

Highly Efficacious Nontoxic Preclinical Treatment for Advanced Metastatic Breast Cancer Using Combination Oral UFT-Cyclophosphamide Metronomic Chemotherapy

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Abstract

Metronomic antiangiogenic chemotherapy, the prolonged administration of relatively low drug doses, at close regular intervals with no significant breaks, has been mainly studied at the preclinical level using single chemotherapeutic drugs, frequently in combination with a targeted antiangiogenic drug, and almost always evaluated on primary localized tumors. We tested a "doublet" combination metronomic chemotherapy treatment using two oral drugs, UFT, a 5-fluorouracil (5-FU) prodrug administered by gavage, and cyclophosphamide, for efficacy and toxicity in a new mouse model of advanced, terminal, metastatic human breast cancer. The optimal biological dose of each drug was first determined by effects on levels of circulating endothelial progenitor cells as a surrogate marker for angiogenesis, which was assessed to be 15 mg/kg for UFT and 20 mg/kg for cyclophosphamide. A combination treatment was then evaluated in mice with advanced metastatic disease using a serially selected metastatic variant of the MDA-MB-231 breast cancer-cell line, 231/LM2-4. UFT or cyclophosphamide treatment showed only very modest survival advantages whereas a combination of the two resulted in a remarkable prolongation of survival, with no evidence of overt toxicity despite 140 days of continuous therapy, such that a significant proportion of mice survived for over a year. In contrast, this striking therapeutic effect of the combination treatment was not observed when tested on primary orthotopic tumors. We conclude that combination oral low-dose daily metronomic chemotherapy, using cyclophosphamide and UFT, is superior to monotherapy and seems to be a safe and highly effective experimental antimetastatic therapy, in this case, for advanced metastatic breast cancer. (Cancer Res 2006; 66(7): 3386-91)

Introduction

The concept of low-dose "metronomic" chemotherapy (1-3) is increasingly attracting attention as a "maintenance" type treatment strategy that is particularly well suited for long-term integration with various new targeted therapeutic biologics, especially antiangiogenic drugs (4) and possibly other drugs such as Herceptin (5). Preclinically, metronomic chemotherapy itself or, more commonly, combination with a dedicated targeted antiangiogenic drug, can sometimes bring about striking antitumor effects with

only relatively mild host toxicities (1, 2, 6-8). However, the effect of similar treatments on advanced, late-stage metastatic disease has not been evaluated preclinically although it has moved rapidly into the clinical setting where it is being evaluated primarily in patients with end-stage high volume (and usually refractory) metastatic disease (4, 9); e.g., oral cyclophosphamide plus oral methotrexate for the clinical treatment of metastatic breast cancer (9) or cyclophosphamide and oral etoposide given sequentially, combined with malidomide and celecoxib for clinical treatment of a variety of solid tumor types (10). Preclinical studies of metronomic chemotherapy, on the other hand, have not tested such chemotherapy doublets, one exception being etoposide plus carboplatin (but combined with an antiangiogenic drug) for the preclinical treatment of gliomas (11). Because standard (conventional maximum tolerated dose) chemotherapy treatment regimens frequently involve "doublet" combinations of such drugs, we asked whether significantly enhanced antitumor activity would also be observed using a concurrent all oral combination metronomic chemotherapy regimen involving two different drugs administered chronically on a daily basis with no breaks at optimal biological doses. In addition to cyclophosphamide, we used UFT, which has been successfully used in metronomic-like dosing regimens (e.g., 250 mg/m² daily for two years), for adjuvant treatment of early-stage resected non-small-cell lung cancer patients (4, 12). We tested metronomic UFT in combination with cyclophosphamide, where cyclophosphamide was given in a "slow" and "fast" metronomic regimen [i.e., daily oral low dose therapy interspersed every 6 weeks with a bolus i.p. injection of the drug at approximately one third of the MTD (100 mg/kg)], as previously described (13), and evaluated in a newly developed model of established high-volume (end-stage) breast cancer metastasis.

Materials and Methods

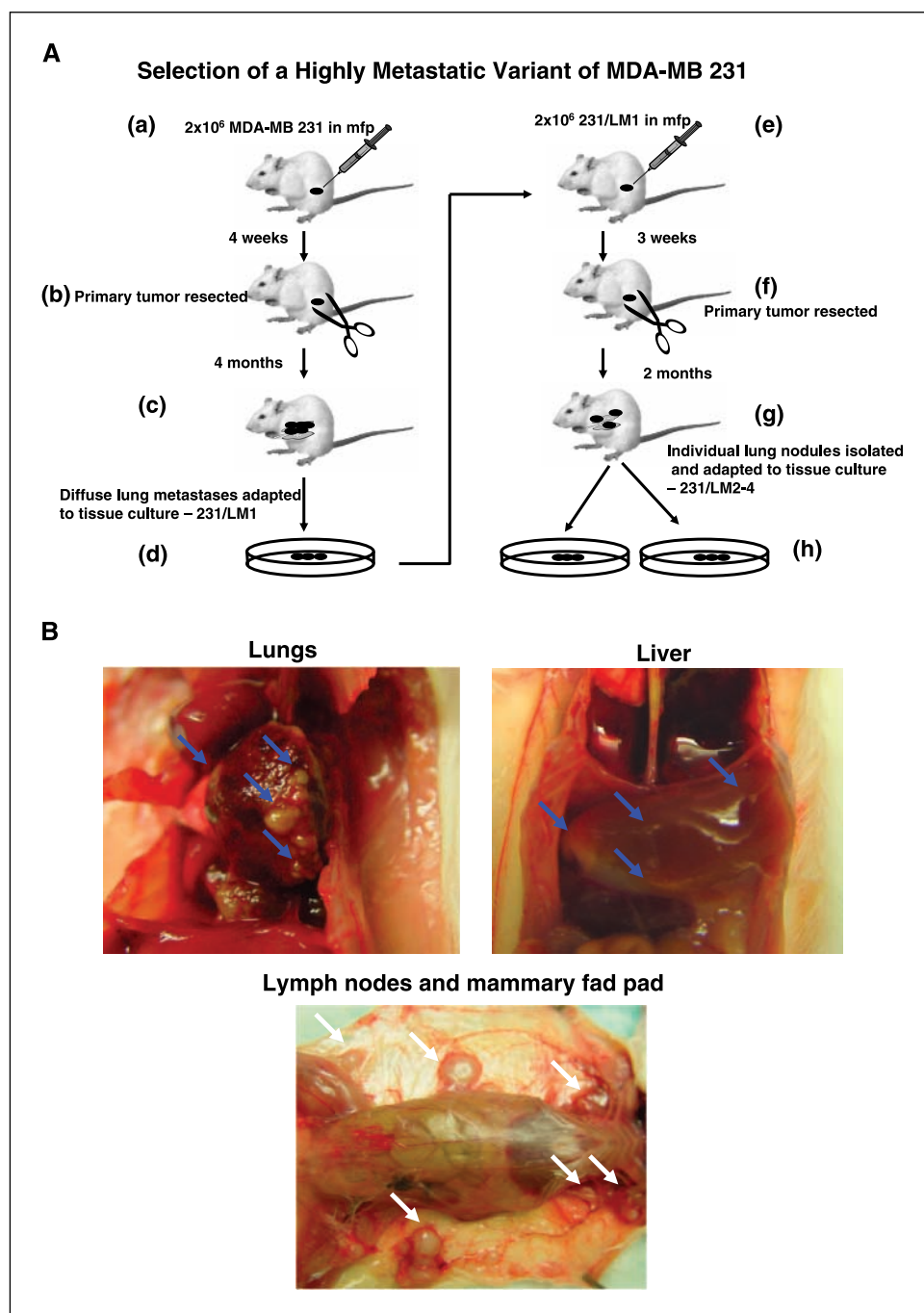
Drugs and schedule. UFT, a 5-FU oral prodrug, consists of a 4:1 molar combination of uracil and tegafur (12, 14). Tegafur, uracil, and the vehicle [hydroxypropylmethyl cellulose (HPMC)] were supplied by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Cyclophosphamide (Baxter Oncology GmbH, Mississauga, Ontario, Canada) was purchased from the institutional pharmacy; it was reconstituted as per instructions of the manufacturer to a stock concentration of 20 mg/mL and administered via drinking water to provide a dose of 20 mg/kg/d cyclophosphamide based on the estimated daily consumption of 3 mL for a 20-g mouse, as previously described (8). UFT was prepared fresh daily just before gavaging by mixing aqueous solutions of tegafur and uracil at a molar ratio of 1:4 in 0.1% HPMC.

Cells and culture conditions. A highly metastatic variant of the MDA-MB-231 human breast cancer cell line called 231/LM2-4 was isolated as described in the Results and Fig. 1. The cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Invitrogen Life Technologies, Inc., Burlington, Ontario, Canada).

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Figure 1. Procedures used for selecting the highly metastatic MDA-MB-231 human breast cancer variant, 231/LM2-4.

MDA-MB-231 cells were injected into the MFPs of SB-17 SCID mice. The 231/LM2-4 variant was obtained subsequent to two rounds of lung metastasis selection in mice, after surgical removal of the primary orthotopically transplanted tumor, as explained in detail in the text and outlined in the diagram. **A**, primary tumors were removed when volumes reached $\sim 400 \text{ mm}^3$. Six weeks later, mice were sacrificed and examined for the presence of macrometastases in the lungs, liver, lymph nodes, and MFPs. **B**, arrows, typical location of metastatic lesions. The sequence for the selection strategy was as follows: (a) 2×10^6 MDA-MB 231 cells were injected into the MFP of adult 6- to 8-week-old female CB17 SCID mice; (b) 4 weeks later, the primary tumor was resected fully by surgery when the tumor volumes were 300 to 500 mm^3 ; (c) at monthly intervals, groups of mice were sacrificed and checked for presence of metastases in the lungs and full-blown diffuse metastatic spread was observed in the mice after 4 months; (d) the whole set of lungs from one mouse was adapted to tissue culture and grown for 3 passages to derive a line referred to as 231/LM1; (e) 2×10^6 231/LM1 cells were injected into the MFP of adult 6- to 8-week-old female CB17 SCID mice; (f) ~ 3 weeks later, the primary tumor was resected when the tumor volume was 300 to 500 mm^3 ; (g) again, at monthly intervals, groups of mice were sacrificed and checked for metastases in the lungs; 2 months after resection of the primary tumor, mice were observed to have numerous macroscopic lung nodules with some spilling into the pleural cavity; (h) several individual lung nodules were isolated and adapted to tissue culture to derive established lines, one of which, 231/LM2-4, was selected for *in vivo* studies. Overall, this selection procedure took almost 9 months to complete. Genotypic analysis of MDA-MB-231 and the 231/LM2-4 variant verified their human origin and lineage relationship (data not shown). Surgical resection of primary tumors was carried out by skin incision and carefully clearing all tumor tissue away from surrounding connective tissue. Weekly weight assessment was used as a surrogate marker for toxicity. The mice were sacrificed when tumor sizes reached 1.7 cm^3 .



Assessment of circulating endothelial progenitor cells by flow cytometry as a surrogate marker to determine the optimal biological dose for metronomic UFT therapy. This was done as previously described (7, 15, 16) using four-color flow cytometry. Circulating endothelial progenitor (CEP) cells were defined as CD45^- , VEGFR-2^+ , CD117^+ , and CD13^+ . The absolute number of CEPs was calculated as the percentage of events collected in CEP enumeration gates multiplied by the total white blood cell (WBC) count.

***In vivo* tumor growth assessment and survival.** Metastatic 231/LM2-4 cells were orthotopically implanted into the right inguinal mammary fat pads (MFP; ref. 17) of CB17 severe combined immunodeficient (SCID) mice. Therapy was initiated either when the "primary" intra-MFP tumor attained a size of 150 to 200 mm^3 or when extensive metastases had developed

21 days after a 400- mm^3 primary tumor was resected (as described in the text).

Statistical analysis. Results are reported as mean \pm SD. Statistical significance of differences, where indicated, was assessed by one-way ANOVA followed by the Student-Newman-Keul's test and/or two-tailed Student's *t* test using PRISM (v.4, GraphPad, San Diego, CA) and Excel (v.2000, Microsoft Office, Santa Rosa, CA). The level of significance was set at $P < 0.05$.

Results

Selection of a highly metastatic variant 231/LM2-4 of the human MDA-MB-231 human breast cancer. As one of the major

goals of our study was to investigate the effects of metronomic chemotherapy on end-stage metastatic disease, we first developed a new preclinical mouse model that would better reflect the clinical situation compared with most models currently used. We chose the MDA-MB-231 human breast cancer based partly on previous experience in our lab using orthotopically transplanted MDA-MB-231 tumors for metronomic chemotherapy testing studies (8). Selection of an aggressively metastatic variant was achieved as described in detail in Fig. 1, and required two important procedures. First, orthotopic transplantation into the MFP has to be done because ectopic/subcutaneous human tumor xenograft transplants almost never give rise to distant metastases, whereas orthotopic transplants can sometimes do so (18). Second, surgical removal of the primary MFP tumors is usually necessary some time after their establishment to prolong survival times, thus allowing previously seeded metastatic cells from the primary tumor sufficient time to grow into macroscopic metastases. Such individual metastases could then be serially transplanted to select for a progressively aggressive metastatic variant, as described by Fidler (18).

When 231/LM2-4 variant cells were injected into the MFPs and the resultant tumors surgically removed ~1 month later, extensive visceral modular metastases were observed in the lungs, liver, and draining lymph nodes 3 to 3.5 weeks after surgery, as shown in Fig. 1B. Mice became moribund and had to be sacrificed ~8 to 10 weeks later (i.e., between 5 and 7 weeks after surgery).

Determination of the optimal biological dose for metronomic chemotherapy. We recently reported that levels of CEPs can be used as a surrogate for angiogenesis and thus as a biomarker to monitor targeted antiangiogenic drug activity (16). Recently, we extended this finding to several different metronomic chemotherapy regimens (13, 15); i.e., the optimal biological dose is the dose that causes the maximum decline in viable CEPs (15).

Using this approach, we estimated the optimal biological dose for UFT administered orally by gavage on a daily basis to be ~15 mg/kg/d both in normal non-tumor-bearing and EMT/6 tumor-bearing BALB/c mice (Fig. 2A and B, respectively). In both cases, levels of viable CEPs were significantly different. In contrast, the levels of WBCs were not significantly different from control at the estimated optimal biological dose (15 mg/kg/d UFT).

Effects of single agent cyclophosphamide or UFT metronomic chemotherapy versus the combination on survival of mice with advanced metastatic disease or localized primary tumors. We first tested metronomic UFT and cyclophosphamide on primary orthotopically transplanted tumors to determine the effects in a more conventional model of therapy. The 231/LM2-4 variant was injected into MFPs of female CB-17 SCID mice ($n = 7-9$ animals per group). When tumors reached a size of 150 to 200 mm³, treatment was initiated as indicated in Fig. 3A. Treatment with the 15 mg/kg/d dose UFT had no obvious antitumor effect as assessed by tumor volume changes. Only the treatment with cyclophosphamide showed tumor growth delay. The metronomic cyclophosphamide treatment consisted of daily administration of ~20 mg/kg administered through the drinking water (8) punctuated by bolus i.p. injections of the drug (150 mg/kg) every 6 weeks (i.e., fast plus slow metronomic chemotherapy; ref. 13). No substantial weight loss or other signs of toxicity were observed in any treatment group (data not shown). Mice treated with 0.1% HPMC (vehicle control) and 15 mg/kg/d dose UFT had to be sacrificed at day 46 due to tumor size reaching 1.7 cm³. In a repeat experiment ($n = 5$ mice per group), the mice were analyzed for the presence of visible metastases at the time of sacrifice of both vehicle control and UFT-treated groups. Tissues or organs such as lung and bone were collected and analyzed for the presence of micrometastases as summarized in Table 1. In the 0.1% HPMC therapy group, the formation of macroscopic metastases in sites such as the lymph nodes, lungs, and liver were observed.

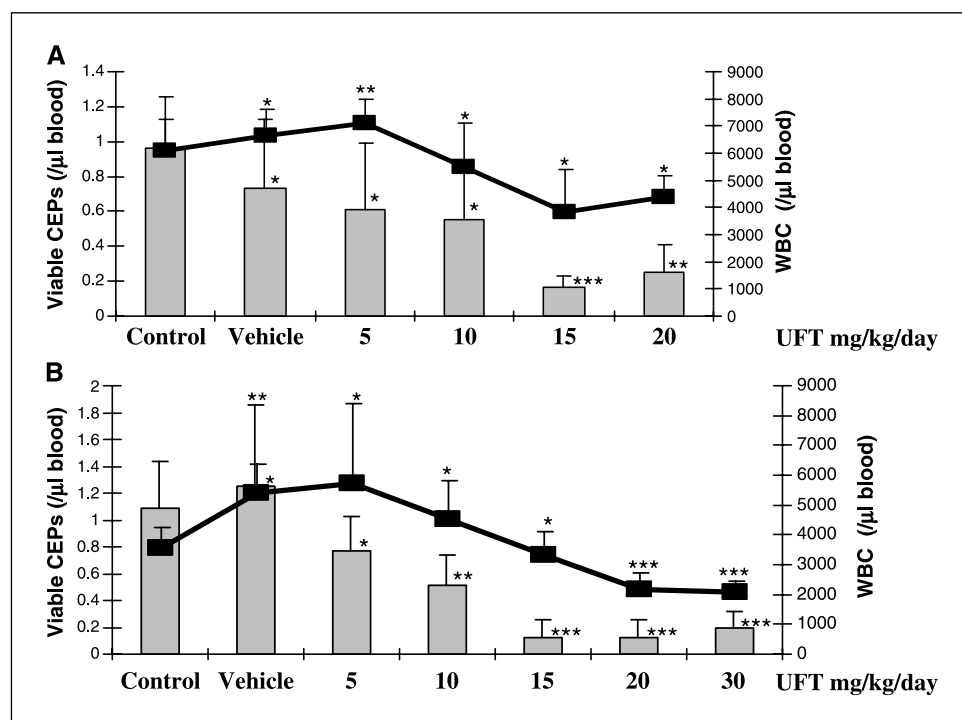


Figure 2. Evaluation of viable CEPs in BALB/c mice treated daily with escalating doses of UFT. Six- to 8-week-old normal (non-tumor-bearing) BALB/c (A) or EMT/6 tumor-bearing BALB/c (B) mice were treated orally by gavage for 1 week with UFT in escalating doses as indicated in the figure. One week later, mice were sacrificed and blood was drawn by cardiac puncture to evaluate the viable CEP levels (bar graph) and WBC counts (line graph) as previously described (7, 15, 16). Vehicle control, 0.1% HPMC. *, $P > 0.05$; **, $0.05 > P > 0.01$; ***, $P < 0.01$ from control.

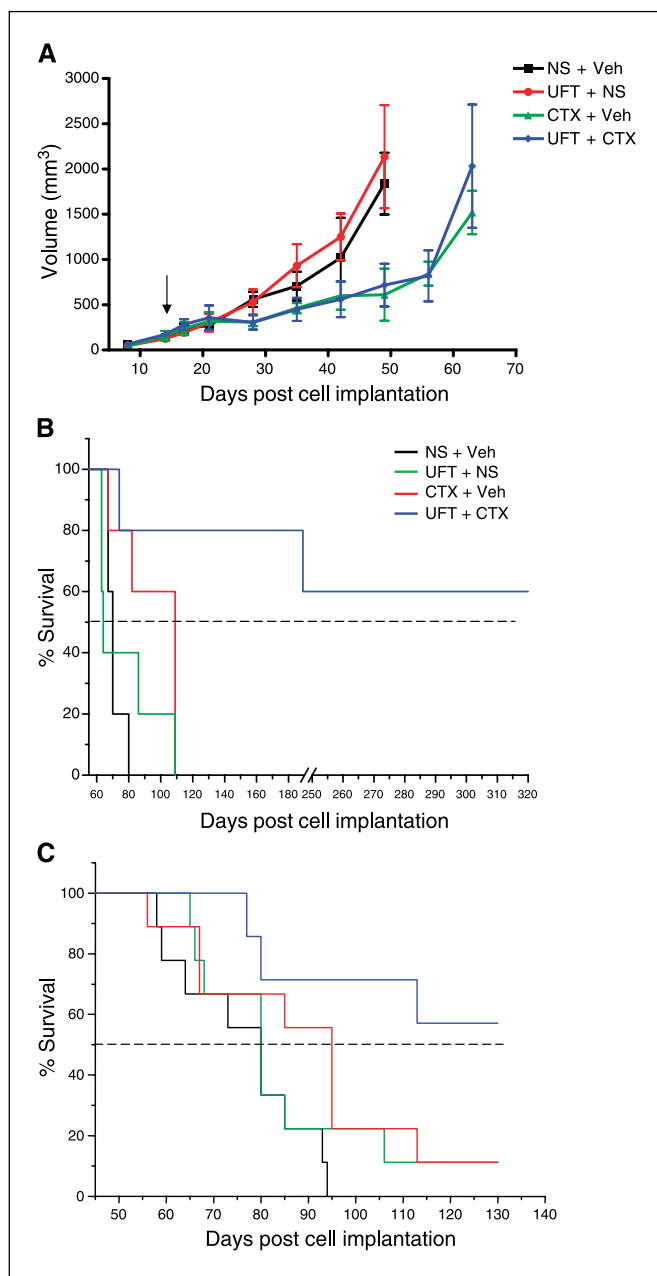


Figure 3. Chronic combination oral metronomic low-dose cyclophosphamide (CTX) and UFT prolongs survival of mice with advanced metastatic disease. **A**, 231/LM2-4 human breast metastatic variant cells were orthotopically injected into the MFPs of 6- to 8-week-old SCID mice. When tumors reached volumes of ~ 200 mm³, treatment with vehicle control, 15 mg/kg/d UFT by gavage, 20 mg/kg/d cyclophosphamide through the drinking water, or a combination of cyclophosphamide and UFT treatments was initiated. Tumors were measured weekly and tumor volume was plotted accordingly. *Arrow*, time of initiation of treatment. Experiment A was repeated ($n = 5$ mice per group) for tissue pathology evaluation as indicated in Table 1. **B** and **C**, 6-week-old SCID mice were recipients of 231/LM2-4 transplanted cells. When tumors reached 400 mm³ (which took ~ 3 weeks) primary tumors were surgically removed. Treatment with vehicle control, 15 mg/kg/d UFT by gavage, 20 mg/kg/d cyclophosphamide through the drinking water, or the daily combination of metronomic UFT and cyclophosphamide, was initiated 3 weeks after surgery on a daily nonstop basis. For example, in the experiment shown in **(B)**, the duration of the therapy was 140 days and was initiated on day 43, 3 weeks after surgery, with termination at day 183. Mice were monitored frequently according to institutional guidelines. A Kaplan-Meier survival curve was plotted accordingly for all treated groups as indicated in the figure. **A** and **C**, $n = 7$ -9 mice per group; **B**, $n = 5$ mice per group. NS, normal saline; Veh, vehicle control (0.1% HPMC).

Histologic analysis in this group showed the presence of micro-metastases in lung to varying degrees among different mice; some mice had extensive deposits of tumor whereas others only showed occasional tumor foci. In contrast, we could not detect metastases, even by histology, in any of the five mice treated with 15 mg/kg/d UFT therapy groups. Thus, counterintuitively, the metronomic UFT treatment seemed to have little or no effect on primary tumor volumes/growth whereas it seemed to have an effect on metastatic disease. Furthermore, the study of metastases in the two drug combination groups showed that two animals from the 15 mg/kg/d UFT + cyclophosphamide combination therapy group died and the rest of the animals were sacrificed at day 71 because they were moribund. Histopathologic analysis of mice from the combination group revealed that at least one mouse died as a result of the development of extensive macrometastases; the remaining mice, at the time of sacrifice, did not show evidence of any macrometastases. The low-dose oral metronomic schedule of cyclophosphamide seemed to reduce the tumor volume and slow down the development of metastases (Fig. 3A; Table 1).

In the next experiments involving treatment of advanced metastases (Fig. 3B and C), the MDA-MB-231/LM2-4 variant was injected orthotopically and this time allowed to attain a size of 400 mm³ ($n = 5$ and $n = 7$ -9 per group, respectively). At that point, the resultant tumors were resected; initiation of the metronomic chemotherapy treatments was delayed for another 21 days to allow the establishment of extensive macroscopic metastases in multiple organ sites. Using survival as an end point, 15 mg/kg/d UFT therapy alone did not have any significant effect on the mice; the low-dose cyclophosphamide monotherapy group showed a small effect on prolonging survival, with a median survival time of 109 days, as opposed to mice from vehicle control group, which had a median survival of only 70 days. However, remarkably, the prolonged combination treatment (15 mg/kg/d UFT + 20 mg/kg/d cyclophosphamide) caused almost complete resolution of advanced metastatic disease and greatly prolonged survival of the mice. Indeed, several of the mice were alive and healthy-looking 4.5 months after the combination therapy was terminated.

Discussion

Our results suggest that similar to many doublet combination conventional MTD chemotherapy regimens, a doublet low-dose metronomic chemotherapy combination—in this case, UFT and cyclophosphamide—is more efficacious than the respective monotherapies. Also very encouraging is the relatively nontoxic nature of the combination therapy, which was administered continuously for 140 days with no breaks, and the remarkable antimetastatic efficacy, considering that treatment was initiated at a near-terminal state of advanced high-volume visceral metastases. An unexpected observation was the relative lack of efficacy of the combination therapy on the same tumor cell line when grown and treated as a localized primary orthotopic tumor transplant, suggesting a preferential, selective, antimetastatic effect of the therapy. Whether this phenomenon is related to the efficacy of UFT on small (i.e., not yet established) metastases versus large established primary tumor masses is under investigation.

One potential concern about our results is the use of one tumor model and one set of drugs for the experiments. However, we have recently obtained similar remarkable antimetastatic and survival results using a nontoxic daily low-dose oral cyclophosphamide

Table 1. Pathology evaluation of overall macrometastases and micrometastases in lungs and bones

Group	Macroscopically	Lung	Bone
Normal saline and vehicle (0.1% HPMC)			
Mouse 1	Extensive tumor involvement: ascites, liver, mesentery, lung, axillary lymph nodes	Extensive tumor involvement	Tumor in soft tissue. No tumor in bone marrow
Mouse 2	Axillary lymph nodes	Rare tumor cells seen	No tumor seen
Mouse 3	No tumor seen	Rare tumor cells seen	No tumor seen
Mouse 4	Extensive tumor involvement: ascites, mesentery, axillary lymph nodes	Occasional tumor foci seen	Tumor in soft tissue. No tumor in bone marrow
Mouse 5	n/a	n/a	n/a
UFT 15 mg/kg and normal saline			
Mouse 1	No tumor seen	No tumor seen	No tumor seen
Mouse 2	No tumor seen	No tumor seen	No tumor seen
Mouse 3	No tumor seen	No tumor seen	No tumor seen
Mouse 4	No tumor seen	No tumor seen	No tumor seen
Mouse 5	No tumor seen	No tumor seen	No tumor seen
Cyclophosphamide 20 mg/kg and vehicle (0.1% HPMC)			
Mouse 1	No tumor seen	No tumor seen	No tumor seen
Mouse 2	No tumor seen	No tumor seen	No tumor seen
Mouse 3	No tumor seen	No tumor seen	No tumor seen
Mouse 4	No tumor seen	No tumor seen	No tumor seen
Mouse 5	No tumor seen	No tumor seen	No tumor seen

regimen combined with low-dose vinblastine given i.p. thrice a week, over 160 to 190 days for the treatment of advanced, late-stage human melanoma metastases, again using a selected metastatic variant subline.¹

An obvious question about the results we obtained concerns the mechanism for the combination metronomic chemotherapy results. Metronomic chemotherapy is thought to inhibit tumor growth primarily by inhibition of angiogenesis (4) by direct targeting of dividing endothelial cells in the growing tumor neovasculature (1, 2) and/or CEPs (7, 15). UFT is a prodrug which is metabolized to 5-FU, γ -butyrolactone, and γ -hydroxybutyrate, all three of which have been shown to have antiangiogenic effects (14). However, antiangiogenic drugs or treatments are generally considered to lack the striking kind of efficacy we have observed here when tested in advanced disease settings, either preclinically or clinically, suggesting the possibility of additional mechanisms.

A previous phase II clinical trial testing the combination of oral cyclophosphamide/UFT, but at higher, more conventional doses, in women with metastatic breast cancer reported encouraging results: all (20 of 20) patients showed either partial responses or stable

disease lasting 6 months or longer (19). Such results, when considered along with other metronomic chemotherapy clinical trials (9, 10), and our preclinical results reported herein, suggest that consideration might now be given to pilot studies of low-dose, less-toxic metronomic UFT/cyclophosphamide therapy for advanced metastatic breast cancer. In addition, our results bolster the rationale (as well as perhaps providing a mechanism) for using chronic daily low-dose metronomic regimens in the treatment of early-stage micrometastatic disease (e.g., daily oral UFT at 250 mg/m² for 2 years in respective non-small-cell lung carcinoma; ref. 12). In this regard, the ability of metronomic UFT to block the growth of micrometastases but not established primary tumors (Fig. 3A and Table 1) is especially noteworthy.

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¹ S. Man and P. Xu, unpublished observations.

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