Preclinical Development

Molecular Cancer Therapeutics

Low-Dose Metronomic Oral Dosing of a Prodrug of Gemcitabine (LY2334737) Causes Antitumor Effects in the Absence of Inhibition of Systemic Vasculogenesis

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Abstract

Metronomic chemotherapy refers to the close, regular administration of conventional chemotherapy drugs at relatively low, minimally toxic doses, with no prolonged break periods; it is now showing encouraging results in various phase II clinical trials and is currently undergoing phase III trial evaluation. It is thought to cause antitumor effects primarily by antiangiogenic mechanisms, both locally by targeting endothelial cells of the tumor neovasculature and systemically by effects on bone marrow–derived cells, including circulating endothelial progenitor cells (CEP). Previous studies have shown reduction of CEPs by metronomic administration of a number of different chemotherapeutic drugs, including vinblastine, cyclophosphamide, paclitaxel, topotecan, and tegafur plus uracil (UFT). However in addition to, or even instead of, antiangiogenic effects, metronomic chemotherapy may cause suppression of tumor growth by other mechanisms such as stimulating cytotoxic T-cell responses or by direct antitumor effects. Here we report results evaluating the properties of metronomic administration of an oral prodrug of gemcitabine LY2334737 in nontumor–bearing mice and in preclinical models of human ovarian (SKOV3-13) and breast cancer (LM2-4) xenografts. Through daily gavage (at 6 mg/kg/d), the schedules tested were devoid of toxicity and caused antitumor effects; however, a suppressive effect on CEPs was not detected. Unexpectedly, metronomic LY2334737 administration caused increased blood flow in luciferase-tagged LM2-4 tumor xenografts, and this effect, readily measured using contrast micro-ultrasound, coincided with a relative increase in tumor bioluminescence. These results highlight the possibility of significant antitumor effects mediated by metronomic administration of some chemotherapy drugs without a concomitant inhibition of systemic angiogenesis. *Mol Cancer Ther; 11(3); 680–9. ©2011 AACR.*

Introduction

The original reports of low-dose metronomic chemotherapy (1, 2) highlighted the antiangiogenic basis for the antitumor effects of administering a chemotherapy drug in this fashion—at very close regular intervals (e.g., daily) using relatively low (i.e., minimally toxic) doses and with no prolonged interruptions (3). Integration with an antiangiogenic agent such as TNP-470 or anti-VEGFR-2 monoclonal antibodies (mAb) with metronomic chemotherapy can cause enhanced antitumor effects, which are sometimes remarkable (1, 2) and accompanied by minimal overt toxic side effects in preclinical models (2, 4). As a result of these potential benefits, a number of phase II clinical trials have been initiated; the results of a number of these trials have shown very encouraging results (5), for example, daily low-dose cyclophosphamide (CTX) and bevacizumab (the anti-VEGF mAb) for the treatment of recurrent, refractory ovarian cancer (6), daily low-dose oral CTX and letrozole, an aromatase inhibitor, for the treatment of metastatic breast cancer in elderly women (7), daily low-dose oral CTX with daily low-dose capecitabine, a 5-FU prodrug, in combination with bevacizumab for the treatment of metastatic breast cancer (8), and daily metronomic capecitabine with weekly gemcitabine and daily sorafenib in renal cell carcinoma (9). At least 4 randomized phase III trials of various metronomic chemotherapy regimens are currently in progress (www.clinicaltrials.gov).

With respect to the antiangiogenic basis of administering chemotherapy in a low-dose frequent manner, a number of studies have shown that some of the endothelial cells of the expanding neovasculature of tumor undergo apoptosis as a result of exposure to metronomic chemotherapy (1, 2), which presumably leads to

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the reduction in microvessel density, as reported in some studies (1, 10). In addition, proangiogenic/vasculogenic bone marrow–derived cells [circulating endothelial progenitor cells (CEP)] can be targeted by various metronomic chemotherapy regimens (11–14). Indeed, this property—reduction in CEPs—has been exploited as a surrogate pharmacodynamic biomarker in mice to determine the optimal biologic/therapeutic dose of many different drugs for metronomic chemotherapy studies, including vinblastine, vinorelbine, cisplatin, CTX, and paclitaxel (15), a nanoparticle formulation of paclitaxel (13) and UFT (tegafur plus uracil), a 5-FU prodrug (14). However there are indications that metronomic chemotherapy may involve additional or even alternative mechanisms (3, 5). For example, low-dose CTX has been known to stimulate the immune system, primarily by targeting the regulatory T cells, thus augmenting the activity of cytotoxic T lymphocytes as well as other types of killer cells, for example, lymphokine-activated killer cells (16). This may explain the ability of metronomic CTX to enhance the activity of antitumor vaccines (17). Direct tumor cell effects caused by metronomic chemotherapy may also be a contributing factor in some cases. For example, we previously reported that the daily administration of a doublet combination of 2 different chemotherapy drugs—CTX and UFT—caused exceptional long-term survival of mice with established advanced (high volume) visceral metastatic disease—in which therapy was initiated one month after resection of primary orthotopic human breast cancer xenografts (14). It is unusual for drugs that only have antiangiogenic effects to bring about such a potent therapeutic effect, suggesting the possibility of a direct antitumor cell effect. Direct targeting of relatively small numbers of cancer stem cells could conceivably cause such an effect, for which there is some preliminary evidence (18). Finally, there are reports that certain chemotherapy drugs administered *in vivo* to tumor-bearing mice can target expression of tumor-associated hypoxia-inducible factor-1α—a major driver of angiogenesis (19, 20) and also many other tumor cell properties involved in growth and progression (21)

Given the obvious advantage, or even necessity for using oral chemotherapy drugs in clinical or even preclinical metronomic chemotherapy experiments, we have placed considerable emphasis on the study of such agents, for example, CTX, UFT (14, 22), or oral topotecan (23), and evaluating them either as monotherapies (e.g., CTX; ref. 11) or as doublet treatment combinations, for example, CTX plus UFT (14), or in combination with targeted biologic antiangiogenic agents (23).

The purpose of this study was to evaluate the properties of an orally bioavailable prodrug of gemcitabine LY2334737 (2′-deoxy-2′,2′-difluoro-N-(1-oxo-2-propylpentyl)-cytidine, hemi p-toluenesulfonic acid hemihydrate; Eli Lilly) from a metronomic chemotherapy perspective. LY2334737 is a gemcitabine analog with an amide-linked valproate (24). The prodrug is orally absorbed intact and slowly releases gemcitabine systemically over an extended time period, consistent with formation rate–limited kinetics. We found that this drug can be safely administered at repetitive low doses for prolonged periods with no long drug-free breaks and cause antitumor effects. The antitumor effects were observed in 2 xenograft models; the LM2-4 triple-negative human breast cancer model (14) and the SKOV3-13 human ovarian carcinoma (23). However, unlike the chemotherapy drugs we have previously studied, it did not cause systemic suppression of CEPs, nor a drop in tumor microvessel density, suggesting involvement of mechanisms largely independent of angiogenesis/vasculogenesis inhibition. As such, this drug might be particularly suitable for combination with other chemotherapeutic drugs that are known to induce anti-angiogenic effects, including inhibition of CEPs, as the combination of two such agents may have nonoverlapping, complimentary mechanisms of action.

Materials and Methods

Drug preparation

Gemcitabine hydrochloride was purchased from the pharmacy at the Sunnybrook Health Sciences Center and prepared in sterile saline immediately before i.p. administration. LY2334737 (Eli Lilly) was prepared at 2.0 mg/mL in which 54% was gemcitabine. It was prepared and diluted as necessary in sodium phosphate (0.1 mol/L, pH 6.0) every week and stored in the dark at 4 degrees until administered by gavage.

Metronomic dosing of the LY2334737 prodrug and of cyclophosphamide

Female Balb/cJ mice were purchased at 6 to 8 weeks of age from Jackson laboratories and allowed to acclimatize for 2 weeks before their use in experiments. Mice were divided into 8 groups (4 mice per group) and treated with different doses of LY2334737 per oral daily, diluent control per oral daily, or gemcitabine HCl administered i.p. every 3 days. On day 28, mice were bled and white blood count and viable CEP analysis was carried out, as previously described by us (15), and that we previously used to identify the optimal metronomic dose of different chemotherapeutic agents (12–14). CEP analysis was calculated as the percentage of CEPs relative to the total white blood cell count. Cyclophosphamide was administered as an i.p. bolus (100 mg/kg) on day 1, followed by continuous 20 mg/kg/d dosing in the drinking water as previously described (22, 25).

Statistical analysis

Data are presented as mean ± SD. Statistical significance of differences was assessed by one-way ANOVA, followed by Newman–Keuls ad hoc statistical test using GraphPad Prism 4 software. Differences between all groups were compared with each other and were considered significant at values of $P < 0.05$.

Cell lines

We previously described the cell lines used in this study, namely the human LM2-4 breast cancer cell line (14) and the human SKOV3-13 ovarian cancer cell line (23).

Orthotopic tumor models, intratumoral blood flow analysis, and luciferase imaging

LM2-4 cells were cotransfected using the pGL3-luciferase (Promega) and pSVII neo vectors and selected in G418. One high luciferase expressing clone, termed LM2-4luc$^+$, was subsequently implanted in the inguinal mammary fat pad of 6- to 8-week-old female severe-combined immunodeficient (SCID) mice (2×10^6 cells in 50 microliter volume) as previously described (14, 26, 27). Tumor blood flow analysis was done by high-frequency microultrasound functional imaging, as essentially described previously in the study by Franco and colleagues (28). The SKOV3-13 ovarian model was previously described in Hashimoto and colleagues (23).

Mice were administered luciferin (a 15 mg/mL stock was made up in PBS and administered i.p. to mice at 150 mg/kg) and were imaged 10 to 12 minutes later by first anaesthetizing and then imaging, described in Hashimoto and colleagues (23), in a IVIS200 Xenogen.

Tumor microvessel density

Microvessel density was evaluated as described elsewhere (28, 29). Briefly, tumors were removed and cut into pieces and then one piece per tumor was immediately frozen on dry ice in Tissue-Tek OCT Compound (Miles Inc.) and kept protected from light at −70°C. For microvessel density, vessels in the frozen sections were immunostained with an anti-CD31 antibody (1:200; BD Pharmingen) and its secondary Cy3-conjugated donkey antirat antibody (1:200; Jackson ImmunoResearch Laboratories Inc.).

Image acquisition and analysis

Tumor sections were visualized under a Carl Zeiss Axioplan 2 microscope (Carl Zeiss Canada Inc.), using bright field and the appropriate fluorescence filters. Images were captured with a Zeiss Axiocam digital camera connected to the microscope using AxioVision 3.0 software. The number of fields per tumor sample varied from 2 to 8, depending on the tumor size, and a representative portion of tumor area was analyzed for each tumor section. Magnification of ×200 was used for the CD31 immunostaining to clearly identify vessel structures (n = 2–8 field per tumor sample). For the analysis of microvessel density immunostaining using anti-CD31, the total number of vascular structures (CD31 positive) per field was counted.

Angiogenesis and tumor health panel analysis

Analysis was carried out on paraffin embedded LM2-4 tumor sections. Tumor blocks were sectioned as 3-μm slices onto standard microscope slides. Slides were deparaffinized, rehydrated, and antigen retrieval was done followed by blocking with Protein Block (Dako) for 30 minutes. For the tumor health panel, slides were stained with a combination of Hoechst 33324 (Invitrogen), rat antimouse CD34 (Biolegend)/antirat Alexa-488 (Invitrogen), rabbit anti-Ki67 (NeoMarkers)/antirabbit Alexa 647 (Invitrogen), and TUNEL TMR (Roche). For the angiogenesis panel, slides were stained with a combination of Hoechst 33324 (Invitrogen), rat antimouse CD34 (Biolegend)/antirat Alexa-488 (Invitrogen), rabbit anti-GLUT1 (Chemicon)/antirabbit Alexa 647 (Invitrogen), and mouse anti-Smooth Muscle Actin/Cy3 (Sigma). Slides were imaged using an iCys Laser Scanning Cytometer (CompuCyte) and a Marianas Digital Imaging Workstation configured with a Zeiss Axiovert 200M inverted fluorescence microscope (Intelligent Imaging Innovations). Quantitative data comparisons of treatment groups were done using the Student t test analysis in JMP statistics software (SAS).

Results

Toxicity analysis and impact on CEP levels of metronomic LY2334737

To determine the optimal biologic dose of LY2334737 given metronomically, we administered the prodrug of gemcitabine daily by gavage to female Balb/c mice for 28 days (Fig. 1A). Doses used were 2, 4, and 6 mg/kg (Fig. 1). For comparative purposes mice were also administered gemcitabine i.p., given every 3 days at doses of 40, 70, 120, or 160 mg/kg for a total of 9 cycles of dosing. On day 28, mice were bled and CEP analysis was carried out (Fig. 1C). We found no significant impact on host weight (as an indicator of toxicity) in any of the LY2334737 treatment groups throughout the course of the experiment. We similarly did not observe any impact of gemcitabine i.p. on mouse weights (Fig. 1B). Surprisingly, daily dosing of LY2334737 also had no significant impact on CEP numbers as shown in Fig. 1C. Furthermore, white blood cell (WBC) counts confirmed the relative absence of host toxicity in this experiment (Fig. 1C). Taken together, these results suggested that LY2334737 could be the first example of a chemotherapy drug which when given metronomically is not only minimally or nontoxic but also has little or no impact on systemic angiogenesis (i.e., as measured by CEPs). It is also important to note that we used CEPs as a surrogate marker (12) for evaluating the optimal metronomic dose of LY2334737 (although our test proved inconclusive); a more accurate evaluation of the precise impact of metronomic LY2334737 on CEPs may require additional studies, with a larger number of mice per group.

Impact on CEP levels and toxicity analysis of escalating doses of daily LY2334737 administration

We assessed increasing concentrations of daily doses of LY2334737 over a 7-day treatment period, with doses up to 20 mg/kg/d. We found that even within this brief

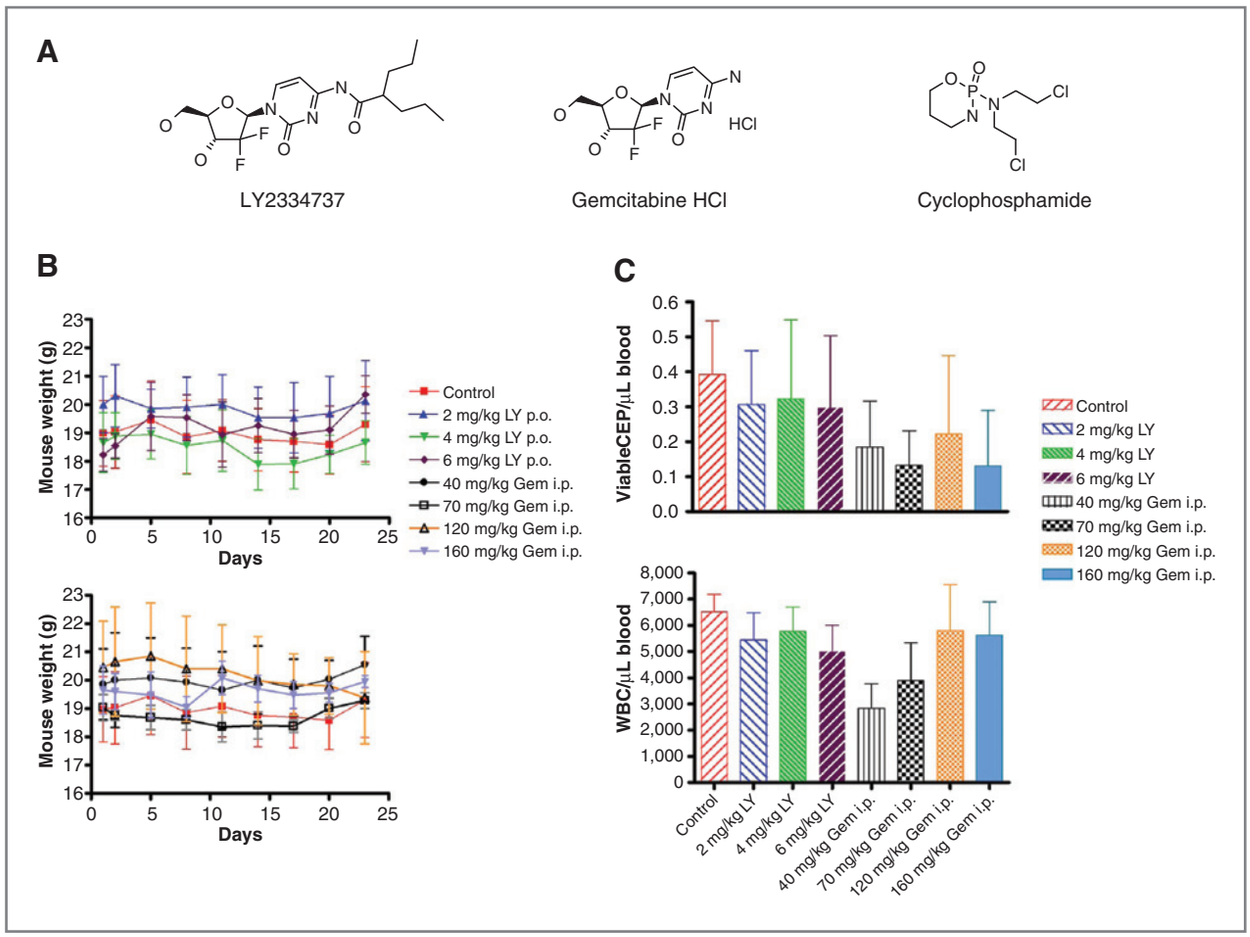

Figure 1. Impact of the prodrug of gemcitabine LY2334737 (LY) or gemcitabine HCl administration on mouse weights as well as CEP and WBCs counts in female Balb/c mice. Gemcitabine (Gem) was given i.p. (at doses of 40, 70, 120, or 160 mg/kg) every 3 days (starting on day 1, then on days 4, 7, etc.) up to day 25 when the last dose was administered. LY2334737 was given daily by gavage (at doses of 2, 4, and 6 mg/kg), and for all groups mice were bled on day 28. A, chemical structure of LY2334737, gemcitabine, and CTX. B, no significant weight loss was observed in all groups throughout the treatment period. p.o., orally. C, viable CEP (top) and WBC (bottom) count for mice treated with the indicated doses of LY2334737 or gemcitabine (Gem), showing no significant impact of LY2334737 on CEP numbers (no statistically significant difference between the groups), in the absence of overt toxicity as determined by relative WBC (no statistically significant difference was found between WBC for controls and for LY2334737-treated groups; the 40 mg/kg gem i.p. treated group was significantly lower than controls, $P < 0.01$).

time frame, the higher doses (>10 mg/kg) were sufficient to induce toxicity, as evidenced by the severe weight loss (see Fig. 2A). However, whereas for doses of 15 and 20 mg/kg, the CEP analysis was complicated by the evident high host toxicity, we again did not observe an impact on CEP levels when daily nontoxic doses (<10 mg/kg) of LY2334737 were administered (Fig. 2B). Taken together, these results suggested that daily LY2334737 doses that do not cause overt toxicity (i.e., 6–8 mg/kg/d dose range) fail to significantly impact CEP numbers, regardless of whether they are administered for a 7-day period or longer (i.e., 28 days as shown for 6 mg/kg in Fig. 1B). At the same time, we cannot exclude that metronomic LY2334737 may be toxic to CEPs; thus, for example, average CEP levels in the 6 mg/kg/d LY2334737 group were slightly lower than controls (Fig. 2B). Also, at 10 mg/kg/d LY2334737 a relative drop in CEPs was observed, although this effect was not specific to that cell type because we also observed a drop in WBC counts. Gemcitabine given every 3 days i.p. (Fig. 2B) is toxic to CEPs, although further studies with a larger number of mice per group are necessary to more clearly define that effect. Furthermore, we cannot exclude that the different impact that gemcitabine and LY2334737 had on CEP levels could be due to the fact that the drugs were administered differently, that is, gemcitabine was administered i.p. every 3 days, whereas LY2334737 was administered daily by gavage.

Impact of metronomic LY2334737 on tumor growth and on intratumoral microvessel density

We evaluated the impact of LY2334737 on tumor-driven angiogenesis, by assessing microvessel density in implanted tumors treated with LY2334737-based regimens. As a tumor model, we used the human breast

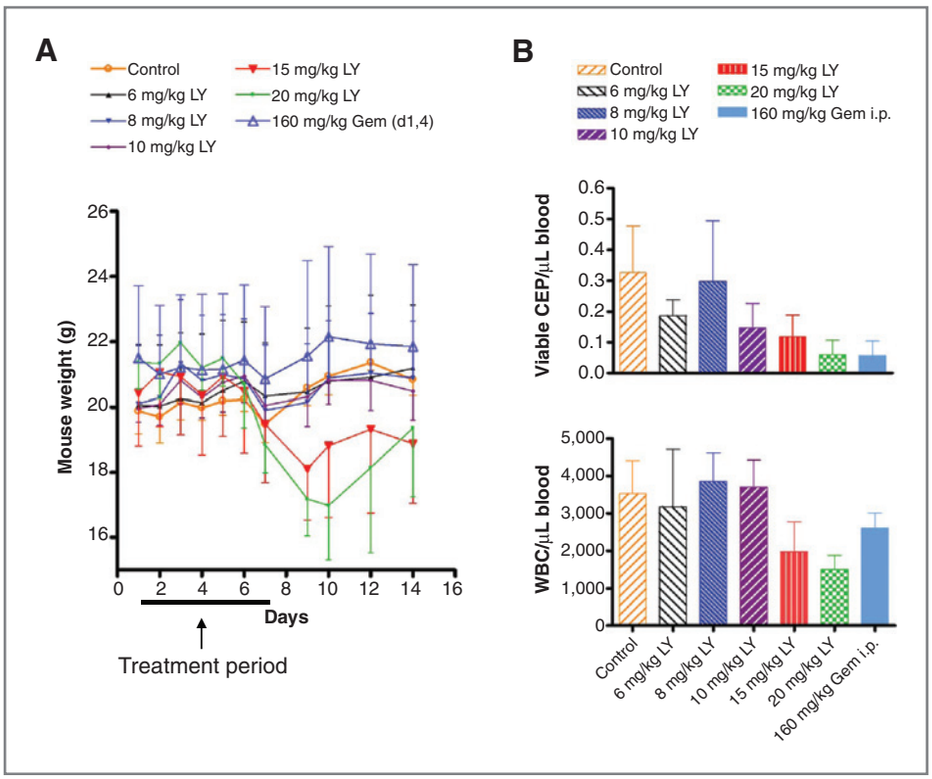

Figure 2. Empirical determination of MTDs of LY2334737 as given by daily administration by gavage. Balb/c mice were treated for 7 days with the indicated levels of LY2334737 (administered daily) or 160 mg/kg gemcitabine HCl (Gem) administered i.p. on days 1 and 4. A, severe weight loss was observed in doses higher than 10 mg/kg of LY2334737, indicating the upper limit that can be dosed of LY2334737 daily; at the end of the treatment period, the mice were bled for CEP analysis. B, impact of the 7-day dosing regimen on CEPs and WBCs, showing that daily doses of LY2334737 at 6 to 8 mg/kg/d did not significantly impact CEP numbers. A reduction in CEP numbers was evident at higher doses (e.g., 15 and 20 mg/kg/d; these were significantly lower, $P < 0.05$, compared with controls and compared with the 8 mg/kg LY2334737-treated group), but this was concomitant with excessive weight loss (shown in A), which was further confirmed by a drop in WBC (a significant difference in WBC, $P < 0.05$, was observed between 20 mg/kg LY2334737-treated mice and controls). LY, LY2334737.

cancer cell line LM2-4luc$^+$ (which was tagged with luciferase) grown orthotopically in SCID mice. We have previously used this model to assess the effectiveness of other metronomic chemotherapy regimens, for example, metronomic CTX (14). As a positive control treatment we used a bolus plus low-dose CTX protocol, which we previously showed to effectively inhibit tumor-driven angiogenesis (25). LY2334737 was administered at daily doses of 6 and 8 mg/kg. In a separate group, gemcitabine was administered i.p. at a dose of 160 mg/kg given every 3 days. We also tested combinations of LY2334737 plus the metronomic CTX protocol, in view of the fact that some drugs can elicit certain antitumor (or antiangiogenic) responses only when they are combined with other treatment regimens (14). Tumor growth was monitored by caliper measurements, and therapies were initiated when the tumors reached an average size of 250 mm^3. All treatment caused antitumor effects compared with the control saline-treated group (Fig. 3A) during the 2-week treatment period. The mice were then sacrificed and the tumors were removed and sectioned for microvessel density analysis. As can be seen from Fig. 3B, the combination of bolus plus low-dose CTX with 8 mg/kg LY2334737 showed some toxicity as indicated by weight loss. This was also observed, to a lesser extent, with the combination of bolus plus low-dose CTX and 6 mg/kg LY2334737. It should be noted that drugs such as gemcitabine may exert different levels of toxicity depending on the tumor that is implanted in the host (30). In this regard, it is noteworthy that the prodrug given at either 6 or 8 mg/kg did not produce significant toxicity (defined as causing >10% body weight loss in the course of treatment) in either nontumor–bearing Balb/c mice or in SCID mice bearing LM2-4luc$^+$ tumors.

Microvessel density analysis of the tumor sections from this experiment showed that bolus plus low-dose CTX (used as a positive control) led to a reduction in the number of vessels compared with saline-treated controls (Supplementary Fig. S1). However, for all LY2334737-treated groups as well as the groups treated with combinations of LY2334737 and bolus plus low-dose CTX, we did not detect any reduction in the number of tumor vessels (Supplementary Fig. S1). In fact there seemed to be a slight increase in the number of vessels in these groups compared with controls. Thus in the treated group, there was no major impact on the number of

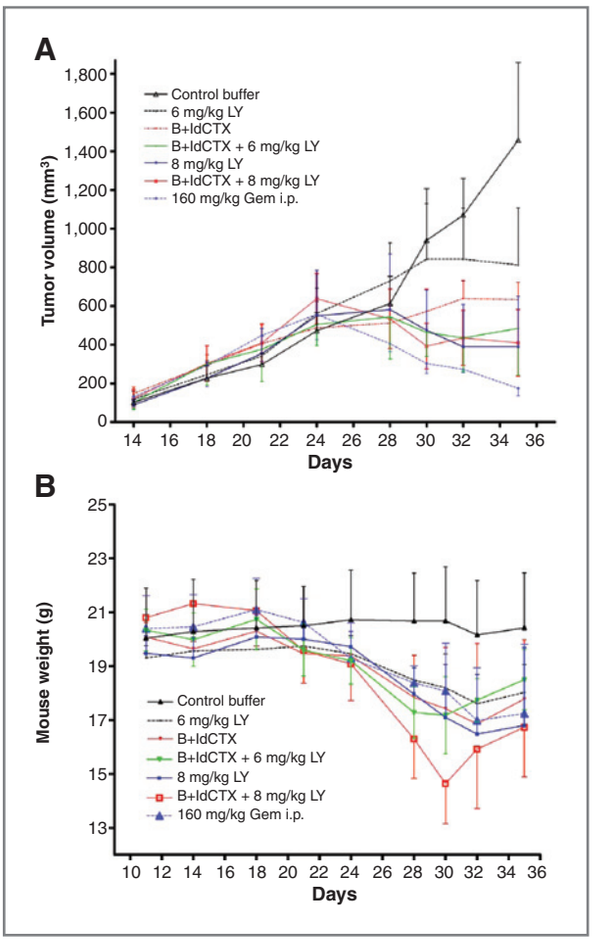

Figure 3. Impact of LY2334737-based regimens on human breast tumors orthotopically implanted in SCID mice. The human LM2-4luc$^+$ cell line was implanted orthotopically in female SCID mice that were treated with the daily indicated doses of LY2334737, either alone or in combination with a CTX regimen. Cyclophosphamide was administered as an i.p. bolus (100 mg/kg) on day 1, followed by continuous 20 mg/kg/d dosing in the drinking water (B+ld CTX). An additional group was included that received gemcitabine i.p. treatment (160 mg/kg given every 3 days). A, tumors treated for 2 weeks (days 21–35) until controls reached 1,500 mm^3, at which point mice were sacrificed. B, mouse weights as a measure of host toxicity, indicating toxicity of the combination regimens, particularly with the combination of LY2334737 (8 mg/kg), together with the bolus plus low-dose CTX treatment. B+ld CTX, bolus plus low-dose CTX; LY, LY2334737.

vessels. Therefore, metronomic LY2334737 does not seem to impact tumor angiogenesis. Quantitative fluorescence imaging of these tumors confirms these treatment effects on vascular density (Supplementary Fig. S2). In addition, treatment with LY2334737 alone results in a decrease in large vessels, an increase in small vessels, and an increase in the vessel normalization index (Supplementary Fig. S2C). The increase in a normalization phenotype (assessed by a decreased tortuosity, increased pericyte coverage, and decreased hypoxia) suggested that metronomic LY2334737 treatment modulates vessel stability and functionality.

Increased blood flow in orthotopically implanted LM2-4luc$^+$ tumors treated with metronomic dosing of prodrug of gemcitabine LY2334737

To evaluate the impact of metronomic LY2334737 dosing on intratumoral blood flow, LM2-4luc$^+$ cells were orthotopically implanted into the mammary fat pad of SCID mice. LY2334737 treatment (8 mg/kg) was started when tumors reached an average size of 250 mm^3—and before the first administration of LY2334737, we measured pretreatment blood flow in all tumors (by intravenous injection of the ultrasound contrast agent)—see Fig. 4A and B. Blood flow measurements were then taken after 1 week of LY2334737 treatment (by intravenous injection of contrast agent) and a second measurement was taken after 3 weeks of treatment—see Fig. 4B and C. We observed that LY2334737 treatment caused an increase in blood flow in LM2-4luc$^+$ tumors (compared with vehicle-treated controls—see Fig. 4C) one week after treatment, and this difference increased 3 weeks into the treatment schedule (see Supplementary Movie files 1–4, as well as Fig. 4C). Caliper measurements showed that, as expected, LY2334737 monotherapy caused the tumors to be growth inhibited compared with controls (Fig. 4A). And yet, paradoxically, the smaller LY2334737-treated tumors showed equal or greater luciferase luminescence than the control tumors (Fig. 4D and E). This observation is consistent with the possibility that the increased intratumoral blood flow in the (smaller) LY2334737-treated tumors produced a more effective delivery of the luciferin substrate.

Antitumor efficacy of LY2334737 regimens on an orthotopic human ovarian cancer model in SCID mice

To confirm the antitumor effects of LY2334737-based regimens and to exclude the possibility that our observations were a peculiarity of the LM2-4 model, we decided to test the effect of LY2334737 administration on another human tumor model. We chose to use an orthotopic human ovarian cancer model that we recently developed (23). The model consists of a clone of human SKOV-3 cells called SKOV-3-13 injected i.p. into SCID mice, which results in the cells eventually growing i.p. as both solid tumors and ascites. Two weeks after tumor implantation, the mice were randomized into 4 groups, which were then treated with LY2334737 monotherapy (6 mg/kg/d), vehicle control, low-dose CTX, or the combination of LY2334737 plus low-dose CTX. Luminescence imaging indicated a significant antitumor effect of the LY2334737-based regimens, compared with controls (Fig. 5A). Furthermore, the resulting survival curve showed that LY2334737 monotherapy increased the survival of mice compared with controls (Fig. 5B). We also noted that the combination of low-dose CTX plus LY2334737 did not increase survival beyond that observed with LY2334737 alone. This ovarian model consists principally of ascites (i.e., a tumor cell suspension in the peritoneal cavity), and because

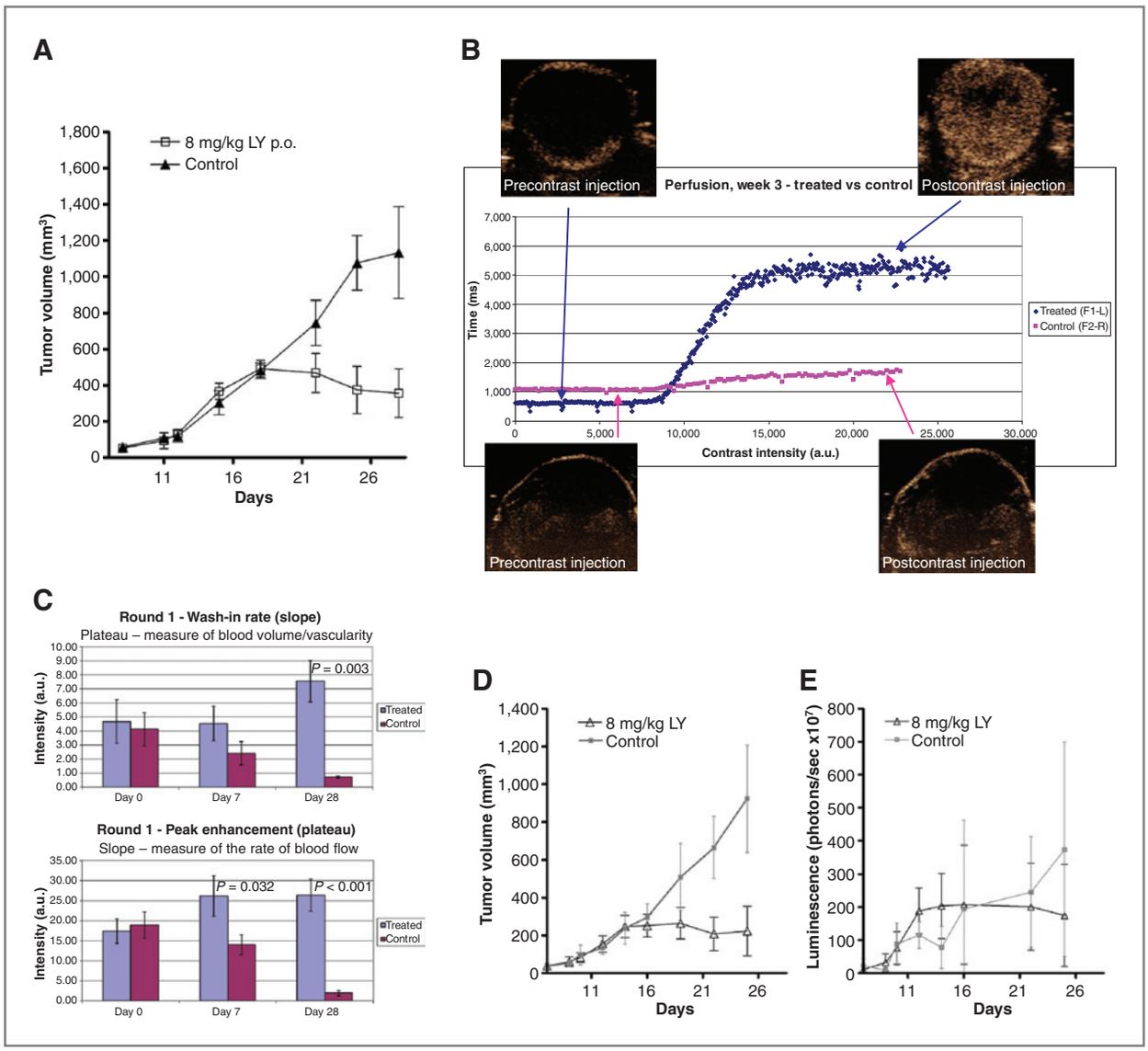

Figure 4. Tumor growth, intratumoral blood flow analysis, and luminescence analysis of LM2-4luc$^+$ tumors treated with LY2334737-based regimens. LM2-4luc$^+$ tumors growing in SCID mice treated with oral prodrug of gemcitabine (LY2334737, 8 mg/kg/d). LM2-4luc$^+$ cells were implanted into SCID mice and LY2334737 monotherapy (or vehicle alone control) was initiated 2 weeks later (arrow). A, LY2334737 treatment significantly inhibited tumor growth compared with controls. p.o., orally. B, blood flow measurement analysis of LM2-4luc$^+$ tumors using contrast micro-ultrasound. Analysis is presented as the plateau (proportional to blood volume) or slope (proportional to speed of tumor blood flow) values. Measurements were taken before treatment initiation and at 1 week and at 3 weeks after treatment commenced. The data show that blood flow increased in LY2334737-treated (8 mg/kg/d) tumors relative to the controls. Micro-ultrasound images and a plot of contrast wash-in from one LY2334737-treated mouse and one vehicle control mouse are shown. Data were assessed by the plateau value and/or the slope of the curve generated. a.u., arbitrary unit. C, summary of the analysis of micro-ultrasound intratumoral blood flow data, showing an increase in blood flow after 1 week and after 3 weeks of LY2334737 treatment compared with controls. Data are of 5 mice per group, and statistical analysis was done by a 2-way ANOVA with Holm-Sidak multiple comparison test. D and E, tumor growth analysis suggested that luminescence did not correlate well with tumor caliper measurements in this model after LY2334737 treatment was initiated. We therefore set up another primary tumor growth curve (D), showing the impact of LY2334737 with a parallel assessment of luminescence (E) in the respective tumors. The results showed an initial discrepancy between the caliper measurements and luminescence data, presumably as a consequence of altered blood flow in LY2334737-treated tumors. LY, LY2334737.

the luciferase substrate in this experiment was administered directly into the peritoneal cavity, the luminescence data correlated well with the resulting survival curve, and the imaging of this model was effectively independent of blood circulation. Thus, taken together, these results indicated that although the growth of solid tumors treated with metronomic LY2334737 may not correlate with luminescence data (i.e., Fig. 4D and E), such a discrepancy is not evident in models in which tumors grow as malignant ascites.

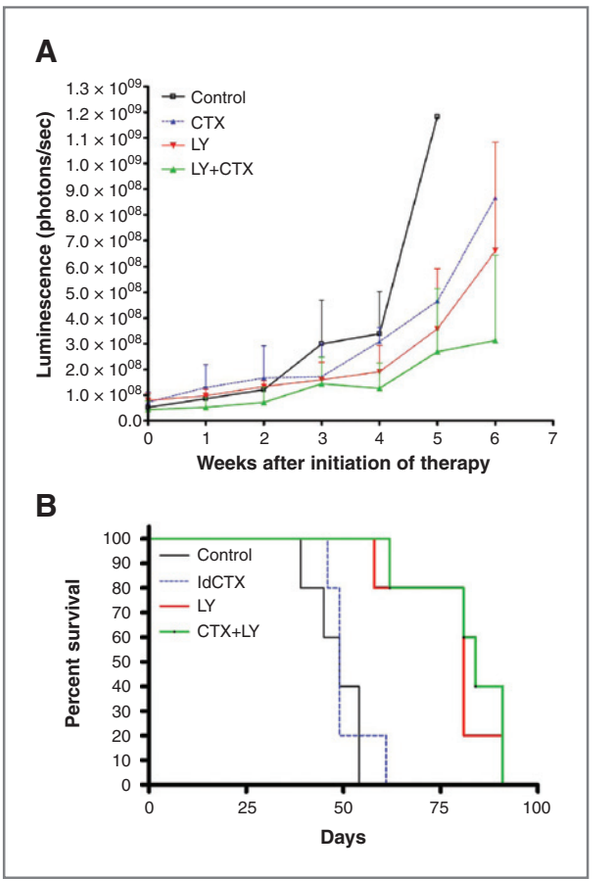

Figure 5. Impact of prodrug of gemcitabine (LY2334737) regimens on SKOV3-13 ovarian cancer cells grown in SCID mice. SKOV3-13 cells were injected i.p. and treatment commenced 2 weeks later. Luminescence data are shown for all treatment groups (A), and the increased survival of mice treated with LY2334737 regimens compared with controls (B).

Discussion

Metronomic chemotherapy is emerging as a potentially important new therapeutic strategy for the treatment of a variety of solid tumors (5–8). Effective metronomic scheduling relies on the prospective identification of a dose at which a chemotherapeutic agent can be administered in a close repetitive fashion with no prolonged breaks, with minimal toxicity. In preclinical mouse studies, determination of the optimal metronomic dose (OMD), that is, the most effective biologic dose at which a chemotherapeutic agent can be administered in a metronomic fashion is the equivalent to the minimum dose administered over a week-long period required to elicit the maximal reduction in CEP numbers (25). Using CEP levels as a pharmacodynamic readout, the OMD was previously determined for several different drugs such as CTX, cisplatin, and UFT among others—and in all cases, the determined OMD using this approach did not cause significant host toxicity, even after very prolonged treatment (14, 23, 31). Here we report the first example of a chemotherapeutic drug we have used that can effectively be administered in a metronomic nontoxic fashion, at doses that have little or no significant impact on CEP numbers (although a comprehensive analysis of the impact of LY2334737 on CEPs was not carried out and is beyond the scope of this study). We also show that nontoxic metronomic doses of the prodrug of gemcitabine LY2334737 that do not suppress CEP levels can nonetheless have an impact on intratumoral blood flow and suppress tumor growth. In other words, CEP analysis failed to predict the effective metronomic dose of LY2334737, which was assessed by us to be in the range of 6 to 8 mg/kg.

The decision to study an oral prodrug of gemcitabine was based on the obvious suitability of such drugs for the frequent (even daily) dosing associated with metronomic chemotherapy regimens in the clinic (5). Second, systemically administered gemcitabine was recently reported to have antitumor (and antiangiogenic) properties when administered in a low-dose, daily, metronomic fashion (32). Third, the observations reported in this study are particularly interesting when considering the announced failure in 2008 of a randomized phase III clinical trial in pancreatic cancer treated with weekly maximum tolerated dose (MTD) gemcitabine plus biweekly bevacizumab, despite prior encouraging phase II data (33). Thus, because metronomic or metronomic-like chemotherapy in some cases has been shown to be effective against tumors that have acquired resistance to MTD chemotherapy of the same drug (1, 34), it is conceivable that metronomic gemcitabine could prove effective in situations in which MTD gemcitabine no longer has activity. In this regard, there is some limited clinical evidence that administering gemcitabine at doses significantly lower than the MTD—in which the dose is continuously titrated using grade 1 neutropenia as a biomarker for dosing (35, 36)—may be less toxic and equally, or even more effective than MTD gemcitabine (35, 36).

Overall, our prodrug-mediated results are important for 3 reasons. First, they stress that caution is needed when assessing only CEP numbers for determining the OMD, even preclinically, and that additional methods may need to be developed to evaluate the most effective dose for certain drugs such as LY2334737. Second, they imply the existence of a new subclass of chemotherapeutic drugs, that is, drugs that can be effectively administered in a metronomic fashion without impacting CEP levels (a clear distinction from most other chemotherapeutic drugs, e.g., CTX, UFT, which we have tested). Third, they provide further evidence that multiple mechanisms, including altered rate of intratumoral blood flow, likely contribute to the antitumor effects that result from metronomic chemotherapy (e.g., by enhancing the intratumoral delivery of anticancer agents), some of which are unrelated to the inhibition of systemic angiogenesis. Thus, in theory, the comparison between metronomic scheduling of drugs such as CTX (which impacts CEPs) and LY2334737 (which does not) should in part reveal mechanisms of action of metronomic chemotherapy that are independent

of inhibition of systemic vasculogenesis or angiogenesis. Our results also raise the question of whether it may be advantageous to combine metronomic chemotherapy using 2 classes of drugs that differ by their mode of action. For example, in this study, we tested one such combination—using metronomic CTX and LY2334737—that has different impacts on CEP levels.

An interesting and unexpected aspect of our results is the finding of increased tumor blood flow induced by the metronomic oral gemcitabine treatment, as detected by high-resolution microultrasound imaging. This could conceivably lead to selectively increased levels of intratumoral gemcitabine, despite the lower daily doses of drug administered, although this was not assessed by us. If true, this may be related to metronomic gemcitabine induced vessel normalization, a phenomenon induced by various antiangiogenic drugs (37, 38) and postulated to increase intratumoral delivery and distribution of coadministered chemotherapy (37, 38). In this regard, a number of other investigators have recently reported circumstances in which metronomic chemotherapy using drugs such as CTX or gemcitabine can induce vessel normalization and increase perfusion, as well as transiently decrease levels of tumor hypoxia (39–41). As such, this could add another therapeutic dimension to the multiple antitumor mechanisms of action associated with metronomic chemotherapy (5). Future studies will have to be done to determine whether the increase in blood flow can be observed with metronomic doses of other drugs (including metronomic gemcitabine administered i.p.), and in tumor models other than the human LM2-4 breast cancer.

In summary, our results with the prodrug of gemcitabine LY2334737 highlight the fact that it cannot be assumed that all chemotherapeutics drugs administered metronomically will have the same biologic impact, in this case, on the levels of CEPs. Identifying the different classes of agents, in terms of their modes of action when dosed metronomically, and the subsequent testing of combinations of the different classes should lead to a better understanding of what constitutes optimal metronomic chemotherapy regimens.

Disclosure of Potential Conflicts of Interest

J. Stewart and M. Uhlik are current employees of Eli Lilly and Company, and A. Dantzig was a past employee of Eli Lilly and Company during the time these studies were done. F. S. Foster is a consultant to VisualSonics.

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