A Comparative Analysis of Low-Dose Metronomic Cyclophosphamide Reveals Absent or Low-Grade Toxicity on Tissues Highly Sensitive to the Toxic Effects of Maximum Tolerated Dose Regimens

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ABSTRACT

The survival benefits of traditional maximum tolerated dose (MTD) cytotoxic therapy have been modest for the treatment of most types of metastatic malignancy and, moreover, often come with increased acute and chronic toxicity. Recent studies have demonstrated that the frequent administration of comparatively low doses of cytotoxic agents, with no extended breaks [low-dose metronomic (LDM) chemotherapy], may not only be at least as efficient as MTD therapy but also less toxic. This coincides with an apparent selectivity for “activated” endothelial cells of the tumor vasculature. However, the impact of LDM chemotherapy on the most sensitive target cell populations normally affected by MTD therapy (i.e., bone marrow progenitors, gut mucosa, and hair follicle cells) has not been analyzed in experimental detail. Therefore, we compared effects of LDM and MTD cyclophosphamide (CTX) on bone marrow and gut mucosa. Furthermore, we studied the potential impact of LDM CTX on angiogenesis in the context of wound healing and evidence of organ toxicity. We show absent or moderate hematologic and intestinal toxicity of LDM as opposed to MTD CTX. Of note was the finding of sustained lymphopenia, which is not unexpected given the use of CTX as immuno-suppressive drug. There was no negative impact on wound healing or evidence of organ toxicity. LDM offers clear safety advantages over conventional MTD chemotherapy and therefore would appear to be ideal for long-term combination therapy with targeted antiangiogenic drugs.

INTRODUCTION

Shortly after the introduction of cytotoxic chemotherapy for the management of neoplastic diseases more than half a century ago, it became evident that frequently observed impressive responses were mostly short lived and that relapsing tumors that initially responded to chemotherapy became drug resistant. Several approaches were developed to try to overcome the development of drug resistance, such as dose escalation of single agents and multidrug combinations, development of cytotoxic drugs with new modes of action, and by pushing the limits of the maximal tolerated dose (MTD) through improving supportive care measures, growth factor and stem cell support. Although some of these approaches were validated recently (e.g., bone marrow progenitors, gut mucosa, and hair follicle cells) has not been analyzed in experimental detail. Therefore, we compared effects of LDM and MTD cyclophosphamide (CTX) on bone marrow and gut mucosa. Furthermore, we studied the potential impact of LDM CTX on angiogenesis in the context of wound healing and evidence of organ toxicity. We show absent or moderate hematologic and intestinal toxicity of LDM as opposed to MTD CTX. Of note was the finding of sustained lymphopenia, which is not unexpected given the use of CTX as immuno-suppressive drug. There was no negative impact on wound healing or evidence of organ toxicity. LDM offers clear safety advantages over conventional MTD chemotherapy and therefore would appear to be ideal for long-term combination therapy with targeted antiangiogenic drugs.

that more drug is better has resulted in a relative neglect in other important parameters, such as the timing and duration of cytotoxic chemotherapy (3, 4). In this regard, the frequent administration of comparatively low doses of cytotoxic agents, with no extended breaks, sometimes called metronomic chemotherapy (5), may not target tumor cells directly, as is primarily the case for the cyclic MTD approach, but indirectly via inhibiting angiogenesis and vasculogen-esis (2). As such, low-dose metronomic (LDM) chemotherapy may offer several advantages over the MTD approach, as shown by various preclinical studies (e.g., reduced toxicity, treatment response irrespective of the resistance profile of the tumor cell population, and the potential for long-term combination therapy with targeted drugs; Refs. 6, 7).

The low toxicity profile of LDM chemotherapy coincides with an apparent selectivity for “activated” endothelial cells of the tumor’s growing neovasculature (8, 9). Several in vitro studies have shown that endothelial cells are much more sensitive, sometimes by one or more orders of magnitude, to exposure of low concentrations of different cytotoxic drugs when compared with many tumor cell lines, fibroblasts of various origin, smooth muscle cells, macrophages, astrocytes, and mammary and prostate epithelial cells (10–12, unpublished observations). However, no data are yet available regarding the comparative sensitivity of the target cell populations that are normally most sensitive to MTD chemotherapy (i.e., bone marrow progenitors, gut mucosa, and hair follicle cells). Moreover, a thorough preclinical toxicity analysis of LDM therapy beyond the documentation of changes in body weight as a surrogate marker for toxicity is not available despite the fact that LDM chemotherapy is rapidly entering the clinical trial arena (13–17).

Therefore, the purpose of the present study was to study the effects of cyclophosphamide (CTX), clinically the most advanced drug in the setting of LDM chemotherapy, on bone marrow function and intestinal mucosa (13–15). Given a similar toxicity pattern for successive therapy cycles of MTD CTX, the observation period for MTD experiments was 3 weeks (corresponding to one treatment cycle). In the case of LDM CTX, the observation period spanned 42 days for hematologic and intestinal parameters. This period was chosen based on the finding that PC-3 xenograft growth could be controlled completely for at least 42 days with LDM CTX (18). We also studied the potential impact of LDM CTX on wound healing, an angiogenesis-dependent process, and morphologic and/or functional alterations of various organs (in particular, the bladder) in animals exposed to LDM CTX for more prolonged periods. The experiments comparing the effects of LDM versus MTD CTX were performed in non-tumor-bearing C.B-17 severe combined immunodeficient (SCID) and BALB/cJ mice (immunodeficient and immunocompetent mouse strains, respectively, often used for tumor experiments) because the presence of a tumor is known to influence bone marrow function, and treated and control animals also would necessarily have different tumor sizes, making interpretation of the obtained data extremely difficult (19). Our results show an unambiguous advantage with

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respects to reduced toxicity when the LDM CTX regimen, known to be significantly efficacious in several different preclinical tumor models, is used.

MATERIALS AND METHODS

Drugs

CTX (Procytox) is manufactured in the form of a white powder by Baxter Oncology GmbH (Toronto, Ontario, Canada) and was purchased from the hospital pharmacy (Sunnybrook & Women’s College Health Sciences Centre, Toronto, Ontario, Canada). The drug is reconstituted according to manufacturer’s instructions to a stock concentration of 20 mg/ml by the addition of sterile distilled H2O, stored at 4°C in the dark, and used within 1 week. DC101 is a neutralizing rat monoclonal antibody directed against the mouse type 2 receptor for vascular endothelial growth factor (VEGF), known as flk-1 or VEGFR-2 (ImClone Systems, Inc, New York, NY).

CTX Treatment Regimens

LDM Schedule. Mice were housed five per cage and given 200 ml water, which was changed twice per week. To achieve as close as possible a dose of 20 mg/kg/day of CTX, a volume of 1.33 ml of the CTX stock solution was added to 200 ml water. Control mice were given an equivalent volume of normal saline, the only nonmedicinal ingredient in the CTX powder (18). Standard Therapy (MTD). BALB/cJ mice were given 150 mg/kg CTX i.p. every other day for a total of three doses (6). C.B-17 SCID mice were administered 150 mg/kg CTX i.p. on day 0 and 100 mg/kg on days 2 and 4, respectively (18). The reduced total amount of CTX tolerated by C.B-17 SCID mice is explained by the defect defining the SCID phenotype, a mutation in the gene coding for the DNA-PKcs implicated in the repair of DNA damage, which renders such mice more susceptible to DNA-damaging agents (20).

Mice

Female BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME); male C.B-17 SCID mice were from Charles River Canada (St. Constant, Quebec, Canada); and NIH Swiss nude male mice were from Taconic (Taconic Town, NY). All of the experiments were started with 6–8-week-old adult mice, all sex and age matched, and performed with three animals per group unless otherwise indicated. All of the procedures were done in accordance with institutional and national guidelines.

Matrigel Plug Perfusion Assay

The Matrigel plug perfusion assay was performed as described previously, with minor modifications (21). Briefly, 0.5 ml Matrigel (Collaborative Biomedical Products, Bedford, MA) supplemented with 500 mg/ml of basic fibroblast growth factor (R&D Systems Inc., Minneapolis, MN) were injected s.c. on day 0 into both flanks of three female BALB/cJ mice per group. As a negative control, three mice were injected with Matrigel alone. Mice undergoing treatment, which was initiated on day 4, received normal saline p.o. and i.p. (positive control), normal saline p.o. and CTX i.p. (3 × 150 mg/kg every other day), CTX 20 mg/kg/day p.o. and saline i.p., and DC101 2 × 800 μg/mouse i.p. on days 4 and 7 as a positive treatment control. At day 10, all of the mice were injected i.v. with 0.2 ml of 25 mg/ml FITC-dextran (Sigma, St. Louis, MO). Plasma samples were collected, and Matrigel plugs were removed and incubated at 37°C overnight with Dispase (Collaborative Biomedical Products, Bedford, MA) and homogenized. Fluorescence readings were obtained using an FL600 Fluorescence Plate Reader (Biotec Instruments, Winooski, VT), and angiogenic response was expressed as the ratio of Matrigel plug fluorescence/plasma fluorescence. The ratio of the positive control minus background (= negative control) was set as 100%.

Wound Healing Assay

A circular full-thickness skin wound, including the panniculus carnosus, was created under isoflurane anesthesia on the shaved back of female BALB/cJ mice. Wounds were neither dressed nor sutured. Mice were initially housed individually in sterile microisolator cages with surgical pads to prevent infection and irritation. After retraction of the wound edges 24 h later, the wound area was measured daily by means of Vernier calipers comparing mice receiving an oral regimen of 20 mg/kg/day CTX versus normal saline (n = 5 per group). The wound area was calculated as follows: width × length × π/2.

Differential Blood Counts

Mice were anesthetized with isoflurane and bled by cardiac puncture. The blood samples were collected in Vacutainer K2-EDTA tubes (Becton Dickenson, Franklin Lakes, NJ). An aliquot was removed, diluted in 3% acetic acid, and the total WBC count (WBC) determined using a hemocytometer. Blood smears were prepared by the wedge slide technique, fixed with 95% methanol, and stained using the Wright-Giemsa method. For the differential blood counts, 200 cells/blood smear (in case of severe leukopenia, 100 cells/blood smear) were analyzed.

Ex Vivo Bone Marrow Proliferation Assay

After removing both femur bones, the distal end was trimmed, and the bone marrow was flushed from the other side with DMEM supplemented with 10% fetal bovine serum (Invitrogen Corp., Burlington, Ontario, Canada) with a 23-gauge needle. After the cells were washed twice and the nucleated cell count determined as outlined previously, 105 cells/well were seeded into 96-well tissue culture plates (Becton Dickinson) and incubated in a humidified atmosphere of 5% CO2 at 37°C for 1 h. Thereafter, cells were pulsed for 4 h with 2 μCi/well of methyl-[3H]-thymidine (Amersham Biosciences, Piscataway, NJ) and frozen and stored at −20°C until further analysis. After thawing and harvesting, UniFilter GF/C plates (Perkin-Elmer, Boston, MA) were read in a TopCount NXT microplate scintillation counter (Packard, Meriden, CT). All of the experiments were performed with eight replicates per mouse.

Colonizing Unit Granulocyte/Granulocyte-Monocyte Assay

Nucleated bone marrow cells were prepared, counted as outlined previously, and resuspended in Iscove’s modified Dulbecco’s medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 2% fetal bovine serum. A total of 15 × 10⁶ nucleated cells/ml of Methocult GF M35434 medium (Stemcell Technologies), which supports the growth of colony-forming units granulocyte (CFU-G), colony-forming units monocyte (CFU-M), and colony-forming units granulocyte-monocyte (CFU-GM), were dispensed in six-well plates (Becton Dickinson) and incubated in a humidified atmosphere of 5% CO2 at 37°C for 7 days, after which colonies were counted. The experiments were performed in duplicate.

Urinalysis

Urine was collected as described previously (18). Briefly, single mice involved in ongoing treatment experiments were placed in a “metabolic” cage lined with 96-well plates for 2 h, after which the urine was collected and stored at −70°C before analysis. Urinalysis was performed with Chemistrip 10 reagent strips (Roche Diagnostics, Laval, Quebec, Canada) following the manufacturer’s protocol.

H&E and Ki-67 Staining

Organs were removed, fixed overnight in phosphate-buffered formalin 10%, and transferred to ethanol 70% before embedding in paraffin. Femur specimens were decalcified in EDTA 15% (pH 8.0) for 5 days before additional processing. H&E staining was performed using standard procedures. For immunohistochemistry of small intestine specimens, paraffin sections were deparaffinized in xylene and gradually rehydrated. Antigen was retrieved by boiling the samples in 1 mM EDTA (pH 7.5) for 20 min and was detected using the Histostain-SP rabbit kit following the manufacturer’s instructions (Zymed, South San Francisco, CA). The primary antibody, NCL-Ki67p, which recognizes proliferating cells, was applied for 3 h at room temperature (Novocastra Laboratories Ltd, Newcastle, UK; Ref. 22). Fifty epithelial cells from the lowest point of the crypt in the direction of the tip of the villus were analyzed for Ki-67 positivity (six crypts/mouse), and the results were expressed as proliferation index (percentage of Ki-67-positive cells).

Statistical Analysis

Results are reported as mean ± SD or SE. Statistical significance of differences was assessed by ANOVA, followed by the Student-Newman-Keuls
test or Wilcoxon signed-rank test (as indicated) using PRISM software (version 4.00; GraphPad, San Diego, CA). The level of significance was set at \( P < 0.05 \).

RESULTS

Significant Inhibition of Angiogenesis by MTD and LDM CTX in the Matrigel Plug Perfusion Assay. LDM and MTD CTX exert a significant and comparable inhibition of angiogenesis (36.9% and 37.6%, respectively; \( P < 0.05 \) for LDM and MTD CTX versus positive control; LDM versus MTD, \( P > 0.05 \)) as shown here for BALB/cJ mice (Fig. 1), although not to the same extent as DC101 (70.8%), the rat monoclonal antibody directed against murine VEGFR-2. Similar results were obtained recently for the C57BL/6 strain (8). Therefore, LDM and MTD CTX demonstrate their antiangiogenic potential in mouse strains, which differ in their potential to support an angiogenic response (23).

Differential Bone Marrow Toxicity of MTD and LDM CTX. Bone marrow toxicity after MTD CTX follows the classical pattern that governs the conventional cyclic administration of cytotoxic drugs in immunodeficient C.B-17 SCID and immunocompetent BALB/cJ mice (24, 25). A general assessment of bone marrow proliferative activity was performed by an ex vivo methyl-[\(^{3}\)H]-thymidine incorporation assay. One day after the last MTD CTX dose, proliferation is massively suppressed, followed by restoration at day 10 and a rebound in the case of C.B-17 SCID mice at day 21 (for the BALB/cJ strain, the rebound likely takes place before day 21; Fig. 2, A and B). Regarding neutrophil and monocyte progenitors, MTD CTX leads to a seemingly counterintuitive increase in CFU-G/GM at day 5 in both strains, which was more pronounced in C.B-17 SCID mice (Fig. 2, C and D). However, CTX is known to spare early myeloid progenitor cells, affecting preferentially the maturation compartment (26). Therefore, concomitant with a decrease in the total number of nucleated bone marrow cells (Fig. 2, E and F), the remaining nucleated cells are enriched in early progenitor cells. During recovery and expansion of the maturation compartment, the relative number of CFU-G/GM decreases below control values.

In contrast to the results obtained with MTD, LDM CTX has no impact on methyl-[\(^{3}\)H]-thymidine incorporation of an unselected population of bone marrow cells (Fig. 2, A and B), and the total number of nucleated bone marrow cells harvested is only initially decreased and again is normal from day 21 onward (Fig. 2, E and F). Intriguingly, in C.B-17 SCID mice there is no evidence for any toxicity in the myeloid maturation compartment (Fig. 2D). Moreover, in BALB/cJ mice, a moderate increase in CFU-G/GM is normalized at day 42 (Fig. 2C).

Together, MTD CTX produces an initial depression of bone marrow function, followed by a rebound as expected. However, bone marrow toxicity of LDM CTX is only minor and transient. The differences seen between the two mouse strains analyzed is most probably explained by C.B-17 SCID mice harboring a mutation in the gene coding for the DNA-PKcs implicated in the repair of DNA damage, resulting in different repair kinetics of DNA double-strand breaks, the main type of DNA damage induced by CTX (20).

Differential Peripheral Blood Counts Reveal Moderate, but Sustained, Lymphopenia in BALB/cJ Mice Treated with LDM CTX. As shown previously with respect to bone marrow, MTD CTX is followed by a classical pattern of alterations in the peripheral WBC. Neutrophil, lymphocyte, and macrophage counts initially are massively reduced, followed by a rebound (Fig. 3, A–C). Unlike MTD, LDM CTX affects WBC less dramatically. However (and different from neutrophils), the number of lymphocytes and monocytes remains below normal levels during the study period (Fig. 3, A–C). In particular, we observe a sustained lymphopenia with values decreased by ~40%. Because of the nature of the C.B-17 SCID phenotype, this type of analysis was not performed in this strain.

LDM CTX Transiently Decreases Gut Mucosal Proliferation. Although gastrointestinal toxicity is not dose limiting in the case of CTX, the rapidly proliferating intestinal lining is a common site of side effects during cytotoxic therapy, including CTX. The proliferation index of small intestinal mucosa, as assessed by Ki-67 staining, was significantly decreased by LDM and MTD CTX at day 5 (for both, \( P < 0.001 \) versus control), not surprisingly to a greater extent by MTD CTX (\( P < 0.05 \) for MTD versus LDM; Fig. 4). Ten days after treatment initiation, the proliferation index returned to normal in both groups, with a rebound phenomenon at day 21 in the case of MTD CTX. Of note, the proliferation index at 6 weeks was still in the normal range in the LDM group. Concordantly, diarrhea or morphologic alterations of the intestinal lining were not seen in long-term treatment experiments.

LDM CTX Is Devoid of Urologic Side Effects and Morphologic Evidence of Toxicity in Various Organ Systems. Urologic side effects, in particular hemorrhagic cystitis, are a known consequence of the administration of CTX in humans and mice (27, 28). However, in bladder tissue sections of nude mice from human xenograft experiments treated for extended periods (up to 81 days) with 20 mg/kg/day of oral CTX, morphologic evidence of toxicity and hemorrhagic cystitis was absent. Correspondingly, urinary bladder edema, and leukocyturia was without evidence of hemoglobinuric/erythrocyturic or proteinuric (data not shown).

In addition, analysis of H&E-stained sections of heart, lung, liver, pancreas, skeletal muscle, bone marrow, and small and large bowel revealed no overt tissue toxicity (data not shown).

Weight Course as Surrogate Marker for Toxicity. Weight often is used as a surrogate marker for treatment-related toxicity in preclinical tumor models. Similar to the hematologic phenomena described previously, MTD CTX leads to a transient weight loss that recovered until the end of the cycle (Fig. 5, A and B). Weight gain after MTD CTX is protracted in C.B-17 SCID mice compared with BALB/cJ mice. As opposed to MTD CTX, the administration of LDM CTX does not cause weight loss but leads to a certain delay in weight gain.

Wound Closure Is Not Affected by LDM CTX in a Skin Excision Model. Because angiogenesis is an integral part of wound healing and because cancer patients often need surgical interventions, we asked whether LDM CTX might inhibit wound healing (29). In a full-thickness skin excision model in BALB/cJ mice, wound closure
kinetics were not significantly influenced by LDM CTX as compared with animals receiving normal saline in the drinking water (Fig. 6).

Interestingly, although hair regrowth was comparable in the scar area, it was delayed in the surrounding zone, which was shaved in preparation for the experiment (data not shown). At no time was a similar phenomenon observed in C.B-17 SCID mice shaved for our xenograft experiments, which might point to a differential strain susceptibility. However, we never observed overt hair loss, neither during this wound-healing experiment in BALB/cJ nor during the course of the many long-term treatment experiments with LDM CTX in C.B-17 SCID mice undertaken in our laboratory (7, 8, 18, 21).

Studies exploring the effects of LDM CTX on embryonic angiogenesis were not performed considering that a single dose of 20 mg/kg CTX is teratogenic and CTX is embryolethal at higher doses (30).

**DISCUSSION**

Although it has been known for some time that conventional cytotoxic drugs can have antiangiogenic activities (31, 32), it was only discovered recently by Browder et al. (6) and confirmed by Klement et al. (21) that such antiangiogenic potential can be optimized by administering comparatively low doses in a regular, so-called metronomic manner for prolonged periods (5). By this frequent or even continuous LDM type of administration, the same drug may be at least as effective, if not superior, compared with the conventional cyclic application of MTD (6). Moreover, the effect may be reinforced by coadministration of targeted antiangiogenic drugs (6, 21).

By various assays, we show here for the first time that toxic side effects of 20 mg/kg/day CTX given orally via the drinking water, an efficacious antiangiogenic and antitumor regimen, are minor and transient in nature with respect to bone marrow progenitor and intestinal epithelial cells, which are among the most sensitive cellular targets traditionally associated with the use of cytotoxic agents (18). This result is seemingly consistent with previously reported in vitro studies showing a many-fold higher sensitivity of endothelial cells of various origins to different cytotoxic drugs when compared with a number of other cell types tested (10–12). The basis for this endo
thelial cell ultrasensitivity to LDM cytotoxic drug exposure is being unraveled. For example, Bocci et al. (8) showed that the specificity derives at least partially from the induction or up-regulation of thrombospondin-1, an endogenous angiogenesis inhibitor, by LDM CTX protocols. In addition to the antiangiogenic effects, LDM regimens also are capable of potent and sustained suppression of the mobilization from the bone marrow of endothelial cell progenitors, and therefore vasculogenesis, as opposed to MTD schedules, which initially suppress but then promote the mobilization of these precursors during long break periods (9).

Using body weight changes as a surrogate marker for toxicity, various LDM regimens have been shown to be devoid of severe toxic side effects in preclinical models (18, 21). In the present study, we demonstrate the superior toxicity profile of LDM (20 mg/kg/day) as opposed to MTD CTX regarding hematologic and intestinal toxicity and weight gain. Moreover, a histologic analysis of several parenchymal organs reveals no tissue damage in tumor-bearing animals treated with LDM CTX for periods up to 81 days. In particular, we note the absence of bladder toxicity as shown morphologically and by urinalysis (to be discussed). In addition, the LDM CTX regimen used has no negative impact on wound healing, an angiogenesis-dependent process (29). Of note, the total CTX dose administered in 3 weeks is similar in the MTD and LDM regimens (450 and 420 mg/kg, respectively).

Recently published clinical trials using metronomic dosing of CTX (13, 14) and other alkylating agents such as trofosfamide (16) and treosulfan (17) confirm the excellent tolerance of such regimens. Colleoni et al. (13) described an LDM regimen of 50 mg/day CTX

Fig. 4. Small intestinal mucosa of BALB/cJ mice was stained for Ki-67 expression, and the percentage of positive cells was expressed as proliferation index (mean ± SEM). Control mice (white bars; n = 12) and mice treated with maximal tolerated dose (gray bars) or low-dose metronomic (black bars) cyclophosphamide (n = 3 for each group/time point after treatment initiation as indicated). *** P < 0.001 versus control.

Fig. 3. A–C, differential WBC counts from Wright-Giemsa-stained peripheral blood smears obtained from BALB/cJ mice (n = 3/group/time point after treatment initiation as indicated). Results are expressed as percentage change compared with the corresponding normal values (n = 12). Maximal tolerated dose cyclophosphamide, gray bars; low-dose metronomic cyclophosphamide, black bars; (A) neutrophil counts; (B) lymphocyte counts; and (C) monocyte counts.

Fig. 5. Weight gain of control mice (white bars), mice treated with maximal tolerated dose (gray bars) or low-dose metronomic (black bars) cyclophosphamide.

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hemorrhagic cystitis, gonadal dysfunction, mild to moderate hair loss, complications are more common (33). Other side effects include
superior in controlling the underlying disease compared with the
daily doses of mostly 2 mg/kg/day. In this setting, oral CTX is
combined with methotrexate (2
/1003 length
/1003 with a Vernier caliper, and the area (mm²) was calculated as follows: \( \frac{1}{2} \times \text{width} \times \pi \). Control mice (white bars) and low-dose metronomic cyclophosphamide-treated mice (black bars); n = 5 per group; Wilcoxon signed-rank test \( P > 0.05 \).

Unfortunately, the studies by Colleoni et al. and Glode et al. do not present data regarding lymphocyte counts and infectious complications. In light of our findings of sustained lymphopenia, the possibility of a certain immunosuppressive potential of LDM CTX cannot be discarded. This is not completely unexpected. First, besides its antineoplastic properties, CTX also is commonly used as an immunosuppressive drug, e.g., for vasculitis syndromes, either as i.v. pulse or in daily doses of mostly 2 mg/kg/day. In this setting, oral CTX is superior in controlling the underlying disease compared with the cyclic administration, but it also is more toxic. In particular, infectious complications are more common (33). Other side effects include hemorrhagic cystitis, gonadal dysfunction, mild to moderate hair loss, and occasional secondary neoplasias (34). The daily dose of CTX used in the published LDM CTX trials was significantly <2 mg/kg/day, which might explain the seemingly lower incidence of infections and the absence of other complications mentioned previously that are common in patients receiving CTX for immunosuppressive purposes.

Second, a recent publication shows that weekly administration of cytotoxic drugs, were given orally 50 mg/day CTX combined with 1 mg/d dexamethasone. Hematotoxicity again was mild, and no unexpected side effects were observed (14).

Finally, can be further reduced without sacrificing antiangiogenic efficacy by the combination of CTX with targeted antiangiogenic compounds. Alternatively, other groups of cytotoxic drugs with less immunosuppressive activity may be used in LDM regimens. Although low doses of CTX (and other cytotoxic agents) have been shown to have immunopotentiating properties, such an effect is not expected with additional decrease of the daily dose of CTX. In this regard, most of these immunopotentiating regimens of CTX use single, not repetitive, low doses of CTX (36). Of note Hermans et al. (35) have demonstrated that under certain conditions LDM CTX protocols can be successfully combined with immunotherapy protocols despite their immunosuppressive side effects, such that the combined effects of LDM CTX and immunotherapy are superior to either monotherapy or MTD CTX with or without immunotherapy.

Preclinically, LDM regimens demonstrate their full potential when used in combination with other agents in a long-term fashion (6, 21). Such long-term combination treatments can be realized because of the low toxicity profile of LDM chemotherapeutic drug regimens and consequently the possibility of protracted administration. Therefore, LDM regimens present an ideal platform for combination treatments not only with antiangiogenic and antivascular compounds but perhaps also targeted agents with indirect antiangiogenic activities, such as the anti-ErbB2-blocking antibody Herceptin, which by their nature should be administered in a long-term fashion. Ironically, even radiotherapy and probably MTD chemotherapy may be meaningful combination partners for LDM regimens as long as the advantage of low toxicity is not significantly compromised (37, 38).

In summary, our results show a clear safety advantage for LDM chemotherapy using CTX when comparing the effects of this type of dosing regimen with conventional MTD chemotherapy regimens on rapidly turning-over tissues. Although a potential major advantage, especially when integrated with targeted antiangiogenic drugs, several challenges must be overcome to realize the full benefits of this therapeutic approach in the clinic, such as defining the optimal low dose and schedule for a given chemotherapeutic drug and dealing with the issue of patient compliance when oral drugs are used (39).

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**REFERENCES**


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* Unpublished observations.


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