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 antitumor therapy, in this case, for advanced metastatic breast cancer.

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 maldonide 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).
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−/CD0, 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 mm³ or when extensive metastases had developed 21 days after a 400-mm³ primary tumor was resected (as described in the text).

Statistical analysis. Results are reported as mean ± 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](https://example.com/figure2.png)
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 macro-
metastases. The low-dose oral metronomic schedule of cyclophos-
phamide 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 mono-
therapies. 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 antimitastatic
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, antimitastatic 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 antimitastatic and survival
results using a nontoxic daily low-dose oral cyclophosphamide

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**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).
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, g-hydroxybutyrate, all three of which have opposite effects on the mobilization and viability of circulating endothelial progenitor cells (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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References


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

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<tr>
<th>Group</th>
<th>Macroposcopically</th>
<th>Lung</th>
<th>Bone</th>
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<td>Normal saline and vehicle (0.1% HPMC)</td>
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<tr>
<td>Mouse 1</td>
<td>Extensive tumor involvement: ascites, liver, mesentry, lung, axillary lymph nodes</td>
<td>Extensive tumor involvement</td>
<td>Tumor in soft tissue.</td>
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<tr>
<td>Mouse 2</td>
<td>Axillary lymph nodes</td>
<td>Rare tumor cells seen</td>
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</tr>
<tr>
<td>Mouse 3</td>
<td>No tumor seen</td>
<td>Rare tumor cells seen</td>
<td>No tumor seen</td>
</tr>
<tr>
<td>Mouse 4</td>
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<td>Occasional tumor foci seen</td>
<td>Tumor in soft tissue.</td>
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<td>UFT 15 mg/kg and normal saline</td>
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<tr>
<td>Mouse 1</td>
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<td>Mouse 2</td>
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1 S. Man and P. Xu, unpublished observations.

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142x342-g-hydroxybutyrate, all three of which have opposite effects on the mobilization and viability of circulating endothelial progenitor cells.
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