Low-dose metronomic oral dosing of a prodrug of Gemcitabine (LY2334737) causes anti-tumor 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 anti-tumor 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 (CEPs). 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 anti-tumor effects. Here we report results evaluating the properties of metronomic administration of an oral prodrug of gemcitabine LY2334737 (LY) in non tumor-bearing mice, and in preclinical models of human ovarian (SKOV3-13) and breast cancer (LM2-4) xenografts. Through daily gavage (at 6mg/kg/day) the schedules tested were devoid of toxicity and caused anti-tumor effects; however, a suppressive effect on CEPs was not detected. Unexpectedly metronomic LY administration caused increased blood flow in luciferase-tagged LM2-4 tumor xenografts; and this effect coincided with a relative increase in tumor bioluminescence. These results highlight the possibility of significant anti-tumor effects mediated by metronomic administration of some chemotherapy drugs without a concomitant inhibition of systemic angiogenesis.
Introduction

The original reports of low-dose metronomic chemotherapy(1, 2) highlighted the antiangiogenic basis for the anti-tumor 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 with metronomic chemotherapy can cause enhanced anti-tumor 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), e.g. daily low dose cyclophosphamide and bevacizumab (the anti-VEGF monoclonal antibody) for the treatment of recurrent, refractory ovarian cancer(6), daily low-dose oral cyclophosphamide and letrozole, an aromatase inhibitor, for the treatment of metastatic breast cancer in elderly women(7), daily low dose oral cyclophosphamide 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 four 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 tumor’s expanding neovasculature undergo apoptosis as a result of exposure to metronomic chemotherapy(1, 2) which presumably leads to the reduction in microvessel
density as reported in some studies(1, 10). In addition, proangiogenic/vasculogenic bone marrow-derived cells (CEPs) 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, cyclophosphamide 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 cyclophosphamide has been known to stimulate the immune system, primarily by targeting the regulatory (T reg) cells thus augmenting the activity of cytotoxic T lymphocytes (CTLs) as well as other types of killer cells e.g. lymphokine-activated killer (LAK) cells(16). This may explain the ability of metronomic cyclophosphamide to enhance the activity of anti-tumor 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 two different chemotherapy drugs- cyclophosphamide and UFT – caused exceptional long term survival of mice with established advanced (high volume) visceral metastatic disease – where therapy was initiated one month after resection of primary orthotopic human breast cancer xenografts(14). It is unusual for drugs which only have antiangiogenic effects to bring about such a potent therapeutic effect, suggesting the possibility of a direct anti-tumor 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-1 (HIF-1α) – a major driver of angiogenesis(19, 20) but 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, e.g. cyclophosphamide, UFT(14, 22), or oral topotecan(23), and evaluating them either as monotherapies (e.g. cyclophosphamide(11)) or as doublet treatment combinations e.g. cyclophosphamide plus UFT(14), or in combination with targeted biologic antiangiogenic agents(23).

The purpose of the present study was to evaluate the properties of an orally bioavailable prodrug of gemcitabine LY2334737 (Eli Lilly, Indianapolis) 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 anti-tumor effects. The anti-tumor effects were observed in two 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 which are known to induce antiangiogenic effects, including inhibition of CEPs, as the combination of two such agents may have non-overlapping, complimentary mechanisms of action.
Materials and Methods

Drug Preparation. Gemcitabine hydrochloride was purchased from the pharmacy at the Sunnybrook Health Sciences Centre and made up in sterile saline immediately prior to i.p. administration. LY2334737 (LY, 2’-deoxy-2’,2’-difluoro-N-(1-oxo-2-propylpentyl)-cytidine, hemi p-toluenesulfonic acid hemihydrate, Eli Lilly, Indianapolis) was made up (2.0mg/ml) of which 54% is gemcitabine. It was prepared and diluted as necessary in sodium phosphate (0.1M, pH6.0) every week, and stored in the dark at 4 degrees until administered by gavage.

Metronomic dosing of the LY prodrug, and of Cyclophosphamide. Female Balb/cJ mice were purchased at 6-8 weeks of age from Jackson laboratories and allowed to acclimatize for 2 weeks before used in experiments. Mice were divided into 8 groups (4 mice/group) and treated with different doses of LY p.o daily, diluent control p.o. 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 count. Cyclophosphamide was administered as an i.p. bolus (100mg/kg) on day 1 followed by continuous 20mg/kg/day dosing in the drinking water as previously described(22, 25).

Statistical Analysis. Data are presented as mean ± standard deviation (SD). Statistical significance of differences was assessed by one way ANOVA, followed by Newman-
Keuls ad-hoc statistical test using GraphPad Prism 4 software (La Jolla, CA). Differences between all groups were compared to 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 co-transfected 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 6-8 week old female SCID mice (2x10^6 cells in 50 microlitre volume) as previously described(14, 26, 27).

Tumor blood flow analysis was performed by high-frequency micro-ultrasound functional imaging as essentially described previously in the study by Franco et al (28). The SKOV3-13 ovarian model was previously described in Hashimoto et al(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 10-12 minutes later by first anaesthetizing and then imaging, described in Hashimoto et al(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., Elkhart, IN) 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, San Diego, CA) and its secondary Cy3-conjugated donkey anti-rat antibody (1:200, Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

**Image acquisition and analysis**

Tumor sections were visualized under a Carl Zeiss Axioplan 2 microscope (Carl Zeiss Canada Inc. Toronto, ON, Canada), 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 200X was used for the CD31 immunostaining to clearly identify vessel structures (n = 2-8 field / 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 performed 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 anti-mouse CD34 (Biolegend)/anti-rat Alexa-488 (Invitrogen), rabbit anti-Ki67 (NeoMarkers)/anti-rabbit
Alexa 647 (Invitrogen), and TUNEL TMR (Roche). For the Angiogenesis Panel, slides were stained with a combination of Hoechst 33324 (Invitrogen), rat anti-mouse CD34 (Biolegend)/anti-rat Alexa-488 (Invitrogen), rabbit anti-GLUT1 (Chemicon)/anti-rabbit 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 performed using the Student’s *t*-test analysis in JMP statistics software (SAS).
RESULTS

Toxicity analysis and impact on CEP levels of metronomic LY. To determine the optimal biologic dose (OBD) of LY given metronomically, we administered the prodrug of gemcitabine daily by gavage to female Balb/c mice for 28 days. Doses used were 2mg/kg, 4mg/kg and 6mg/kg (Fig 1a). For comparative purposes mice were also administered gemcitabine i.p., given every three days at doses of 40, 70 120 or 160mg/kg for a total of 9 cycles of dosing. On day 28 mice were bled and CEP analysis was carried out (Fig 1b). We found no significant impact on host weight (as an indicator of toxicity) in any of the LY treatment groups throughout the course of the experiment. We similarly did not observe any impact of gemcitabine i.p. on mouse weights (Fig. 1a). Surprisingly, daily dosing of LY also had no significant impact on CEP numbers as shown in Fig. 1b. Furthermore, white blood cell counts (WBC) confirmed the relative absence of host toxicity in this experiment (Fig 1b). Taken together, these results suggested that LY could be the first example of a chemotherapy drug which when given metronomically is not only minimally or non-toxic, 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 LY (although out test proved inconclusive); a more accurate evaluation of the precise impact of metronomic LY 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 LY administration. We assessed increasing concentrations of daily doses of LY over a 7-day treatment period, with doses up to 20mg/kg/day. We found that even within this brief
time frame, the higher doses (>10mg/kg) were sufficient to induce toxicity as evidenced by the severe weight loss (see Fig 2a). However, whereas for doses of 15mg/kg and 20mg/kg the CEP analysis was complicated by the evident high host toxicity, we again did not observe an impact on CEP levels when daily non-toxic doses (<10mg/kg) of LY were administered (Fig 2b). Taken together, these results suggest that daily LY doses that do not cause overt toxicity (i.e. 6-8mg/kg/day 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 6mg/kg in Fig 1b). At the same time, we cannot exclude that metronomic LY may be toxic to CEPs; thus, for example, average CEP levels in the 6mg/kg/day LY group were slightly lower than controls (Fig 2b). Also, at 10mg/kg/day LY a relative drop in CEPs was observed, although this effect was not specific to that cell type since we also observed a drop in white blood counts (WBC). 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 LY had on CEP levels could be due to the fact that the drugs were administered differently; i.e., gemcitabine was administered i.p. every 3 days, whereas LY was administered daily by gavage.

Impact of metronomic LY on tumor growth and on intratumoral microvessel density. We evaluated the impact of LY on tumor driven angiogenesis, by assessing microvessel density in implanted tumors treated with LY based regimens. As a tumor model we used the human breast cancer cell line LM2/4luc+ (which was tagged with luciferase) grown orthotopically in SCID mice. We have previously employed this model
to assess the effectiveness of other metronomic chemotherapy regimens, e.g. metronomic cyclophosphamide (CTX)(14). As a positive control treatment we employed a bolus plus low dose CTX protocol, which we previously showed to effectively inhibit tumor driven angiogenesis(25). LY was administered at daily doses of 6mg/kg and 8mg/kg. In a separate group, gemcitabine was administered i.p. at a dose of 160mg/kg given every 3 days. We also tested combinations of LY plus the metronomic CTX protocol, in view of the fact that some drugs can elicit certain anti-tumor (or anti-angiogenic) 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 250mm³. All treatment caused anti-tumor effects compared to the control saline treated group (Fig 3a) during the two 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 8mg/kg LY 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 6mg/kg LY. 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 6mg/kg or 8mg/kg did not produce significant toxicity (defined as causing >10% body weight loss in the course of treatment) in either non-tumor bearing Balb/c mice, nor 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 to saline treated controls (Supplementary Figure 1). However, for all
LY treated groups as well as the groups treated with combinations of LY and bolus plus low-dose CTX, we did not detect any reduction in the number of tumor vessels (Supplementary Figure 1). In fact there appeared to be a slight increase in the number of vessels in these groups compared to controls. Thus in the treated group there was no major impact on the number of vessels. Therefore, metronomic LY does not seem to impact tumor angiogenesis. Quantitative fluorescence imaging of these tumors confirms these treatment effects on vascular density (Supplementary Figure 2). Additionally, treatment with LY alone results in a decrease in large vessels, an increase in small vessels, and an increase in the Vessel Normalization Index (Supplementary Figure 2C). The increase in a normalization phenotype (assessed by a decreased tortuosity, increased pericyte coverage, and decreased hypoxia) suggests that metronomic LY treatment modulates vessel stability and functionality.

**Increased blood flow in orthotopically implanted LM2-4luc+ tumors treated with metronomic dosing of prodrug of gemcitabine LY.**

To evaluate the impact of metronomic LY dosing on intratumoral blood flow, LM2-4luc+ cells were orthotopically implanted into the mammary fat pad of SCID mice. LY treatment (6mg/kg) was started when tumors reached an average size of 250mm$^3$ – and prior to the first administration of LY we measured pre-treatment blood flow in all tumors (by i.v. injection of the contrast agent) – see Fig 4a, and Fig 4b. Blood flow measurements were then taken after one week of LY treatment (by i.v. injection of contrast agent), and a second measurement was taken after three weeks of treatment – see Fig 4b and Fig 4c. We observed that LY treatment caused an *increase* in blood flow in
LM2-4luc+ tumors (compared to vehicle treated controls - see Fig 4c) one week after treatment, and this difference increased three weeks into the treatment schedule (see Movie files 1-4, as well as Fig 4c). Caliper measurements showed that, as expected, LY monotherapy caused the tumors to be growth inhibited compared to controls (Fig 4a). And yet, paradoxically, the smaller, LY-treated tumors, showed equal or greater luciferase luminescence than the control tumors (Fig 4d-e). This observation is consistent with the possibility that the increased intratumoral blood flow in the (smaller) LY-treated tumors produced a more effective delivery of the luciferin substrate.

**Anti-tumor efficacy of LY regimens on an orthotopic human ovarian cancer model in SCID mice.** To confirm the anti-tumor effects of LY 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 LY 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 intraperitoneally into SCID mice, which results in the cells eventually growing intraperitoneally as both solid tumors and ascites. Two weeks after tumor implantation the mice were randomized into four groups which were then treated with LY monotherapy (6mg/kg/day), vehicle control, low-dose CTX, or the combination of LY plus low-dose CTX. Luminescence imaging indicated a significant anti-tumor effect of the LY based regimens, compared to controls (Fig. 5a). Furthermore, the resulting survival curve showed that LY monotherapy increased the survival of mice compared to controls (Fig 5b). We also noted that the combination of low-dose CTX plus LY did not increase survival beyond that observed
with LY alone. This ovarian model consists principally of ascites (i.e. a tumor cell suspension in the peritoneal cavity), and because 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 indicate that while the growth of solid tumors treated with metronomic LY may not correlate with luminescence data (i.e. Fig. 4d-e), such a discrepancy is not evident in models where tumors grow as malignant ascites.
Discussion

Metronomic chemotherapy is emerging as a potentially important new therapeutic strategy for the treatment of a variety of solid tumors (6-8). 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), i.e., the most effective biological 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 non-toxic fashion, at doses that have little or no significant impact on CEP numbers (although a comprehensive analysis of the impact of LY on CEPs was not carried out, and is beyond the scope of this study). We also show that non-toxic 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 LY which was assessed by us to be in the range of 6-8mg/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 anti-tumor (and anti-angiogenic) 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, since metronomic or metronomic-like chemotherapy in some cases has been shown to be effective against tumors which have acquired resistance to MTD chemotherapy of the same drug(1, 34), it is conceivable that metronomic gemcitabine could prove effective in situations where 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 – where 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 three 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, i.e. 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 anti-tumor effects that result from
metronomic chemotherapy (e.g., by enhancing the intra-tumoral 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 LY (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 two classes of drugs that differ by their mode of action. For example, in this study we tested one such combination – using metronomic CTX and LY which have 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 intra-tumoral 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 intra-tumoral delivery and distribution of co-administered chemotherapy(37, 38). In this regard a number of other investigators have recently reported circumstances where metronomic chemotherapy using drugs such as cyclophosphamide or gemcitabine can induce vessel normalization and increase perfusion and transiently decrease levels of tumor hypoxia(39-41). As such this could add another therapeutic dimension to the multiple anti-tumor mechanisms of action associated with metronomic chemotherapy(5). Future studies will have to determine if 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 biological 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.

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References:


Figure Legends

**Fig 1.** Impact of the prodrug of gemcitabine LY2334737 (LY) or Gemcitabine HCl administration on mouse weights as well as CEP and White Blood Cell (WBC) counts in female Balb/c mice. Gemcitabine (Gem) was given i.p. (at doses of 40, 70, 120 or 160mg/kg) every three days (starting on day 1, then on days 4, 7 etc) up to day 25 when the last dose was administered. LY was given daily by gavage (at doses of 2, 4 and 6mg/Kg), and for all groups mice were bled on day 28.  

a) Chemical structure of LY2334737, Gemcitabine, and cyclophosphamide.  
b) No significant weight loss was observed in all groups throughout the treatment period.  
c) Viable CEP (top) and WBC (bottom) count for mice treated with the indicated doses of LY or Gemcitabine (Gem), showing no significant impact of LY 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 LY treated groups; the 40mg/kg Gem i.p treated group was significantly lower than controls, p<0.01).

**Fig 2a.** Empirical determination of maximum tolerated doses of LY as given by daily administration by gavage. Balb/c mice were treated for 7 days with the indicated levels of LY (administered daily), or 160mg/kg Gemcitabine HCl (Gem) administered i.p on days 1 and 4.  
a) Severe weight loss was observed in doses higher than 10mg/kg of LY, indicating the upper limit that can be dosed of LY 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 LY at 6-8mg/kg/day did not significantly
impact CEP numbers. A reduction in CEP numbers was evident at higher doses (e.g., 15 and 20 mg/kg/day; these were significantly lower, \(p<0.05\), compared to controls, and compared to the 8 mg/kg LY 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 LY treated mice and controls).

**Fig 3a.** Impact of LY based regimens on human breast tumors orthotopically implanted in SCID mice. The human LM2-4luc+ cell line was implanted orthotopically in female SCID mice which were treated with the daily indicated doses of LY either alone or in combination with a cyclophosphamide regimen. Cyclophosphamide was administered as an i.p bolus (100 mg/kg) on day 1 followed by continuous 20 mg/kg/day dosing in the drinking water (B+ld CTX). An additional group was included which received Gemcitabine i.p treatment (160 mg/Kg given every 3 days). a) Tumors treated for two weeks (days 21-35), until controls reached 1500 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 LY (8 mg/Kg) together with the bolus plus low dose CTX treatment.

**Fig 4a.** Tumor growth, intratumoral blood flow analysis, and luminescence analysis of LM2-4luc+ tumors treated with LY based regimens. LM2-4luc+ tumors growing in SCID mice treated with oral prodrug of gemcitabine (LY, 6 mg/kg/day). LM2-4luc+ cells were implanted into SCID mice and LY monotherapy (or vehicle alone control) was initiated
two weeks later (arrow). **a)** LY treatment significantly inhibited tumor growth compared to controls. **b)** Blood flow measurement analysis in LM2-4luc+ tumors. Analysis is presented as Plateau or Slope values. Measurements were taken before treatment initiation, and at one week and at three weeks after treatment commenced. The data show that blood flow increased in LY (6mg/kg/day) treated tumors relative to the controls. Data from one LY treated mouse and one vehicle control mouse are shown. Data are assessed by the plateau value and/or the slope of the curve generated. **c)** Summary of the analysis of intratumoral blood flow data. Showing an increase in blood flow after 1 week, and after 3 weeks of LY treatment, compared to controls. Data is of 5 mice per group, and statistical analysis was done by a 2 way ANOVA with Holm Sidak multiple comparison test. **d-e)** Tumor growth analysis suggested that luminescence did not correlate well with tumor caliper measurements in this model after LY treatment was initiated. We therefore set up another (4d) primary tumor growth curve, showing the impact of LY with a (4e) parallel assessment of luminescence 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 LY treated tumors.

**Fig 5.** Impact of prodrug of gemcitabine (LY) regimens on SKOV3-13 ovarian cancer cells grown in SCID mice. SKOV3-13 cells were injected intraperitoneally and treatment commenced 2 weeks later. **a)** Showing luminescence data for all treatment groups and, **b)** the increased survival of mice treated with LY regimens compared to controls.
Fig 3a

Fig 3b

B+IdCTX = Bolus plus low-dose cyclophosphamide
Perfusion, Week 3 - Treated Vs Control

Pre-contrast injection  Post-contrast injection

4b

Treated (F1-L)  Control (F2-R)

Pre-contrast injection  Post-contrast injection

4c

Round 1 - Wash-in Rate (Slope)
Plateau – measure of blood volume / vascularity

Round 1 - Peak Enhancement (Plateau)
Slope – measure of the rate of blood flow

4d

Tumor volume (mm$^3$)

8mg/kg LY  Control

4e

Luminescence (photons/sec x 10$^5$)

8mg/kg LY  Control
Low-dose metronomic oral dosing of a prodrug of Gemcitabine (LY2334737) causes anti-tumor effects in the absence of inhibition of systemic vasculogenesis

Giulio Francia, Yuval Shaked, Kae Hashimoto, et al.

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