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Advances in Brief

Maximum Tolerable Dose and Low-Dose Metronomic Chemotherapy Have Opposite Effects on the Mobilization and Viability of Circulating Endothelial Progenitor Cells[1]

Francesco Bertolini,[2] Saki Paul, Patrizia Mancuso, Silvia Monestiroli, Alberto Gobbi, Yuval Shaked, and Robert S. Kerbel

Division of Hematology-Oncology, Department of Medicine [F. B., S. P., P. M.] and Department of Experimental Oncology, IFOM-Fondazione Italiana per la Ricerca sul Cancro Institute of Molecular Oncology [S. M., A. G.], European Institute of Oncology, 20141 Milan, Italy; Molecular and Cell Biology Research, Sunnybrook and Women's College Health Sciences Centre, Department of Medical Biophysics, University of Toronto, Toronto, Ontario, M4N 3M5 Canada [Y. S., R. S. K.]

Abstract

There is growing evidence that vasculogenesis (progenitor cell-derived generation of new blood vessels) is required for the growth of some neoplastic diseases. Here we show that the administration of cyclophosphamide (CTX) at the maximum tolerable dose with 21-day breaks or at more frequent low-dose (metronomic) schedules have opposite effects on the mobilization and viability of circulating endothelial progenitors (CEPs) in immunodeficient mice bearing human lymphoma cells. Animals treated with the maximum tolerable dose CTX experienced a robust CEP mobilization a few days after the end of a cycle of drug administration, and tumors rapidly became drug resistant. Conversely, the administration of metronomic CTX was associated with a consistent decrease in CEP numbers and viability and with more durable inhibition of tumor growth. Our findings suggest that metronomic low-dose chemotherapy regimens are particularly promising for avoiding CEP mobilization and, hence, to potentially reduce vasculogenesis-dependent mechanisms of tumor growth.

Introduction

A number of recent experimental observations have suggested that the growth of some types of cancer may depend on vasculogenesis (*i.e.*, progenitor-cell dependent generation of new blood vessels) and not just angiogenesis (*i.e.*, mature endothelial-cell dependent generation of new blood vessels) alone. The presence of marrow-derived CEP[3] able to participate in adult vasculogenesis was first demonstrated by Asahara *et al.* (1) in a murine model and by Shi *et al.* (2) in a canine bone marrow transplantation model in which a dacron graft was colonized by donor endothelial cells. Regarding neoplastic diseases, the first proof of principle of the pivotal role of CEP in tumor growth was recently obtained in the $Id1^{+/-}$ $Id3^{-/-}$ murine model (3). In these mice, most tumors failed to grow progressively, if at all, and any tumor growth present remained at a quiescent state because of the lack of an adequate vascularization. Conversely, in $Id1^{+/-}$ $Id3^{-/-}$ mice transplanted with wild-type $Id1^{+/+}$ $Id3^{+/+}$ bone marrow cells, vasculogenesis and tumor progression were similar to wild-type mice. In addition to these studies, de Bont *et al.* (4) found that mobilized human CD34+ progenitor cells enhance tumor growth in a preclinical murine lymphoma model, Bolontrade *et al.* (5) demonstrated that CEP-induced vasculogenesis plays a role in a preclinical murine model of Ewing's sarcoma, and Shirakawa *et al.* (6) provided evidence of postnatal vasculogenesis in breast-cancer-bearing mice. Along this line, we have demonstrated recently that CECs and their CEP subsets increase in some preclinical cancer models (7–8), as well as in cancer patients (9).

It is well known that during the break periods between successive cycles of MTD chemotherapy, including CTX, there can be a marked mobilization of hematopoietic progenitors from the marrow into the peripheral blood circulation, as part of an adaptive response to the chemotherapy induced myelosuppression (10). This raises several questions. First, would the levels of CEPs mobilized also show a similar decrease followed by a rapid recovery and increase shortly after MTD chemotherapy; second, might it be the case that such increase are not observed when the same drug is administered more frequently at lower doses, *i.e.*, metronomically? If so, this might be suggestive of a mechanism by which low-dose metronomic chemotherapy regimens suppress angiogenesis without causing an increase in the severity of undesirable side effects normally associated with traditional cytotoxic chemotherapy regimens, such as myelosuppression (11–13). It may also explain part of the basis of the robust repair process to damaged tumor endothelial cells that takes place during the long break periods between successive cycles of MTD chemotherapy (11).

The purpose of this study was to investigate the effects of MTD *versus* metronomic regimens of the same chemotherapeutic drug (CTX) on CEP kinetics and viability using two preclinical models of human lymphoma xenografts. In particular, we were interested in evaluating whether one of the mechanisms of the angiogenic effects of metronomic CTX might be a sustained suppression of the mobilization and/or viability of bone-marrow-derived CEPs. We found strikingly different effects on MTD *versus* metronomic CTX on CEP mobilization and viability, which could have implications for the clinical use of metronomic chemotherapy regimens should similar findings apply in patients.

Materials and Methods

Animal Models. NOD/SCID mice of 6–8 weeks of age received i.p. injections of 10×10^6 Namalwa or Granta 519 cells (ATCC, Manassas, VA) as we have described previously (7–8, 14). Namalwa cells derived from an EBV+ Burkitt's lymphoma, Granta 519 cells were derived from a mantle cell lymphoma in leukemic transformation. Animals were evaluated for tumor growth every other day, tumors were measured by calipers, and the formula [width^2 $\times$ length $\times$ 0.52] was applied for approximating the volume of a spheroid (7–8). Tumor-bearing NOD/SCID mice ($n = 12$ per study group), were treated with CTX at the MTD for NOD/SCID mice (75 mg/kg on days 3,

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[2] To whom requests for reprints should be addressed, at Division of Hematology-Oncology European Institute of Oncology via Ripamonti 435, 20141 Milan, Italy. Phone: 39-02-57489535; Fax: 39-02-57489537; E-mail: francesco.bertolini@ieo.it.

[3] The abbreviations used are: CEP, circulating endothelial progenitor; CTX, cyclophosphamide; MTD, maximum tolerable dose; CEC, circulating endothelial cell; VEGF, vascular endothelial growth factor; FC, flow cytometry; 7AAD, 7-aminoactinomycin D.

5 and 7, or 225 mg/kg/cycle of therapy repeated every 21 days; see Ref. 12) or a metronomic low-dose schedule dose according to Browder *et al.* (170 mg/kg every 6 days; see Ref. 11) i.p. in a site remote from the inoculated tumor or a metronomic low-dose schedule of CTX in drinking water according to Man *et al.* (~20 mg/kg/day, Ref. 12). Circulating levels of VEGF were measured by a commercially available assay as reported previously (7).

All procedures involving animals were performed in accordance with national and international laws and policies.

CEC and CEP Measurement by FC. Mice were bled from retro-orbital sinus for CEC and CEP evaluation, which was performed by enumeration using four-color FC. Monoclonal antibodies reacting with CD45 were used to exclude hematopoietic cells; CECs and their CEP subset were depicted as described previously (7–8) using the endothelial murine markers VEGF receptor 2 fetal liver kinase 1, CD13, and CD117 (PharMingen BD, San Diego, CA). Nuclear staining (Procount; BD Biosciences, San Jose, CA) was used on some occasions to exclude the possibility that platelets or cell debris hampered the accuracy of CEC and CEP enumeration (8–9). After RBC lysis, cell suspensions were evaluated by a FACSCalibur (BD Biosciences) using analysis gates designed to exclude dead cells, platelets, and debris. After acquisition of at least 100,000 cells/sample, analyses were considered as informative when adequate numbers of events (*i.e.,* >50, typically 100–200) were collected in the CEC and CEP enumeration gates. Percentages of stained cells were determined and compared with appropriate negative controls. Positive staining was defined as being greater than nonspecific background staining, and 7AAD was used to enumerate viable, apoptotic, and dead cells (15).

Evaluation of CEP Clonogenic Potential. At the time of sacrifice, animals were evaluated for the presence of clonogenic CEP as we described previously (8). A total of 0.5×10^6 nucleated cells were seeded in Petri dishes coated previously with fibronectin in the presence of collagen gel, EC medium, 12.5% FCS and 12.5% horse serum supplemented with VEGF (100 ng/ml) and basic fibroblast growth factor (5 ng/ml). Cells were cultured at 37°C. After 2 weeks of culture, colonies with endothelial morphology (elongated, sprouting or spindle cells) were enumerated and sub-clones established by picking colonies and resuspending the cells in the presence of VEGF and basic fibroblast growth factor. Fresh medium and cytokines were added weekly. After a 4-week culture, when seeded cells showed endothelial characteristics (patterned, tubular networks, sometimes multinucleate cells), the endothelial phenotype ($CD45^-$, $CD31^+$) of cultured cells was evaluated and confirmed by FC.

Statistical Analysis. Statistical comparisons were performed using the *t* test, ANOVA, and linear regression when data were normally distributed, and the nonparametric analyses of Spearman and Mann-Whitney when data were not normally distributed. Values of $P < 0.05$ were considered statistically significant.

Results

CEC and CEP enumeration by FC are depicted in Fig. 1. In our previous work (7–8), CECs were enumerated as $CD45^-VEGFR2^+$ cells, and the CEP subset was enumerated as $CD45^-VEGFR2^+CD117^+$ cells. Consistently with what we have observed previously (8), CD117 expression was found in 29–88% of CEC. In the present study, we found that the frequency of $CD45^-VEGFR2^+CD117^+$ CEP was similar to that of $CD45^-CD13^+$ $CD117^+$ CEP (Fig. 1, *D* and *E*). Furthermore, as shown in Fig. 1, >98% of $CD45^-CD13^+$ CECs were $CD45^-VEGFR2^+$. In addition to the hematopoietic compartment, CD13 is also found in non-hematopoietic tissues, and its novel expression by endothelial cells of angiogenic, but not normal, vasculature has been described recently (16). Treatment of animals with CD13 inhibitors significantly impaired neovascularization and xenograft tumor growth, indicating that CD13 plays an important functional role in vasculogenesis and identifying it as a critical regulator of angiogenesis (16). $VEGFR2^+CD13^+$ cells included 40–100% of the $CD45^-$ cell population. In the $CD45^-$ cell fraction, the frequency of cells that expressed VEGFR2 but not CD13 (or *vice versa*) was always <5%. Regarding CD117 (c-kit), the crucial role of c-kit expression in hematopoietic and endothelial progenitors has been recently underlined by Heissig *et al.* (17).

To study CEP viability, considering that a FITC-conjugated anti-VEGFR2 antibody was not available for the present studies, we enumerated viable CEP as $CD45^-CD13^+$ $CD117^+7AAD^-$ cells. The relative frequency of CEC and CEP in tumor-free NOD/SCID mice evaluated as controls and in tumor-bearing mice evaluated at the time

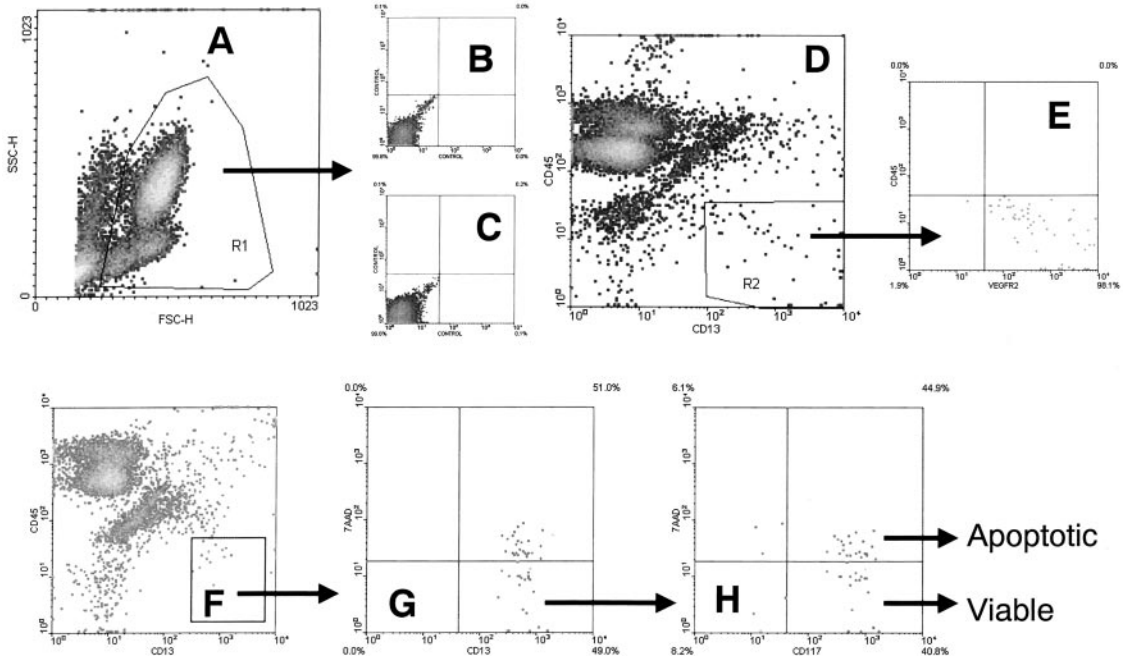

Fig. 1. *A–G*, representative FC evaluation of CEC and CEP enumeration and viability. *A* shows the gate used to exclude platelets, dead cells, and debris. *B* shows the Ig-PE and Ig-PerCP negative controls. *C* shows the Ig-FITC and Ig-PerCP negative controls. *D* and *E* show that >98% of $CD45^-CD13^+$ (*D*) CECs were $CD45^-$ $VEGFR2^+$ (*E*). *F* shows the gate on $CD45^-CD13^+$ cells used to evaluate CEC ($CD45^-CD13^+CD117^-$) and CEP ($CD45^-CD13^+CD117^+$) frequency and their viability according to 7AAD expression in *G* and *H*. *Panels* represent the same sample evaluated with negative controls (*A–C*), FITC-anti-CD13, PE-anti-VEGFR2, PerCP-anti-CD45, and APC-anti-CD117 (*D* and *E*), and FITC-anti-CD13, PE-anti-CD45, 7AAD, and APC-anti-CD117 (*F–H*).

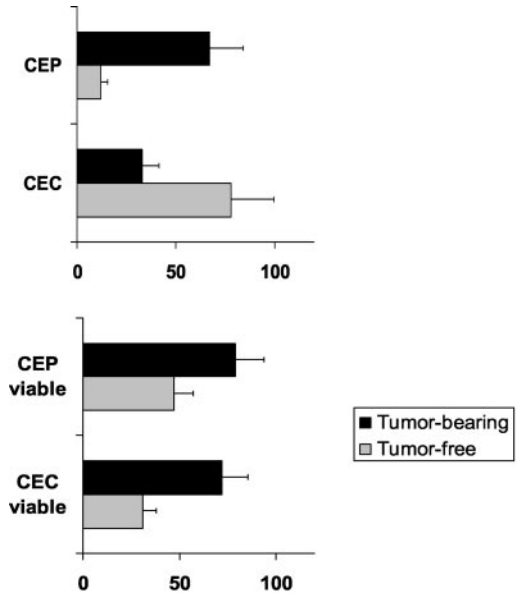

Fig. 2. *Top*, frequency of $CD117^-$ (*CEC*) and $CD117^+$ (*CEP*) $CD45^-CD13^+$ cells in tumor-free NOD/SCID mice evaluated as controls and in Namalwa tumor-bearing mice evaluated at sacrifice. *Bottom*, frequency of viable CEC and CEP in tumor-free and tumor-bearing NOD/SCID mice.

of sacrifice is indicated in Fig. 2. CEP frequency and viability was significantly increased in tumor-bearing animals ($P < 0.01$).

As shown in Figs. 3 (Namalwa-bearing mice) and 4 (Granta 519-bearing mice), CEP increase in untreated animals paralleled tumor growth in both models of human lymphoma. As described previously (7–9, 18), this might be attributable to the increase of tumor-derived VEGF production paralleling tumor growth in size. As we observed and reported in our previous study (7), a positive correlation was found in tumor-bearing mice between CEC and human VEGF ($r = 0.532$, $P = 0.04$). However, it is unlikely that the increase in VEGF levels can itself explain CEP mobilization kinetics. In fact, marrow homing and mobilization in the peripheral blood of hematopoietic and/or endothelial progenitors is known to rely on complex (and still poorly understood) molecular pathways where integrins and multiple cell-to-cell and cell-to-matrix machinery, in addition to cytokine and chemokine levels, play a dominant role (19–20). We are currently measuring VEGF levels in cancer patients treated with MTD and metronomic CTX to better understand the relative contribution of this cytokine in CEP mobilization.

In both lymphoma models, the first course of MTD CTX delayed but did not prevent tumor growth, and a dramatic increase in CEP was observed a few days after MTD CTX. The CEP increase after MTD was not attributable solely to tumor growth, because, in both models, its magnitude was significantly higher than that observed in control animals, which, at the same time, already had significantly larger tumors. In MTD CTX-treated animals, the subsequent courses of MTD CTX did not reduce tumor growth. In marked contrast, in metronomic CTX-treated animals, CEPs were not increased, and their viability was significantly reduced when compared with MTD-treated animals (Figs. 3 and 4).

In comparison with MTD-treated animals, tumor growth was significantly delayed in Namalwa-bearing mice treated with metronomic CTX administered daily in drinking water. Tumor growth was not observed up to day 40 (*i.e.*, 1 week after therapy discontinuation) in Namalwa-bearing mice treated with metronomic CTX administered on a weekly basis according the method of Browder *et al.* (170 mg/kg every 6 days; see Ref. 11). In these mice, when therapy was discontinued, CEP increase paralleled tumor growth, but the CEP mobilization effect observed after MTD CTX was not seen. In Granta 519-bearing mice, observed up to day 90, tumor growth (and, in turn, CEP increase) was abrogated by metronomic CTX [administered either according to Browder *et al.* (11) or according to Man *et al.* (12)], but not by MTD CTX.

In CEP clonogenic assays, cultures of tumor-bearing mice treated with MTD CTX and evaluated at sacrifice generated a mean of 14 ± 7 colonies. In sharp contrast, cultures of tumor-bearing mice treated with metronomic CTX generated a mean of 1 ± 2 colonies ($P = 0.001$).

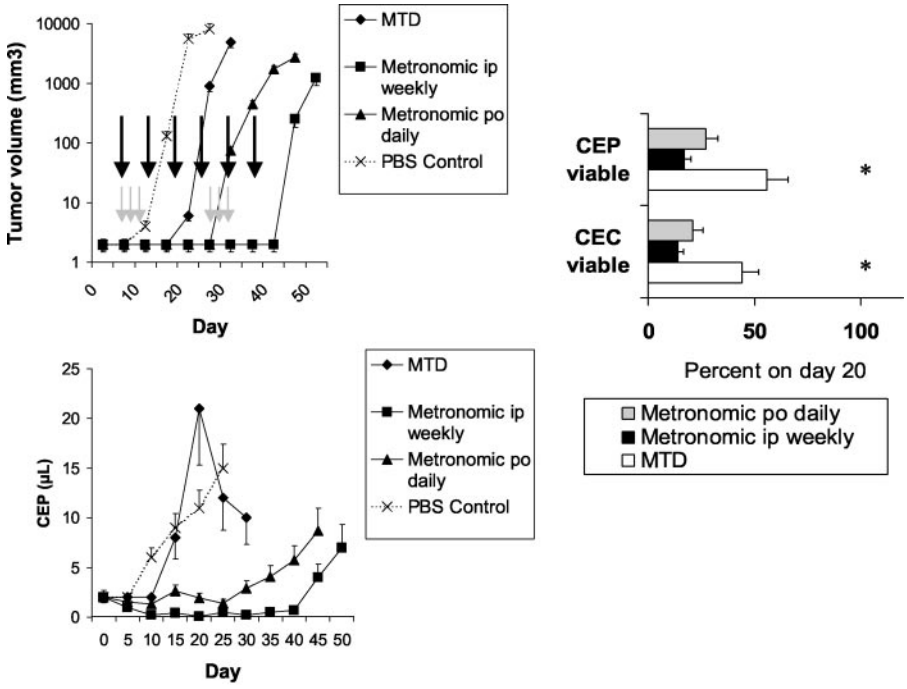

Fig. 3. Tumor volume, day of MTD (*gray arrows*) and metronomic (*black arrows*) treatment and CEP kinetics in Namalwa-injected NOD/SCID mice. The *panel* on the *right* indicates the frequency of viable (7AAD-) CEC and CEP on day 20. Results are expressed as means $\pm$ 1 SD (*bars*); $*P < 0.01$.

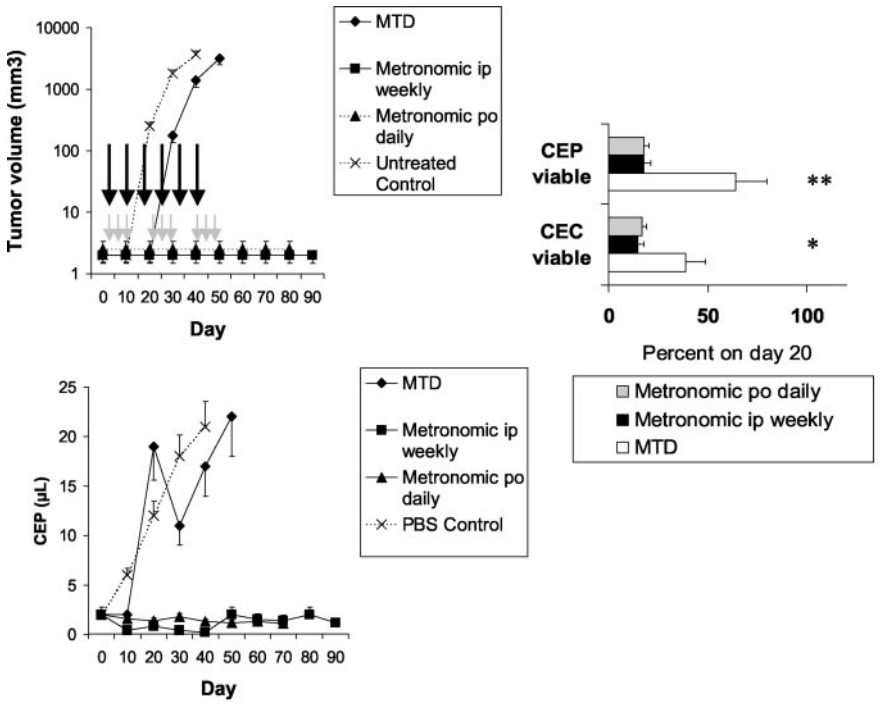

Fig. 4. Tumor volume, day of MTD (*gray arrows*) and metronomic (*black arrows*) treatment and CEP kinetics in Granta 519-injected NOD/SCID mice. The *panel* on the *right* indicates the frequency of viable (7AAD-) CEC and CEP on day 20. Results are expressed as means ± 1 SD (*bars*); $*P < 0.01$; $**P < 0.001$.

Discussion

These results support the novel finding that MTD and metronomic chemotherapy have opposite effects on CEP mobilization and viability. MTD CTX mobilized viable CEPs, whereas metronomic CTX did not mobilize CEPs and, moreover, increased the frequency of apoptotic CEP. Although a formal demonstration of an obligatory role for CEP mediated vasculogenesis was not carried out in this study, it is highly likely that such cells do in fact make a significant contribution to the growth of the lymphoma models we have studied. For example, growth of other lymphoma cell lines is strongly suppressed in Id mutant mice, which are deficient in CEP-mediated vasculogenesis (3), and previous studies in our laboratory have shown that continuous infusion of endostatin inhibits CEP mobilization and tumor growth in one preclinical lymphoma model (8).

Our results strengthen the conclusion that the antitumor effects of low-dose metronomic chemotherapy are attributable, at least in part, to a mechanism involving inhibition of tumor blood vessel formation. In addition to antiangiogenic mechanisms in which fully differentiated endothelial cells are growth-inhibited and/or killed by metronomic low-dose chemotherapy (11), an antivasculogenic process may also be involved that is mediated through effects on reducing CEP mobilization and viability. It is also interesting to consider whether MTD chemotherapy may sometimes accelerate tumor (re)growth and drug resistance by increased mobilization of CEPs. This may also help explain the robust reversal of the damage inflicted by MTD CTX on tumor blood vessel endothelial cells as noted by Browder *et al.* (11). An influx of mobilized CEPs during the rest periods between cycles of MTD therapy may replace damaged or killed endothelial cells. In this regard, evaluating the mobilization, viability, and levels of CEPs detected in cancer patients treated with low-dose metronomic chemotherapy regimens, *e.g.*, daily low-dose oral CTX and twice-a-week oral methotrexate in breast cancer (21) or leukeran in lymphoma[4] will be of considerable interest. Such studies, which we are currently undertaking, may provide a surrogate marker to monitor the antivasculogenic effects of metronomic chemotherapy protocols.

[4] P. Mancuso and F. Bertolini, manuscript in preparation.

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