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PRECLINICAL STUDIES

Preclinical analysis of resistance and cross-resistance to low-dose metronomic chemotherapy

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Summary Low-dose metronomic chemotherapy is an emerging form of chemotherapy with distinct mechanisms of action from conventional chemotherapy (e.g., antiangiogenesis). Although developed to overcome resistance to conventional chemotherapy, metronomic chemotherapy is subject to resistance on its own. However, there is a paucity of information on mechanisms of resistance, on cross-resistance between metronomic regimens using different cytotoxic drugs, and on cross-resistance between metronomic versus conventional chemotherapy, or versus targeted antiangiogenic therapy. Herein we show that PC-3 human prostate cancer xenografts were sensitive to both metronomic cyclophosphamide and metronomic docetaxel, but resistant to metronomic topotecan. Conventional docetaxel was only moderately active in parental PC-3 and in metronomic cyclophosphamide resistant PC-3 tumors. However, in metronomic cyclophosphamide resistant PC-3 tumors combining conventional docetaxel or bolus cyclophosphamide therapy with continued metronomic cyclophosphamide was superior to each treatment alone. Furthermore, bevacizumab had single-agent activity against metronomic cyclophosphamide resistant PC-3 tumors. Microarray analyses identified altered regulation of protein translation as a potential mechanism of resistance to metronomic cyclophosphamide. Our results suggest that sensitivity to metronomic chemotherapy regimens using different cytotoxic drugs not only depends on shared mechanisms of action such as antiangiogenesis, but also on as yet unknown additional antitumor effects that appear to be drug-specific. As clinically observed with targeted antiangiogenic agents, the continued use of metronomic chemotherapy beyond progression may amplify the effects of added second-line therapies or vice versa. However, metronomic chemotherapy is no different from other systemic therapies in that predictive biomarkers will be essential to fully exploit this novel use of conventional chemotherapeutics.

Keywords Low-dose metronomic chemotherapy · Maximum tolerated dose chemotherapy · Drug resistance · Prostate cancer · Cyclophosphamide · Docetaxel

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Introduction

In order to maximize direct tumor cell kill, conventional chemotherapy is administered intermittently at the maximum tolerated dose, followed by treatment-free intervals to enable recovery of host tissues (e.g., bone marrow and intestinal mucosa) from therapy-induced toxic side-effects. In contrast, a different use of conventional chemotherapeutic agents has been described recently, i.e., low-dose metronomic chemotherapy, the frequent and uninterrupted long-term administration of small doses of cytotoxic drugs [1, 2]. Metronomic chemotherapy is characterized by the near-absence of acute high-grade side-effects, combined with promising antitumor effects seen in a number of phase II

trials involving a broad range of tumor types, including studies of metronomic oral cyclophosphamide in prostate cancer [2–4]. Furthermore, the use of metronomic-like chemotherapy regimens improved overall survival in phase III trials of early lung and breast cancer [5, 6]. Since the majority of metronomic regimens are based on oral chemotherapeutics, metronomic chemotherapy is particularly suitable for outpatient use. Moreover, in view of the very beneficial toxicity profile, metronomic chemotherapy can be considered for elderly and frail patients that otherwise would not be candidates for conventional, maximum tolerated dose chemotherapy [7–10]. All in all, metronomic chemotherapy is emerging as a novel treatment alternative and is currently being clinically studied in at least six randomized phase III trials in either the adjuvant setting, in patients with advanced cancer, the elderly and frail, and as a maintenance treatment strategy following conventional induction chemotherapy [2, 11] (http://clinicaltrials.gov).

While the effects of conventional chemotherapy are primarily directed towards the cancer cell population, the antitumor activities of metronomic chemotherapy are thought to be mainly mediated through antiangiogenic effects [1]. In addition, recent evidence highlights the immunomodulatory potential of metronomic chemotherapy, whereas the importance of direct anti-cancer cell effects (e.g., interference with the hypoxia-induced factor 1α pathway, and targeting of cancer stem cells) remains to be determined [2, 4, 12, 13].

Initial preclinical studies by Browder et al. suggested that metronomic cyclophosphamide therapy is able to overcome resistance to conventional cyclophosphamide chemotherapy [14]. Furthermore, the antiangiogenic basis of metronomic chemotherapy would indicate that such regimens might be beneficial for the treatment of a broad range of tumor types. On the other hand, there is preclinical and clinical evidence showing intrinsic or acquired resistance to metronomic therapy [2, 3, 15, 16]. However, taking into consideration the different mechanisms of action of metronomic versus conventional chemotherapy, we speculated that likewise different mechanisms of resistance to metronomic versus conventional chemotherapy would be operative. In this regard, we recently showed in human prostate and breast cancer models that acquired resistance to metronomic cyclophosphamide chemotherapy is not associated with cross-resistance to maximum tolerated dose cyclophosphamide [15].

Herein we expanded on our previous studies by addressing three questions. First, does response to metronomic cyclophosphamide therapy predict response to metronomic regimens utilizing a different chemotherapeutic agent? Second, does in vivo acquired resistance to metronomic cyclophosphamide convey cross-resistance to second-line therapies, including to targeted antiangiogenic drugs such as vascular endothelial growth factor pathway inhibitors? Third, are there specific gene expression changes associated with resistance to metronomic cyclophosphamide?

Briefly, our results show that sensitivity to a metronomic regimen using a given drug (e.g., cyclophosphamide) does not necessarily predict response to another metronomic chemotherapy regimen utilizing a different chemotherapy drug (i.e., topotecan). Both treatment-naïve and metronomic cyclophosphamide resistant PC-3 prostate cancer xenografts responded poorly to maximum tolerated dose docetaxel, but maximum tolerated docetaxel combined with continued metronomic cyclophosphamide was superior compared to each treatment used alone in metronomic cyclophosphamide resistant tumors. In addition, metronomic cyclophosphamide resistant PC-3 xenografts responded well to anti-vascular endothelial growth factor antibody therapy using bevacizumab. Finally, altered regulation of protein translation appears to contribute to resistance to metronomic chemotherapy.

Materials and methods

Materials

Cyclophosphamide and docetaxel were obtained from the institutional pharmacy. Bevacizumab was kindly provided by Genentech Inc. (South San Francisco, CA), and oral topotecan was supplied by GlaxoSmithKline Inc. (Collegeville, PA). All drugs were reconstituted as per manufacturer's instructions.

Animal procedures and treatment regimens

PC-3 human prostate cancer cells (ATCC, Manassas, VA) were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C in DMEM supplemented with 5 % fetal bovine serum. Metronomic cyclophosphamide resistant PC-3 cells (i.e., LCR1.1) and according control cells (i.e., NS1.1) were derived as previously described [15]. For tumor generation, 2×10^6 cells were injected subcutaneously into the flanks of 6–8 week old, 25–30 g male athymic nude mice (Harlan, Indianapolis, IN). Tumor size was assessed weekly using calipers and by applying the formula ($0.5\times[L \times W^2]$), where L and W represent the largest and the smallest tumor diameter, respectively [17]. We obtained serial mouse body weights as a measure of treatment toxicity. All animal procedures were performed according to institutionally approved animal care guidelines (Sunnybrook Research Institute Animal Care Committee). The mice were housed in cohorts of 5 mice per cage under specific pathogen free conditions.

In first-line treatment experiments, we initiated therapy at an average tumor size of around 200 mm^3. In second-line

treatment experiments, PC-3 xenograft tumors of 200 mm^3 were subjected to metronomic cyclophosphamide chemotherapy until they reached an average size of around 750 mm^3, at which point second-line therapy was initiated.

We used the following regimens, either as monotherapies or in combination ($n \geq 5$ mice per treatment cohort): (1) metronomic cyclophosphamide: 20 mg/kg/d administered continuously through the drinking water [18]; (2) maximum tolerated dose cyclophosphamide: 100 mg/kg on days 1, 3 and 5 intraperitoneally (21-day treatment cycles) [18]; (3) bolus dose cyclophosphamide: 150 mg/kg given intraperitoneally every 21 days [19]; (4) metronomic docetaxel: 10 mg/kg twice a week intraperitoneally (Man and Kerbel, unpublished observation); (5) maximum tolerated dose docetaxel: 25 mg/kg intraperitoneally every 21 days (as converted from the conventional dose in humans [20]); (6) metronomic oral topotecan: 1 mg/kg by daily gavage [21]; and (7) bevacizumab 100 μg/mouse twice a week intraperitoneally [22]. Of note, for all metronomic regimens we used the optimal biological dose of the respective agent obtained by dose–response analysis of treatment-associated changes of the number of circulating endothelial progenitor cells, as previously reported [23].

cDNA microarray analyses

Ten μg of total RNA obtained from advanced LCR1.1 tumors (~1,500 mm^3), and of NS1.1 tumors ($n=3$ each) were labeled with cyanine dyes (Cy5 and Cy3)(Amersham Biosciences/GE Health Care Life Sciences, Baie d'Urfe, QC/Canada) and hybridized to Human 19 k v8 cDNA arrays on the Advalytix SlideBooster™ (Advalytix, Germany) using DIG Easy Hyb hybridization solution (Roche Applied Sciences, Indianapolis, IN) at the University Health Network Microarray Centre (http://www.microarrays.ca; Toronto, ON/Canada). The slides were scanned on the Agilent G2565BA scanner (Santa Clara, CA) and quantified using ArrayVision v.8.0 (Imaging Research Inc./GE Health Care Life Sciences). The data and images were then loaded into GeneTraffic™ (Iobion Informatics, Toronto, ON/Canada) for normalization (using the Lowess, sub-grid method) and filtering to remove spots that were flagged, mostly due to signals near background levels. A list of all genes that were not flagged was subjected to one-class Significance Analysis of Microarrays (SAM; version 2.20, Stanford University) [24]. In a second SAM, we only included genes that had absolute values of $\log_2$ ratio ≥ 1 in at least 1 observation.

The combined list of differentially regulated genes from both analyses was uploaded and mapped to genes in the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7, http://david.abcc.ncifcrf.gov/) [25, 26] for gene enrichment and functional annotation analyses. We applied default settings except that the EASE threshold (p value of a modified Fisher Exact test for gene enrichment) was set at 0.05.

Statistical analyses

Results are reported as mean ± standard deviation unless otherwise indicated. Statistical significance of observed differences was assessed using the PRISM Version 4.00 software (GraphPad, San Diego, CA) and by applying a two-sided level of significance set at $p<0.05$.

Results

First-line therapies of established PC-3 tumors: studying intrinsic resistance

PC-3 human prostate xenograft tumors are exquisitely sensitive to metronomic chemotherapy applying the alkylating agent cyclophosphamide at 20 mg/kg/day given through the drinking water (Fig. 1a), notably a regimen that is very well tolerated (Fig. 1b) and that is of translational relevance [3, 15, 18]. To test treatment sensitivity to other classes of chemotherapeutic agents, first we treated PC-3 tumor bearing mice with metronomic docetaxel monotherapy. While conventional docetaxel is the standard of care first-line chemotherapy in advanced prostate cancer [27], metronomic regimens containing docetaxel have shown activity in a number of phase I/II clinical trials of prostate and other tumor types [28–31]. In addition, metronomic protocols of docetaxel, or of other taxanes, have been successfully applied in breast, gastric and ovarian cancer models [32–35]. In the PC-3 model, metronomic docetaxel resulted in a significant growth delay that was comparable to the effects of metronomic cyclophosphamide, whereas combining docetaxel and cyclophosphamide in one metronomic regimen was not superior to each regimen alone (Fig. 1a). Unexpectedly, mice started to lose weight and became lethargic after 3 weeks of metronomic docetaxel monotherapy (Fig. 1b), thus they had to be sacrificed as per institutional animal care guidelines. The weight loss was even more pronounced in mice receiving combined metronomic docetaxel and cyclophosphamide chemotherapy (Fig. 1b). At necropsy, most metronomic docetaxel treated mice were found to have generalized large bowel distension with signs of fecal impaction (Fig. 2a). The droppings of mice subjected to metronomic docetaxel were not only visibly larger but the average dry weight per dropping was also significantly increased around twofold (Fig. 2b). Of note, we did not observe such a phenomenon in mice treated with maximum tolerated dose docetaxel, with metronomic cyclophosphamide alone, or with a combination of maximum tolerated dose docetaxel plus metronomic cyclophosphamide. Despite a negative impact of maximum tolerated dose

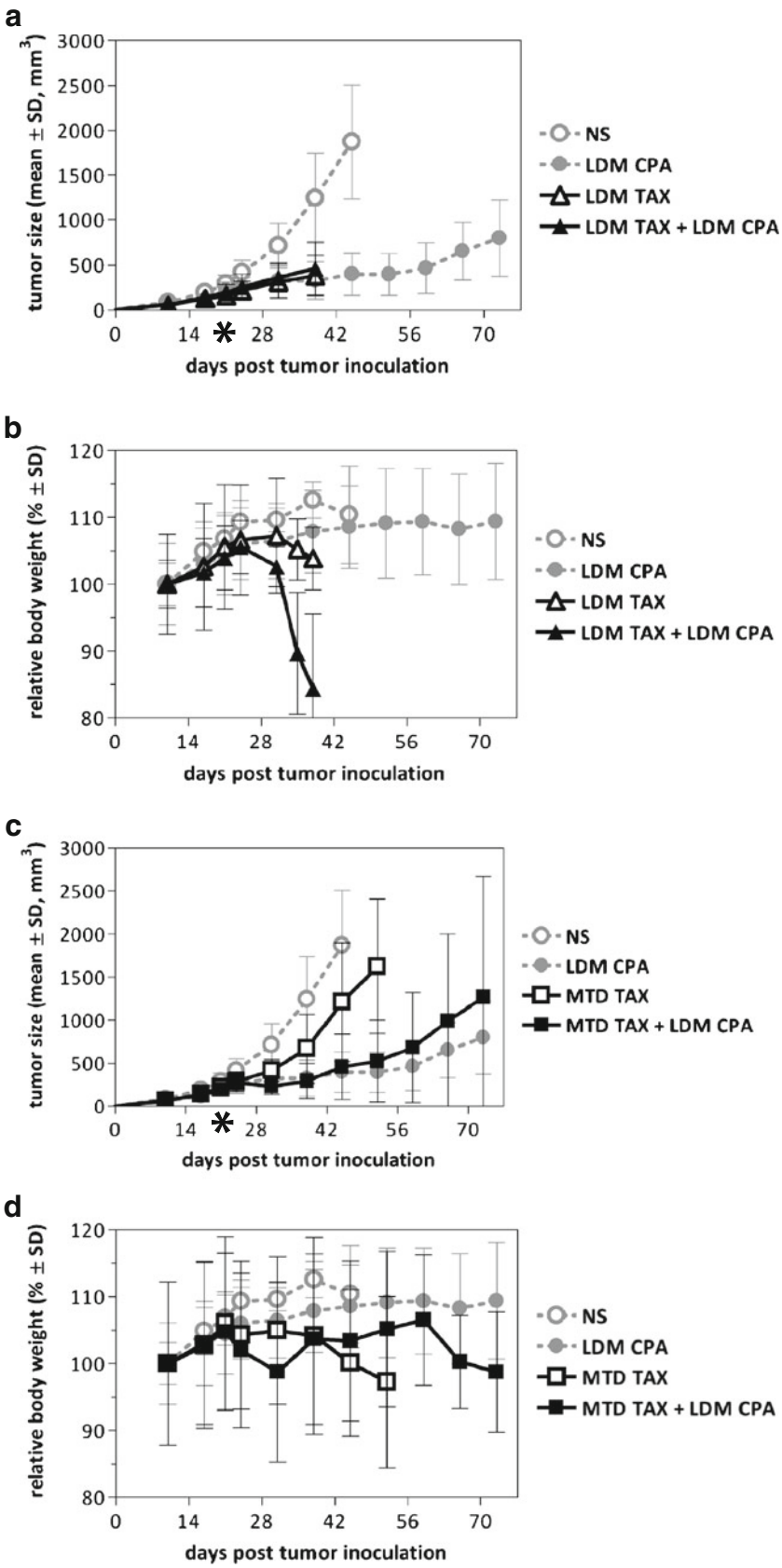

Fig. 1 Effects of metronomic versus conventional docetaxel chemotherapy on PC-3 human prostate cancer xenograft tumors: **a** Low-dose metronomic cyclophosphamide or docetaxel monotherapy (LDM CPA and LDM TAX, respectively) resulted in similar anti-PC-3 effects (both $p=0.006$ versus normal saline (NS) control on day 38), which were not further enhanced by combined cyclophosphamide/docetaxel metronomic therapy ($p=0.012$ versus NS on day 38). **b** Mice treated with metronomic docetaxel, and even more so, mice subjected to combined metronomic docetaxel/cyclophosphamide therapy experienced weight loss and became lethargic after 3 weeks of treatment. **c** Maximum tolerated dose docetaxel was only moderately active in PC-3 tumors (MTD TAX; $p=0.081$ versus NS on day 38). Combined MTD TAX and LDM CPA were not more active than LDM CPA alone ($p=0.009$ and $p=0.004$, respectively, versus NS on day 38). **d** Despite treatment-associated weight fluctuations, MTD TAX was better tolerated than LDM TAX based on body weight changes over time. * treatment start at day 21, average tumors size of 212±89 mm^3

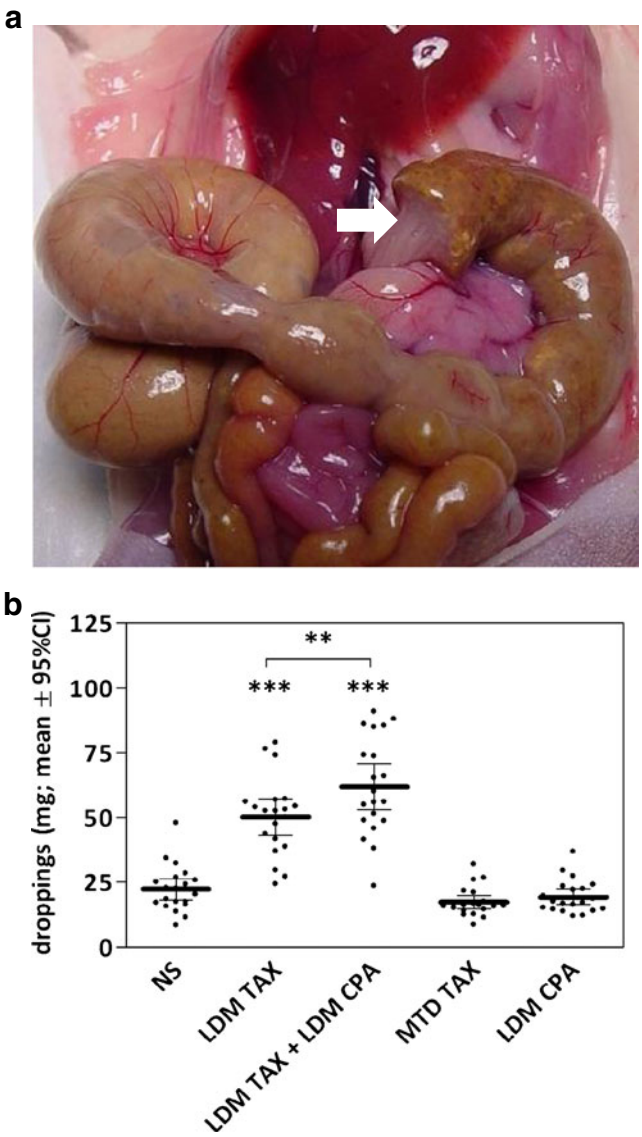

Fig. 2 Fecal impaction and intestinal stasis associated with metronomic docetaxel chemotherapy. **a** After 3 weeks of therapy, mice subjected to metronomic docetaxel became lethargic and started to lose weight. Necropsy revealed intestinal stasis and fecal impaction (arrowhead) in the majority of animals. **b** The droppings of mice subjected to metronomic docetaxel (LDM TAX) were visibly larger, and their average dry weight was increased more than twofold compared to other treatment cohorts (NS: normal saline; MTD TAX: maximum tolerated dose docetaxel, LDM CPA: metronomic cyclophosphamide). ***, $p<0.001$; **, $p<0.01$

docetaxel therapy on mouse weight gain (Fig. 1d), treatment tolerance of conventional docetaxel was superior to metronomic docetaxel, without signs of lethargy or intestinal toxicity. As previously reported, the antitumor effects of maximum tolerated dose docetaxel on PC-3 xenografts were modest (Fig. 1c) [36, 37]. Furthermore, the combination of maximum tolerated dose docetaxel with metronomic cyclophosphamide was not superior to metronomic cyclophosphamide therapy alone (Fig. 1c).

Next, we decided to test the antitumor activity of oral metronomic topotecan. Successful metronomic applications of this topoisomerase I inhibitor have been described in colorectal, ovarian and pediatric tumor models [21, 38–40]. However, the metronomic oral topotecan regimen we applied failed to show antitumor activity towards established primary PC-3 xenograft tumors (Fig. 3a). Otherwise, we could confirm the excellent tolerance of this treatment protocol (Fig. 3b).

Treatment of PC-3 xenograft tumors resistant to metronomic cyclophosphamide: overcoming acquired resistance

Despite an initial marked response to therapy, PC-3 xenograft tumors subjected to metronomic cyclophosphamide eventually progress [15]. In an attempt to define potential second-line therapies, we decided to switch mice with recurrent PC-3 tumors on metronomic cyclophosphamide therapy to various other cyclophosphamide regimens, to maximum tolerated dose docetaxel, or to bevacizumab therapy. In previous experiments we showed that PC-3 variants with stable, acquired resistance to metronomic cyclophosphamide therapy remained sensitive to maximum tolerated dose cyclophosphamide treatment when the cells were re-implanted into new hosts [15]. Herein, to expand on these findings and to more closely replicate a clinical scenario, we subjected PC-3 tumor

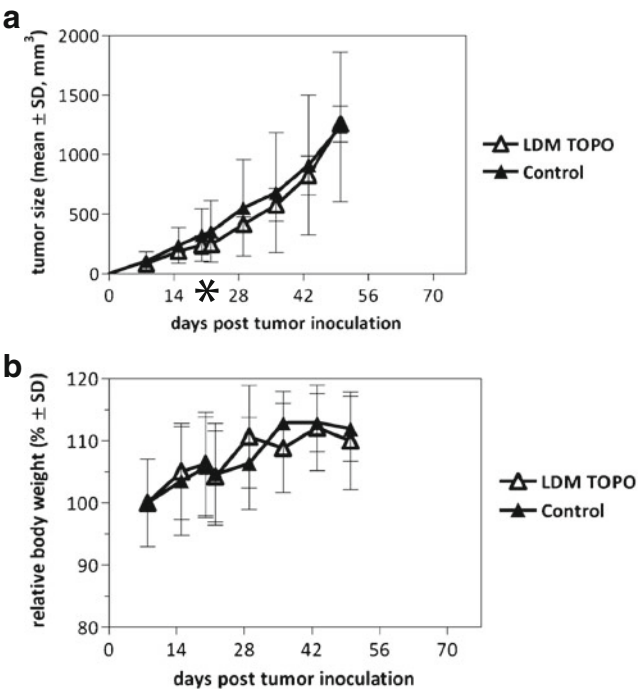

Fig. 3 Oral metronomic topotecan therapy does not suppress primary PC-3 tumor growth. **a** Oral metronomic topotecan (LDM TOPO) does not affect PC-3 tumor growth compared to control gavage (Control). **b** As suggested by serial weight measurements, LDM TOPO is very well tolerated. * treatment start at day 21, average tumors size of 212± 89 mm^3

bearing mice to metronomic cyclophosphamide therapy until recurrence to an average size of around 750 mm^3. Next, we compared the tumor growth pattern of cohorts of mice maintained on metronomic cyclophosphamide versus mice switched either to (i) saline control, or to (ii) maximum tolerated dose cyclophosphamide (300 mg/kg per 21-day cycle), to (iii) a previously described bolus dose cyclophosphamide regimen (150 mg/kg per 21-day cycle) [19], or to (iv) combined bolus dose and metronomic cyclophosphamide (Fig. 4a). Metronomic cyclophosphamide therapy beyond the point of acquired resistance was not significantly superior to normal saline control therapy. In contrast, maximum tolerated dose cyclophosphamide resulted in initial tumor regression followed by delayed progression (Fig. 4a). While the cyclophosphamide bolus dose regimen was less effective than the maximum tolerated dose cyclophosphamide protocol, the

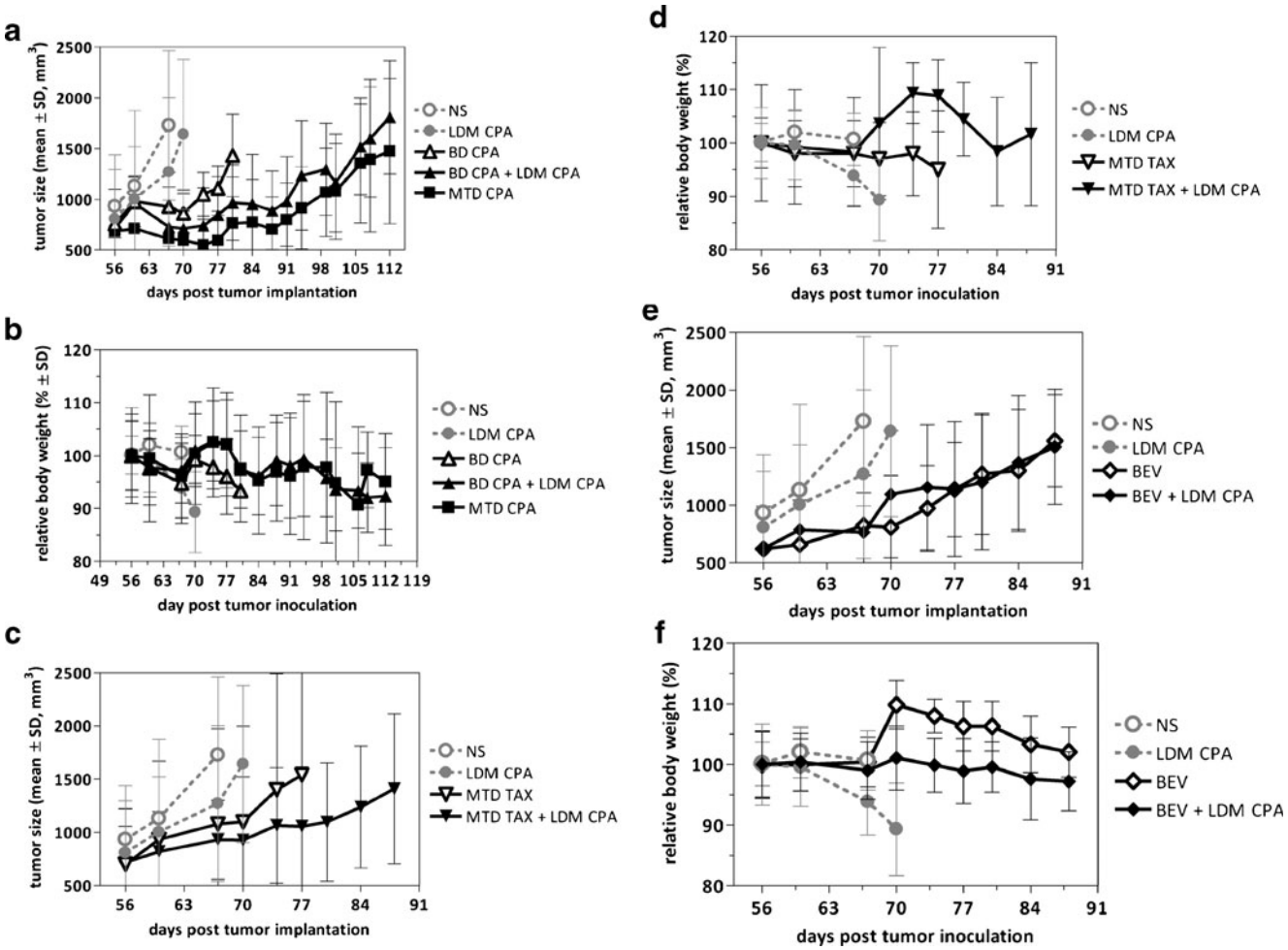

Fig. 4 Second-line therapies in PC-3 tumors with in vivo acquired resistance to metronomic cyclophosphamide. **a** Continuation of low-dose metronomic cyclophosphamide (LDM CPA) beyond progression (i.e., at an average tumor size of around 750 mm^3) was not beneficial (p=0.658 on day 67 compared to normal saline (NS) control). However, compared to NS, bolus dose cyclophosphamide alone (BD CPA), and combined bolus dose and metronomic cyclophosphamide (BD CPA + LDM CPA) delayed the time to an average tumor size of 1500 mm^3 by around 14 or 42 days, respectively, even though the degree of initial tumor regression did not reach statistical significance (BD CPA versus NS p=0.189 on day 67; BD CPA + LDM CPA versus NS p=0.072 on day 67). Of note, PC-3 tumors with in vivo acquired resistance to LDM CPA remained highly sensitive to maximum tolerated dose cyclophosphamide (MTD CPA versus NS p=0.037 on day 67). **b** With increasing tumor and treatment burden there was a trend for progressive weight loss that remained though less than 10 % of the starting weight (i.e., weight at initiation of second-line therapy). **c** Neither maximum tolerated dose docetaxel (MTD TAX), nor MTD TAX combined with metronomic cyclophosphamide (MTD TAX + LDM CPA), resulted in significant early tumor growth delay (p= 0.429 and 0.196, respectively, versus NS on day 67). However, the time to an average tumor burden per mouse of around 1500 mm^3 increased by around 10 days (MTD TAX) or 21 days (MTD TAX and LDM CPA). **d** As per serial weight measurements, MTD TAX and MTD TAX + LDM CPA were well tolerated. **e** Treatment with bevacizumab (BEV) or bevacizumab combined with metronomic cyclophosphamide (BEV + LDM CPA) did not produce significant early antitumor effects (p=0.115 and 0.076, respectively, on day 67), but the time to an average tumor size of around 1500 mm^3 was increased by 21 days. BEV + LDM CPA was not superior to BEV monotherapy. **f** Both bevacizumab treatment regimens were well tolerated

combination of bolus dose and metronomic cyclophosphamide resulted in similar tumor growth suppression as the maximum tolerated dose cyclophosphamide regimen (Fig. 4a). All treatments were associated with gradual weight loss coinciding with increasing tumor burden. The weight curves also documented transient weight losses associated with both maximum tolerated dose and bolus dose cyclophosphamide administration (Fig. 4b).

Next, we tested the impact of maximum tolerated dose docetaxel on metronomic cyclophosphamide resistant PC-3 tumors. Similar to the modest antitumor activity seen in treatment-naïve PC-3 tumors (Fig. 1c), maximum tolerated dose docetaxel resulted in a growth delay of a few days only in metronomic cyclophosphamide resistant PC-3 xenografts (Fig. 4c). However, the antitumor effects of maximum tolerated dose docetaxel combined with metronomic cyclophosphamide were superior compared to each treatment alone.

Bevacizumab does not significantly affect the growth of treatment-naïve PC-3 tumors [36]. However, we previously showed that bevacizumab is able to overcome acquired resistance of erbB-2 expressing MDA-MB-231 breast cancer xenograft tumors to combined metronomic cyclophosphamide and trastuzumab (anti-erbB-2 antibody) therapy [41]. Therefore, we decided to study the use of second-line bevacizumab therapy in metronomic cyclophosphamide resistant PC-3 tumors. While bevacizumab monotherapy impaired PC-3 tumor progression, no further enhancement of this antitumor activity was seen with the addition of metronomic cyclophosphamide (Fig. 4e).

cDNA microarray analyses

To obtain molecular insights into the mechanisms of resistance to metronomic cyclophosphamide chemotherapy, we performed cDNA microarray analyses comparing LCR1.1 with NS1.1 tumors [15], as depicted in Fig. 5a. The normalized and filtered expression data was subjected to one-class SAM under two conditions [24]. First, when all genes were included for SAM, 22 were found to be significantly upregulated in LCR1.1 tumors (Supplemental Table 1). None were negatively regulated. Second, we included only genes with absolute values of log2 ratio≥1 in at least one observation for SAM. This analysis revealed 26 upregulated genes, but again no downregulated genes (Supplemental Table 2). These two lists were merged to yield a total of 41 upregulated genes in LCR1.1 tumors (Table 1). Only few of the 41 genes have been clearly associated with cancer in general, or prostate cancer in particular, such as JAK1 and FLI1 [42–45].

Next, the combined list of genes (Table 1) was imported into the DAVID database for gene enrichment and functional annotation analyses [25, 26]. While the list of upregulated genes in LCR1.1 was significantly enriched for 8 different gene ontology terms, only the term encompassing 'translation, ribosomal structure and biogenesis' maintained significance following Bonferroni correction for multiple testing (Table 2). Genes related to translation regulation (i.e., EIF2B1, EIF253, IMP3, PES1) were 17-fold enriched. Interestingly, PES1 contains a BRCT domain commonly found in proteins regulating cell cycle checkpoints following DNA damage, including the breast cancer gene BRCA1 [46]. The BRCT domain feature was around 40-fold enriched in our combined list of upregulated genes. Of note, metronomic cyclophosphamide resistance does not appear to be associated with significant differential changes in pro-angiogenic factors such as vascular endothelial growth factor, or endogenous angiogenesis inhibitors that amplify the antiangiogenic effects of metronomic cyclophosphamide, such as thrombospondin-1 or pigment epithelium-derived factor (Fig. 5b) [47, 48].

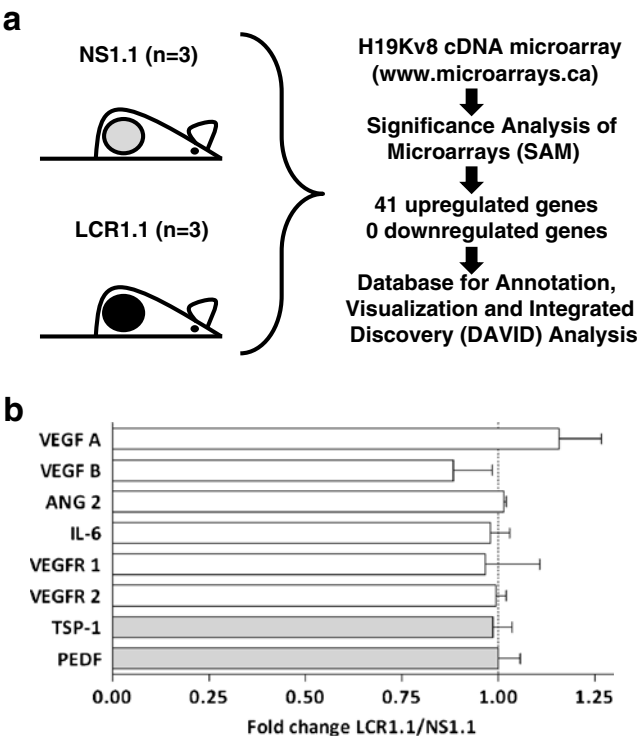

Fig. 5 cDNA microarray analyses of LCR1.1 versus NS1.1 tumors. **a** Total RNA of metronomic cyclophosphamide resistant LCR1.1 tumors, and of NS1.1 control tumors were subjected to cDNA microarray analyses followed by Significance Analysis of Microarrays (SAM) and gene enrichment as well as functional annotation analyses. **b** Neither pro-angiogenic genes (open bars) nor endogenous angiogenesis inhibitors previously implicated in metronomic chemotherapy effects (shaded bars) were differentially regulated (SAM, $p>0.05$) in LCR1.1 versus NS1.1 tumors. VEGF A/B: vascular endothelial growth factor A/B; VEGFR1/2: VEGF receptor 1/2; ANG 2: angiopoietin 2; IL-6: interleukin 6; TSP-1: thrombospondin 1; PEDF; pigment epithelium-derived factor

Table 1 Significantly upregulated genes in metronomic cyclophosphamide resistant PC-3 tumors (* genes identified in both SAM analyses)

Rank	UniGene cluster	Gene identifier	Name	Fold induction
1*	Hs.503743	GRIA4	Glutamate receptor, ionotrophic, AMPA 4	2.676
2*	Hs.438823	KCNH2	Potassium voltage-gated channel, subfamily H (eag-related), member 2	2.497
3*	Hs.4302	SLC29A4	Solute carrier family 29 (nucleoside transporters), member 4	2.378
4*	Hs.486508	SLC2A12	Solute carrier family 2 (facilitated glucose transporter), member 12	2.346
5*	Hs.505545	SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	2.235
6*	Hs.182982	GOLGA8A	Golgi autoantigen, golgin subfamily a, 8A	2.129
7	Hs.83293	ANKIB1	Ankyrin repeat and IBR domain containing 1	2.085
8	Hs.42644	GLRX3	Glutaredoxin 3	2.028
9*	Hs.571466	CSMD1	CUB and Sushi multiple domains 1	1.919
10	Hs.170131	NFIC	Nuclear factor I/C (CCAAT-binding transcription factor)	1.778
11	Hs.435771	API5	Apoptosis inhibitor 5	1.636
12	Hs.369762	ENOSF1	Enolase superfamily member 1	1.625
13	Hs.538374	ENTPD1-AS1	ENTPD1 antisense RNA 1	1.613
14	Hs.8015	USP21	Ubiquitin specific peptidase 21	1.602
15	Hs.207538	JAK1	Janus kinase 1 (a protein tyrosine kinase)	1.602
16	Hs.78592	EIF2B1	Eukaryotic translation initiation factor 2B, subunit 1 alpha, 26 kDa	1.580
17	Hs.524461	SP1	Sp1 transcription factor	1.569
18	Hs.75249	ARL6IP5	ADP-ribosylation factor-like 6 interacting protein	1.569
19	Hs.135270	CRMP1	Collapsin response mediator protein 1	1.569
20	Hs.487036	MYO5C	Myosin VC	1.558
21	Hs.632119	CHRM1	Cholinergic receptor, muscarinic 1	1.558
22	Hs.513043	IMP3	IMP3, U3 small nucleolar ribonucleoprotein, homolog	1.526
23	Hs.425427	LYAR	Hypothetical protein FLJ20425	1.505
24	Hs.516032	HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit (HADHA)	1.495
25	Hs.42502	F2RL2	Coagulation factor II (thrombin) receptor-like 2	1.474
26	Hs.105642	GNB1L	Guanine nucleotide binding protein (G protein), beta polypeptide 1-like	1.474
27	Hs.631520	ANKRD23	Ankyrin repeat domain 39	1.464
28	Hs.570423	SAMSN1	SAM domain, SH3 domain and nuclear localisation signals, 1	1.464
29	Hs.24763	RANBP1	RAN binding protein 1	1.454
30	Hs.443881	PAXIP1	PAX interacting (with transcription-activation domain) protein 1	1.444
31	Hs.504281	FLI1	Friend leukemia virus integration 1	1.434
32	Hs.356416	CBX7	CBX7 chromobox homolog 7	1.434
33	Hs.539684	EIF2S3	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52 kDa	1.414
34	Hs.517543	PES1	Pescadillo homolog 1, containing BRCT domain	1.395
35	Hs.334603	REPS1	RALBP1 associated Eps domain containing 1	1.376
36	Hs.444767	KIF13B	Kinesin family member 13B	1.366
37	Hs.511477	ZNF280D	Suppressor of hairy wing homolog 4	1.347
38	Hs.643483	CALM2	Calmodulin 2 (phosphorylase kinase, delta)	1.292
39	Hs.9728	ARMCX1	Armadillo repeat containing, X-linked 1	1.266
40	Hs.643496	ATG10	ATG10 autophagy related 10 homolog	1.240
41	Hs.705699	TMEM50A	TMEM50A transmembrane protein 50A	1.240

Discussion

Since the first use of antifolates and nitrogen mustard in the 1940s, the field of cancer chemotherapy has rapidly evolved with the development and approval of numerous classes of cytotoxic agents with distinct mechanisms of actions, and the capability of safely administering increasingly higher doses of chemotherapeutics alone or in combination [49]. Nonetheless,

Table 2 Gene ontology analysis

Category	Term	*P* value	Fold enrichment	Bonferroni
COG_ONTOLOGY	Translation, ribosomal structure and biogenesis	0.0071	16.957	0.0211
GOTERM_CC_FAT	Intracellular non-membrane-bounded organelle	0.0088	2.188	0.6301
GOTERM_CC_FAT	Non-membrane-bounded organelle	0.0088	2.188	0.6301
KEGG_PATHWAY	Neuroactive ligand-receptor interaction	0.0228	9.932	0.3246
GOTERM_CC_FAT	Cytoskeletal part	0.0402	2.984	0.9899
GOTERM_CC_FAT	Dendrite	0.0430	8.713	0.9927
INTERPRO	BRCT domain	0.0437	43.383	0.9917
SMART	BRCT domain	0.0440	42.032	0.7036
GOTERM_MF_FAT	Phosphoinositide phospholipase C activity	0.0452	41.546	0.9988

therapeutic resistance remains one of the major obstacles in systemic cancer therapy to date, and that includes also targeted agents [50, 51]. Furthermore, maximum tolerated dose chemotherapy administration is especially challenging in the elderly and frail due to treatment-associated acute toxic side-effects. In contrast, low-dose metronomic chemotherapy is generally very well tolerated while showing promising antitumor efficacy [2]. However, as is the case with maximum tolerated dose chemotherapy, therapeutic resistance is likely to limit the benefits of metronomic chemotherapy. In addition, there are concerns that metronomic chemotherapy might facilitate the development of resistance to conventional chemotherapy or to antiangiogenic therapies [15].

We recently showed that stable, in vivo acquired resistance to metronomic cyclophosphamide therapy is phenotypically distinct from resistance to maximum tolerated dose cyclophosphamide [15]. In other words, PC-3 human prostate cancer cells that acquired resistance to metronomic cyclophosphamide remained sensitive to maximum tolerated doses of cyclophosphamide. On the other hand, it is important to note that PC-3 tumor variants resistant to maximum tolerated dose cyclophosphamide still responded to metronomic cyclophosphamide, albeit to lesser degrees than parental PC-3 [15].

Herein, we expanded on these previous findings. First, we show that the PC-3 model is not universally responsive to metronomic chemotherapy regimens. While parental PC-3 tumors respond well to metronomic cyclophosphamide and to metronomic docetaxel, they are resistant to metronomic oral topotecan. This contrasts with significant antitumor effects of metronomic topotecan reported in preclinical studies of ovarian and colon cancer, in neuroblastoma, osteosarcoma and rhabdomyosarcoma models [21, 38, 39], and in renal cell cancer (Jedeszko and Kerbel, unpublished observations). Interestingly, Aljuffali et al. recently showed significant anti-PC-3 effects of topotecan given continuously by means of a micro-osmotic pump [52]. Using this subcutaneous method of drug administration, a dose as low as 0.1 mg/kg/day of topotecan enabled PC-3 tumor stabilization over the entire treatment period of 4 weeks. Second, the degree of response to maximum tolerated doses of a given chemotherapeutic agent does not necessarily predict response to the same drug used metronomically. In fact, while PC-3 tumors are exquisitely sensitive both to maximum tolerated dose cyclophosphamide and to low-dose metronomic cyclophosphamide, we observed significant antitumor activity of metronomic docetaxel but only a modest benefit of maximum tolerated dose docetaxel. Third, discontinuation of metronomic cyclophosphamide upon acquired therapeutic resistance does not result in tumor growth acceleration. The latter possibility of 'rebound' tumor growth after therapy is stopped has been raised as a concern for patients undergoing targeted antiangiogenic therapies [53, 54]. Fourth, metronomic cyclophosphamide therapy continued beyond the point at which resistance develops is still beneficial in combination with intermittent administration of maximum tolerated dose docetaxel, or bolus dose cyclophosphamide. Finally, the use of metronomic docetaxel resulted in unexpected toxicity, i.e., intestinal stasis and fecal impaction, the nature of which remains to be further investigated. Others have safely administered similar metronomic docetaxel regimens preclinically, albeit using different mouse strains [32–35]. Of note, the cumulative dose of docetaxel administered using the metronomic regimen applied herein (i.e., 60 mg/kg/21 days) was higher than the docetaxel dose of the maximum tolerated dose regimen (i.e., 25 mg/kg/21 days). While the intestinal toxicity seen in our mice could be related to cumulative docetaxel exposure, increasing the dosing frequency while decreasing individual doses at the same time [35], or using implantable continuous-release formulations of docetaxel [55, 56] could be ways to reduce the cumulative dose of docetaxel, possibly without compromising in terms of antitumor effects. However, it is reassuring that intestinal stasis has not been described as a consequence of metronomic docetaxel administration in patients, although intestinal obstruction is considered a very rare clinical side-effect of conventional docetaxel chemotherapy [28–31].

In the absence of robust biomarker guidance, the clinical translation of the metronomic treatment concept is challenging due to the empirical nature by which the metronomic dose and the dosing interval are chosen [2]. Our findings highlight another challenge. Although our studies have been restricted to the PC-3 tumor model and three distinct chemotherapy drugs, the findings suggest that the type of chemotherapy agent chosen for metronomic purposes matters as a function of the tumor to be treated. Furthermore, the therapeutic sensitivity to a given cytotoxic drug used conventionally does not appear to be closely associated with the potential benefit of using the same drug metronomically. This is not entirely unexpected considering the distinct mechanisms of action proposed for metronomic versus conventional chemotherapy [4].

With the exception of its use as monotherapy for adjuvant treatment of early stage micrometastatic disease, metronomic chemotherapy is unlikely to be used alone, especially in situations of rapid tumor progression of advanced stage cancer [5, 6]. However, due to the generally excellent safety profile, metronomic chemotherapy can be added not only safely but also very successfully to other treatment modalities [2, 4]. In this respect, we have shown previously that metronomic cyclophosphamide combined with intermittent bolus cyclophosphamide is superior compared to each treatment alone [19]. In addition, we report here for the first time the same observation for tumors with acquired resistance to metronomic cyclophosphamide monotherapy.

The antiangiogenic activities of metronomic chemotherapy make such regimens an interesting combination partner for targeted antivascular agents such as vascular endothelial growth factor inhibitors [57]. Such combinations are expected to be associated with a lower risk of high-grade adverse events such as myelosuppression and thromboembolic complications, the risk of which is known to increase when antivascular agents are combined with conventional, maximum tolerated dose chemotherapy [58, 59]. Our studies suggest that metronomic chemotherapy and targeted antivascular agents may also be used sequentially. In fact, metronomic cyclophosphamide resistant PC-3 xenografts were sensitive to bevacizumab therapy, even though parental PC-3 tumors do not respond to bevacizumab [36]. These findings are in line with results reported by du Manoir et al. [41]. By studying 231-H2N tumor xenografts (an erbB-2/HER2 expressing MDA-MB-231 breast cancer cell variant) with in vivo acquired resistance to metronomic cyclophosphamide combined with trastuzumab (an anti- erbB-2/HER2 monoclonal antibody), they showed that such resistant tumors were sensitive to adding bevacizumab. On the other hand, in our metronomic cyclophosphamide resistant PC-3 tumors continuing metronomic cyclophosphamide in addition to starting bevacizumab was not superior to bevacizumab alone, even though upfront combinations of metronomic cyclophosphamide and anti-VEGF agents have shown to be beneficial in the PC-3 model [18].

Thus, it appears that despite acquired resistance to metronomic cyclophosphamide monotherapy the continuation of metronomic cylophophosphamide is beneficial when combined with conventional docetaxel or bolus cyclophosphamide administration. Two non-mutually exclusive explanations for these findings might apply. It remains to be seen if metronomic cyclophosphamide is sensitizing tumors to conventional chemotherapy even if these tumors are resistant to metronomic cyclophosphamide monotherapy. Alternatively, conventional chemotherapy may be able to re-sensitize metronomic cyclophosphamide resistant PC-3 tumors to metronomic cyclophosphamide administration. However, such postulated cross-sensitization seems not to take place when metronomic cyclophosphamide is used in conjunction with bevacizumab.

Resistance to antiangiogenic agents targeting the vascular endothelial growth factor pathway is mediated by a number of mechanisms such as: (1) evasive resistance by activation of redundant angiogenic pathways; (2) vascular remodeling resulting in comparatively mature vessels that tend to be less responsive to antiangiogenic treatments compared to newly formed immature capillaries; (3) vascular co-option, the enhanced ability for infiltrative tumor progression into the surrounding normal tissue that depends on the pre-existing vasculature of this normal tissue rather than neoangiogenesis; and (4) reduced vascular dependence, the successful tumor cell adaptation to the hostile microenvironment resulting from long-term antiangiogenic therapy, characterized by oxygen, nutrient and growth factor deprivation [60]. In the PC-3 model resistance to metronomic cyclophosphamide appears to be mediated primarily by reduced vascular dependence [17]. Accordingly, our microarray analyses do not reveal significant expression changes of pro-angiogenic molecules or of endogenous angiogenesis inhibitors in LCR1.1 versus NS1.1 tumors. Although this may be considered inconsistent with the response of metronomic cyclophosphamide resistant PC-3 tumors to bevacizumab, our experimental setup cannot exclude the possibility of increased sensitivity of tumor endothelial cells to vascular endothelial growth factor deprivation in metronomic cyclophosphamide resistant versus treatment-naïve PC-3 tumors.

Microarray analyses of LCR1.1 tumors revealed potentially actionable expression changes such as JAK1 upregulation [43, 61]. In addition, our data indicate that resistance to metronomic cyclophosphamide chemotherapy is associated with altered regulation of protein translation. In comparative proteome analyses, Thoenes et al. identified thioredoxin containing protein 5, cathepsin B and annexin A3 as possible mediators of resistance of PC-3 derived tumors to metronomic cyclophosphamide [16]. None of these genes were found to be differentially regulated in our analyses. Using the same PC-3

models Kubisch et al. performed cDNA microarray analyses that revealed an association of resistance to metronomic cyclophosphamide with three gene ontologies: complement and coagulation cascade, axon guidance and steroid biosynthesis [62]. The lack of overlap in the gene expression changes seen in these two studies compared to our analyses may result from methodological differences. In addition, the cyclophosphamide resistant PC-3 variants used by Thoenes et al. and Kubisch et al. were derived by using a weekly metronomic cyclophosphamide regimen [14], whereas we obtained the LCR1.1 variant by administering cyclophosphamide continuously via the drinking water [18].

In conclusion, as seen with other systemic treatment modalities, metronomic chemotherapy is not devoid of intrinsic or acquired resistance. Herein, we have expanded our previous findings that resistance to metronomic versus conventional chemotherapy is distinct. Altered regulation of protein translation and anti-coagulation amongst others may contribute to resistance to metronomic cyclophosphamide therapy. A better understanding of the molecular mechanisms of such resistance is expected to lead the way to overcome or delay therapeutic resistance to metronomic chemotherapy using cyclophosphamide and possibly also other chemotherapeutic drugs. Such efforts will need to include tumor models other than PC-3.

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Conflict of interest The authors declare that they have no conflict of interest.

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