Low-dose Metronomic Combined with Intermittent Bolus-dose Cyclophosphamide Is an Effective Long-term Chemotherapy Treatment Strategy

Yuval Shaked, Urban Emmenegger, Giulio Francia, Limor Chen, Christina R. Lee, Shan Man, Armen Paraghamian, Yaacov Ben-David, and Robert S. Kerbel

Department of Molecular and Cellular Biology, Sunnybrook and Women's College Health Sciences Centre and the Department of Medical Biophysics, University of Toronto, Ontario, Canada

Abstract

Metronomic chemotherapy refers to the close, regular administration of comparatively low doses of cytotoxic drugs, with minimal or no drug-free breaks, over prolonged periods. It is thought to have an antiangiogenic basis. However, whereas surprisingly durable and potent tumor responses have been observed in a number of preclinical tumor models, relapses usually eventually occur using this type of treatment strategy. We therefore decided to test modified metronomic chemotherapy regimens that might significantly delay such relapses, but still maintain modest and acceptable toxicity profiles. Here, we show that repeated administration of bolus doses (BDs) of cyclophosphamide every 3 or 6 weeks, combined with a daily oral low-dose metronomic (LDM) regimen (20 mg/kg/d cyclophosphamide), improves efficacy and significantly delays progression of transplanted PC-3 human prostate cancer xenografts, syngeneic transplanted EMT-6 breast tumors, and “spontaneous” murine erythroleukemia. Efficacy was superior whereas toxicity was mild and comparable to the LDM regimen, the latter assessed by body weight, neutrophil, lymphocyte, and total white blood counts. Antiangiogenic activity, measured by reduction in circulating endothelial precursor cells, revealed that the greatest degree of suppression occurred using the combination treatment. Overall, our results indicate that the administration of intermittent BD combined with chronic oral LDM cyclophosphamide is a potent treatment regimen for controlling tumor growth, which has a low toxicity profile, over prolonged periods of time.

Introduction

Metronomic chemotherapy refers to the chronic administration of comparatively low doses of cytotoxic drugs at close, regular intervals, with no prolonged drug-free interruptions (1). This form of “dose-dense” chemotherapy, however, is not necessarily designed with the intention of substantially increasing cumulative drug-dose over time, i.e., it is not always dose-intense; as such it minimizes host toxic side effects and the resultant need for supportive care treatment (1). Despite the reduced toxicity in comparison with maximum tolerated dose (MTD) regimens, results of preclinical studies have sometimes shown surprisingly potent and durable antitumor effects in a number of models, including transplantable human lymphoma (2), human prostate carcinoma (3), and human non-small cell lung carcinoma xenografts (4) using cyclophosphamide or trofosfamide. However, similar to virtually all anticancer therapies, relapses nevertheless eventually occur in most cases. By way of example, daily continuous administration of cyclophosphamide through the drinking water can cause initial regression followed by long-term stable disease of PC-3 human cancer xenografts; the “stable disease” lasts about 6 to 8 weeks, which is followed by rapid tumor progression despite continuation of therapy (3). The basis for such relapses is unknown (3, 5), although they can be delayed in other models by combining the metronomic chemotherapy regimen with another agent such as a targeted antiangiogenic drug, where the two drugs can be coadministered for long periods of time (6–8).

Another possible strategy that can be used to improve the durability of metronomic chemotherapy responses is to initiate metronomic dosing as a follow-up long-term “maintenance” therapy after a short up-front course of MTD of the same drug (7, 9) which has been called “chemo-switching” by Pietras and Hanahan (8). We asked whether an alternative approach might also have promise, namely, intermittent bolus injections of a drug such as cyclophosphamide, but at doses well below the MTD (e.g., 1/3 MTD), given at long intervals (3- or 6-weeks), combined with the daily oral low-dose approach. In this regard, Browder et al. administered cyclophosphamide metronomically at 1/3 the MTD on a weekly basis and found that it induced significant and long-term tumor growth control of several transplanted mouse tumor cell lines (6). However, toxicity requiring supportive care measures was noted after long-term therapy. We asked whether the degree of toxicity may be minimized by reducing the frequency of the bolus drug administration, but the efficacy increased by combining it with minimally or nontoxic daily low-dose oral cyclophosphamide. We tested this combination treatment strategy using three different tumor models involving both spontaneous (i.e., nontransplanted) hematologic (“liquid”) and transplanted solid tumor models. Because metronomic chemotherapy has an antiangiogenic basis (1, 6), we assessed the effects of various chemotherapy regimens for antiangiogenic activity using vascular endothelial growth factor receptor-2 (VEGFR-2) circulating endothelial progenitor cells (CEPs) as a surrogate marker, which we recently described for targeted antiangiogenic drugs (10). Our results show marked increases in antitumor and antiangiogenic activities, accompanied by only modest toxicity, when using the combined intermittent bolus dose (BD) plus low-dose metronomic (LDM) cyclophosphamide treatment regimen.
Materials and Methods

Cyclophosphamide administration and treatment schedules. Cyclophosphamide (Procytix, Baxter Corp., Toronto, ON, Canada) was administered either orally (p.o.) via the drinking water at an approximate dose of 20 mg/kg/d as previously described (3), or i.p. at a dose of 150 mg/kg every 3 or 6 weeks as indicated. In the MTD control groups, a total dose (per cycle) of 450 mg/kg cyclophosphamide (3 \times 150 \text{ mg/kg}) on days 1, 3, and 5 was administered i.p. followed by a 2-week break, after which the 6-day cycle of treatment was repeated, as previously published (6, 11). Control mice received corresponding amounts of the cyclophosphamide diluent (normal saline), p.o. or i.p. as required.

Tumor inoculation/induction and assessment of disease progression. PC-3 human prostate cancer cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in DMEM supplemented with 5% FCS. Cells (2 \times 10^6) were injected s.c. into the right flank of 6- to 8-week-old male NIH Swiss athymic nude mice. EMT-6/CTX mouse breast tumor cells (2 \times 10^6) were transplanted s.c. into the flanks of 6- to 8-week-old female BALB/c. EMT-6/CTX is a subline of EMT-6 selected for acquired resistance to cyclophosphamide by multiple exposures to high doses of the drug in vivo, as described by Teicher et al. (12). Tumor size was assessed by means of Vernier scale calipers and the formula (width^2 \times length) \times 0.5. Once tumors reached 200 mm^3, treatment was initiated in groups of five to eight animals. Murine erythroleukemia was induced by the injection of Friend murine leukemia virus (F-MuLV) as previously described (13). Briefly, Friend viral lysates were injected i.p. into age-matched BALB/cj neonates 1 day after birth. Weaning of all offspring took place 3 weeks post-birth after which they were randomly separated into treatment groups. Disease progression was followed by weekly hematocrit measurements. Fifty to 60 \mu L of tail vein blood was centrifuged in heparinized capillary tubes at 1,400 \times g in an Ultra Micro-Hematocrit centrifuge and the percentage of RBC was calculated by use of a hematocrit gauge. All animal work was carried out according to institutional guidelines.

Toxicity assessment. Body weight and signs of animal distress were assessed and documented on a weekly basis, if not more frequently. For total white and differential (lymphocyte and neutrophil) blood counts, blood was collected from the lateral tail vein. Smears were prepared by the wedge slide technique followed by standard Wright’s staining, and analyzed under a light microscope. Differential WBC counts were not assessed in the EMT-6 tumor model because an elevated number of circulating tumor cells has already appeared in the bloodstream in the early stage of disease.

Evaluation of circulating endothelial progenitor cells by flow cytometry. Evaluation of viable CEPs was carried out on blood collected by either cardiac puncture or from the orbital sinus (as indicated in the text), followed by enumeration using four-color flow cytometry, as previously described (2). Briefly, anti-CD13, anti-flk-1 (vascular endothelial growth factor receptor-2), anti-CD45, and anti-CD117 fluorochrome-labeled antibodies were purchased from BD Biosciences, Canada. Nuclear staining (Procunt, BD, San Jose, CA) was conducted to exclude the possibility of platelets or cellular debris interfering with the accuracy of CEP enumeration (14, 15). After red cell lysis, cell suspensions were evaluated by a FACSCalibur cell analyzer and CellQuest Pro acquisition to acquire at least 100,000 events per sample in order to analyze the percentage of CEPs. The absolute number of CEPs was then calculated as the percentage of the events that were collected in the CEP enumeration gates, multiplied by the total white cell count (WBC). 7-Aminoactinomycin D was used to enumerate viable versus apoptotic and dead cells (16).

Statistical analysis. Results are reported as the mean \pm SD. Statistical significance of differences was assessed by the Student’s t test, using Microsoft Office 2000 Excel or GraphPad Prism 4.00 software. The level of significance was set at \( P < 0.05 \).

Results

Intermittent bolus dose combined with daily low-dose cyclophosphamide improves tumor control in solid and hematologic tumor models. As previously reported, PC-3 xenograft–bearing mice treated with LDM cyclophosphamide administered through the drinking water on a daily nonstop basis exhibit an initial regression phase followed by a 6- to 8-week period (long-term) of stable disease, after which the tumors relapse in spite of continuous treatment (3). We sought to determine whether concomitant administration of BD and LDM cyclophosphamide might prevent or significantly delay such relapses, presumably by targeting both the tumor cell and the vascular compartment of the growing tumor. To do so, mice with established (~200 mm^3) PC-3 human prostate cancer xenograft tumors were treated with bolus injections of 150 mg/kg of cyclophosphamide administered i.p. every 3 weeks which was accompanied by ~20 mg/kg/d of cyclophosphamide (i.e., the LDM cyclophosphamide regimen) administered continuously through the drinking water without any breaks. The interval of 3 weeks was chosen based on preliminary experiments, studying PC-3 tumor growth kinetics after administration of 150 mg/kg of cyclophosphamide. The dose of 150 mg/kg was intentionally chosen to keep it well below the MTD (i.e., 3 \times 150 \text{ mg/kg every 2 days per 6-day cycle}) in order not to compromise the favorable toxicity profile of the LDM cyclophosphamide regimen, in contrast to the very toxic MTD regimen. Figure 1A summarizes the results indicating a striking superiority of both combined BD + LDM and MTD cyclophosphamide regimens over either the BD or LDM cyclophosphamide monotherapies. Similar results were obtained using the aggressive syngeneic EMT-6/CTX murine breast tumor model (Fig. 1B), i.e., both BD + LDM and MTD regimens induced significantly improved tumor growth delays.

In addition, to test whether the combination BD + LDM regimen also has an impact on a hematologic malignancy, treatment was initiated in mice with advanced stage erythroleukemia, i.e., when a significant reduction in hematocrit levels was observed (~4-week-old mice) due to progressive replacement of the spleen with malignant erythroid cells. A prolonged median survival time of 2 weeks was obtained using the LDM cyclophosphamide regimen alone compared with the untreated group; this was reflected by a transient stabilization of the hematocrit levels. Subsequently, a marked hematocrit decline was observed after 6 weeks of treatment and mice had to be sacrificed due to severe anemia as the growing erythroleukemic cells displaced normal erythropoiesis (Fig. 1C and D, respectively). Based on results of preliminary experiments, BD injections were administered every 6 weeks in this model, and were found to be more effective in terms of delaying tumor relapse than the LDM monotherapy (prolongation of the median survival by 4 weeks). However, the combination BD + LDM cyclophosphamide regimen resulted in an unprecedented prolongation of median survival by 12 weeks. More importantly, the MTD regimen was not nearly as effective in this model, and mice had to be sacrificed after two complete cycles of MTD cyclophosphamide regimen due to severe body weight loss. Overall, the results presented for all three models suggest superior efficacy and potent antitumor activity of the BD + LDM cyclophosphamide regimen, although complete tumor eradication was not achieved in any of the models tested.

The bolus dose + low-dose metronomic cyclophosphamide regimen exhibits similar hematotoxicity in comparison with the low-dose metronomic cyclophosphamide regimen, but has less host toxicity than the maximum tolerated dose cyclophosphamide regimen. By applying the BD + LDM regimen, the total dose of cyclophosphamide during a 3-week period is actually
BD + LDM improves treatment efficacy and delays relapse in both solid and hematologic malignancies. A, PC-3 human xenograft; B, EMT-6/CTX murine breast cancer; C and D, “spontaneous” murine erythroleukemia model were treated with various cyclophosphamide regimens. For solid tumor models, tumor volumes were measured on a weekly (A) or biweekly (B) basis, using Vernier scale calipers. For the erythroleukemia model, tumor growth (C) was assessed by hematocrit measurements as described in Materials and Methods, and survival (D) was plotted using Kaplan-Meier analysis. Arrows, bolus administration (i.e., either BD or MTD regimens): full arrows, BD and MTD regimens in solid tumor models (A and B; 150 mg/kg and 3 × 150 mg/kg, respectively); in liquid tumor model (C), full arrow for MTD and dashed arrows for BD regimens. The LDM cyclophosphamide regimen (i.e., 20 mg/kg/d) was administered through the drinking water in all groups when treatment was initiated as described in the text. Of note, the BD cyclophosphamide regimen was not assessed in the EMT-6/CTX tumor model.
somewhat higher than the 450 mg/kg of the MTD schedule (570 and 495 mg/kg for the treatment of PC-3 xenografts and Friend virus erythroleukemia, respectively). Given the known myelo- and immunosuppressive potential of cyclophosphamide, we therefore assessed various hematologic variables, and body weight as variables of host toxicity as previously described (7, 11). The results in Fig. 2 show that in both PC-3 and erythroleukemia tumor models, the LDM cyclophosphamide regimen results in a drop in the total WBC at day 42 (Fig. 2A and B, respectively), which is mostly due to lymphopenia, as previously described (11). Similar

Figure 2. Toxicity evaluation in both solid and liquid tumor models treated with cyclophosphamide in various regimens. Hematologic toxicity: one drop of blood (from tail vein) was taken after 42 days of treatment for both PC-3 human xenograft (A), and erythroleukemia (B) models (n ≥ 5/group), in order to assess the lymphocyte, neutrophil and total white blood counts, using a blood smear technique. Constitutional toxicity measured by body weight changes: All mice in each group were weighed on a weekly basis in the PC-3 (C), EMT-6/CTX (D) and erythroleukemia (E) tumor models (n ≥ 5/group). Results are presented as a percentage of change in body weight from the baseline, when treatment was initiated. C and D, arrows, either MTD or BD cyclophosphamide regimens. E, full arrows, MTD cyclophosphamide dosing; dashed arrows, BD cyclophosphamide. LDM cyclophosphamide regimen was administered when treatment was initiated in all groups, and was given continuously as described in the text. Differential WBC counts and BD cyclophosphamide regimen was not assessed in the EMT-6/CTX tumor model.
results of hematotoxicity were observed after each complete cycle (i.e., at days 84 and 147) for both the PC-3 and erythroleukemia tumor models (data not shown). Interestingly, the BD cyclophosphamide monotherapy seems to be devoid of such potentially immunosuppressive activity. Accordingly, in combination with LDM cyclophosphamide (i.e., BD + LDM cyclophosphamide), this regimen exhibits lymphopenia similar to the LDM monotherapy regimen. The MTD cyclophosphamide results in a similar degree of immunosuppression as the LDM cyclophosphamide. Erythropoiesis was not significantly affected by any of the treatments in the PC-3 model as evidenced by stable hematocrit levels (data not shown). In addition, we observed less pronounced body weight loss in the PC-3, EMT-6/CTX and erythroleukemia models with the combination treatment regimen (i.e., BD + LDM cyclophosphamide), as opposed to the marked changes occurring with the MTD cyclophosphamide regimen. The latter showed a body weight loss of 8%, 7%, and 13% for PC-3, EMT-6/CTX and erythroleukemia models, respectively, during the first treatment cycle (Fig. 2C, D, and E, respectively). It is worthwhile mentioning that in the case of MTD scheduling in all tumor models, the mice recovered from their weight loss at the end of each cycle [except for the case of PC-3 tumor model when the mice did not recover from the sixth MTD cycle (Fig. 2C)]. Furthermore, in the case of erythroleukemic mice, the MTD group had to be sacrificed after two complete cycles of treatment due to weight loss exceeding 15%, whereas the BD + LDM regimen was not significantly different from the remaining treatment groups (Fig. 2E). Because we have shown recently that BALB/cf recover completely by the end of one MTD cyclophosphamide cycle (11) and nonleukemic BALB/cf mice tolerate at least four MTD cyclophosphamide cycles, this suggests that leukemia progression is the primary factor responsible for the severe weight loss observed. Overall, the results in Fig. 2 indicate that in all tumor models tested the combination approach is feasible, and less toxic than the respective MTD regimen.

The bolus dose + low-dose metronomic regimen causes a sustained reduction in the viability of circulating endothelial progenitor cells. A recent study showed that the administration of LDM cyclophosphamide either at 170 mg/kg/wk or at 20 mg/kg/d (administered through the drinking water) causes a sustained reduction in viable CEPs in comparison with the MTD cyclophosphamide regimen (which caused a marked drop followed by a rapid rebound during the break period), in two human lymphoma xenograft models (2). The suppression in mobilization and viability of such cells indicates, at least in part, the involvement of an antiangiogenic/antivasculogenic response (2). This conclusion is reinforced by recent results validating CEPs as a surrogate marker of angiogenesis in mice (10). We therefore asked whether the levels of CEPs remain suppressed even when the LDM cyclophosphamide regimen is administered for prolonged periods of time, and whether the combination treatment (i.e., BD + LDM) regimen has the same, or even better, antiangiogenic effect as the LDM regimen, i.e., equal or more sustained suppressed levels of CEPs. The results in Fig. 3 show that in the case of PC-3 tumor model, after 1 week of treatment, the levels of viable CEPs decreased in all treated groups; however, a relative increase in viable CEPs was observed after 12 weeks of treatment in the LDM cyclophosphamide group in comparison with the BD + LDM regimen (Fig. 3A). In contrast, in the erythroleukemia model, after 1 week of treatment, both LDM and BD + LDM regimens were significantly lower in comparison with the untreated control group. However, after 6 weeks of treatment, we observed relatively lower but not significantly different suppressed levels of viable CEPs using the BD + LDM regimen (Fig. 3B). The differences in CEP levels between these

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1 Y. Shaked, unpublished observations.
tumor models are likely due to the blood sampling at different time points (12 and 6 weeks, respectively) and perhaps the use of two different classes of malignancies representing solid or liquid tumors. However, in the case of treatment using the BD + LDM regimens—in both the PC-3 and erythroleukemia models—we observed relative reduced levels of viable CEPs, at both 6 and 12 weeks. Interestingly, the BD monotherapy regimen showed reduced viable CEPs similar in magnitude to the BD + LDM regimen, even after 12 weeks of treatment in the PC-3 tumor-bearing mice as opposed to the BD group in the erythroleukemia model where the levels of viable CEPs were significantly higher than the BD + LDM regimen. Moreover, we must note that at the levels of viable CEPs in the MTD groups do not represent an antiangiogenic effect by definition, because the levels of such cells depend on the time of sampling (i.e., reduced at the time of drug administration but increased during the later stages of the drug-free break period) as previously described (2). Overall, these results suggest that suppression of angiogenesis may be a mechanism, at least in part, for the delayed tumor relapses using the modified combination regimen (BD + LDM).

Discussion

There are a number of ways that the antitumor activity of a given metronomic chemotherapy drug regimen can be improved: (a) by an up-front short course of a higher cumulative dose of the drug such as one or two cycles of the MTD, i.e., so-called chemo-switching (8); (b) by concurrent combination with a targeted antiangiogenic drug (6, 7), or some other type of drug such as a tumor vaccine (17); (c) by combinations of a and b (7, 8); (d) by determining, in theory, the optimal biological metronomic dose using a surrogate marker such as CEPs, as we recently showed for targeted antiangiogenic drugs (10); (e) and possibly, by combining two different chemotherapeutic drugs administered in low-dose metronomic fashion such as cyclophosphamide and methotrexate (18). Our results now suggest another possibility: by combining intermittent BD injections of using much lower than MTDs, along with much more frequent (daily, in this case) oral administration of relatively low doses of the same drug. This might be likened to the combined use of “fast” plus “slow” metronomic chemotherapies.

There are several possible explanations for the superior antitumor activity of the BD + LDM cyclophosphamide regimen. First, the bolus of 150 mg/kg might reinforce the antiangiogenic effects of LDM cyclophosphamide. Indeed, a similar BD (170 mg/kg) was administered, but every 6 days, by Browder et al. who found that this regimen induced potent antiangiogenic activity and induced major antitumor effects on tumors selected in vivo for high levels of acquired resistance to cyclophosphamide (6). Moreover, the demonstration of more sustained suppression of CEPs with the combination treatment when compared with the LDM cyclophosphamide monotherapy regimen suggests a more efficacious anti-“vasculogenic” effect. Second, LDM cyclophosphamide increases tumor hypoxia,8 which may further be aggravated by increased oxygen consumption due to bolus cyclophosphamide induced DNA repair activity. As such, the anoxia/hypoxia-mediated antitumor effects might be increased. Third, BD cyclophosphamide might simply exert a direct tumor debulking effect on drug-sensitive tumor cells. Fourth, the LDM chemotherapy might induce normalization of the tumor neovasculature (19), which in turn might increase delivery into the tumor of the injected BD cyclophosphamide (19). Finally, although LDM cyclophosphamide is thought to work primarily through antiangiogenic mechanisms, it might (also) exert some chemosensitizing effects or slow down tumor cell repopulation kinetics that have been observed to take place between BD chemotherapeutic drug administrations (20).

Not unexpectedly, the improved antitumor activity of the combination of BD + LDM cyclophosphamide comes at the price of some increased toxicity. However, the BD + LDM protocol is clearly superior when tested in the erythroleukemia model compared with a standard MTD cyclophosphamide regimen. In the PC-3 model, where the BD was given every 3 weeks instead of every 6 weeks, the combination regimen was more toxic, but nevertheless still compares favorably with the MTD protocol. The aggressive EMT-6/CTX tumor model is clearly the least responsive. Nevertheless, we still found that the impact of BD + LDM cyclophosphamide regimen is similar to the MTD cyclophosphamide regimen but with less toxicity (as indicated in Fig. 2D). Of note is the fact that the mice had to be sacrificed according to institutional guidelines probably before the full “metronomic” effect comes into play. Thus, taken together, it would seem that the BD + LDM regimen is at least as effective, but less toxic than the MTD regimen in all models tested (either solid or liquid tumors). It is important to point out that the combination therapy does not aggravate immunosuppression, a side effect of LDM cyclophosphamide that we recently described, and which may be of potential clinical relevance (11). Preliminary experiments with lower BD cyclophosphamide doses still show significant antiangiogenic activity but not to the same extent we observed with the BD + LDM regimen, but clearly found to be less toxic.8 In clinical terms, it is therefore conceivable that the BD might be titrated according to an individual’s tolerance/toxicity (21).

As opposed to the approach of adding antiangiogenic compounds such as bevacizumab to conventional MTD chemotherapy regimens, our approach offers potentially reduced acute toxicities (22), and reduced costs when drugs such as cyclophosphamide are used. It remains to be determined whether the tumor being treated must have some inherent sensitivity to the drug used for bolus administration in order to obtain the superior antitumor effects we have observed. Other questions which need to be addressed include whether the combination of LDM cyclophosphamide with bolus administration of other drugs, such as taxanes (which are known for their own antiangiogenic properties, e.g., refs. 23–25) would be similarly beneficial, and whether the combination of targeted antiangiogenic drugs will improve the effects of the type of sequential metronomic chemotherapy we have tested, in a manner similar, in principle, to the chemo-switching protocol described recently by Pietras and Hanahan (8).

The modified regimen we tested has a therapeutic advantage over MTD regimens, at the very least in terms of toxicity. Of the models we tested, only one, the PC-3 tumor xenograft model, did not result in a significant difference in the median survival of BD + LDM versus MTD treatment groups (data not shown). However, it should be noted that only very minor host toxicity measured by body weight fluctuations was observed in the BD + LDM group. Furthermore, in the erythroleukemia model, we

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3 Y. Shaked and U. Emmenegger, unpublished observations.
observed a significant difference in the median survival of BD + LDM compared with the MTD regimen, and in the absence of general toxicity. Although in the BD + LDM regimen, the cumulative dose was greater than in the respective MTD regimen (570 and 450 mg/kg/cycle, respectively), it nevertheless had less toxic effects. Thus, our modified regimen is a potentially useful regimen to consider for clinical evaluation.

In summary, interspersing bolus injections of higher, but still moderate, relatively nontoxic doses of cyclophosphamide, spaced every 3 or 6 weeks, along with uninterrupted daily low-dose oral cyclophosphamide seems to significantly improve the antitumor treatment efficacy of the daily low-dose oral metronomic regimen, but without inducing unacceptable toxicity. Preliminary evidence indicates that LDM cyclophosphamide regimen can have striking activity on prolonging the survival of mice with advanced metastatic breast cancer when it is combined with a second oral metronomic chemotherapy protocol involving daily oral administration of UFT, i.e., uracil plus tegafur, a 5-fluourouracil prodrug which can be administered clinically at low doses to patients every day for 2 years with no breaks as a beneficial (active) adjuvant treatment of early stage non–small cell lung cancer (26).

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