Evidence Implicating Immunological Host Effects in the Efficacy of Metronomic Low-Dose Chemotherapy

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Abstract

Conventional chemotherapy drugs administered at a maximum tolerated dose (MTD) remains the backbone for treating most cancers. Low-dose metronomic (LDM) chemotherapy, which utilizes lower, less toxic, doses given on a close regular basis over prolonged periods, is an alternative and better tolerated potential strategy to improve chemotherapy. LDM chemotherapy has been evaluated preclinically and clinically and has shown therapeutic benefit, in both early and advanced stage metastatic disease, especially when used as a maintenance therapy. However, knowledge about the antitumor mechanisms by which LDM chemotherapy acts remain limited. Here we characterized the effects of LDM and MTD capecitabine therapy on tumor and host cells using high-throughput systems approaches involving mass spectrometry flow cytometry and automated cell imaging followed by *in vivo* analyses of

Introduction

Conventional chemotherapy is commonly administered at maximum tolerated doses (MTD). This usually requires extended breaks (e.g., 2–3 weeks) to allow recovery from associated toxic side effects. While MTD chemotherapy can be very effective in the short term in causing significant tumor shrinkages, the subsequent survival benefits in patients with advanced metastatic disease are often very limited (1, 2). Previous studies have shown that an induction of rapid host responses to MTD chemotherapy, comprised of the mobilization and subsequent tumor "homing" of proangiogenic bone marrow-derived cell (BMDC) popula-

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such therapies. An increase in myeloid and T regulatory cells and a decrease in NK and T cytotoxic cells were found in MTDcapecitabine-treated tumors compared with LDM-capecitbinetreated tumors. Plasma from MTD capecitabine-treated mice induced a more tumorigenic and metastatic profile in both breast and colon carcinoma cells than plasma from mice treated with LDM capecitabine. These results correlated, in part, with *in vivo* studies using models of human or mouse advanced metastatic disease, where the therapeutic advantage of MTD capecitabine was limited despite a substantial initial antitumor activity found in the primary tumor setting. Overall these results implicate a possible contribution of immunologic host effects in accounting for the therapeutic limitations of MTD compared with LDM capecitabine. *Cancer Res; 76(20); 5983–93.* ©2016 AACR.

tions to the treated tumor, can result in accelerated tumor cell repopulation, thus explaining, at least in part, some of the limited effects or recurrence of tumors after therapy (3-5). In particular, endothelial progenitor cells, and various myeloid cells or macrophages were shown to home to the tumor site and induce tumor angiogenesis following therapy (5-7). This reactive BMDC host response is accompanied by a systemic induction of various cytokine and growth factors, which contribute not only to tumor regrowth but also to metastasis (8, 9). Plasma obtained from nontumor-bearing mice that had been treated in vivo with MTD paclitaxel, for example, can cause increased migration and invasion of tumor cells when tested in vitro. In addition, normal mice "preconditioned" by paclitaxel and subsequently given an intravenous injection of Lewis lung carcinoma cells succumb to pulmonary metastasis earlier than control mice (9). These results further suggest that reactive host responses following MTD chemotherapy can potentially generate tumor growth-promoting prometastatic effects, therefore negating or even completely blunting the desired antitumor cell activity of the drug used.

One strategy to avoid or prevent these undesirable reactive host responses is the continuous (sometimes daily) administration of chemotherapy drugs in lower doses with no extended break periods. This is referred to as low-dose metronomic (LDM) chemotherapy (10-12). The first proposed mechanism to explain the antitumor effects of LDM chemotherapy was inhibition of tumor angiogenesis (1, 10, 11, 13). Moreover, recent studies demonstrated that low-dose cyclophosphamide stimulates the immune system (14–18), and possibly targets cancer stem cells (19). Preclinically, LDM chemotherapy can sometimes cause

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remarkable antitumor effects (10), even when treating advanced visceral metastatic disease (10, 20, 21). In addition, it has been shown that plasma from LDM gemcitabine–treated mice reduced the invasive properties of pancreatic tumor cells *in vitro* otherwise promoted by plasma from MTD gemcitabine (22), suggesting that unlike MTD chemotherapy, LDM chemotherapy may not cause acute reactive host protumorigenic/prometastatic effects.

LDM chemotherapy regimens have moved into advanced (phase III) clinical trial evaluation (23, 24). For example, a randomized first-line metastatic colorectal cancer phase III trial, CAIRO3, evaluated the therapeutic efficacy of continuous lower dose daily oral capecitabine administered in combination with bevacizumab as a maintenance therapy following induction treatment with a standard regimen of capecitabine, oxaliplatin, and bevacizumab (CAPOX-B), compared with the standard control arm where the induction therapy was followed by observation only. Patients treated with maintenance LDM capecitabine combined with bevacizumab had increased progression-free survival (PFS) compared with observation only (23).

Here, we compared the therapeutic benefit of LDM and MTD capecitabine regimens using high-throughput systems-level comparison of host and tumor cell characteristics assessed in response to the same drug. Moreover, we combined these therapies with LDM cyclophosphamide to evaluate the benefit of the two-drug combination, which is being tested clinically in breast cancer patients as a doublet metronomic chemotherapy (25).

Materials and Methods

Cell lines

MDA-MB-231/LM2-4 is an aggressive spontaneously metastatic variant of the MDA-MB-231 human breast carcinoma cell line selected in vivo (26). The parental line was originally obtained from Dr. Jeff Lemontt (Genzyme) in 2000. EMT-6/CDDP is a cisplatin-resistant variant of EMT-6 murine breast carcinoma cells that was selected in vivo (originally provided by Dr. Beverly Teicher (National Cancer Institute, Rockville, MD) in 1991; ref. 27). MCF-7 human breast carcinoma as well as HT-29 and HCT-116 human colon carcinoma cell lines were purchased from the ATCC in October 2009. All human cell lines were last authenticated in 2013 by Genetica DNA Laboratories (a LabCorp Specialty Testing Group); using analytic procedures for DNA extraction, PCR, and capillary electrophoresis on a 3130xl genetic analyzer (Applied Biosystems), the results of which were confirmed by known repository cell line databases with a match of over 80%. All cells were grown in RPMI1640 medium supplemented with 10% FBS (Hyclone). The cells were passaged in culture for no more than 4 months after being thawed from authenticated stocks, and were regularly tested and found to be mycoplasma-free (EZ-PCR Mycoplasma Test Kit, Biological industries)

Tumor models

Female yellow fluorescent protein (YFP) SCID mice (6–8 weeks old) were bred in-house from breeding pairs originally provided by Dr. Janusz Rak (McGill University, Montreal, Canada). The patient-derived "triple-negative" breast carcinoma xenograft (PDX) tumor HCI-002 line was provided by Dr. Alana Welm (Huntsman Cancer Institute, University of Utah, Salt Lake City, UT) and was propagated in YFP-SCID mice by serial passage, where tumor tissue pieces (2–5 mm³ in size) were implanted in the mammary fat pads of mice, as described previously (28, 29).

To set up the advanced stage metastatic disease model, 2×10^6 LM2-4 cells were orthotopically injected into 6-week-old female CB-17 SCID (Charles River Laboratories), as described previously (26). To establish the colon carcinoma tumor model, $3-5 \text{ mm}^3$ pieces of HT-29 subcutaneous tumors (tumor cells tagged with luciferase) were orthotopically sutured to the cecum of 6-weekold male YFP-SCID mice as described previously (30). Tumor growth was assessed by IVIS, a live animal imaging system, in which the overall bioluminescence in each mouse was measured, as described previously (30, 31). To generate primary syngeneic tumors, EMT-6/CDDP cells (0.5×10^6) were implanted into the mammary fat pads of 8-week-old Balb/c mice (Harlan Laboratories). To model metastatic disease, orthotopic primary EMT-6/CDDP tumors were resected when they reached 150-200 mm³ and survival was monitored. Procedures involving animals and their care were conducted in accordance with the animal care guidelines of Sunnybrook Health Science Centre (Toronto, Canada) and the Technion (Haifa, Israel).

Drug doses and schedules

Cyclophosphamide (Baxter Oncology GmbH) was reconstituted at a stock concentration of 20 mg/mL to administer a dose of 20 mg/kg/day through the drinking water (32). Capecitabine (LC Laboratories) was prepared in a 100-mL solution containing 2 g Klucel LF (Ashland), 100 µL Tween-80, 90 mg Methyl P, and 10 mg Propyl P (Sigma). Treatment was initiated when primary tumors reached a size of 500 mm³ or 200 mm³ in the HCI-002 and EMT-6/CDDP tumor models, respectively. In the LM2-4 and EMT-6/CDDP metastatic models, treatment was initiated 3 weeks and 3 days after tumor resection, respectively. In the HT-29 tumor model, treatment was initiated 3 weeks after tumor implantation. To determine the optimal biological dose of capecitabine in a LDM chemotherapy regimen, recipient 8- to 10week-old nontumor-bearing Balb/c mice were treated with escalating doses of capecitabine by daily gavage. After one week, blood was drawn by retro-orbital sinus bleed, and evaluated for viable circulating endothelial progenitor cells (CEP) levels. The dose causing maximal suppression of viable CEPs in peripheral blood with limited toxicity was adopted as the optimal dose for LDM, as described previously (13), and was achieved at the dose of 100 mg/kg (Supplementary Fig. S1). The MTD regimen of capecitabine was determined to be a 21-day cycle involving 400 mg/ kg/day for 4 days followed by 17-day drug-free break period based on drug toxicity and tolerability.

Mass cytometry acquisition and analysis

HCI-002 tumors were excised from mice on day 11 after they were treated with capecitabine and/or cyclophosphamide. Tumors were prepared as single-cell suspensions, as described previously (3, 33). Cells obtained from each tumor in the same group were pooled (n = 5-6 tumors/group). Three million cells were immunostained with a mixture of metal-tagged antibodies (Supplementary Table S1). All antibodies were conjugated using the MAXPAR reagent (Fluidigm Inc.). Rhodium and iridium intercalators were used to identify live/dead cells. Cells were washed twice with PBS, fixed in 1.6% formaldehyde (Sigma-Aldrich), washed again in ultrapure H₂O, and acquired by CyTOF mass cytometry system (DVS Sciences). The analysis of data was performed using Cyto Spanning Tree Progression of Density Normalized Events (SPADE algorithm) on Cytobank database, as described previously (34, 35). Additional details are provided in Supplementary Materials online. Samples were validated by multiparametrical flow cytometry techniques. SPADE analysis was set on computing 100 clusters from the CD45⁺ cells. Data are presented using SPADE Cytobank online software. For each group, CD45 expression intensity is determined by color, and the number of events in each cluster is represented by the circle size.

High-throughput imaging analysis

Images collected from the IC200 (Vala Sciences) system were analyzed by Matlab software followed by analysis steps illustrated in Supplementary Fig. S2. Additional details are provided in Supplementary Materials online.

Invasion and migration assay

Invasion and migration assays were performed using Boyden chambers in line with a previous study (9). LM2-4 or HCT-116 cells (2×10^5) from overnight culture in serum-free DMEM were added to the top part of the chamber, while the bottom part was filled with 700 µL of serum-free DMEM supplemented with 5% plasma obtained from mice as indicated in the text. After 24 hours, the assay was stopped by fixing the cells with 4% paraformalde-hyde, and then stained with 0.5% crystal violet. The filters were visualized under bright field microscope. The percentage of cell coverage was quantified by measuring the number of positive pixels per field of at least five random fields at ×100 magnification.

Tissue immunostaining

Tissue immunostaining was performed as described previously (5). Details are provided in Supplementary Materials online.

Statistical analysis

Data are presented as mean \pm SD. Statistical significance of differences was assessed by one-way ANOVA, followed by Tukey post hoc statistical test or two-tailed Student t test, using GraphPad Prism 5 software. P < 0.05 was used as the threshold of statistical significance. χ^2 test for goodness of fit was used to assess significance in the number of colonies across different groups (P <0.05). For the analysis of colony area, the Kruskal-Wallis nonparametric test followed by Dunn post hoc test with Bonferroni correction was used to assess statistical significance, and the results presented as difference compared with control. A χ^2 test of independence P < 0.05 with Bonferroni correction was performed to assess statistical significance between phenotypic cluster profiles for different treatment groups. The Fisher exact test was used for calculating significance between clusters across treatments in the CyTOF experiment. Differences between all groups were compared with each other or were compared with control and were considered significant at values of *, P < 0.05; **, P <0.01, and ***, *P* < 0.001.

Results

BMDCs expressing protumorigenic-associated surface markers colonize tumors in mice treated with MTD versus LDM capecitabine

To study the colonization of host cells in the capecitabinetreated tumors, we used high-throughput multiplexed mass cytometry-based flow cytometry technique, CyTOF, which characterizes changes in multiple cell surface markers representing different cell subpopulations (36). To this end, HCI-002 PDX-bearing SCID mice were treated with capecitabine using either MTD or LDM regimens, with or without LDM cyclophosphamide. After 11 days, tumors were resected and dissociated into single-cell suspensions, and analyzed by CyTOF after samples were pooled (n = 5-6 tumors per treatment group), using a panel of antibodies (Supplementary Table S1). The panel included B- and T-cell markers that were only used as internal negative controls for the purpose of this experiment, as SCID mice are deficient in B and T cells. The CyTOF data was analyzed by gating on host-derived (murine) CD45⁺ cells as the backbone of the SPADE clusters from which 100 unsupervised clusters were generated as the output. CD45⁺ cells were chosen to ensure that our SPADE analysis solely focused on BMDCs of murine origin and excluded human tumor cells. The SPADE analysis of BMDC-colonizing tumors from all treated groups revealed a significant enrichment of myeloid-derived suppressor cell (MDSC) subpopulations in MTD capecitabine group (13/23 clusters), whereas in LDM capecitabine and/or control groups there was a significant enrichment in macrophages and monocytic cell subpopulations (16/21 clusters) and NK-cell subpopulations (2/2 clusters; Fig. 1A). The changes found in the percentage of MDSCs, NK cells, and macrophages were validated by flow cytometry (Supplementary Fig. S3A). Moreover, parallel results were obtained when analyzing the colonization of BMDCs in EMT-6/CDDP orthotopic primary breast carcinoma tumors following treatment with the above indicated regimens, using a multiparametric flow cytometry technique. In this tumor model, additional immune cells were analyzed including B, T, and regulatory T cells. A significantly lower percentage of regulatory T cells and a significantly higher percentage of cytotoxic T cells were observed using LDM capecitabine compared with MTD capecitabine therapy, similar to previous reports using cyclophosphamide or gemcitabine (15, 16). No significant differences were observed in T helper and B cells in any of the treatment groups (Supplementary Fig. S3B).

To further assess the potential protumorigenic nature of the different BMDC subpopulations, we selected a subset of 12 surface markers (described in Supplementary Table S2) from Supplementary Table S1, which are known to associate with protumorigenic/prometastatic activities. Subsequently, we analyzed the changes in their expression across clusters and/or changes in the number of cells collected in each cluster when comparing the MTD and LDM capecitabine regimens. Increased expression of such surface markers and/or increased number of cells per cluster expressing those specific markers in all BMDC populations, were summarized in a Venn diagram (Fig. 1B). Specifically, CD93, Gr-1, CD115, CD11b, and CD206 were upregulated in tumors treated with MTD capecitabine compared with LDM capecitabine, while CD11c and CD49b were downregulated. Surface markers such as F4/80, CD138, CD205, CD34, and CD44 revealed either a mixed expression and/or population-size pattern. Notably, the changes observed in cluster size found in MTD and LDM capecitabine regimens did not substantially change when the LDM cyclophosphamide treatment was added, suggesting that LDM cyclophosphamide had little effect on the type of host cells colonizing treated tumors (Fig. 1A, and data not shown). These results suggest that the host BMDC response to capecitabine therapy is altered towards facilitating a more tumorigenic and metastatic phenotype when the drug is administered using the MTD regimen compared with the LDM regimen.



A distinct colony-forming capacity and prometastatic properties induced in response to plasma from mice treated with MTD versus LDM capecitabine

Previous studies indicated that plasma from mice treated with chemotherapy may affect tumor cell-aggressive properties (9, 22). We sought to determine the effects of plasma from mice treated with MTD and LDM capecitabine regimens on the tumorigenic potential of breast and colon carcinoma cells by assessing changes in colony formation. To do this, nontumor-bearing Balb/c mice were treated with MTD or LDM capecitabine regimens with or without LDM cyclophosphamide. After one week, mice were sacrificed and plasma was obtained. The plasma (5%) was used on a modified colony formation assay. We used MCF7 breast carcinoma and HT29 colon carcinoma cells, as these cells form colonies in soft agar. Colonies formed within a week were imaged using an automated microscope. Images were then analyzed to identify individual colonies.

In the presence of plasma from MTD capecitabine–treated mice, more colonies were formed (P < 0.05) with larger colony areas (P < 0.05, n = 96 fields/group) compared with control and LDM capecitabine for both MCF7 and HT-29 cells (Fig. 2A and B).

Colonies from LDM cyclophosphamide treatment groups, regardless of whether the capecitabine therapy was LDM or MTD, were smaller in size and number compared with the control (Fig. 2B). To examine additional morphology changes between colonies in the different treatment groups, the shape features described in Supplementary Table S3 were measured for all of the colonies (>200 colonies for each treatment). Unsupervised multivariate clustering was then used to obtain 6 phenotypic clusters (the number of treatment groups). Clustering of colonies was automated and unbiased, segregating colonies by phenotypic characteristics. The profile for MTD capecitabine was significantly different than the control while there were no significant changes in the profiles of LDM capecitabine treatment groups compared with control for both cell lines (Fig. 2C). Furthermore, there were substantial differences in the colonies after MTD and LDM treatments. For example, in MCF-7 cells treatment with plasma from MTD capecitabine resulted in fewer type 1, more type 3, and fewer type 5 colonies than LDM capecitabine (consecutively numbered in descending order from the top). Hellinger similarity was used to examine the relationships between the different phenotypic profiles and quantify the differences in treatment profiles

Figure 1.

BMDCs colonizing breast tumors following MTD, but not LDM capecitabine, express more surface markers commonly associated with increased metastasis. A, six-to-eight week old female YFP SCID mice (n =5-6 mice/group) were orthotopically implanted with pieces of HCI-002 human breast carcinoma. When tumors reached 500 mm³ treatment with MTD or LDM capecitabine (CPB) regimens with or without LDM cvclophosphamide (CTX) was initiated. Subsequently, tumors were resected on day 11 and prepared as single-cell suspensions. Cells were immunostained and acquired by CVTOF The results were analyzed by SPADE using 100 unsupervised computational clustering of all CD45⁺ cell populations. The size and color of clusters (bubbles) indicate the number of events and the expression of CD45, respectively, Arrows, MDSC, NK cells, and macrophages/monocytes populations. **B**, a Venn diagram of MTD capecitabine versus LDM capecitabine demonstrates the changes in the expression of surface markers and the number of cells per cluster expressing the designated markers known to be associated with metastasis, as indicated in Supplementary Table S2. *. surface markers known to be associated with metastasis but were unchanged or exhibited a mixed pattern of expression and/or number of cells between MTD and LDM capecitabine regimens.

Figure 2.

Plasma from mice treated with MTD capecitabine increased surrogates in vitro of tumor cell tumorigenic potential when compared with plasma from mice treated with LDM capecitabine. Nontumor-bearing Balb/c mice treated with MTD or LDM capecitabine (CPB), with or without LDM cyclophosphamide (CTX), were bled a week later, and plasma was separated. Plasma (5%) was used in a colony formation assay involving MCF-7 and HT-29 cells. A, representative images of colonies from the different groups. Scale bar, 100 um. B. high content imaging analysis of the colonies. The color range in side bar represents number of colonies per treatment group, and the y-axis is the relative area of each colony (ratio of the area of each colony to the median area of the untreated control in each experiment; n = 96fields/group). The box plot of the relative area focuses colonies that are relatively larger with respect to untreated control. C, unsupervised clustering of colonies using an affinity propagation algorithm with the number of clusters set to 6 to identify colonies that have similar morphologic phenotypes. Each color represents one cluster, and the height of the bar (y-axis) represents the fraction of the colonies in that treatment group with the phenotypic characteristics of that specific cluster. χ^2 test of independence was employed to access significance of colony proportions for the different treatment groups across different morphologic phenotypes. D, relationship of the phenotypes to each other presented as a distance map based on Hellinger similarity between treatment profiles, plotted and displayed using a multidimensional scaling algorithm. *, P < 0.05 from plasma control.



(displayed as a multidimensional scaling plot in Fig. 2D). In this representation, the more similar two treatment groups are phenotypically, the closer they are in the plot. In both cell lines, the colonies from MTD capecitabine group are most distant and significantly different from the control group while the LDM capecitabine group is closer to and not significantly different from the controls (Fig. 2C and D). This result demonstrates that based on multiple morphologic measures the colonies from the LDM capecitabine group are more like the untreated control than the MTD capecitabine group. Surprisingly, the colonies from both cell lines treated with doublet LDM capecitabine and cyclophosphamide are also significantly different from both the untreated controls and MTD treatment. Moreover, the doublet LDM capecitabine and cyclophosphamide are also furthest away from the

MTD capecitabine treatment suggesting that by multiple morphologic criteria the colonies that formed are the most dissimilar. This may be in part related to the smaller size of the doublet LDM capecitabine and cyclophosphamide colonies noted above. To further evaluate the metastatic properties of tumor cells in response to the plasma from the different treatment groups, we used the Boyden chamber assay to assess LM2-4 and HCT-116 cell migration and invasion properties. A significant increase in tumor cell invasion and migration was observed in MTD capecitabine compared with control or LDM capecitabine regimen. These results were more pronounced using the LM2-4 cells compared with HCT-116 cells. However, the addition of LDM cyclophosphamide to capecitabine therapy resulted in a mixed metastatic pattern (Fig. 3). Taken together, the results in Figs. 2



Figure 3.

Increased migration and invasion properties of tumor cells in response to exposure to plasma obtained from MTD but not LDM capecitabine (CPB)-treated mice. Nontumor-bearing Balb/c mice treated with MTD or LDM capecitabine, with or without LDM cyclophosphamide (CTX), were bled a week later, and plasma was separated. Plasma (5%) was used to assess the migration and invasion properties of LM2-4 and HCT-116 cells. Representative images of tumor cell coverage are provided (left), with quantification (right). *, P < 0.05; **, P < 0.01; ***, P < 0.001; as assessed by one-way ANOVA followed by Tukey *post hoc* test.

and 3 suggest that exposure to plasma from MTD capecitabinetreated mice gives rise to phenotypically distinct sets of colonies and increased cellular migration and invasion properties compared with plasma from LDM capecitabine-treated mice. Furthermore, the increase in the number and size of colonies in the presence of plasma from MTD capecitabine-treated mice is suggestive that the MTD capecitabine regimen may have a greater potential to promote tumor growth and metastasis properties compared with plasma from the LDM-treated mice.

MTD capecitabine therapy partially delays primary tumor growth, but has little effect on survival compared with LDM capecitabine therapy

To assess whether the high-throughput systems-level *in vitro* results correlated with and thus translate to meaningful differences in therapeutic benefit, we evaluated the antitumor efficacy of MTD and LDM capecitabine in the HCI-002, EMT-6/CDDP,

and HT-29 primary orthotopic tumor models. Treatment with capecitabine using the MTD or LDM regimens was initiated when tumors reached 500 mm³, 200 mm³, and 3 weeks after implantation, respectively, with or without LDM cyclophosphamide. Mice treated with MTD capecitabine, alone, or with LDM cyclophosphamide therapy exhibited delayed tumor growth compared with mice treated with LDM capecitabine, or vehicle control. Furthermore, while LDM capecitabine exhibited little therapeutic activity, the combination of doublet LDM capecitabine and cyclophosphamide resulted in a greater antitumor activity in both EMT-6/CDDP and HT-29 tumor models. LDM cyclophosphamide, on its own, also resulted in a substantial antitumor activity in those tumor models (Fig. 4A–C).

In a parallel experiment, the HCI-002 tumors were removed 11 days after treatment was initiated, and tumor sections were stained for apoptosis and necrotic markers. Necrotic tissue and apoptosis were significantly higher in mice treated with MTD



Figure 4.

Increased apoptosis and necrosis in tumors from mice treated with MTD capecitabine when compared with all other treatment groups. **A-C**, tumor growth was assessed in mice bearing HCI-002 breast cancer PDX (n = 4-5 mice/group; **A**), murine syngeneic breast carcinoma (EMT-6/CDDP; n = 4-5 mice/group; **B**), or human colon carcinoma (HT-29; n = 3-6 mice/group; **C**). Treatment with capecitabine (CPB) using MTD or LDM regimens with or without LDM cyclophosphamide (CTX) was initiated when tumors reached 500 mm³, 200 mm³, and 3 weeks after tumor implantation, respectively. **D-F**, the HCI-002 tumors were resected 10 days after treatment was initiated, and tumor sections were stained for necrosis and apoptosis (**D**) using TUNEL (**E**) and cleaved caspase-3 staining (**F**). Quantifications are provided (n = 4 fields/tumor). *, P < 0.05; **, P < 0.01; ***, P < 0.001 as assessed by one-way ANOVA followed by Tukey *post hoc* test.

capecitabine compared with all other treatment groups. Notably, the concurrent LDM cyclophosphamide treatment group suppressed the effects of MTD capecitabine (Fig. 3D–F and Supplementary Fig. S4). Overall, these results suggest that MTD therapy results in greater initial tumor cell kill. However, tumors from MTD capecitabine displayed higher rates of proliferating cells (Ki-67) than all other treatment groups, while control tumors exhibited the highest cell proliferation rate (Fig. 5), in line with the increased *in vitro* tumor colony formation (Fig. 2A). This is consistent with previous studies demonstrating that angiogenesis and the colonization of specific BMDCs in MTD-treated tumors may contribute to tumor cell repopulation (3, 4, 6). Notably, a significant decrease in angiogenesis in HCI-002

and EMT-6/CDDP tumor models was observed in LDM compared with MTD capecitabine therapy, in accordance with previous studies (Supplementary Fig. S5; refs. 10, 11). Thus, MTD capecitabine treatment results in greater tumor cell apoptosis, but also in an elevated proliferation rate that is characteristic of tumor cell repopulation.

We next assessed the therapeutic benefit of MTD and LDM capecitabine regimens in the advanced metastatic treatment setting. To this end, mice were implanted with LM2-4 or EMT-6/CDDP, human and murine breast carcinoma cells, respectively. Established primary tumors were then resected and treatment with MTD or LDM capecitabine with or without LDM cyclophosphamide was initiated 3 weeks and 3 days later, respectively.



Figure 5.

Cell proliferation in tumors treated with MTD versus LDM capecitabine (CPB) with or without LDM cyclophosphamide (CTX). **A**, HCI-002 PDX primary tumor sections were stained with Ki67 (green) for the evaluation of cell proliferation. DAPI was used to counterstain nuclei. Scale bar, 200 μ m. **B**, a quantification of the percentage of Ki67⁺ pixels is provided (n = 4-5 fields/tumor). *, P < 0.05 as assessed by one-way ANOVA followed by Tukey *post hoc* test.

Survival of mice was monitored over time. While there was no significant difference in the mortality rates between LDM capecitabine, MTD capecitabine, and control in the two tumor models tested, the addition of LDM cyclophosphamide to LDM capecitabine significantly increased the survival rate compared with control (Fig. 6). Of note, in mice bearing orthotopic HT29 primary tumors that were sacrificed at endpoint, the number of visible metastases in the liver was substantially greater in mice treated with MTD capecitabine than mice treated with LDM capecitabine. This is consistent with the higher invasiveness and migration of HCT-116 cells stimulated *in vitro* with plasma from MTD versus LDM capecitabine–treated mice (Fig. 3).

Discussion

Previous studies have shown that in addition to the expected antitumor effects of chemotherapy, such treatments may also include host effects that can contribute to tumor regrowth, angiogenesis, and even metastasis, which may reduce or even nullify the overall desired net impact of inhibiting tumor growth and metastasis (37-39). For example, CEPs are markedly increased in the bloodstream within 24 hours of MTD paclitaxel chemotherapy. Such cells can preferentially home to the treated tumor site and promote angiogenesis thus facilitating tumor regrowth (3). In contrast, this effect on CEPs is avoided or even suppressed when using a LDM therapy protocol, for example, using cyclophosphamide (40). These results suggest that there are distinct, even opposite, host response effects induced by MTD versus LDM regimens (11, 41). Moreover, such differential effects are not limited only to CEP mobilization, as they have also been shown to

variably modulate metastasis. Increased invasion and migration of pancreatic tumor cells were observed *in vitro* in the presence of plasma from mice treated with MTD gemcitabine compared with mice treated with LDM gemcitabine (22). We observed similar results when using capecitabine. To better understand the basis of such differential effects, in this case using capecitabine, we employed an integrative systems biology approach, combining high-throughput analysis of surface markers expressed on host cell subpopulations, automated imaging of tumor cell colonies, along with *in vivo* validation using preclinical models of orthotopic primary tumor growth and metastatic disease.

Dissociated orthotopic primary tumors were subjected to highthroughput mass spectrometry multiplex CyTOF analysis, and the results showed that MTD capecitabine-treated tumors were colonized by more BMDC subpopulations, especially MDSCs. On the other hand, NK cells and macrophages were significantly reduced. The surface markers expressed by BMDCs were associated with protumorigenic and metastatic cell phenotypes compared with BMDCs associated with LDM capecitabine treatment. Indeed, a previous study indicated that MDSCs are decreased in tumors treated with a LDM gemcitabine regimen compared with MTD gemcitabine (22, 42). Furthermore, LDM cyclophosphamide regimens can stimulate the immune system by decreasing the number of regulatory T cells (16). In our study, we found that the percentage of regulatory T cells was significantly reduced, whereas cytotoxic T cells were significantly increased in mice treated with LDM compared with MTD capecitabine, indicating that LDM chemotherapy may contribute to immune stimulation (14, 16). We also used an automated imaging approach to study surrogate markers of tumorigenic potential by means of tumor cell colony formation in soft



Extended survival of mice with metastatic breast cancer disease following treatment with doublet LDM capecitabine (CPB) and cyclophosphamide (CTX). LM2-4 (**A**) or EMT-6/CDDP (**B**) cells (2×10^6 , 0.2×10^6 , respectively) were orthotopically implanted into the mammary fat pad of YFP SCID (n = 8-10 mice/group) and Balb/c (n = 6-7 mice/group) mice, respectively. Tumors were surgically resected, and treatment with MTD or LDM capecitabine regimens was initiated with or without LDM cyclophosphamide. Survival was monitored and Kaplan-Meier survival curves were plotted.



agar following MTD versus LDM capecitabine therapies. Cultured tumor cells exposed to plasma obtained from tumor-free mice that had been treated with MTD capecitabine compared with LDM capecitabine showed an increase in both colony number and size. Furthermore, the characteristics of both MCF-7 and HT-29 colony morphology in plasma from MTD capecitabine-treated and LDM cyclophosphamide-treated animals were phenotypically most distant from each other. Finally, the Hellinger distance of MTD capecitabine to control is larger than the distance between MTD capecitabine in combination with LDM cyclophosphamide and the control in both cell lines. This suggests that concurrent LDM cyclophosphamide therapy was able to counteract the protumorigenic host effects of MTD capecitabine, at least when assessing the secondary impact of host effects on tumor cell characteristics in the breast cancer models. These results together with the survival advantage of doublet LDM capecitabine and cyclophosphamide in LM2-4 and EMT-6/CDDP metastatic mice, and the decrease in MDSCs suggest a possible consensus between the various techniques assessing differential host effects induced by MTD versus LDM treatments in these breast tumor models (but not in the colon carcinoma tumor model tested).

In the clinical CAIRO3 phase III trial, the beneficial effects of maintenance therapy using metronomic capectiabine (plus bevacizumab) (23) could conceivably be due to the inhibition of any

delayed secondary protumorigenic effects generated by the induction (conventional) therapy despite its initial therapeutic activity. Thus, here we used LDM cyclophosphamide therapy as an "addon" treatment having possible antiangiogenic activity, as reported previously (32). This drug combination enhanced outcomes in advanced metastatic disease treatment settings compared with capecitabine monotherapy regardless of the dose used, while in the primary tumor setting the therapeutic impact observed was not improved compared with capecitabine monotherapy using both MTD and LDM regimens, in line with previous studies (26, 42).

Overall, our results have provided a rationale for further evaluation of low-dose chemotherapy drug combinations. In addition, the unique methodology used in this preclinical study can be incorporated into future clinical trial evaluation of cancer treatments, whereby the systematic evaluation of host and tumor effects in response to a cancer therapy could conceivably suggest potential predictive biomarkers for clinical outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Shaked, E. Pham, S. Man, D.W. Andrews Development of methodology: E. Pham, S. Man, D.W. Andrews Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Shaked, E. Pham, K. Magidey, S. Man, F. Wu, V. Miller, D.W. Andrews

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Shaked, E. Pham, S. Hariharan, K. Magidey, O. Bevar-Katz, D.W. Andrews

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Study supervision: Y. Shaked

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