

Antitumor Effects in Mice of Low-dose (Metronomic) Cyclophosphamide Administered Continuously through the Drinking Water¹

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Abstract

A number of recent preclinical studies have sparked interest in the concept of exploiting conventional chemotherapeutic drugs as antiangiogenic. Such antiangiogenic activity is achieved or optimized by metronomic-dosing protocols in which the drug is given at comparatively low doses using a frequent schedule of administration (*e.g.*, once to three times per week) with no breaks, particularly when combined with an endothelial cell-specific antiangiogenic drug. The use of *p.o.* chemotherapeutic drugs is particularly suitable for this type of treatment strategy. We tested one such drug, cyclophosphamide (CTX), in a protocol wherein the drug was administered to mice at low doses, of ~10–40 mg/kg on a daily basis through the drinking water. CTX is typically given *p.o.* to patients, but it has almost always been injected when treating preclinical mouse tumor models. We found *p.o.* CTX to be a safe and convenient treatment with significant antitumor efficacy. Growth delays were observed for human orthotopic breast or ectopic colon cancer xenografts in nude or SCID mice. Established PC3 human prostate tumor xenografts could be induced to almost fully regress, remaining virtually nonpalpable for ≥2 months of continuous therapy, after which tumors began to grow progressively. These re-emergent tumors were not found to be drug resistant when tested in new hosts, using the same treatment protocol. Regression of spontaneously arising, late-stage pancreatic islet cell carcinomas in Rip Tag transgenic mice was also observed. The effects of continuous *p.o.* CTX treatment were enhanced significantly in an orthotopic, metastatic breast cancer xenograft model when used in combination with an antivascular endothelial growth factor receptor-2 blocking antibody. Maximum tolerated dose levels established for other mouse strains proved highly toxic to SCID mice, whereas daily *p.o.* low-dose regimens of CTX were well tolerated. Taken together, the results demonstrate the feasibility of delivering CTX in a *p.o.* metronomic chemotherapy regimen, which proved safe, reasonably efficacious, and potentially applicable to chronic treatment. Such a regimen may be particularly well suited for integration with antiangiogenic drugs.

Introduction

Conventional cytotoxic anticancer chemotherapeutic drugs were developed with the intent of treating cancer by direct killing, or inhibition of growth, of cycling tumor cells. Recently, however, there has been considerable interest in the notion of exploiting such drugs as angiogenesis inhibitors (1, 2). The rationale is based on the fact that

virtually all classes of cancer chemotherapeutic drugs are designed to damage DNA or disrupt microtubules of dividing cells and that endothelial cell division takes place during new blood vessel formation, including tumor angiogenesis (3, 4). Moreover, for reasons that are unclear, cycling endothelial cells may actually be more sensitive to certain chemotherapeutic drugs than other types of normal cell or tumor cells (5–7). Successfully targeting genetically stable, activated host endothelial cells in tumor vessels may have the advantage of a reduced susceptibility to drug resistance mechanisms driven by the types of genetic instabilities normally associated with the cancer cell genome (6, 8, 9), although certain tumor cell genetic mutations may, in some cases, influence the response to antiangiogenic therapy (10).

Browder *et al.* (9) reported that the potential antiangiogenic effects of a chemotherapeutic drug, such as CTX,³ may not be realized if the drug is administered in the conventional fashion, *i.e.*, at the MTD with long rest periods (*e.g.*, 2–3 weeks) between successive cycles of drug therapy. Such extended rest periods allow recovery of tumor endothelial cells from the damage inflicted by the chemotherapy (9). However, by administering the drug more frequently, *e.g.*, weekly, at lower doses (obligated to avoid myelosuppression), the recovery/repair process could be compromised, thus improving the antiangiogenic effects (9). Indeed, mouse tumors selected for resistance to CTX in a conventional MTD schedule were nevertheless sensitive if the regimen was switched to a lower-dose, more frequent schedule (9). This type of regimen, called “metronomic” dosing (11), or “antiangiogenic chemotherapy” (9), appears to have interesting precedents in the clinic (2), *e.g.*, significant percentages of breast or ovarian cancer patients who stopped responding to a once-every-3 week MTD taxane regimen have been reported to respond to a weekly, lower dose regimen of the same drug (reviewed in Ref. 2).

The effects of metronomic chemotherapy regimens can also be improved significantly by concurrent administration of a drug targeted to angiogenic endothelial cells, such as a monoclonal antibody to the VEGF R2, the small molecule TNP-470, the PEX protein fragment of MMP-2, or antibodies to endoglin (6, 12, 13). These observations suggest a logic for integrating chemotherapy with antiangiogenic drugs so as to improve efficacy while circumventing the acute undesirable toxic side effects associated with standard or high-dose chemotherapy.

Interest in assessing low-dose metronomic chemotherapy regimens in the clinic (14) has also been stimulated by the availability of a number of *p.o.* chemotherapy drugs, such as CTX, along with some encouraging preliminary clinical trial results using such drugs, *e.g.*, methotrexate, plus CTX (15). Given the recent results of several preclinical metronomic chemotherapy studies in which drugs, such as

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³ The abbreviations used are: CTX, cyclophosphamide; MTD, maximum tolerated dose; VEGF R2, vascular endothelial cell growth factor, type 2 receptor; hCG, human chorionic gonadotropin; FBS, fetal bovine serum; *p.o.*, per os (oral).

CTX (9, 13, 16), vinblastine (6), taxol (7), cisplatin (7), etoposide (12), or carboplatin (12), and topotecan (17) were administered by injection once to three times a week, we asked whether a p.o. drug in humans, such as CTX, can be chronically and safely administered in a similar (if not more frequent) but more convenient basis in mice, namely p.o., through drinking water. We report here that p.o. delivery is indeed possible, even in SCID mice, which are hypersensitive to the toxic effects of standard dosing schedules of CTX, and show that the effects of such a regimen can be improved by concurrent administration of an antiangiogenic drug, namely the anti-VEGF R2 antibody, DC101.

Materials and Methods

Tumor Cell Lines, Tumors, and Animals

The PC-3 human prostate and HT-29 human colon cancer cell lines were purchased from American Type Culture Collection (Rockville, MD). The WM115 and WM 239a human melanoma cell lines were obtained from Dr. Meenhard Herlyn. MDA-MB 231 is a human breast cancer cell line that was originally obtained from Dr. Jeff Lemontt (Genzyme Corp.), whereas MDA-MB 435, also a human breast cancer line, was originally obtained from Dr. Dalia Cohen (Novartis, East Hanover, NJ). HT-29 and MDA-MB 435 were transfected with pCleo-hCG (a gift from Dr. Bert Vogelstein, Johns Hopkins, Baltimore, MD) to generate HT-29hCG20 and 435hCG17. The pCleo-hCG gene expression construct was used to generate cell lines that secrete the β -subunit of hCG, which can be used as a soluble surrogate marker of tumor burden (18). WM115, WM239a, and MDA-MB 231 were maintained in tissue culture in RPMI 1640 supplemented with 5% FBS, and the other cell lines were grown in DMEM supplemented with 5% FBS. All cell lines were grown as monolayer cultures to 75% confluence, detached, made into single cell suspensions using 0.05% trypsin-EDTA (Invitrogen Canada, Inc., Burlington, Ontario, Canada), and washed once with complete medium and twice with FBS-free medium. Cells were counted and adjusted to the appropriate concentration for injection.

Male or female CB-17 SCID mice and NIH Swiss nude mice were purchased from Taconic (German Town, NY). PC-3 and HT29hCG20 were injected s.c. in the right flank at $2 \times 10^6/0.2$ ml and $5 \times 10^6/0.2$ ml, respectively, of each mouse. Two million/50 μ l WM115 and WM239a cells were injected subdermally in the right flank; $2 \times 10^6/50$ μ l MDA-MB 231 and MDA-MB-435hCG17 cells were implanted orthotopically into the mammary fat pad. After cell implantation, the mice were randomized into groups of five or six and monitored on a weekly basis for tumor growth. Perpendicular tumor diameters were measured using Vernier scale calipers, and tumor volume was calculated using the formula $[(x^2 \times y)/2]$ for an ellipsoid. When the primary tumor volume reached ~ 150 – 200 mm³, therapy was initiated. Institutional guidelines were followed for maintenance of animals and end point of tumor studies.

We also studied the Rip-Tag transgenic mouse model of *de novo* pancreatic islet carcinogenesis, wherein the large and small T antigen oncogenes of SV40 (SV40 Tag) are targeted to pancreatic β cells using an insulin gene's regulatory elements (19). Mice die of tumor burden at 13–14 weeks of age. A regression trial was performed in which therapy was initiated at week 12, as described in "Results," and elsewhere (20).

Drugs and Therapeutic Schedules

1. Low-dose Metronomic Schedule of Continuous p.o. Administration of Cyclophosphamide. CTX (Procytox) is manufactured in the form of a white powder by Carter-Horner, Inc. (Mississauga, Ontario, Canada) and purchased from the hospital pharmacy (Sunnybrook & Women's College Health Sciences Centre, Toronto, Ontario, Canada); it is reconstituted as per manufacturer's instructions to a stock concentration of 20 mg/ml by the addition of sterile distilled H₂O. The reconstituted CTX was stored at 4°C in the dark and used within the week. Mice were housed five per cage and given 200 ml of water (with/without CTX), which is changed twice per week. Each mouse is estimated to drink 1.5 ml of water/10 grams of body weight/day (21, 22). To achieve as close as possible a dose of 10, 20, 25, or 40 mg/kg CTX,

0.66, 1.25, 1.67, or 2.67 ml, respectively, of the stock solution were added to 200 ml of water. Control mice are given an equivalent volume of normal saline in their drinking water because this is the only nonmedicinal ingredient in the powder. Weight loss was used as a parameter for toxicity; the mice were weighed once per week to determine any toxic effect of the drug. An estimated dose between 20 and 25 mg/kg/day was used in most experiments, whereas doses of 10 or 40 mg/kg/day were used in others.

2. Standard Therapy with CTX. The schedule was a 21-day cycle of either 100 or 150 mg/kg CTX, administered i.p. once every other day over 6 days (*i.e.*, three injections), followed by 2 weeks of rest (9).

3. Administration of DC101 monoclonal anti-VEGF R2/flk-1 Antibody. DC101 is a neutralizing monoclonal antibody against the mouse VEGF R2 (flk-1) (6). The DC101 was administered at 800 μ g/mouse i.p. twice per week.

Transfection. The method of assessing the tumor burden *in vivo* through measurement of urinary β -hCG by Shih (18) was adapted to independently verify the tumor volume as measured by using Vernier scale calipers. HT-29 and MDA-MB 435 cells were transfected with the pCleo-hCG and selected with neomycin. Medium was collected from individual clones and measured for hCG expression using the PATHOZYME-free β -hCG measurement kit (Omega Diagnostics, Ltd., Scotland, United Kingdom).

Measurement of hCG in Mouse Urine. Urine collection was carried out once per week at the same time that the tumors were measured. Each group of mice was placed in a "metabolic cage" lined with 96-well plates for 2 h, after which the urine in the wells were collected and pooled. The samples were stored at -80°C until the termination of the experiment when all of the samples were assayed together. The PATHOZYME-free β -hCG kit was used to measure the amount of hCG in the samples, and a Creatinine kit (Sigma Canada) was used to normalize the hCG readings. The levels were expressed as hCG units/mg Creatinine.

Statistical Analysis. The results (mean values \pm SD) of all of the experiments were subjected to statistical analysis by ANOVA, followed by the Student-Newman-Keuls test, using the GraphPad Prism software package v. 3.0 (GraphPad Software, Inc., San Diego, CA). The level of significance was set at $P < 0.05$.

Results

When CTX was administered continuously in the drinking water of xenotransplanted female NIH Swiss nude mice carrying tumors derived from human breast (435hCG17), colon (HT-29hCG20), and melanoma (WM115 and WM239a) cell lines, significant growth delays were observed, as early as 14–21 days ($P < 0.05$) after initiation of therapy. The results are shown in Fig. 1A for a derivative of HT29, called HT29hCG20; the results for 435hCG17, WM115, and WM239a tumors were very similar (data not shown). The changes in tumor volume measurements were confirmed by using secreted β -hCG in the urine as a surrogate marker of relative tumor burden (18) for HT29 colon tumor line engineered to express β -hCG, as shown in Fig. 1B. In addition, even more impressive antitumor responses were observed for s.c. PC-3 tumors growing in male NIH Swiss nude mice (Fig. 1, C and D); p.o. CTX produced almost complete regression of palpable tumor mass, followed by a long period (almost 60 days) of no net expansion in tumor mass during therapy, after which the tumors began to grow progressively while on therapy. When such "recurrent" tumors (*e.g.*, the cell line called '00.15,5–6) were excised, cultured for two to three passages, and then reimplanted into a new group of mice, they again responded to the low-dose p.o. metronomic schedule of CTX in a manner similar to the parental PC3 cells (Fig. 1D), suggesting that the tumor cells *per se* had not acquired resistance to the CTX therapy. This experiment has been repeated three times, and the results are similar.

We next asked whether the growth delays observed in the human tumor xenograft models could be enhanced if the CTX treatment was combined with an antiangiogenic drug, the anti-VEGF R2 antibody, DC101. Fig. 2 shows the results when the breast cancer cell line MDA-MB 231 was growing in CB-17 SCID mice after injection of

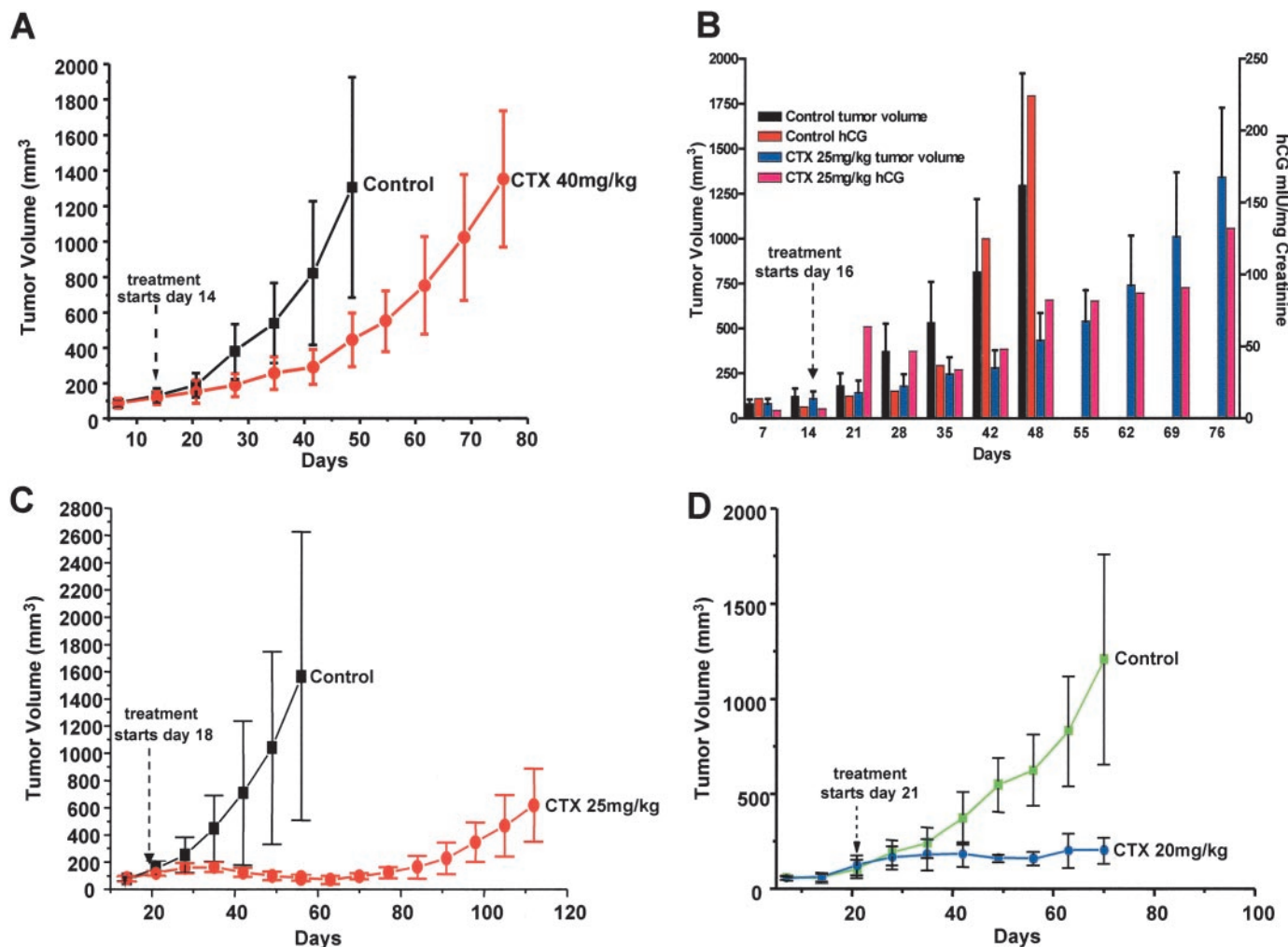


Fig. 1. Antitumor effects of continuous low-dose (~40 mg/kg/day) CTX administered through the drinking water of NIH Swiss nude mice. *A*, the tumor growth delay effect on established s.c. HT29 human colon carcinoma HT29hCG20 xenografts; very similar growth delay effects were noted on established MDA-MB-435 human breast cancer cell xenografts implanted orthotopically (using a dose of 25 mg/kg/day) and established WM115 and WM239 human implanted melanoma xenografts s.c. using a dose of 40 mg/kg/day (data not shown). *B*, noninvasive assessment of relative tumor burdens of s.c. human colon HT29hCG20 xenografts in NIH Swiss nude mice assessed by tumor volume as measured by Vernier scale calipers *versus* urinary β -hCG levels. The measurement of β -hCG is of pooled urine samples from each group of mice. *C*, effect of continuous low-dose CTX (25 mg/kg/day) on established s.c. PC3 human prostate cancer xenografts. The tumors fully regressed and then remained dormant (nonpalpable) for almost 2 months of continuous therapy, after which they began to relapse, around day 85. In *D*, when relapsing PC3 variant tumors were removed from the p.o. CTX-treated mice, established as cell lines in culture, and re injected within 3–4 weeks into new hosts, such variants, *e.g.*, cell line 00.15.5–6, responded to the p.o. CTX therapy in a similar fashion as the parental population, suggesting the tumor cells are not stable drug-resistant subpopulations.

cells into the mammary fat pads of female mice. This orthotopic transplantation method resulted in the formation of extensive metastases in the lungs, liver, and lymph nodes. In these experiments, we compared p.o., low-dose CTX with a conventional, MTD protocol. The MTD, established in other mouse strains, involved 150 mg/kg CTX injected i.p. once every day for 6 days for a total dose of 450 mg/kg/cycle. In an initial experiment, the standard (MTD) therapy worked equally well, if not better, than the low-dose CTX in initially reducing tumor size. However, this “standard” MTD of CTX proved extremely toxic to SCID mice, resulting in severe weight loss and death of mice after 1 week of treatment (Fig. 2, *top* and *middle panels*). In striking contrast, no weight loss or other signs of toxicity were observed in the group of SCID mice treated with ~25 mg/kg/day for 50 days nonstop of therapy using the p.o. method of administration, which represents approximately at least three times the cumulative dose of standard dose therapy.

Because the 450 mg/cycle regimen was acutely lethal to SCID mice, we repeated the experiment with six mice per group using a lower bolus dose that represented a true MTD regimen for SCID mice,

evaluating its effects in comparison with a continuous p.o. low-dose regimen, used alone, or in combination with concurrent DC101 antibody treatment. The results are shown in the bottom panel of Fig. 2. The MTD treatment consisted of 100 mg/kg CTX injected every 2 days over a 6-day period for a total dose of 300 mg/kg/cycle, followed by a 2-week rest period. In this experiment, we used an estimated dose of 20 mg/kg/day for the continuous p.o. low-dose regimen. DC101 was administered twice per week. As shown in Fig. 2, both the p.o. low-dose CTX treatment and DC101 treatment groups had a prolongation of survival of almost 30 days compared with the untreated control group. Mice in the control group, because of their moribund state, had to be sacrificed at approximately day 55 after tumor cell injection, compared with day 83 for the DC101 or low-dose CTX groups. Mice in the MTD CTX group had to be sacrificed ~1 week earlier. The two drug combination treatment groups (*i.e.*, CTX + DC101) showed enhanced survival, with the low-dose CTX + DC101 the superior of the two regimens. Thus, all six mice in the MTD CTX + DC101 group died between days 82 and 100, whereas all of the mice in the low-dose CTX + DC101 group were

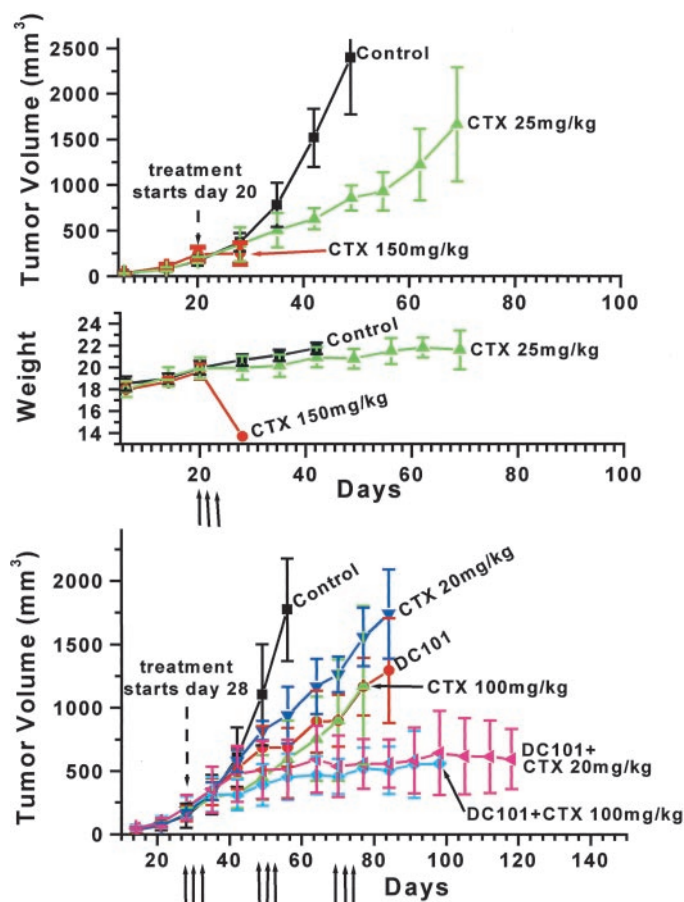


Fig. 2. *Top*, the response of established orthotopic MDA-MB-231 breast tumor xenografts to contrasting CTX therapy regimens. CTX 25 mg/kg refers to a continuous low-dose p.o. (administered via the drinking water) regimen. Mice were sacrificed at day 70. CTX 150 mg/kg refers to a MTD regimen in which CTX was injected i.p. every other day over 6 days for a total dose of 450 mg/kg/cycle therapy. *Vertical arrows below the middle panel*, the time of injections. This panel also shows body weight of tumor-bearing control and CTX-treated mice. Note the rapid decline in body weight is the group treated with the 450 mg/kg/cycle (150 mg/kg/injection) CTX. *Bottom panel*, the efforts of a p.o. low-dose CTX regimen at ~20 mg/kg/day versus an MTD regimen consisting of 100 mg/kg/injection (300 mg/kg/cycle) MTD CTX therapy. Three injections (*vertical arrowheads*) comprised one cycle of therapy, which was followed by a 2-week rest period. DC101, the DC101 anti-VEGF R2 antibody treatment, which was administered twice a week at a dose of 800 μ g/mouse. All mice in the MTD CTX + DC101 group died between days 82 and 100. In contrast, all mice in the low-dose + DC101 treatment group were alive at day 120. Thus, prolongation of survival was maximal in the low-dose CTX + DC101 treatment group. These mice were also found to be largely free of lymph node, lung, and liver metastases, in contrast to the other treatment groups and control.⁴

alive at day 120. Aside from toxicity of the CTX treatment, another factor that may have influenced survival was the formation of extensive metastases in sites, such as the lymph nodes, lungs (macroscopic lesions), and liver (mostly microscopic lesions). It is of interest that we could not detect metastases, even by histology, in any of the six mice treated with the combination low-dose CTX/DC101 therapy group, when those mice were sacrificed at day 125.

Finally, we also found the p.o. CTX protocol in this case, used at 10 mg/kg/day, caused modest regressions of spontaneously arising pancreatic islet tumors in Rip-Tag transgenic mice (Fig. 3). Treatment was initiated when mice were 12 weeks of age, *i.e.*, a so-called "regression trial" of advanced stage tumors (20). Mice normally die by weeks 13–14. As shown in Fig. 3, tumors were smaller at week 16 than at week 12. Half the treated mice were still alive at week 16, whereas all sham-treated mice were dead by 14 weeks. For compar-

ison, a low-dose vinblastine protocol (6) was also evaluated, demonstrating no such efficacy.

Discussion

The motivation to test the antitumor effects of cyclophosphamide administered to mice on a daily basis through the drinking water was based on several considerations: (a) p.o. bioavailable drugs are well suited for protocols in which the drugs are administered frequently, as often as once a day (2, 15); (b) CTX itself has been used in this fashion clinically, *e.g.*, in daily administration at low doses for prolonged periods, with no breaks, in metastatic breast cancer patients, where few significant toxic side effects were noted (15); and (c) CTX has shown positive responses both preclinically (9, 13, 16) and clinically (15) in the context of metronomic/antiangiogenic chemotherapy protocols. In the first preclinical study, by Browder *et al.* (9), the drug was injected at one-third of the MTD i.p., once a week, whereas in the clinical study by Colleoni *et al.* (15), it was given daily, in the form of a 50-mg tablet. We chose to mimic aspects of the clinical study in mice. However, rather than giving the drug invasively by gavage every day, we tested the effects of a noninvasive protocol wherein it was supplied in the drinking water. As far as we are aware, this has not been reported previously.

The results we obtained are encouraging in several respects. The antitumor efficacy results were obvious, ranging from significant tumor growth delays and prolongation of survival to actual regressions of established s.c. (ectopic), transplanted tumors (*e.g.*, the PC3 prostate carcinoma), or spontaneous pancreatic islet cell tumors arising in Rip Tag transgenic mice. Furthermore, the antitumor effects were not restricted to ectopic (s.c.) growing primary tumors. Thus, orthotopic human breast cancers (and the resultant distant lung and liver metastases in such mice) were affected by the daily p.o. low-dose CTX therapy. Toxic side effects, such as loss of body weight, were not apparent, even when the drug was ingested every day for 3 months in SCID mice, despite the fact that these mice appear to be so sensitive to the same drug given the MTDs for other strains of mice (*e.g.*, 450 mg/cycle) they died within a week.

An obvious experimental drawback of p.o. drugs given by the drinking water to mice, as opposed to gavaging, is that the exact amounts of drug the animals receive are not known. Estimates of drug dose are based on the average daily amount of water (3 ml for a 20-gram mouse) that mice normally drink. Pyruvate kinase studies to examine levels of CTX pro-drug and its active toxic metabolites 4-hydroperoxycyclophosphamide and acrolein (23–25) are under way.

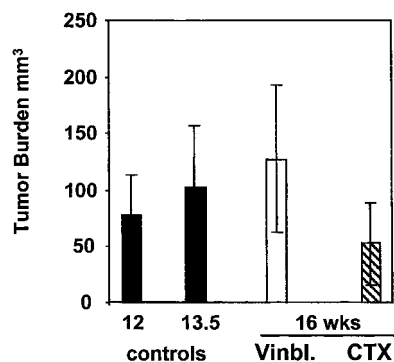


Fig. 3. Regression trial (20) of continuous p.o. CTX (10 mg/kg/day), administered through the drinking water or vinblastine injected every 3 days at 1.5 mg/m², as described previously (6) in transgenic mice developing pancreatic islet cell carcinomas (19). Therapy was initiated when mice reached 12 weeks of age. Untreated mice died by week 16. The p.o. CTX protocol induced tumor regressions. Both the p.o. CTX and injected vinblastine protocols caused prolongation of survival of similar magnitude (data not shown).

⁴ S. Man *et al.*, unpublished observations.

Although CTX could be detected, the levels of the active metabolites were below the limit of detection of the assay.⁵

An important question raised by our results is whether the metronomic cyclophosphamide protocol we tested is specifically antiangiogenic, and if so, whether this property accounts for all the antitumor activity observed. We have recently obtained evidence using the Matrigel perfusion assay (6) that the oral cyclophosphamide schedule can suppress angiogenesis.⁶ However, it is unclear whether the chronic CTX regimen affects tumor cell growth/survival directly. We are currently addressing this issue in our ongoing studies. Also noteworthy is our observation regarding PC3 tumors, which began to grow again after an initial regression and a prolonged period of no net tumor growth. This could be interpreted as a developing form of tumor cell resistance against CTX. However, our finding that these tumors, when passaged and reimplanted into new host mice, show no sign of acquired resistance, may be an indication of a CTX-altered host mechanism such as altered drug metabolism by host liver enzymes (16). Alternatively, the susceptibility of endothelial cells may be affected by prolonged exposure to CTX, resulting in a reduced antiangiogenic effect of CTX. It should be noted that there is no direct experimental evidence for either of these possibilities. Nevertheless, this phenomenon of apparent resistance to CTX deserves further study.

Similar to our results published previously (6), as recently confirmed by others (12, 13, 17), we found that the addition of an antiangiogenic drug, DC101, to the low-dose p.o. cyclophosphamide protocol significantly increased antitumor efficacy and prolonged survival in an orthotopic breast cancer model. We are currently extending the p.o. CTX regimen to tumor cell lines preselected for high levels of acquired cyclophosphamide resistance and using quantitative assays to measure changes in metastatic tumor burden, such as the β -hCG method described by Shih *et al.* (18), as confirmed in this paper (Fig. 1B).

In summary, chronic administration of cyclophosphamide at low doses on a daily basis through the drinking water of mice appears to be a relatively safe and efficacious form of low-dose metronomic therapy. It will be of interest to determine whether other p.o. chemotherapeutic drugs can be similarly efficacious when delivered in comparable fashion, using nontoxic metronomic dosing schedules, especially in combination with other classes of antiangiogenic drugs. Finally, further delineation of the target cells of metronomic chemotherapy will be important, as there is prospect that such regimens are targeting multiple cell types of tumors (26), including endothelial cells and drug-sensitive tumor cells, as well as other constituents that contribute to the tumor phenotype.

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⁵ S. Ludeman and M. Colvin, unpublished observations.

⁶ S. Man and R. S. Kerbel, unpublished observations.