Influence of Formulation Vehicle on Metronomic Taxane Chemotherapy: Albumin-Bound versus Cremophor EL–Based Paclitaxel

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Abstract

Purpose: Low-dose metronomic chemotherapy treatments, especially when combined with 'dedicated' antiangiogenic agents, can induce significant antitumor activity without serious toxicity in various preclinical models. It remains unclear, however, whether some cytotoxic drugs are better suited for metronomic regimens than others. Paclitaxel appears to be a strong candidate for metronomic chemotherapy given its ability to inhibit endothelial cell functions relevant to angiogenesis in vitro at extraordinarily low concentrations and broad-spectrum antitumor activity. Clinically relevant concentrations of the formulation vehicle cremophor EL in Taxol, however, were previously reported to nullify the antiangiogenic effect of paclitaxel, the result of which would hamper its usefulness in metronomic regimens. We hypothesized that ABI-007, a cremophor EL–free, albumin-bound, 130-nm form of paclitaxel, could potentially alleviate this problem.

Experimental Design: The antiangiogenic activity of ABI-007 was assessed by multiple in vitro assays. The in vivo optimal dose of ABI-007 for metronomic chemotherapy was determined by measuring circulating endothelial progenitors in peripheral blood. The antitumor effects of metronomic and maximum tolerated dose ABI-007 and Taxol were then evaluated and compared in severe combined immunodeficient mice bearing human MDA-MD-231 breast cancer and PC3 prostate cancer xenografts.

Results: ABI-007 significantly inhibited rat aortic microvessel outgrowth, human endothelial cell proliferation, and tube formation. The optimal metronomic dose of ABI-007 was determined to be between 3 and 10 mg/kg. Metronomic ABI-007 but not Taxol, significantly suppressed tumor growth in both xenograft models. Furthermore, the antitumor effect of minimally toxic metronomic ABI-007 approximated that of the maximum tolerated dose of Taxol.

Conclusions: Our results underscore the influence of formulation vehicles on the selection of cytotoxic drugs for metronomic chemotherapy.

An alternative dosing regimen to pulsatile maximum tolerated dose (MTD) or "dose dense" and dose-intensive chemotherapy is "metronomic chemotherapy": the frequent administration of such drugs at close regular intervals with no prolonged breaks over long periods of time (1). The reduced toxicity and comparable or even increased efficacy of metronomic regimens compared with some MTD counterparts have been shown in a number of preclinical models (2, 3). In addition, metronomic chemotherapy regimens are particularly well suited for long-term combination with relatively non–toxic-targeted biological therapeutics especially antiangiogenic drugs (4, 5), sometimes being used after an initial short course of MTD chemotherapy (i.e., "chemo-switching" protocols; refs. 4, 6). Some phase II clinical trials have been reported with encouraging results, both in terms of antitumor efficacy and reduced toxicity, although such results need to be validated in larger randomized phase II or III trials (7–9). These trials have involved combinations of two metronomically given drugs [e.g., cyclophosphamide and methotrexate (7) or cyclophosphamide and etoposide (8)] given orally on a daily basis, or similar protocols in combination with a drug such as bevacizumab (Avastin), the anti-vascular endothelial growth factor antibody (9).

Virtually every class of chemotherapeutic drug has been reported to have antiangiogenic properties (10), which in many cases can be amplified by metronomic dosing schedules (1).

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Received 12/16/05; revised 4/21/06; accepted 5/10/06.

Grant support: National Cancer Institute of Canada (NCIC), Canadian Institutes of Health Research (R.S. Kerbel) and Terry Fox Research Grant for New Investigators funded through the NCIC.

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Note: S.S.W. Ng and Y. Shaked are recipients of postdoctoral fellowships from the Canadian Institutes of Health Research.

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doi:10.1158/1078-0432.CCR-05-2762

However, the selection of chemotherapeutic drugs for metronomic regimens remains somewhat arbitrary. It is not clear whether some agents or classes of agents are better suited for metronomic chemotherapy than others. Taxanes, such as paclitaxel, would seem to be excellent candidates based on the finding that ultra low (e.g., picomolar) concentrations of paclitaxel can selectively inhibit endothelial functions relevant to angiogenesis, or even kill such cells (11–15). In addition, long-term metronomic chemotherapy using microtubule-inhibiting taxanes as opposed to mutagenic and hence potentially carcinogenic DNA damaging drugs, such as alkylating agents, could also be an advantage, especially in patients receiving adjuvant metronomic therapy regimens over long periods of time for early-stage disease (16). Furthermore, taxane metronomic chemotherapy may be useful to combine with metronomic chemotherapy using another class of drug, such as anti-metabolites (e.g., UFT, the oral 5-fluorouracil prodrug; ref. 16). For example, we have recently reported that a concurrent combination of daily oral low-dose UFT and cyclophosphamide can successfully control highly advanced visceral metastases of human breast cancer in immunodeficient mice, whereas UFT or cyclophosphamide used alone could not (17). However, clinically relevant concentrations of the formulation vehicle cremophor EL in Taxol were previously reported to nullify the antiangiogenic activity of paclitaxel, suggesting that this agent or other anticancer drugs formulated in cremophor EL and other commonly used solubilization vehicles, such as polysorbate 80 (Tween 80), may need to be used at much higher doses than anticipated to achieve effective metronomic chemotherapy (18). As such, the advantage of reduced acute serious side effects associated with low-dose paclitaxel regimens versus conventional MTD paclitaxel may be compromised. Clearly, the presence of cremophor EL in Taxol hampers the use of paclitaxel in metronomic chemotherapy. This may explain, for example, the results of Klement et al. (19), in which metronomic Taxol on its own had little obvious effects on transplanted primary human breast tumors in several models.

ABI-007 (Abraxane), a novel cremophor EL–free, albumin-bound, 130-nm form of paclitaxel, was developed to retain the therapeutic benefits of paclitaxel but eliminate cremophor EL–associated toxicities in the Taxol formulation. Several clinical trials have shown the improved pharmacokinetic and toxicity profiles as well as therapeutic efficacy of ABI-007 over Taxol in MTD regimens (20–24). We hypothesized that the cremophor EL–free nature of ABI-007 may render paclitaxel-based metronomic chemotherapy feasible. With this in mind, the primary objective of this study was to evaluate the therapeutic potential of paclitaxel-based metronomic regimens using ABI-007.

Materials and Methods

Drugs. Taxol injection containing paclitaxel at 6 mg/mL in a mixture of cremophor EL and ethanol USP (1:1 v/v; Bristol-Myers Squibb Canada, Montreal, Canada) was purchased from the local hospital pharmacy. ABI-007 was obtained from American BioScience (Santa Monica, CA).

Rat aortic ring assay. Twelve-well tissue culture plates were coated with 250 μL of Matrigel (Collaborative Biomedical Products, Bedford, MA) and allowed to gel for 30 minutes at 37°C and 5% CO_2. Thoracic aortas were excised from 8- to 10-week-old male Sprague-Dawley rats. Following removal of fibroadipose tissues, the aortas were cut into 1-mm-long cross-sections, placed on Matrigel-coated wells, and covered with an additional 250 μL of Matrigel. After the second layer of Matrigel had set, the rings were covered with EGM-II and incubated overnight at 37°C and 5% CO_2. EGM-II consists of endothelial cell basal medium (EBM-II; Cambrex, Walkersville, MD) plus endothelial cell growth factors provided as the EGM-II Bulletkit (Cambrex). The culture medium was subsequently changed to EBM-II supplemented with 2% fetal bovine serum, 0.25 μg/mL amphotericin B, and 10 μg/mL gentamicin. Aortic rings were treated with EBM-II containing the vehicle (0.9% saline/albumin), carboxyamidotriazole (12 μg/mL), or ABI-007 (0.05-10 nmol/L paclitaxel) for 4 days and photographed on the fifth day using a ×2.5 objective. Carboxyamidotriazole, a known antiangiogenic agent, was used at higher than clinically achievable concentration as positive control (25). Experiments were repeated four times using aortas from four different rats. The area of angiogenic sprouting, reported in square pixels, was quantified using Adobe Photoshop 6.0.

Endothelial cell proliferation assay. Human umbilical vein endothelial cells (HUVEC; Cambrex) were maintained in EGM-II at 37°C and 5% CO_2. HUVECs were seeded onto 12-well plates at a density of 30,000 per well and allowed to attach overnight. The culture medium was then aspirated, and fresh culture medium containing either the vehicle (0.9% saline/albumin), or ABI-007 (0.05-10 nmol/L paclitaxel) was added to each well. After 48 hours, cells were trypsinized and counted with a Coulter Z1 counter (Coulter Corp., Hialeah, FL). All experiments were repeated thrice.

Endothelial cell tube formation assay. Eight-well slide chambers were coated with 150 μL of Matrigel and allowed to gel at 37°C and 5% CO_2 for 30 minutes. HUVECs were then seeded at 30,000 per well in EGM-II containing either the vehicle (0.9% saline/albumin) or ABI-007 (0.05-10 nmol/L paclitaxel) and incubated at 37°C and 5% CO_2 for 16 hours. After incubation, slides were washed in PBS, fixed in 100% methanol for 10 seconds, and stained with DiffQuick solution II (Dade Behring, Inc., Newark, DE) for 2 minutes. To analyze tube formation, each well was digitally photographed using a ×2.5 objective. A threshold level was set to mask the stained tubes. The corresponding area was measured as the number of pixels using MetaMorph software (Universal Imaging, Downingtown, PA). Experiments were repeated thrice.

Determination of the* in vivo *optimal biological dose of ABI-007 by measuring circulating endothelial cells and circulating endothelial progenitors. Six- to 8-week-old female BALB/cJ mice were randomized into the following eight groups (n = 5 each): untreated, treated with i.p. bolus injections of either the drug vehicle (0.9% saline/albumin), or ABI-007 at 1, 3, 6, 10, 15, or 30 mg/kg paclitaxel daily for 7 days. At the end of the treatment period, blood samples were drawn by cardiac puncture and collected in EDTA-containing vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Circulating endothelial cells (CEC) and circulating endothelial progenitors (CEP) were enumerated using four-color flow cytometry as previously described (3, 26). Monoclonal antibodies specific for CD45 were used to exclude $CD45^+$ hematopoietic cells. CECs and their CEP subset were depicted using the murine endothelial markers fetal liver kinase 1/vascular endothelial growth factor receptor 2, CD13, and CD117 (BD PharMingen, San Diego, CA). Nuclear staining (Procount; BD Biosciences, San Jose, CA) was done to exclude the possibility of platelets or cellular debris interfering with the accuracy of CEC and CEP enumeration (27, 28). After red cell lysis, cell suspensions were evaluated by a FACSCalibur (BD Biosciences) using analysis gates designed to exclude dead cells, platelets, and debris. At least 100,000 events per sample were obtained to analyze the percentage of CECs and CEPs. The absolute number of CECs and CEPs was then calculated as the percentage of the events collected in the CEC and CEP enumeration gates multiplied by the total white

cell count. Percentages of stained cells were determined and compared with the appropriate negative controls. Positive staining was defined as being greater than nonspecific background staining. 7-Amino-actinomycin D was used to enumerate viable versus apoptotic and dead cells (29).

Human tumor xenograft therapy studies. Human prostate cancer cell line PC3 and human breast cancer cell line MBA-MD-231 were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). All animal experiments were done in accordance with institutional guidelines for animal welfare. PC3 (5×10^6 cells) were injected s.c. into 6- to 8-week-old male severe combined immunodeficient mice, whereas MDA-MB-231 (2×10^6 cells) were implanted orthotopically into the mammary fat pad of female severe combined immunodeficient mice. When the primary tumor volume reached ~150 to 200 mm^3, animals were randomized into eight groups (n = 5-10 per group). Each group was treated with either 0.9% saline/albumin vehicle control, cremophor EL vehicle control, metronomic Taxol (1.3 mg/kg, i.p., qd), metronomic ABI-007 (3, 6, or 10 mg/kg paclitaxel, i.p., qd), MTD Taxol (13 mg/kg, i.v., qd×5, 1 cycle), or MTD ABI-007 (30 mg/kg paclitaxel, i.v., qd×5, 1 cycle). Perpendicular tumor diameters were measured with a caliper once a week, and their volumes were calculated using the formula $\pi/6 \times a \times b^2$, where a is the longest dimension of the tumor, and b is the width. At the end of the treatment period, blood samples were drawn by cardiac puncture from mice in all groups and collected in EDTA-containing vacutainer tubes (Becton Dickinson). CECs and CEPs were enumerated as described above. Tumors were harvested, snap frozen in optimum cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) in liquid nitrogen and subsequently processed for immunofluorescence staining.

Detection and quantification of intratumoral microvessel density. Five-micrometer-thick sections obtained from each frozen tumor were stained with H&E for histologic examination. For detection of microvessels, sections were stained with a rat anti-mouse CD31/platelet/endothelial cell adhesion molecule 1 antibody (1:1,000; BD PharMingen) followed by a Texas Red–conjugated goat anti-rat secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). A single microvessel was defined as a discrete cluster or single cell stained positive for CD31/platelet/endothelial cell adhesion molecule 1, and the presence of a lumen was not required for scoring as a microvessel. The microvessel density for each tumor was expressed as the average count of the three most densely stained fields identified with a ×20 objective on a Zeiss AxioVision 3.0 fluorescence microscopic imaging system. Four to five different tumors per each vehicle control or treatment group were analyzed.

In vivo *angiogenesis evaluation.* The Matrigel plug perfusion assay was done with minor modifications as previously described (4). Briefly, 0.5 mL Matrigel supplemented with 500 ng/mL of basic fibroblast growth factor (R&D Systems, Inc., Minneapolis, MN) was injected s.c. on day 0 into the flanks of 10-week-old female BALB/cJ mice. On day 3, animals were randomly assigned to eight groups (n = 5 each). Each group was treated with either 0.9% saline/albumin vehicle control, cremophor EL vehicle control, metronomic Taxol (1.3 mg/kg, i.p., qd), metronomic ABI-007 (3, 6, or 10 mg/kg paclitaxel, i.p., qd), MTD Taxol (13 mg/kg, i.v., qd×5), or MTD ABI-007 (30 mg/kg paclitaxel, i.v., qdx5). As a negative control, five additional female BALB/cJ mice of similar age were injected with Matrigel alone. On day 10, all animals were injected i.v. with 0.2 mL of 25 mg/mL FITC-dextran (Sigma, St. Louis, MO). Plasma samples were subsequently collected. Matrigel plugs were removed, incubated with Dispase (Collaborative Biomedical Products, Bedford, MA) overnight at 37°C, and then homogenized. Fluorescence readings were obtained using a FL600 fluorescence plate reader (Biotech Instruments, Winooski, VT). Angiogenic response was expressed as the ratio of Matrigel plug fluorescence to plasma fluorescence.

Statistics. All results were presented as mean ± SE. Comparisons were made with one-way ANOVA followed by Student Newman-Keuls or Dunnett's test with $P < 0.05$ as the criterion for statistical significance. Correlation between viable CEP levels in peripheral blood and intratumoral microvessel density in the human tumor xenografts studies was examined using the nonparametric Spearman test with the level of significance set at $P < 0.05$.

Results

Rat aortic angiogenesis, HUVEC proliferation, and tube formation in response to ABI-007. We first asked whether the antiangiogenic effects of paclitaxel can be effectively delivered by the cremophor EL–free ABI-007 *in vitro*. As shown in Fig. 1A, ABI-007 suppressed aortic microvessel outgrowth in a

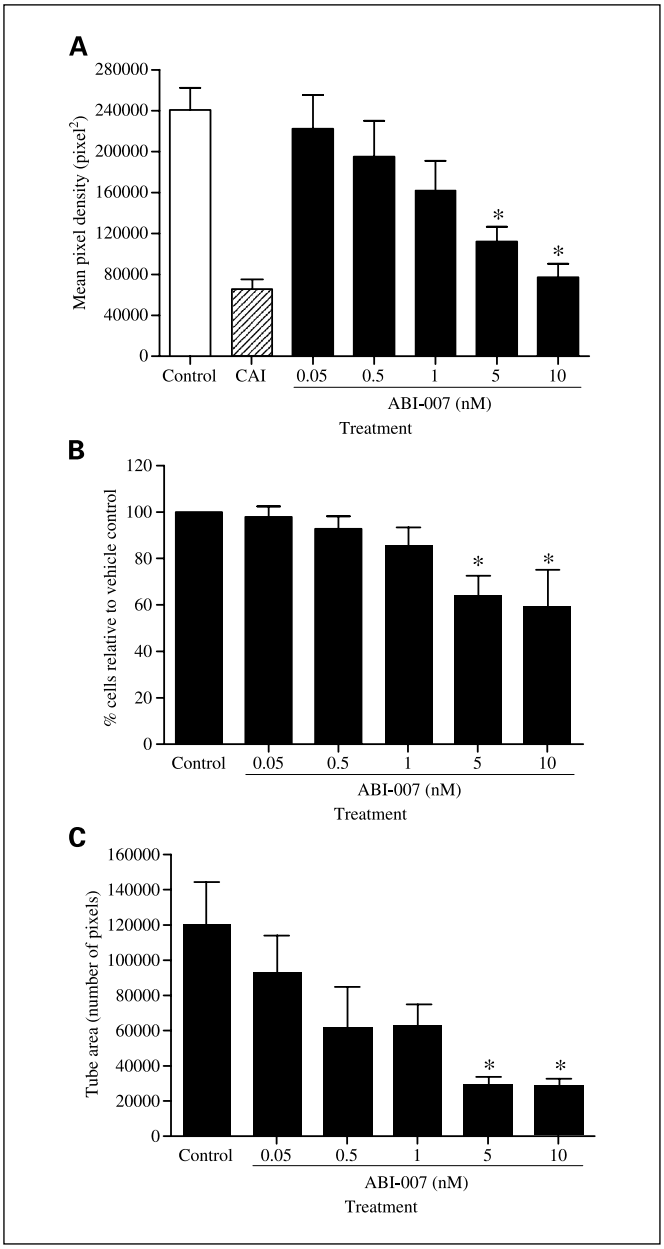

Fig. 1. Effects of ABI-007 on rat aortic ring angiogenesis (*A*), HUVEC proliferation (*B*), and tube formation (*C*). Columns, mean; bars, SE. *, $P < 0.05$, significantly different from the vehicle control. CAI, carboxyamidotriazole.

concentration-dependent manner relative to the vehicle control, reaching statistical significance at 5 nmol/L (53% inhibition) and 10 nmol/L (68% inhibition). The amount of albumin present at each concentration of ABI-007 alone did not inhibit angiogenesis (data not shown).

HUVEC proliferation was significantly inhibited by ABI-007 at 5 and 10 nmol/L by 36% and 41%, respectively. ABI-007 also blocked tube formation by 76% at both 5 and 10 nmol/L (Fig. 1B and C).

In vivo *optimal biological dose for metronomic ABI-007.* Next, the optimal dose of ABI-007 to be used in metrnomic chemotherapy was determined. Enumeration of viable CEPs can be used as a surrogate pharmacodynamic marker to establish the optimal biological dose for targeted antiangiogenic drugs (26) and metronomic chemotherapy (30). Figure 2 shows that ABI-007 given i.p. daily for 7 days at 3 and 10 to 30 mg/kg significantly decreased CEP levels in non–tumor-bearing BALB/cJ mice. However, ABI-007 at 10 to 30 mg/kg was associated with a significant reduction of white blood cell count indicative of toxicity (data not shown). Although the reduction of CEP levels by ABI-007 at 6 mg/kg did not reach statistical significance, decrease in white blood cell count was not evident. We, therefore, concluded that the *in vivo* optimal biological dose for metronomic ABI-007 was between 3 and 10 mg/kg. In a preliminary study, metronomic Taxol at 1.3, 3, 6, or 13 mg/kg given i.p. (qdx7) did not significantly reduce viable CEP levels, whereas metronomic Taxol at 30 mg/kg or higher resulted in severe toxicity and eventually mortality in mice (data not shown). It was previously reported that the i.p. administration of Taxol at doses commonly used in the clinic resulted in entrapment of paclitaxel in cremophor EL micelles in the peritoneal cavity and, consequently, insignificant plasma paclitaxel concentration (31). This would explain why doses of metronomic Taxol (1.3, 3, 6, and 13 mg/kg) that did not cause death failed to change viable CEP levels. In this case, the i.p. administration of metronomic Taxol at 1.3 mg/kg would not be any different from that at 13 mg/kg. We, therefore, selected the lower dose (1.3 mg/kg) to minimize the amount of cremophor EL per paclitaxel administration for subsequent experiments.

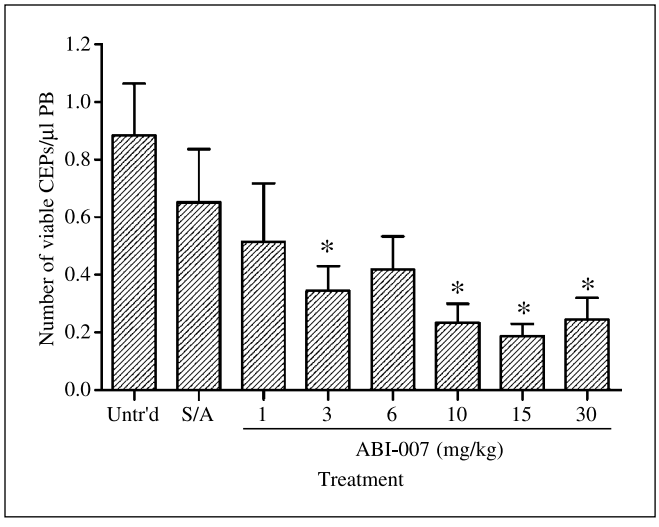

Fig. 2. Determination of optimal biologic dose of ABI-007 for metronomic dosing. Levels of viable CEPs in peripheral blood of BALB/cJ mice in response to escalating doses of ABI-007. Untr'd, untreated control; S/A, saline/albumin vehicle control. Columns, mean; bars, SE. *, $P < 0.05$, significantly different from the untreated control.

Antitumor effects of metronomic and MTD ABI-007 versus Taxol. Metronomic ABI-007 (3, 6, and 10 mg/kg) but not Taxol (1.3 mg/kg) given i.p. daily for 4 weeks significantly inhibited growth of both MDA-MB-231 and PC3 tumors (Fig. 3A and B). Neither ABI-007 nor Taxol given metronomically induced weight loss in mice (Fig. 3C and D). Although MTD ABI-007 (30 mg/kg) suppressed tumor growth more effectively than MTD Taxol (13 mg/kg), significant weight loss was noted with the former, indicating toxicity (Fig. 3C and D). In addition, two of five mice treated with MTD ABI-007 displayed signs of paralysis in one limb 6 days after the last dose of drug. The paralysis was transient and resolved within 24 to 48 hours. Interestingly, the antitumor effect of metronomic ABI-007 at 6 mg/kg approximated that of MTD Taxol in the MDA-MB-231 xenograft model (Fig. 3A). Increasing the dose of metronomic ABI-007 to 10 mg/kg did not seem to confer more pronounced tumor growth inhibition (Fig. 3A). In contrast, metronomic ABI-007 elicited greater antitumor response at 10 mg/kg than at 3 and 6 mg/kg in the PC3 xenografts (Fig. 3B).

Viable CEP levels in response to metronomic and MTD ABI-007 versus Taxol. As illustrated in Fig. 4A, metronomic ABI-007 significantly decreased the levels of viable CEPs in a dose-dependent manner in MDA-MB-231 tumor-bearing mice. Viable CEP levels also exhibited a dose-dependent reduction in response to metronomic ABI-007 in PC3 tumor-bearing mice but reaching statistical significance only at 10 mg/kg (Fig. 4B). Although metronomic Taxol seemed to reduce the levels of CEPs slightly in both xenograft models, the reduction did not reach statistical significance (Fig. 4A and B). Both MTD ABI-007 and MTD Taxol significantly lowered viable CEPs compared with their respective vehicle controls in MDA-MB-231 tumor-bearing mice, whereas only MTD Taxol did so in the PC3 tumor-bearing mice (Fig. 4A and B).

Effects of metronomic and MTD ABI-007 and Taxol on intratumoral microvessel density. In MDA-MB-231 tumors, metronomic ABI-007 at 6 and 10 mg/kg as well as MTD ABI-007 seemed to reduce microvessel density slightly although statistical significance was not reached (Fig. 5A). In PC3 tumors, metronomic ABI-007 at 3 and 10 mg/kg seemed to decrease microvessel density but without reaching statistical significance (Fig. 5A). Interestingly, a significant correlation existed between microvessel density and the levels of viable CEPs in the MDA-MB-231 (Fig. 5B; $r = 0.76$, $P = 0.04$) but not in the PC3 (Fig. 5C; $r = -0.071$, $P = 0.88$) model.

In vivo *antiangiogenic activity of metronomic and MTD ABI-007 versus Taxol.* In the Matrigel plug perfusion assay, metronomic ABI-007 at 6 and 10 mg/kg seemed to decrease angiogenesis, although the inhibition did not reach statistical significance (Fig. 6). Angiogenesis seemed to be unaltered by metronomic ABI-007 at 3 mg/kg, MTD ABI-007, MTD, and metronomic Taxol relative to the respective vehicle controls (Fig. 6). These observations were similar to the intratumoral microvessel density results described above.

Discussion

Our results show that metronomic chemotherapy using albumin-bound nanoparticle paclitaxel (ABI-007), but not

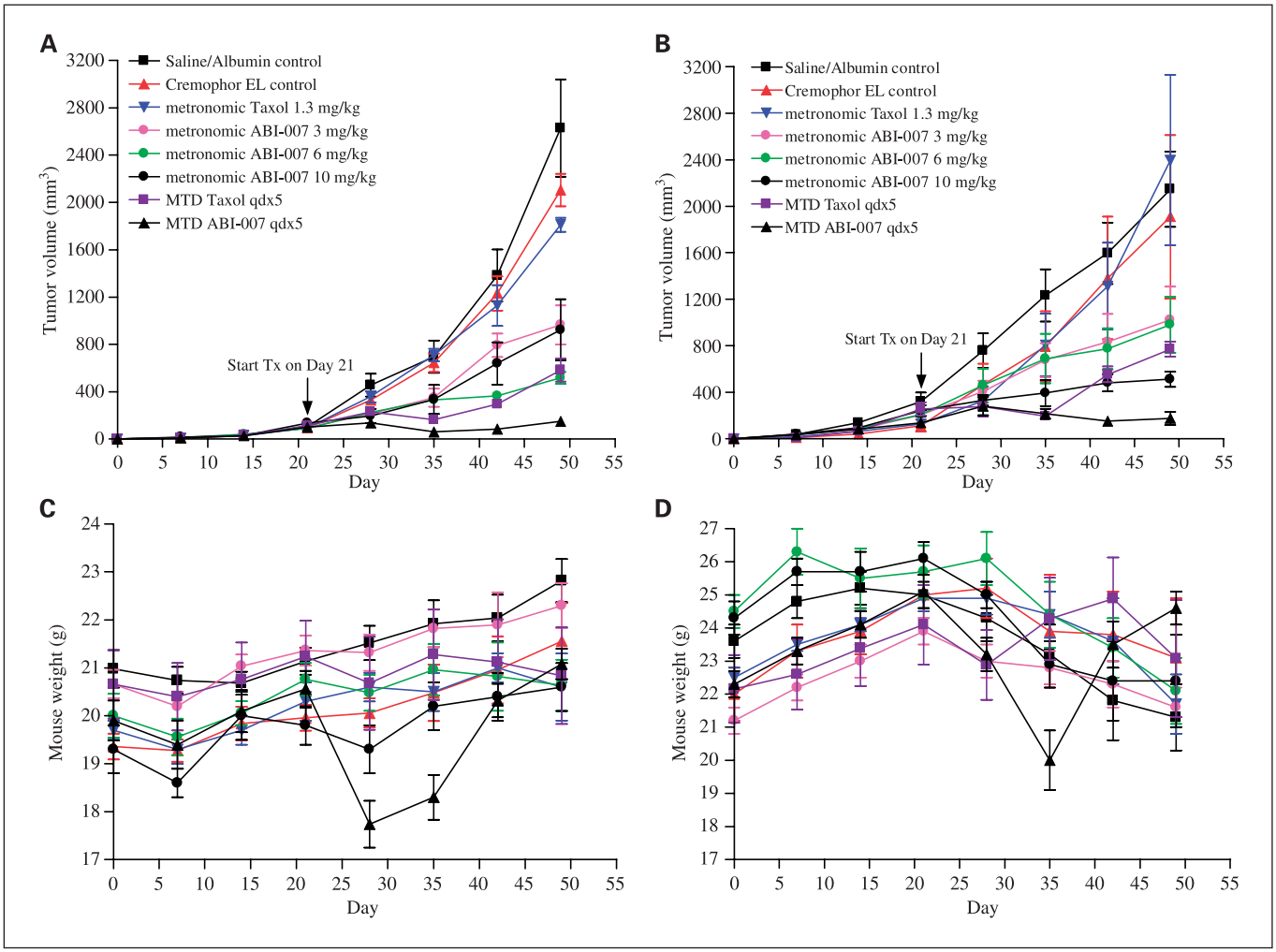

Fig. 3. Effects of ABI-007 and Taxol used in metronomic or MTD regimens on MBA-MD-231 (*A*) and PC3 (*B*) tumor growth and the body weight of MDA-MB-231 (*C*) and PC3 (*D*) tumor-bearing SCID mice. Five to 10 mice were used per control or treatment group. Points, mean; bars, SE.

paclitaxel formulated in cremophor EL (Taxol), exhibits potent *in vivo* antitumor activity. It was previously reported that clinically relevant concentrations of formulation vehicles, such as cremophor EL, nullify the *in vitro* antiangiogenic effect of taxanes (18). Paclitaxel at 4 nmol/L dissolved in DMSO but not in cremophor EL suppressed rat aortic angiogenesis and HUVEC proliferation (18). In the present study, ABI-007 at 5 nmol/L induced responses similar to those elicited by paclitaxel at 4 nmol/L dissolved in DMSO, indicating that the antiangiogenic property of paclitaxel was effectively delivered by cremophor EL–free ABI-007. Desai et al. (32) recently showed that paclitaxel in ABI-007 is actively transported into and across endothelial cells by gp60 (a specific albumin receptor)–mediated caveolar transcytosis, a process that is inhibited by cremophor EL in Taxol.

One cycle of either MTD ABI-007 or MTD Taxol was shown herein to cause marked tumor growth inhibition and transient regression in two different xenograft models (MDA-MB-231 and PC3). However, MTD Taxol–treated tumors rapidly resumed growth 3 weeks after the last dose of drug (day 42), whereas the growth of MTD ABI-007–treated tumors continued to be suppressed. The ability of MTD ABI-007 but not MTD Taxol to significantly reduce viable CEPs in MDA-MB-231 tumor-bearing mice might contribute, at least in part, to the enduring antitumor effect of the former. A higher paclitaxel plasma clearance and a larger volume of distribution for ABI-007 than for Taxol was recently reported in humans (33) as well as more rapid cellular uptake and binding (32), suggesting that paclitaxel in the ABI-007 formulation might be more effective against CEPs than Taxol. Higher intratumoral paclitaxel concentration achieved by the ABI-007 formulation could be another contributing factor (32, 33). We cannot explain why there was a lack of a significant decrease in viable CEPs by MTD ABI-007 in PC3 tumor-bearing mice. The possibility that the s.c. PC3 tumors might be less dependent on recruitment of CEPs for angiogenesis compared with the orthotopic MDA-MB-231 tumors cannot be excluded.

The significant weight loss and transient paralysis associated with MTD ABI-007 treatment and the absence of cremophor EL in the formulation prompted us to explore the feasibility of using ABI-007 in metronomic regimens as a less toxic but still highly effective antitumor alternative, one that would be well suited for long-term combination with other drugs, such as vascular endothelial growth factor–targeted antiangiogenic

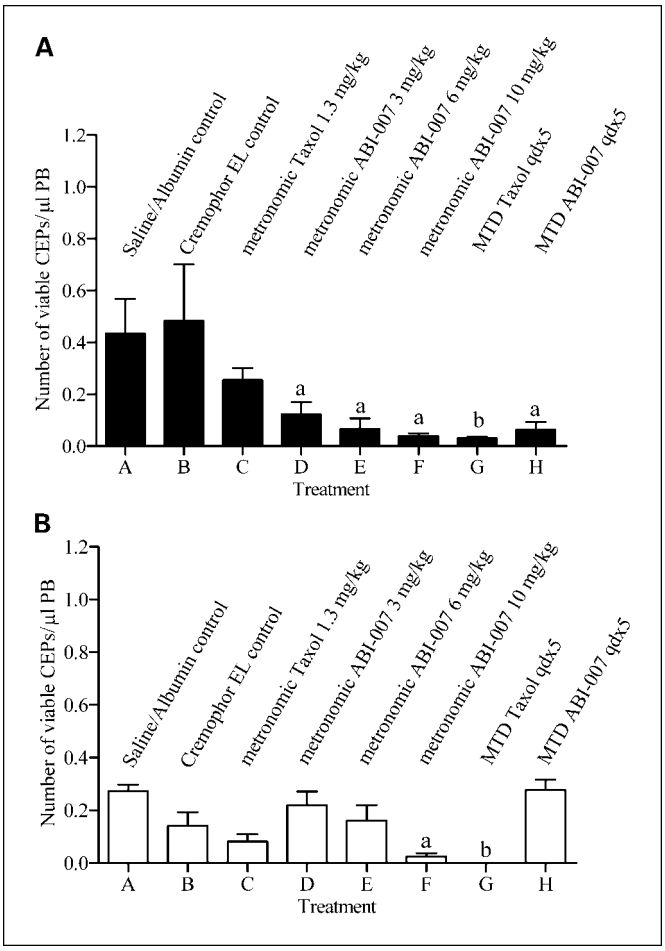

Fig. 4. Changes in the levels of viable CEPs in peripheral blood of MDA-MB231 (*A*) and PC3 (*B*) tumor-bearing severe combined immunodeficient mice after treatment with metronomic or MTD ABI-007 and Taxol. Columns, mean; bars, SE. a, $P < 0.05$, significantly different from saline/albumin vehicle control. b, $P < 0.05$, significantly different from cremophor EL vehicle control.

drugs (4, 6). Naturally, the selection of optimal doses of ABI-007 for such regimen is pivotal to achieving therapeutic efficacy. We have recently shown the potential use of viable CEP levels in peripheral blood as a biomarker to determine the optimal biological doses of antiangiogenic drugs (26) and for metronomic chemotherapy, at least in mice (30). For example, the dose of DC101 (800 μg), a rat monoclonal antibody against mouse vascular endothelial growth factor receptor 2, which induced the lowest levels of viable CEPs also elicited the greatest reduction in tumor volume, whereas dose escalation up to 2,000 μg failed to decrease both variables any further (26). In the current study, metronomic ABI-007 at 3 and 10 to 30 mg/kg significantly reduced viable CEP levels in non–tumor-bearing BALB/cJ mice. However, ABI-007 at 10 to 30 mg/kg was also associated with significant reduction in white blood cell count, indicative of toxicity. We, therefore, deduced that the optimal biological doses for metronomic ABI-007 were between 3 and 10 mg/kg. Indeed, metronomic ABI-007 at 3 to 10 mg/kg given i.p. daily for 4 weeks effectively inhibited MDA-MB-231 and PC3 tumor growth. The observed decrease in tumor volume was accompanied by a dose-dependent reduction in viable CEP levels in both xenograft models. It should also be noted that the total dose of ABI-007 given over 4 weeks in the metronomic regimen, especially in the 10 mg/kg/d treatment group, was substantially higher than that given over 5 days in the MTD regimen (280 versus 150 mg), and yet no significant weight loss was evident in the former. In marked contrast, metronomic Taxol failed to suppress tumor growth or significantly alter viable CEP levels. In all likelihood, this can be attributed to the entrapment of paclitaxel in cremophor micelles in the

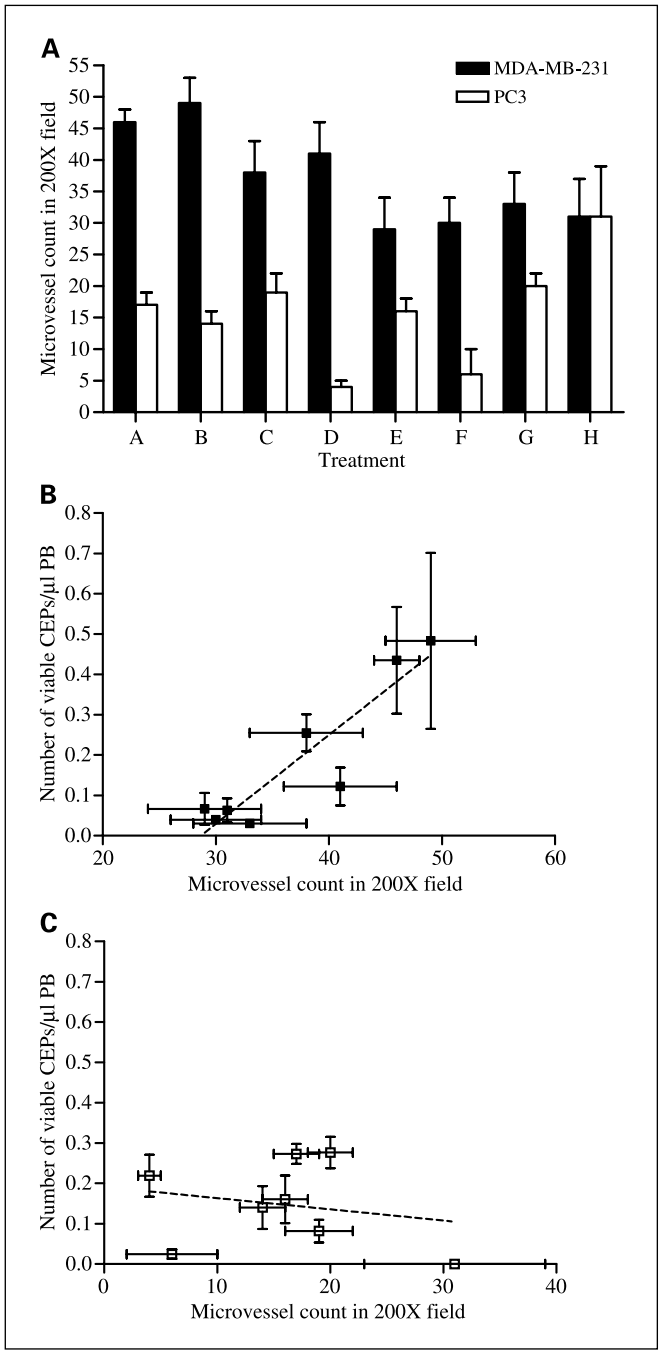

Fig. 5. Intratumoral microvessel density (*A*) of MDA-MB-231 (■) and PC3 (□) xenografts treated with (*A*) saline/albumin control; (*B*) cremophor EL control; (*C*) metronomic 1.3 mg/kg Taxol; (*D, E*, and *F*) metronomic 3, 6, and 10 mg/kg ABI-007, respectively; (*G*) MTD Taxol; and (*H*) MTD ABI-007. Columns, mean; bars, SE. Correlation between intratumoral microvessel density and the number of viable CEPs in peripheral blood in MDA-MB-231 (*B*) and PC3 (*C*) tumor-bearing mice.

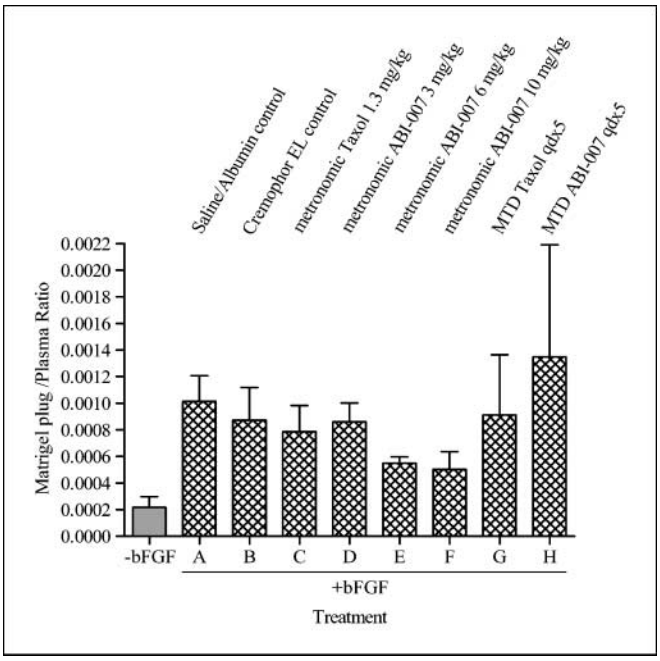

Fig. 6. Effects of metronomic or MTD ABI-007 and Taxol on basic fibroblast growth factor (*bFGF*) – induced angiogenesis in Matrigel plugs injected s.c. into the flanks of BALB/cJ mice. Matrigel implanted without basic fibroblast growth factor (*–bFGF*) served as negative control. Columns, mean; bars, SE.

peritoneal cavity. Gelderblom et al. (31) reported a significant increase in bioavailability and systemic concentrations of i.p. paclitaxel in the absence of cremophor EL in patients. Interestingly, the antitumor effect of metronomic ABI-007 at 6 and 10 mg/kg approximated that of MTD Taxol in MDA-MB-231 and PC3 xenografts, respectively. This observation suggests that metronomic ABI-007 at optimal biological doses may be a more desirable means to administer paclitaxel than MTD Taxol because cremophor EL–associated toxicities are eliminated.

Intratumoral microvessel density seemed to decrease slightly in response to metronomic and MTD ABI-007 but not Taxol, albeit without reaching statistical significance. However, reduction in microvessel density is not always obtained with antiangiogenic agents (34). A significant correlation between microvessel density and the levels of viable CEPs was noted in the MDA-MB-231, but this was not observed in the PC3 xenograft model. The *in vivo* antiangiogenic activity of the various treatment regimens used in the tumor xenograft studies was also evaluated using the Matrigel plug perfusion assay. A trend of decreasing angiogenesis with increasing metronomic ABI-007 doses was evident, whereas MTD Taxol and MTD ABI-007 did not seem to block angiogenesis, although statistical significance was not reached with any treatment. Viable CEP levels may be a more sensitive marker than intratumoral microvessel density or Matrigel plug angiogenesis in the evaluation of response to treatment modalities with an antiangiogenic/antivasculogenic component. Although the mechanistic basis of metronomic chemotherapy is thought to be primarily antiangiogenic (2–4, 30), recent evidence showed that long-term metronomic chemotherapy in mice using cyclophosphamide can also stimulate immune responses by augmenting memory T cells and depleting both regulatory and suppressor T cells (35). We cannot exclude the possibility that metronomic chemotherapy using paclitaxel may also induce similar immunomodulatory responses. It is becoming increasingly clear that metronomic chemotherapy regimens exert multiple effects in addition to angiogenesis inhibition.

In summary, we have shown that paclitaxel in the absence of cremophor EL is a viable and effective drug for metronomic chemotherapy. The therapeutic potential of metronomic ABI-007 given alone or in combination with other anticancer and/or antiangiogenic agents warrants investigation in the clinical setting. Our findings especially underscore the importance of selecting an optimal formulation strategy for cytotoxic drugs (and other investigational agents) to be used in metronomic chemotherapy regimens. Clearly, the potential of the nanoparticle albumin-bound technology extends beyond paclitaxel. For instance, Nab-028, the novel taxane ABI-028 formulated as albumin-bound nanoparticles, showed improved efficacy and lower toxicity than ABI-028 formulated in Tween 80 (36). 17-(Allylamino)-17-demethoxygeldanamycin, a hydrophobic drug that inhibits HSP90 and shows poor tolerability when solubilized in a DMSO-egg lecithin vehicle, was successfully prepared as an albumin-bound nanoparticle formulation suitable for i.v. administration (37). Finally, with respect to the use of taxanes in metronomic chemotherapy regimens, a comparison of nanoparticle-based formulations, such as ABI-007, with orally bioavailable taxanes (e.g., BMS-275183) would seem timely because our results suggest that metronomic chemotherapy with low-dose, cremophor EL–free taxanes should be feasible and effective.

Acknowledgments

We thank Cassandra Cheng for her excellent secretarial assistance.

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