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Intermittent Metronomic Drug Schedule Is Essential for Activating Antitumor Innate Immunity and Tumor Xenograft Regression[1,2]

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Abstract

Metronomic chemotherapy using cyclophosphamide (CPA) is widely associated with antiangiogenesis; however, recent studies implicate other immune-based mechanisms, including antitumor innate immunity, which can induce major tumor regression in implanted brain tumor models. This study demonstrates the critical importance of drug schedule: CPA induced a potent antitumor innate immune response and tumor regression when administered intermittently on a 6-day repeating metronomic schedule but not with the same total exposure to activated CPA administered on an every 3-day schedule or using a daily oral regimen that serves as the basis for many clinical trials of metronomic chemotherapy. Notably, the more frequent metronomic CPA schedules abrogated the antitumor innate immune and therapeutic responses. Further, the innate immune response and antitumor activity both displayed an unusually steep dose-response curve and were not accompanied by antiangiogenesis. The strong recruitment of innate immune cells by the 6-day repeating CPA schedule was not sustained, and tumor regression was abolished, by a moderate (25%) reduction in CPA dose. Moreover, an ~20% increase in CPA dose eliminated the partial tumor regression and weak innate immune cell recruitment seen in a subset of the every 6-day treated tumors. Thus, metronomic drug treatment must be at a sufficiently high dose but also sufficiently well spaced in time to induce strong sustained antitumor immune cell recruitment. Many current clinical metronomic chemotherapeutic protocols employ oral daily low-dose schedules that do not meet these requirements, suggesting that they may benefit from optimization designed to maximize antitumor immune responses.

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Introduction

Metronomic chemotherapy involves the administration of cancer chemotherapeutic drugs at regular intervals, without long breaks, and is thought to yield improved antitumor activity through antiangiogenesis combined with conventional drug cytotoxicity [1–4]. Metronomic schedules investigated in preclinical studies include intermittent drug dosing, e.g., the 6-day repeating metronomic schedule empirically found to be most efficacious by Browder et al. [1], as well as daily oral low-dose treatment regimens, which are proposed to be even more effective in killing tumor endothelial cells [5,6]. Metronomic drug schedules have been evaluated in clinical trials, primarily using daily dosing regimens, with promising results [7–9]. Recent studies have shown that other mechanisms, notably antitumor immunity, may also be activated by metronomic chemotherapy. For example, metronomic administration of gemcitabine and docetaxel restores lymphocyte effector function by suppressing bone marrow–derived suppressor cells [10,11], while paclitaxel, cyclophosphamide (CPA), temozolomide and vinorelbine preferentially deplete regulatory T suppressor cells (Tregs) [12–15]. Furthermore, CPA administration

Abbreviations: CPA, cyclophosphamide; MTD, maximum tolerated dose; NK, natural killer; Prf1, perforin-1

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on an every 6-day metronomic schedule stimulates antitumor innate immune cell recruitment, which is associated with marked regression of implanted brain tumors in both severe combined immunodeficiency (SCID) immune-deficient and immune-competent mice [16]. Tumor regression is incomplete when natural killer (NK) cells are immunodepleted from fully immune-competent mice bearing subcutaneous syngeneic GL261 gliomas, demonstrating the importance of the innate immune system in tumor regression induced by CPA given on an every 6-day metronomic schedule [16].

Metronomic chemotherapy has shown therapeutic benefits beyond those observed with conventional maximum tolerated dose chemotherapy and can induce responses in patients where standard chemotherapy is no longer effective [7,8]. For example, daily metronomic vinorelbine treatment increases progression-free survival and overall survival in elderly patients with metastatic breast cancer [17]. Intermittent metronomic administration of vinorelbine and cisplatin using optimized doses and schedules gives favorable clinical responses in patients with advanced/metastatic non–small cell lung carcinoma [18], and prolonged responses are achieved in patients with refractory cancers given vinorelbine on a thrice weekly metronomic schedule [13]. A striking 65-month progression-free survival was reported in a patient with stage IIIC ovarian cancer given metronomic CPA on a daily schedule without side effects [19], and metronomic irinotecan administration in patients with metastatic colorectal cancer resulted in stable disease despite progression following initial treatment with a standard irinotecan regimen [20]. Furthermore, metronomic regimens generally show reduced toxicity compared to maximum tolerated dose (MTD) regimens, and by avoiding the dose-dense exposure of MTD schedules, metronomic administration of CPA, as well as other agents, avoids immunosuppression and instead allows for antitumor immune activation [21]. While it is often assumed that the antiangiogenic activity of metronomic chemotherapy contributes to these therapeutic responses, other mechanisms, including antitumor immune responses, may also be at work. Little is known, however, about the requirements, in terms of schedule and dose, to induce metronomic chemotherapy-activated antitumor immunity, a prerequisite for translating preclinical studies of immune-activating metronomic chemotherapeutic regimens to the clinic.

Here, we investigated a recently described brain tumor xenograft model of metronomic CPA-induced antitumor innate immunity [16] to determine the schedule and dose requirements for innate immune cell recruitment and activation leading to tumor regression. We found that tumor recruitment and activation of innate immune cells, including macrophages, dendritic cells, and NK cells, by an intermittent 6-day repeating schedule of metronomic CPA is severely compromised when using dose-equivalent but more frequent metronomic CPA schedules, most notably the daily oral CPA regimen that has served as a model for many clinical trials of metronomic chemotherapy. Furthermore, we report an unexpectedly steep dose-response curve for activation of antitumor innate immunity by the every 6-day metronomic schedule, with a strong correlation seen between innate immune cell recruitment and individual tumor regression responses. Together, our findings support the conclusion that to achieve an effective antitumor immune response, metronomic CPA must be at a dose that is sufficiently high to induce tumor or stromal cell damage leading to the activation of antitumor innate immunity but also sufficiently well spaced in time to not ablate responding antitumor immune cells, some of which may be particularly sensitive to drug cytotoxicity.

Materials and Methods

Cell Lines and Reagents

CPA monohydrate was purchased from Sigma Chemical Co (St Louis, MO). FBS and Dulbecco's modified Eagle's medium (DMEM) culture medium were purchased from Invitrogen (Frederick, MD). The rat 9L gliosarcoma cell line was authenticated by and obtained from the Neurosurgery Tissue Bank at University of California, San Francisco (UCSF; San Francisco, CA) and grown at 37°C in a humidified, 5% CO_2 atmosphere in 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin containing DMEM culture medium.

Quantitative Polymerase Chain Reaction Analysis

Methods for isolation of total RNA from frozen tumor tissue, reverse transcription, and quantitative polymerase chain reaction (qPCR) analysis (primers shown in Table W1 and in [16]) are described [16]. Primers designed using Primer Express software (Applied Biosystems, Carlsbad, CA) were evaluated using LaserGene software (DNAStar, Madison, WI) to ensure mouse gene specificity, except as noted. The absence of cross-species amplification was verified by testing each primer pair on a panel comprising rat liver, human umbilical vein endothelial cells (HUVEC), and mouse liver RNA, in addition to rat 9L and human U251 tumor cell RNA. Data were analyzed using the comparative C_T ($\Delta\Delta C_T$) method and are presented as relative levels of each RNA compared to the RNA level in untreated tumors after normalization to the 18S RNA content of each sample.

Tumor Xenograft Studies

Five- to 6-week-old (24-26 g) male ICR/Fox Chase immune-deficient SCID mice (Taconic Farms, Germantown, NY) were housed in the Boston University Laboratory of Animal Care Facility and treated in accordance with approved protocols and federal guidelines. 9L cells (4×10^6 cells) were injected subcutaneous (s.c.) on each posterior flank in 0.2 ml of serum-free DMEM using a 0.5-inch 29-gauge needle and a 0.3-ml insulin syringe. Tumor areas (length × width) were measured twice weekly using Vernier calipers (VWR, Cat. No. 62379-531), and tumor volumes were calculated on the basis of vol = $(\pi/6) * (L * W)^{3/2}$. Tumors were monitored, and treatment groups were normalized (each tumor volume was set to 100%) once average tumor volumes reached ~300 mm^3. Mice were treated with CPA given on the following metronomic schedules: oral dosing [23.3 or 70 mg/kg body weight (BW) per day] [6] or intermittent bolus dosing by intraperitoneal (i.p.) injection (70 mg/kg BW, i.p., every 3 days or 70, 105, 140, or 170 mg/kg BW, i.p., every 6 days) on days indicated in each figure by vertical arrows along the *X*-axis. CPA was given orally through drinking water [5] using 0.7 or 2.1 mg of CPA per 4.5 ml of sterile tap water based on a measured water consumption rate of 0.15 ml/g mouse BW per day. All doses are based on the weight of CPA monohydrate. Tumor sizes and mouse body weights were measured at least twice a week. Tumor growth rates before drug treatment were similar among all normalized groups. The plasma pharmacokinetics of 4-OH–CPA exposure was assayed in 9L tumor-bearing mice given CPA by i.p. injection at 70 or 140 mg/kg BW (n = 4 mice per group). We were unable to obtain consistent 4-OH–CPA plasma pharmacokinetic data for the 23.3 mg/kg BW/day oral dosing (drinking water) group [1x per oral (p.o.) dosing], presumably because of the variable time and volume of the last *ad lib* exposure to CPA. To estimate the pharmacokinetics of *ad lib* drinking water exposure to CPA, we assumed that the mice drink five times per day on average. Accordingly, CPA

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