory pro-angiogenic 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; low-dose metronomic cyclophosphamide at a dose of 50 mg per day costs about USD 10 per month (17). Furthermore, it has the benefit of oral administration and 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, levels of both CEPs and mature circulating endothelial cells (CECs) 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 (TEMs), CD11b+Gr1+ myeloid cells, mesenchymal stem cells (MSCs) and VEGFR1+ hematopoietic progenitor cells (HPCs) (45). Co-recruitment of VEGFR-1+ HPCs together with CEPs has been shown to stabilize tumor vasculature and facilitate CEP incorporation (46). Interestingly, in this regard we have recently found that OXi-4503 not only mobilizes VEGFR2+ CEPs, but also leads to a rise in circulating bone marrow-derived VEGFR1+ HPCs¹. Prolonged repetitive exposure to a low dose of cyclophosphamide appears to specifically target (VEGFR2+) CEPs. Thus, VEGFR1+ cells that are mobilized by OXi-4503 may not be inhibited by metronomic cyclophosphamide. It would be of interest to assess whether adding antibodies against VEGFR1+ HPCs to our combination treatment could perhaps improve tumor response. Additionally, VEGFR1+ cells have been suggested to play a role in the first steps of the development of metastasis by creating a pre-metastatic niche in distant organs, where tumor cells can home (47). Gao et al have shown that after these initiating steps, VEGFR2+ CEPs control the angiogenic switch mediating the progression of lung micrometastasis into macrometastasis (48). These results suggest differential roles for VEGFR1+ HPCs and VEGFR2+ CEPs in metastasis, and a need to block both VEGFR1 and VEGFR2 signaling, as reported in another study showing that only concurrent treatment with neutralizing antibodies against both VEGFR1 and VEGFR2 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 to a situation where both VEGF receptors 1 and 2 are blocked.

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

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

In summary, our results suggest that integration of metronomic chemotherapy with VDA treatment caused potentially enhanced VDA-mediated anti-tumor 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, e.g. using an approach that could possibly serve as a first or second line alternative for targeted drugs, such as bevacizumab.

¹Y. Shaked, unpublished observations

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Figure 1. Evaluation of viable CEP levels in mice treated with metronomic cyclophosphamide (CTX), OXi-4503 (OXi) or a combination of both drugs

Non-tumor bearing nude mice (n=5 per group) were treated with LDM cyclophosphamide (CTX), and OXi-4503. Untreated mice and mice treated with either of the drugs alone were used as controls. 4 hours after OXi-4503 administration, blood was drawn for the evaluation of viable CEPs using five color flow cytometry. Error bars + SD; * 0.01 < p < 0.05; ** p < 0.01 using student's t-test unpaired. Values are compared to control mice, unless indicated otherwise.

Figure 2. Evaluation of anti-tumor effect and body weight in mice treated with metronomic cyclophosphamide (CTX), OXi-4503 (OXi) or a combination of both drugs

(A) Tumor volume of nude mice (n=5 per group) orthotopically implanted with 231/LM2-4 cells, treated with CTX and OXi-4503.

(B) Body weight of nude mice bearing 231/LM2-4 tumors treated with CTX and OXi-4503 (n=5 per group).

(C) Tumor volume of CB-17 SCID mice (n=5 per group) orthotopically implanted with 231/LM2-4 cells, treated with CTX and OXi-4503.

(D) Nude mice (n=4 per group) subdermally implanted with MeWo cells, treated with CTX and OXi-4503.

Grey arrows, start continuous CTX administration; black arrows, OXi-4503 administration.

All error bars + SD. CTX + OXi-4503 values are compared to OXi-4503 alone. * 0.01 < p < 0.05 ; ** p < 0.01 using student's t-test two-tailed unpaired.

Figure 3. Analysis of MVD, perfusion, necrosis, tumor cell proliferation and apoptosis in tumors grown in nude mice after treatment with cyclophosphamide (CTX) and OXi-4503 (OXi) 231/LM2-4 bearing nude mice (400 mm³) were treated with LDM CTX and OXi-4503. Three days after OXi-4503 administration, tumors were harvested and sections were prepared for the assessment of (A) MVD in red and perfusion in blue, scale bar 50 μ m; (B) tumor cell proliferation in red and apoptosis in green, scale bar 50 μ m; (C) necrosis in green on H&E staining, scale bar 100 μ m. Error bars + SD. Values are compared to untreated mice, unless indicated otherwise. * 0.01 < p < 0.05; ** p < 0.01 using student's t-test two-tailed unpaired.

Figure 4. Homing of GFP+ bone marrow-derived cells to tumors

Lethally irradiated nude mice were reconstituted with GFP+ bone marrow and 4 weeks later 231/LM2-4 cells were orthotopically implanted. CTX and OXi-4503 therapy was initiated when tumor volumes reached 400 mm³. Three days after OXi-4503 administration, tumors were removed and GFP+ cells homing to the tumors were analyzed on prepared sections (A), Scale bars, 50 μ m; Parts of the tumors analyzed for the presence of GFP+ cells by flow cytometry (B, left panel). These tumor cells were strained with CD45, CD31 and VEGFR2 to analyze bone marrow-derived GFP+ ECs by flow cytometry ('CEPs' B, right panel). Error bars + SD. Values are compared to untreated mice, unless indicated otherwise. * 0.01 < p < 0.05; ** p < 0.01 using student's t-test two-tailed unpaired.