Tumors That Acquire Resistance to Low-Dose Metronomic Cyclophosphamide Retain Sensitivity to Maximum Tolerated Dose Cyclophosphamide

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Abstract
Low-dose metronomic (LDM) chemotherapy is emerging as an alternative or supplemental dosing strategy to conventional maximum tolerated dose (MTD) chemotherapy. It is characterized primarily, but not exclusively, by anti-angiogenic mechanisms of action and the absence of high-grade adverse effects commonly seen with MTD chemotherapy. However, similar to other anticancer therapies, inherent resistance to LDM chemotherapy is common. Moreover, even tumors that initially respond to metronomic regimens eventually develop resistance through mechanisms that are as yet unknown. Thus, we have developed in vivo models of PC-3 human prostate cancer cells resistant to extended LDM cyclophosphamide therapy. Such PC-3 variants show stable resistance to LDM cyclophosphamide in vivo yet retain in vitro sensitivity to 4-hydroperoxy-cyclophosphamide (precursor of the active cyclophosphamide metabolite 4-hydroxy-cyclophosphamide) and other chemotherapeutic agents, namely, docetaxel and doxorubicin. Moreover, LDM cyclophosphamide–resistant PC-3 variants remain sensitive to MTD cyclophosphamide therapy in vivo. Conversely, PC-3 variants made resistant in vivo to MTD cyclophosphamide show varying levels of resistance to metronomic cyclophosphamide when grown in mice. These results and additional studies of variants of the breast cancer cell line MDA-MB-231 suggest that resistance to LDM cyclophosphamide is a distinct phenomenon from resistance to MTD cyclophosphamide and that LDM cyclophosphamide administration does not select for MTD chemotherapy resistance. As such, our findings have various implications for the clinical use of metronomic chemotherapy.

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Introduction
The concept of antiangiogenic therapy has been validated in a number of phase 3 clinical trials involving advanced, metastatic stages of various tumor types [1–3]. However, the improvements achieved to date with such therapy are only incremental. Inherent or acquired therapeutic resistance is among the reasons for this limited progress [4,5].

Mechanisms of resistance to antiangiogenic therapies that are emerging from preclinical studies seem, in many cases, to be distinct from chemotherapy drug resistance but remain poorly understood. Four major, mutually nonexclusive categories of resistance have been described [4–7]: 1) evasive resistance, i.e., activation of alternative/redundant angiogenic pathways; 2) vascular co-option, i.e., enhanced ability for infiltrative tumor progression depending on the preexisting

Abbreviations: LCR, low-dose metronomic cyclophosphamide–resistant PC-3 variants; LDM, low-dose metronomic (chemotherapy); MCR, maximum tolerated dose cyclophosphamide–resistant PC-3 variants; MTD, maximum tolerated dose (chemotherapy); NS, PC-3 control variants obtained after in vivo passage in mice subjected to normal saline treatment.
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vascularity of the tumor-surrounding host tissue rather than neoangiogenesis; 3) reduced vascular dependence, i.e., successful tumor cell adaptation to the hostile microenvironment resulting from long-term antiangiogenic therapy, characterized by oxygen, nutrient, and growth factor deprivation [8,9]; and 4) rapid vascular remodeling resulting in more mature vessels, which tend to be less responsive to antiangiogenic drugs compared with newly formed immature capillaries. Although the tumor vasculature is the primary target of antiangiogenic therapies, the first three of the aforementioned mechanisms of resistance have in common that tumor cells play a decisive role in contributing to or mediating such resistance, either by facilitating treatment-resistant neoangiogenesis or by obtaining growth characteristics that enable them to progress in the absence of neoangiogenesis.

Whereas MTD chemotherapy affects primarily tumor cells, low-dose metronomic (LDM) chemotherapy (i.e., frequent—often daily—extended administration of small doses of conventional chemotherapeutic drugs without major breaks) is thought to operate primarily through antiangiogenic effects [10], although some additional mechanisms have been implicated [11]. Promising preclinical LDM chemotherapy results [12–16] have been confirmed in an increasing number of phase 2 clinical trials of a broad range of advanced tumor types [11,17–20] and in two randomized phase 3 trials of early stage lung and breast cancer, involving extended (up to 2 years) daily oral tegafur/uracil (i.e., UFT) adjuvant therapy [21,22]. In addition, while a number of phase 3 trials applying LDM regimens in various clinical scenarios are currently recruiting patients (www.clinicaltrials.gov) [23], metronomic cyclophosphamide (± methotrexate) therapy has been adopted as a treatment option for advanced breast cancer by the European Society for Medical Oncology [24]. Aside from clinical benefits of applying LDM regimens, using oral drugs such as cyclophosphamide, UFT, or capecitabine in a metronomic manner comes along with an excellent safety profile [10,11,25,26]. Furthermore, metronomic administration of a given cytotoxic agent may result in beneficial antitumor effects even in tumors resistant to MTD regimens of the same drug [12,27,28]. However, as is the case with other anticancer therapies, inherent or acquired therapeutic resistance is among the limitations of LDM therapy. Moreover, there are concerns that the use of LDM therapy could facilitate rapid therapeutic resistance per se and to subsequent MTD chemotherapy [29,30].

Thus, to enable comparable studies of resistance to LDM versus MTD chemotherapy, we derived variants of the human prostate cancer cell line PC-3, and the human breast cancer cell line MDA-MB-231, made resistant in vivo to either LDM or MTD cyclophosphamide therapy. These two tumor models respond both to LDM and to MTD cyclophosphamide therapy, which makes them ideal to study therapeutic resistance. Our results show that acquired in vivo resistance to metronomic cyclophosphamide is a stable and transplantable (i.e., tumor cell-intrinsic) phenotype. However, resistance to LDM cyclophosphamide does not involve cross-resistance to MTD cyclophosphamide therapy or other cytotoxic agents, namely, docetaxel and doxorubicin. These findings have implications on how to optimize the benefit of LDM regimens in various clinical circumstances.

Materials and Methods

Materials and Reagents

Cyclophosphamide, docetaxel, and doxorubicin were obtained from the institutional pharmacy. 4-OOH-cyclophosphamide was a gift of Dr S.M. Ludeman (Duke University, Durham, NC).

Animal Procedures

PC-3 human prostate cancer cells (ATCC, Manassas, VA) were maintained in a humidified atmosphere of 5% CO₂ at 37°C in Dulbecco’s modified Eagle medium supplemented with 5% fetal bovine serum. A total of 2 × 10⁶ cells were injected subcutaneously into the flanks of 6- to 8-week-old male athymic nude mice (Harlan, Indianapolis, IN). Tumor size was assessed using calipers and the formula \( 0.5 \times L \times W^2 \), where \( L \) and \( W \) represent the largest and the smallest tumor diameter (mm), respectively [31]. Growth inhibition (%) of individual tumors was calculated when the mean tumor size of the control group reached around 1000 mm³ and by applying the formula \( 100 \times (1 – \text{volume of treated tumor} / \text{mean tumor volume in control mice}) \).

We initiated treatment at an average tumor size of 200 mm³ by using the following regimens (n = 5 mice per treatment cohort) [14]: 1) LDM cyclophosphamide: 20 mg/kg per day continuously administered through the drinking water; 2) MTD cyclophosphamide: 150 mg/kg on day 1 and 100 mg/kg on days 3 and 5 administeredintraperitoneally (21-day treatment cycles); and 3) normal saline control.

To derive treatment-resistant PC-3 variants, tumors that had developed therapeutic resistance were resected, dissociated mechanically and enzymatically as previously described [31], and adapted to tissue culture.

The HER-2/neu expressing met2 and LM2-4H2N variants of the human breast cancer cell line MDA-MB-231 were derived and maintained as described previously [32]. To obtain LDM cyclophosphamide–resistant met2, we implanted 2 × 10⁶ met2 cells into the inguinal mammary fat pad of severe combined immunodeficient mice purchased from Charles River Canada (Saint-Constant, Quebec, Canada). Tumors were surgically removed when they reached an average size of 500 mm³ to allow metastatic progression. Three weeks after surgery, treatment with LDM cyclophosphamide was initiated as outlined above. Next, we resected the lungs of mice with treatment-resistant lung metastases to derive LDM cyclophosphamide–resistant met2 cells. By applying the same procedure, we also obtained MTD cyclophosphamide–resistant and lung metastatic variants of LM2-4H2N [32]. Because of the high sensitivity of severe combined immunodeficient mice to alkylating agents such as cyclophosphamide, we reduced the MTD dose to 70 mg/kg administered on days 1, 3, and 5 of each 21-day treatment cycle.

Proliferation Assays

PC-3 and variants were plated in 96-well plates (1500 cells per well), incubated overnight, and then exposed to 4-OOH-cyclophosphamide (a precursor of the active cyclophosphamide metabolite 4-OH-cyclophosphamide) for 6 days with daily medium/drug changes, as described previously [33]. Thereafter, the cells were pulsed for 4 hours with 2 μCi per well of methyl-[³H]-thymidine (Amersham, Piscataway, NJ), frozen, and stored (–20°C). After thawing and harvesting the cells, UniFilter GF/C plates (Perkin-Elmer, Boston, MA) were read in a TopCount NXT microplate scintillation counter (Packard, Meriden, CT).

For proliferation assays of PC-3 treated with docetaxel or doxorubicin and of met2 and LM2-4H2N treated with 4-OOH-cyclophosphamide, we plated 5000 cells per well and added drug-containing medium the next day. Relative cell numbers were assessed 3 days later by methylene blue staining [31].
Figure 1. Derivation of PC-3 variants resistant to LDM cyclophosphamide (CPA) therapy. LDM cyclophosphamide treatment (20 mg/kg per day per os) [14] of subcutaneous PC-3 xenografts was started when tumors reached 200 mm³. After initial regression, tumor growth eventually resumed. Recurrent tumors were harvested and adapted to tissue culture. LDM cyclophosphamide–resistant PC-3 variants (LCR1) were then subjected to another round of in vivo selection yielding LCR2 variants.

Colony Formation Assays
PC-3 and variants were either incubated for 6 days in the presence of 0.5 or 1 μM 4-OOH-cyclophosphamide, or saline control, as outlined above, or for 1 hour at 25, 50, or 100 μM 4-OOH-cyclophosphamide. Thereafter, 4-OOH-cyclophosphamide–containing media were removed, and the cells were rinsed with PBS, trypsinized, and replated at various concentrations. After 12 days, cells were stained with crystal violet, and colonies (>50 cells) were counted. The surviving fraction was calculated as the ratio of the plating efficiency of drug-treated cells to the plating efficiency of untreated cells.

Measurement of Aldehyde Dehydrogenase and Cytochrome 3A4 Activity
Lysates of PC-3 and variants prepared in 100 mM Tris buffer (pH 8.0) were analyzed for their aldehyde dehydrogenase activity using the method by Bostian and Betts, as described previously [34,35]. Cytochrome 3A4 activity was determined in microsomal preparations of PC-3 and variants by applying the P450-Glo CYP3A4 Assay Kit (Promega Corporation, Madison, WI) and following the manufacturer’s instructions [34].

Statistical Analyses
One-way analysis of variance (ANOVA; with Newman-Keuls multiple comparison test) and t tests were performed as indicated, by using PRISM Version 4.00 software (GraphPad, San Diego, CA) and applying a two-sided level of significance set at P < .05.

Results
Derivation of LDM and MTD Cyclophosphamide–Resistant PC-3 Variants
Previous experiments indicated that PC-3 variants derived from small relapsing tumors removed at first signs of progression (i.e., tumors that would be considered as “stable disease” in clinical terms), after an initial response to LDM cyclophosphamide, remain sensitive to the same treatment when implanted into a new host [14]. To obtain resistant variants, we therefore decided to extend the period of treatment for as long as possible and to resect tumors near the end point as per institutional guidelines (~1500 mm³), followed by adaptation to tissue culture as previously described (Figures 1 and 2A) [31]. Such LDM cyclophosphamide–resistant PC-3 variants, or “LCRs,” show resistance to metronomic cyclophosphamide therapy when reimplanted into new mice (Figure 2, B–D). This in vivo resistance phenotype is stable and maintained after prolonged in vitro (10 passages; Figure 2C) and repeat in vivo passages (Figure 2D; LCR2.2). In contrast, in vivo passage by itself does not result in the development of resistance to LDM cyclophosphamide therapy. In fact, PC-3 variants obtained after in vivo passage(s) of PC-3 in normal saline–treated mice, termed “NS,” remain sensitive to LDM cyclophosphamide therapy (Figure 2D; NS1.1 and NS2.4).

By applying a similar procedure as outlined for LDM cyclophosphamide, we also derived the MTD cyclophosphamide–resistant (“MCRs”) PC-3 variants, MCR2 and MCR3. Briefly, after long-term growth suppression of PC-3 xenografts by MTD cyclophosphamide therapy rapid tumor recurrence was observed after a total of six (MCR3) or eight (MCR2) treatment cycles (Figure 3A). At that time, xenograft tissue was resected and adapted to tissue culture. When reimplanted, MCR2 and MCR3 xenograft growth is slowed somewhat in mice subjected to MTD cyclophosphamide therapy compared with mice receiving normal saline control. However, MCR2 and MCR3 tumors do not show the profound and sustained regression seen with the parental PC-3 line (Figure 3, B–D).

In Vitro Resistance and Cross-resistance Testing
To compare in vivo and in vitro (i.e., tumor cell intrinsic) resistance behavior to cyclophosphamide, we first exposed PC-3 and a number of the obtained resistant variants to a MTD-like 1-hour treatment with micromolar doses (1-100 μM) of 4-OOH-cyclophosphamide, followed by plating for a colony formation assay (Figure 4A). Whereas MCR2 and MCR3 showed clear signs of resistance to 4-OOH cyclophosphamide under these circumstances, the responses of LCR1.1 and LCR2.2 were equivalent to corresponding NS1.1 and
NS2.4 control variants. Next, we treated LCR1.1, LCR2.2, corresponding control cells, and MCR2 (the PC-3 variant with the highest degree of 4-OOH-cyclophosphamide resistance as found in the conventional colony formation assay) with low (i.e., ≤1 μM) concentrations of 4-OOH-cyclophosphamide in a metronomic-like 6-day proliferation assay, as described previously [33]. In this assay, neither LCR1.1 nor LCR2.2 showed resistance to 4-OOH-cyclophosphamide. However, the growth of MCR2 was significantly less inhibited by the 6-day 4-OOH-cyclophosphamide treatment than any other cell line tested (Figure 4B). The same panel of PC-3 variants was also subjected to colony formation assays after exposure to the 6-day 4-OOH-cyclophosphamide treatment schedule. Again, MCR2 cells showed superior colony formation properties compared with all other PC-3 variants tested (Figure 4C). In contrast, the responses of LCR1.1 and LCR2.2 were equivalent to corresponding NS1.1 and NS2.4 control variants. Together, these results suggest that in vivo–acquired resistance to metronomic cyclophosphamide therapy does not promote classic drug resistance to cyclophosphamide in PC-3.

To study the phenomenon of differential resistance to LDM versus MTD cyclophosphamide in another tumor model, we compared the growth of met2 and LM2-4H2N human breast cancer cells exposed to 4-OOH-cyclophosphamide with the growth of corresponding variants made resistant to LDM and MTD cyclophosphamide. As detailed in the Materials and Methods section, treatment-refractory met2 and LM2-4H2N cells were derived from spontaneous lung metastases, which had developed under treatment with LDM (met2) or MTD (LM2-4H2N) cyclophosphamide therapy. Similar to PC-3 and LCRs, LDM cyclophosphamide–resistant met2 did not show resistance to 4-OOH-cyclophosphamide when compared with parental met2 in a proliferation assay (Figure 4D). In contrast, MTD cyclophosphamide–resistant LM2-4H2N variants have a proliferative advantage compared with LM2-4H2N when exposed to 4-OOH-cyclophosphamide.

Figure 2. In vivo testing of parental PC-3 and variants with acquired resistance to LDM cyclophosphamide: Established (∼200 mm³) xenografts were treated with LDM cyclophosphamide (▲, 20 mg/kg per day per os), MTD cyclophosphamide (◇, 150 mg/kg intraperitoneally on day 1 and 100 mg/kg on days 3 and 5; 21-day treatment cycles), or saline control (●). (A) PC-3 xenografts were sensitive to LDM and MTD cyclophosphamide (P < .001 saline vs LDM and MTD cyclophosphamide, and P > .05 LDM vs MTD cyclophosphamide, day 79). (B) The selected LCR1.1 variant was resistant to LDM cyclophosphamide therapy (▲, P > .05 vs saline, day 63) but retained sensitivity to MTD cyclophosphamide (◇, P < .05 vs saline and LDM cyclophosphamide, day 63). (C) LCR1.1 resistance to LDM cyclophosphamide (▲) was maintained after 10 in vitro passages of LCR1.1 cells (P > .05 saline vs LDM cyclophosphamide). (D) LDM cyclophosphamide yielded similar tumor growth inhibition in PC-3 and control variants NS1.1 and NS2.4 (i.e., PC-3 passaged in vivo in mice subjected to normal saline). However, the antitumor effects of LDM cyclophosphamide were significantly reduced in LCR1.1 and LCR2.2 variants. *P > .05, **P < .05, ***P < .01 (t test). CPA indicates cyclophosphamide.
The reason why resistance to 4-OH-cyclophosphamide in these cells is not appreciated over a larger drug concentration range remains to be investigated.

Aldehyde dehydrogenase and cytochrome 3A4 are critically involved in cyclophosphamide biotransformation and can contribute to treatment resistance [34,36]. Therefore, we determined aldehyde dehydrogenase activity in lysates of PC-3 and variants. Figure 4E shows that the enzymatic activity of LCR did not differ significantly from the activity measured in PC-3 and NS variants. Otherwise, although increased tumor cell aldehyde dehydrogenase activity has been described as a potential mechanism of resistance to cyclophosphamide through increased drug detoxification, this does not apply to MCR2. Furthermore, despite being reduced in microsomal preparations of LCRs, NS variants and MCR2 compared with PC-3, the activity of cytochrome 3A4 (i.e., an enzyme involved in cyclophosphamide activation) does not seem to correlate with the resistance properties of the various PC-3 variants (Figure 4F).

Next, we asked whether LCRs display signs of resistance to classes of chemotherapeutic drugs other than alkylating agents such as cyclophosphamide. Because taxanes and topoisomerase II inhibitors are commonly used for the treatment of advanced prostate cancer [37,38], we subjected the panel of PC-3 and variants to docetaxel as well as doxorubicin in proliferation assays. Neither LCR1.1 nor LCR2.2 displayed signs of cross-resistance to these two agents (Figure 5, A and B). Given that the described primary mechanisms of resistance to cyclophosphamide, docetaxel, and doxorubicin differ [39], it is not unexpected that MCR2 cells are devoid of cross-resistance to docetaxel or doxorubicin.

**In Vivo Cross-resistance Testing**

The *in vitro* results suggested that LCR xenografts retain sensitivity to MTD cyclophosphamide therapy. Indeed, MTD cyclophosphamide therapy resulted in comparable growth inhibition of PC-3, LCR1.1, and NS1.1 tumors (Figure 5C). Although growth inhibition of LCR2.2 tumors by MTD cyclophosphamide is less pronounced

(Figure 4D). The reason why resistance to 4-OH-cyclophosphamide in these cells is not appreciated over a larger drug concentration range remains to be investigated.
compared with PC-3 xenografts, there is no significant difference in the treatment response of LCR2.2 and the corresponding control cell line NS2.4. The latter suggests that repeated \textit{in vivo} passage rather than previous LDM CPA exposure may be the reason for the slightly reduced antitumor effects of MTD cyclophosphamide in LCR2.2.

Tumor cell lines resistant to MTD doses of a given chemotherapeutic drug have been shown to be sensitive to metronomic administration of the same agent [12, 27]. The resistant lines used in these publications were either obtained by long-term \textit{in vitro} selection, transfection of genes involved in drug resistance (i.e., mdr-1), or administration of

Figure 4. \textit{In vitro} analysis of cyclophosphamide-resistant tumor cell variants. (A) The colony-forming properties of LCR1.1 and LCR2.2 assessed after exposure to high-dose 4-OOH-cyclophosphamide for 1 hour did not differ compared with PC-3 and control variants (i.e., NS1.1 and NS2.4). Conversely, MCR2 and MCR3 colony formation was significantly superior under these conditions. Similarly, proliferation assays (B) and colony-forming assays (C) after metronomic-like treatment with low doses of 4-OOH-cyclophosphamide (≤ 1 μM for 6 days) revealed therapeutic resistance of MCR2. In contrast, the LDM cyclophosphamide–resistant variants LCR1.1 and LCR2.2 did not display such \textit{in vitro} cyclophosphamide resistance. (D) An LDM cyclophosphamide–resistant variant of the human breast cancer cell line met2 does not have a proliferative advantage over parental cells in the presence of 4-OOH-cyclophosphamide, in contrast to MTD cyclophosphamide–resistant LM2-4H2N cells. (E) PC-3, LCR as well as NS variants, and MCR2 did not significantly differ from each other with respect to aldehyde dehydrogenase activity, an enzyme critically involved in cyclophosphamide detoxification. (F) Whereas the activity range of cytochrome 3A4 (a cyclophosphamide activating enzyme) varies around five-fold considering PC-3 and all variants thereof tested, the cytochrome 3A4 activity profile does not seem to be related to the response to LDM and/or MTD cyclophosphamide. ***$P < .001$ (one-way ANOVA with Newman-Keuls multiple comparison test).
supralethal doses of cytotoxics (followed briefly afterward by tumor cell passage into a fresh host). However, to mimic the manner in which patients’ tumors acquire resistance during the course of treatment, our selection process occurred in the same host exposed to repeat MTD cyclophosphamide treatment cycles. We therefore asked whether the derived MCR2 and MCR3 variants retained sensitivity to metronomic cyclophosphamide. Whereas MCR3 tumor xenografts were almost completely resistant to LDM cyclophosphamide therapy, the growth-inhibitory effects of this regimen in MCR2 tumors is around 50% compared with PC-3 (although this result does not reach statistical significance, in part because of high intertumor variability; Figure 5D). Thus, based on these results, we conclude that resistance to MTD chemotherapy using a given drug cannot be overcome universally by applying the same drug in a metronomic manner.

Discussion

Antiangiogenic therapies, including metronomic chemotherapy, are susceptible to inherent or acquired treatment resistance, as is the norm for other anticancer therapies [1,4]. Here we show that cyclophosphamide can generate dramatically different resistance phenotypes, depending on the dose and schedule of treatment. Thus, PC-3 cells made resistant in vivo to MTD cyclophosphamide therapy show stable resistance to cyclophosphamide in vitro and in vivo. Conversely, cells made resistant in vivo to metronomic cyclophosphamide administration retain sensitivity to cyclophosphamide, docetaxel, and doxorubicin in vitro and to MTD cyclophosphamide in vivo. Similarly, LDM cyclophosphamide–resistant met2 breast cancer cells do not show evidence of classic cyclophosphamide resistance.

It is not without precedent that the mode of chemotherapy administration can affect the mechanisms of action and hence of resistance to chemotherapeutics. Indeed, 5-fluorouracil preferentially incorporates into RNA during bolus administration in contrast to preferential incorporation into DNA when applying infusional regimens. This schedule-dependent drug behavior could explain the absence of complete cross-resistance between these regimens [40]. However, our results seen with LDM cyclophosphamide differ from the findings with 5-fluorouracil because both bolus and infusional 5-fluorouracil are usually used at MTD doses. In other words, these 5-fluorouracil regimens are intended to directly target the tumor parenchyma, whereas LDM chemotherapy likely affects primarily the tumor vasculature [10].

Arguments raised against the use of below-MTD, “suboptimal” doses of chemotherapeutic agents include reduced antitumor efficacy.

Figure 5. Cross-resistance testing: Proliferation assays using methylene blue staining of PC-3 variants with acquired resistance to LDM or MTD cyclophosphamide did not reveal evidence for cross-resistance to docetaxel (A) or doxorubicin (B). Interestingly, compared with the other cell variants tested, LCR2.2 is significantly more sensitive to docetaxel (P < .001 at 0.1, 1, and 10 pM of docetaxel, one-way ANOVA). (C) Whereas the in vivo effects of MTD cyclophosphamide therapy were comparable in PC-3, LCR1.1, and NS1.1, the antitumor activities of MTD cyclophosphamide were less pronounced in both LCR2.2 and NS2.4 compared with that in PC-3. However, the comparable therapeutic responses in LCR2.2 and NS2.4 suggest that this phenomenon may be related to repeated in vivo passage. (D) LDM cyclophosphamide–related growth inhibition of MCR2 and MCR3 tumors is reduced compared with PC-3. Because of the large intertumor variability regarding treatment response, the trend with MCR2 did not reach statistical significance. **P < .05, *P < .05 (t test). CPA indicates cyclophosphamide.
and rapid emergence of drug resistance [29,30]. The latter would be of particular concern in cases of combined metronomic and conventional chemotherapy, or the early use of metronomic chemotherapy (i.e., LDM therapy in the [neo]adjuvant setting or as first-line therapy for advanced disease). The absence of classic cyclophosphamide resistance in LCRs and LDM cyclophosphamide–resistant met2 cells reported herein and our previous observations of beneficial combinations of LDM and MTD chemotherapy are therefore very reassuring [41]. Furthermore, whereas LDM cyclophosphamide chemotherapy induces severe hypoxia in PC-3 xenografts and Dunning prostate R3327-AT tumors [31,42], which in turn could contribute to the development of multidrug resistance [43], the lack of cross-resistance of LCRs to docetaxel and doxorubicin does not support this notion in our model. As such, our results challenge concerns expressed that metronomic chemotherapy might compromise the later use of conventional chemotherapy regimens.

When patients are screened for clinical trial eligibility, LDM chemotherapy is usually regarded as a line of cytotoxic therapy equivalent to MTD chemotherapy. In other words, previous LDM therapy could make patients ineligible for certain clinical trials. In the light of the results presented herein, this policy may need to be reconsidered, at least in the case of LDM chemotherapy using cyclophosphamide. However, preliminary findings suggest that the phenomenon of differential resistance to LDM and MTD chemotherapy administration may also apply to other classes of cytotoxic agents such as topoisomerase I inhibitors. For example, human ovarian cancer xenografts that have acquired resistance to long-term daily LDM oral topotecan therapy continue to be responsive to MTD topotecan treatment (K. Hashimoto and R.S. Kerbel, unpublished observations).

Metronomic administration of a given chemotherapeutic agent has been proposed as a means to overcome classic drug resistance to the same agent in vivo [12,27]. However, the resistant variants used in such studies were selected by using either various in vitro selection protocols or repeat ex vivo passages of tumor cells after supraletal doses of cytotoxics (as detailed in the Results section). These methods are distinct from the selection procedure applied herein. Because the nature of the selection procedure can impact on the in vivo behavior of the resulting resistant cell lines [44], this offers a potential explanation why the antitumor effects of LDM cyclophosphamide are very limited in MCR2 and almost nonexistent in MCR3. In fact, MTD chemotherapy usually results not only in direct antitumor effects but also in collateral vascular damage [10]. Although these angiocellular effects are relatively short-lived because of rapid vascular repair [45], if they are recurring, they might contribute to the selection of tumor cells capable of withstanding the consequences of long-term antiangiogenic therapies. This could explain why the benefits of using metronomic regimens and other antiangiogenic strategies are less pronounced in patients who have undergone multiple previous rounds of chemotherapy [46,47].

The results summarized herein contribute to a growing body of (pre)clinical evidence that, under certain circumstances, resistance to antiangiogenic therapies may depend largely on tumor cell intrinsic properties [4,5,48,49]. In fact, the concepts of vascular co-option and reduced vascular dependence suggest that antiangiogenic therapy–refractory tumor progression may occur in the absence of neoangiogenesis. Whereas vascular co-option has been described almost exclusively in malignant astrocytomas [49], the phenomenon of reduced vascular dependence is expected to apply to a broader range of tumor types [31,48,50]. Previous studies with PC-3 xenografts had suggested that reduced vascular dependence contributes to resistance to LDM cyclophosphamide in this tumor model [31]. Furthermore, we showed that the tumor vasculature does not seem to develop resistance to long-term LDM cyclophosphamide therapy [34]. Similarly, LDM cyclophosphamide therapy lacks significant antitumor activity in LCR tumors but decreases microvessel density in LCR tumors to the same extent as seen with PC-3 and NS xenografts (A. Kouri and U. Emmenegger, unpublished observations).

Using the tumor models described in this report, we are currently studying the mechanisms of resistance to LDM compared with MTD cyclophosphamide chemotherapy. Regarding the former, a recent proteomic analysis by Thoenes et al. revealed several interesting candidate genes [51]. Ultimately, the results of such studies may suggest means of improving the antitumor activities of metronomic chemotherapy and reveal candidate markers that predict resistance to such therapy as well as to other antiangiogenic treatment strategies.

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**References**


