Efficacy of Low-Dose Oral Metronomic Dosing of the Prodrug of Gemcitabine, LY2334737, in Human Tumor Xenografts

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Abstract

LY2334737, an oral prodrug of gemcitabine, is cleaved in vivo, releasing gemcitabine and valproic acid. Oral dosing of mice results in absorption of intact prodrug with slow systemic hydrolysis yielding higher plasma levels of LY2334737 than gemcitabine and prolonged gemcitabine exposure. Antitumor activity was evaluated in human colon and lung tumor xenograft models. The dose response for efficacy was examined using 3 metronomic schedules, once-a-day dosing for 14 doses, every other day for 7 doses, and once a day for 7 doses, 7 days rest, followed by an additional 7 days of once-a-day dosing. These schedules gave significant antitumor activity and were well tolerated. Oral gavage of 6 mg/kg LY2334737 daily for 21 days gave equivalent activity to i.v. 240 mg/kg gemcitabine. HCl administered once a week for 3 weeks to mice bearing a patient mesothelioma tumor PXF 1118 or a non–small cell lung cancer tumor LXFE 937. The LXFE 397 tumor possessed elevated expression of the equilibrative nucleoside transporter-1 (ENT1) important for gemcitabine uptake but not prodrug uptake and responded significantly better to treatment with LY2334737 than gemcitabine (P ≤ 0.001). In 3 colon xenografts, antitumor activity of LY2334737 plus a maximally tolerated dose of capecitabine, an oral prodrug of 5-fluorouracil, was significantly greater than either monotherapy. During treatment, the expression of carboxylesterase 2 (CES2) and concentrative nucleoside transporter-3 was induced in HCT-116 tumors; both are needed for the activity of the prodrugs. Thus, metronomic oral low-dose LY2334737 is efficacious, well tolerated, and easily combined with capcitabine for improved efficacy. Elevated CES2 or ENT1 expression may enhance LY2334737 tumor response. Mol Cancer Ther; 12(4): 1–10. ©2013 AACR.

Introduction

Gemzar (gemcitabine.HCl) is an effective chemotherapeutic agent used in the treatment of several solid tumors. Gemcitabine (2',2'-difluorodeoxycytidine) is taken up by cells via nucleoside transporters and is rapidly catalyzed to phosphorylated metabolites that inhibit DNA synthesis. Gemcitabine-5'-diphosphate causes irreversible inhibition of ribonucleotide reductase thereby reducing deoxyribonucleotide triphosphate pools needed for DNA synthesis; gemcitabine 5'-triphosphate is incorporated directly into DNA inhibiting further chain elongation (1–4). In plasma and liver, gemcitabine is rapidly inactivated by cytidine deaminase (CDA) to 2',2'-difluorodeoxycytidine (dFdU). Clinically, Gemzar is administered 1,000 to 1,250 mg/m² as a standard dose of 30-minute intravenous infusion once a week for 3 to 4 weeks. Clinical trials examined lower drug doses given on a more frequent schedule of every third day for 4 weeks in patients with pancreatic cancer and found that a low-dose 30-minute infusion of 250 mg/m² gemcitabine or 150-minute infusion of 10 mg/m²/minute gemcitabine had comparable or improved survival times with better safety and quality of life than the standard regimen (5, 6). Oral administration would permit gemcitabine to be given on a more frequent basis that may further enhance efficacy. Clinical studies of the oral availability of gemcitabine found low systemic exposure due to rapid first pass metabolism to dFdU and gastrointestinal toxicity (7, 8).

The prodrug of gemcitabine, LY2334737, (Fig. 1) was designed to reduce intestinal activation and first-pass deamination with a stable amide linkage of valproate to gemcitabine (9). Oral LY2334737 is absorbed intact and cleaved slowly resulting in high plasma levels of circulating prodrug and prolonged, lower levels of circulating gemcitabine emulating a slow gemcitabine infusion (9, 10). Recently, carboxylesterase 2 (CES2), expressed at high levels in the liver, kidney, and intestine, was identified as the hydrolase responsible for LY2334737 cleavage (11, 12). In vitro, LY2334737 is cytotoxic to CES2-expressing cells capable of intracellular prodrug hydrolysis and more efficacious in a xenograft model expressing the CES2 transgene (11).
"Metronomic dosing" refers to administration of chemotherapy at frequent intervals (e.g., daily) with low, minimally toxic doses (13). In contrast to intravenous delivery of maximally tolerated doses of chemotherapeutic agents, advantages include: (i) direct, continual tumor growth inhibition and (ii) an indirect effect through inhibition of angiogenesis and vasculogenesis (13–15) or, as shown for LY2334737, increased tumor blood flow thereby potentially enhancing drug delivery (16). When metronomically dosed orally once a day for 14 days in the human colon HCT-116 xenograft model, LY2334737 showed excellent antitumor activity that is equivalent to standard high doses of gemcitabine.HCl typically administered intraperitoneally (9). Orthotopic models of human metastatic breast LM2- and ovarian SK-OV-3 cancers also responded well to LY2334737 treatment (16).

The first agent to show improved clinical efficacy with metronomic dosing was capetabine (Xeloda), an oral prodrug of 5-fluorouracil (5-FU). Conversion of capetabine takes place in 3 enzymatic steps, CES2, CDA, and thymidine phosphorylase (17, 18). Metronomic dosing results in low plasma concentrations of capetabine and its intermediates and simulates a low-dose infusion of 5-FU (18). When administered daily, capetabine efficacy is superior to intravenous 5-FU infusion in the treatment of anthracycline-resistant metastatic breast cancer (19) and is approved for colorectal cancer treatment (20). Recent studies of metronomically dosed capetabine with gemcitabine show comparable or modestly improved survival times and good tolerability (21, 22). The present study examines several prodrug properties. Because gemcitabine is a schedule-dependent antitumor agent, the dose response for LY2334737 efficacy in the HCT-116 xenograft model was evaluated using several schedules (23). Additional efficacy studies were conducted with xenografts derived from established human cell lines or patient tumors. Patient tumors that are maintained in mice retain the original tumor histology and are more predictive of clinical drug response (24, 25). LY2334737 was evaluated as a single agent for the treatment of several human patient-derived lung and colon tumors and in combination with capetabine in colon tumor xenografts. Dual oral prodrug therapy served as an opportunity to metronomically dose both gemcitabine and 5-FU. Expression of genes of interest was assessed to gain insight into possible mechanisms for tumor responses.

Materials and Methods

Materials

Gemcitabine.HCl, LY2334737, and LY2334737 hemi-P-toluene sulfonic acid hemihydrate salt were from Eli Lilly and Company. [3H]LY2334737 was custom synthesized; [3H]cytidine (MT615, 20 Ci/mmol) was from Moravek Biochemical. Capetabine (Xeloda) was purchased from Roche or Capital Wholesale Drug Companies. Sigma-Aldrich supplied valproic acid (VPA) and S-(p-nitrobenzyl)-6-thioinosine (NBMPR). Growth media and HEK Ebna were purchased from Invitrogen and FBS from Hyclone. The cell lines, HCT-116, HT-29, and HL-60 were obtained from American Type Culture Collection. Cell lines and patient-derived tumors (Oncotest; ref. 24) were pathogen tested and genetically authenticated by short tandem repeat analysis. Banked master stocks were returned to within approximately 6 months or if inconsistencies in growth behavior were observed.

Cytotoxicity assays

A detailed protocol was previously published (26). HL-60 cells (5,000–10,000/well) were grown 3 days in RPMI-1640 with 10% FBS. The effect of varying VPA concentrations on HL-60 growth was evaluated in the absence or presence of 10 nmol/L gemcitabine. For metronomic treatment paradigms, cells (2,000/well) were plated overnight in McCoy’s 5A (modified) HEPES medium plus 10% FBS. Two-hour drug pulses were used as: (i) a single treatment on day 1 or (ii) multiple consecutive days or (iii) alternate day treatments. Each pulse was followed by drug-free growth medium wash and replacement. Viability was measured with CellTiter 96 AQueous One Solution (Promega).

Xenograft studies

HCT-116 xenograft studies (11, 27) included treatment with LY2334737 administered by oral gavage either as the free base in a vehicle of 1% sodium carboxymethylcellulose, 0.5% sodium lauryl sulfate, 0.05% antifoam, or as the salt of LY2334737 in a vehicle of 1% sodium carboxymethylcellulose, 0.5% sodium lauryl sulfate, 0.05% antifoam, or as the salt of LY2334737 in a vehicle of 0.1 mol/L Na3P04, pH 6; the dose is reported as the free base. Gemcitabine.HCl was administered intraperitoneally (0.2 mL) every third day for a total of 4 doses. For combination studies, capetabine (reconstituted daily in 40 mmol/L citrate buffer, 5% gum Arabic, or deionized water) was combined with LY2334737 before oral administration so mice received one daily dose (0.2 mL). HT-29 and patient tumor-derived studies were conducted at Oncotest GmbH following a published protocol using NMRI nu/nu mice (Harlan; refs. 24, 28). Data for tumor volumes and body weights were analyzed using a nonparametric test.
were analyzed by 2-way ANOVA statistics (27). Drug treatments were considered "well tolerated" if mean body weight loss of the treatment group was 15% or less and was not significantly lower than the vehicle control. In certain studies, tumors were collected and snap frozen at the end of study for transcript analysis. For HCT-116 study, tumors were also obtained 24 hours after the 7th or 14th drug doses.

RNA isolation
RNA was isolated from homogenized xenograft tumors using the Trizol reagent (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen) with on-column DNAseI. RNA yield and purity was determined by A260/A280. First-strand cDNA synthesis used 2 μg RNA and Superscript III reverse transcriptase with Oligo (dT) primers.

Absolute quantitative real-time PCR
Cycling conditions and standard curve methods were used for the ABI7900 HT Instrument (Applied Biosystems) were used. Amplification mixtures contained 25 ng template cDNA and 300 nmol Invented Gene Specific 20 × Assay-on-Demand primers, for CES2 (Hs01077945_m1), concentrative nucleoside transporter (CNT) 1 (CNT1, Hs00984403_m1), CNT3 (Hs00223220_m1), equilibrative nucleoside transporter (ENT) 1 (ENT1, SLC29A1, Hs01085706-m1), ENT2 (SLC29A2, Hs00155426-m1), thymidine phosphorylate (Hs00157317-m1), ribonucleotide reductase subunit M1 (RRM1; Hs00168784_m1), and glyceralddehyde-3-phosphate dehydrogenase (GAPDH; 4326317E; Applied Biosystems). Quadruplicate measurements were made on 3 tumors per group, normalized to GAPDH, and analyzed by one-way ANOVA.

Uptake studies
Following several washes with sodium-free choline chloride-buffered Earle’s balanced salt solution (CBSS), transport of [3H]cytidine or [3H]LY2334737 was measured with Microscint40 and TopCount scintillation counter (Perkin Elmer) and normalized to protein in CBSS by confluent HEK 293 Ebna cells at room temperature in the absence or presence of the ENT1 inhibitor NBMPR (100 nmol/L; ref. 30). Uptake was initiated by the addition of substrate (2–1,000 mol/L) and terminated at 1, 3, 5 d pulse 104.5, 1, 3, d pulse 108.3, every other day 23.8a, b, 10.8, HT-29 colon cell line 23.8a, b, 10.8, HT-29 colon cell line 23.8a, b, 10.8, HT-29 colon cell line

Results

In vitro metronomic dosing
HT-29 and HCT-116 were used to determine whether frequent treatments altered the dose response to gemcitabine. Treatment paradigms modeled dosing regimens used in subsequent xenograft studies and included: (i) a 2-hour drug pulse given only on day 1 to mimic an intravenous bolus treatment, (ii) a 2-hour pulse on consecutive days to mimic once-a-day dosing, or (iii) a 2-hour pulse on alternate days to model every-other-day dosing. The largest EC50 value (Table 1) was observed when cells received a single-drug pulse on day 1; values decreased with each successive daily pulse. When compared with a single exposure, 4 days of sequential treatments enhanced potency 9.5- and 10.8-fold. A 2-hour treatment every other day for 2 or 3 exposures also enhanced drug potency 3.2- to 3.9-fold for HCT-116 and 4.7- to 5.2-fold for HT-29 cells. Potency of gemcitabine was enhanced with metronomic dosing in vitro and was schedule dependent.

Table 1. Cell growth inhibition of 2-hour pulse gemcitabine treatments on consecutive days or every other day

<table>
<thead>
<tr>
<th>Treatment schedule</th>
<th>EC50 [nmol/L]</th>
<th>Potency (-fold)</th>
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</thead>
<tbody>
<tr>
<td>HCT-116 colon cell line</td>
<td></td>
<td></td>
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<tr>
<td>Consecutive days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d pulse</td>
<td>198.8 ± 35.0a</td>
<td>1.0</td>
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<tr>
<td>2 d pulse</td>
<td>58.4 ± 9.1a</td>
<td>3.6</td>
</tr>
<tr>
<td>3 d pulse</td>
<td>31.3 ± 5.6a</td>
<td>7.0</td>
</tr>
<tr>
<td>4 d pulse</td>
<td>21.2 ± 2.1a,b,c,d</td>
<td>9.5</td>
</tr>
<tr>
<td>Every other day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 3 d pulse</td>
<td>67.5 ± 10.9a</td>
<td>3.2</td>
</tr>
<tr>
<td>1, 3, 5 d pulse</td>
<td>56.7 ± 11.6a</td>
<td>3.9</td>
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<tr>
<td>HT-29 colon cell line</td>
<td></td>
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</tr>
<tr>
<td>Consecutive days</td>
<td></td>
<td></td>
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<tr>
<td>1 d pulse</td>
<td>445.2 ± 55.6a</td>
<td>1.0</td>
</tr>
<tr>
<td>2 d pulse</td>
<td>163.9 ± 67.9a</td>
<td>2.6</td>
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<tr>
<td>3 d pulse</td>
<td>80.9 ± 23.7a</td>
<td>6.2</td>
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<tr>
<td>4 d pulse</td>
<td>53.7 ± 23.8a,b,c</td>
<td>10.8</td>
</tr>
<tr>
<td>Every other day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 3 d pulse</td>
<td>108.3 ± 33.8a</td>
<td>4.7</td>
</tr>
<tr>
<td>1, 3, 5 d pulse</td>
<td>104.5 ± 32.3a</td>
<td>5.2</td>
</tr>
</tbody>
</table>

NOTE: Cells were grown for 6 days with varying gemcitabine concentrations with different exposures. A 2-hour drug pulse was given on sequential days for 1, 2, 3, or 4 total treatments or every other day (days 1 and 3 or days 1, 3, and 5). EC50 values are the mean ± SEM of 3 independent experiments each with 2 or 3 replicates. The potency of each treatment is relative to the 1-day treatment. Significance was determined by 2-way ANOVA as P ≤0.05 with a 2-fold or more difference in mean EC50 values.

- aSignificantly different from the single (1 d pulse) treatment.
- bSignificantly different from the 2-day (2 d pulse) treatment.
- cSignificantly different from the 1- and 3-day (1, 3 d pulse) treatment.
- dSignificantly different from the 1-, 3-, and 5-day (1, 3, 5 d pulse) treatment.
intact and shows rate-limited formation of gemcitabine and prolonged gemcitabine exposure (9). Therefore, different treatment regimens were used to evaluate the efficacy of the prodrug as a single agent. The antitumor activity of LY2334737 in the HCT-116 xenograft model was evaluated when dosed once a day for 14 doses; a preliminary summary was published (9). As shown in Fig. 2A, this schedule for LY2334737 had significant antitumor activity at 3.77 mg/kg (P ≤ 0.01) and 7.55 mg/kg LY2334737 (P ≤ 0.001); 1.89 mg/kg was not significantly different from vehicle. The 7.55 mg/kg prodrug dose was as efficacious (67% maximal tumor growth inhibition on day 26) as gemcitabine.HCl administered intraperitoneally (i.p.) at 160 mg/kg every 3 days for 4 doses. There was no significant weight loss (Supplementary Fig. S1) although one animal died in the 7.55 mg/kg dose group. A second study also showed statistically significant tumor suppression by LY2334737 at doses of 6.5 and 5.2 mg/kg, but not at a lower dose level of 3.25 mg/kg LY2334737 (data not shown). A 14% maximum weight loss was observed in the 6.5 mg/kg treatment group. A clear dose–response effect was observed in these studies, and LY2334737 was generally well tolerated (Supplementary Fig. S1). Subsequently, alternative schedules were evaluated that included rest periods between dosing. A schedule of every-other-day dosing for 7 treatments was examined (Fig. 2B). Doses of 6.5 mg/kg (P ≤ 0.05) and 13 mg/kg LY2334737 (P ≤ 0.01) gave significant overall tumor growth inhibition. No deaths and no significant body weight loss occurred at these doses (Supplementary Fig. S1). However, the highest dose of 26 mg/kg was toxic and terminated early.

Figure 2. Efficacy with 3 treatment schedules for metronomic oral dosing of LY2334737 in the human colon HCT-116 xenograft model. Data are the mean ± SEM. Symbols denote statistically significant inhibition: *, P < 0.05; **, P < 0.01; ***, P < 0.001. A, dose response of once-a-day oral administration of LY2334737 for 14 days. Mice (10/group) were dosed by oral gavage with vehicle (~~), 1.89 mg/kg (O), 3.77 mg/kg [ ], or 7.55 mg/kg LY2334737 (~) or 160 mg/kg gemcitabine HCl (ii) i.p. on a schedule of once every 3 days for 4 doses. A summary table of this study was reported (9). B, dose response of every-other-day dosing for 7 doses. Mice (9/group) were treated with vehicle (~~), 6.5 mg/kg (O), 13 mg/kg [ ], or 26 mg/kg LY2334737 (~). Maximum tumor inhibition was 48% on day 37 in the 13 mg/kg group. C, dose response of once-a-day dosing for one week, followed by a week of rest, then once-a-day dosing for the next week for a total of 14 doses. Mice (8/group) were treated with vehicle (~~), 6.0 mg/kg (O), 7.2 mg/kg [ ], or 10.5 mg/kg LY2334737 (terminated early, data not visible). Maximum tumor inhibition was 64% in the 6 mg/kg group.
Another schedule examined alternate weeks of dosing (Fig. 2C). Mice were dosed once-a-day for 7 days, followed by a 1 week rest period, and 7 additional once-a-day doses. The 2 LY2334737 doses (6.0 mg/kg and 7.2 mg/kg) gave overall statistically significant reduction in tumor growth (P \leq 0.001) relative to the vehicle; the 10.5 mg/kg treatment was toxic and terminated early. Body weight loss was observed in both the vehicle and LY2334737 treatment groups during the first week of treatment, but mice recovered during the treatment break and showed no significant weight loss during the second treatment week. One death occurred in each drug treatment group on this schedule. Thus as a single agent, LY2334737 was efficacious and well tolerated on several metronomic schedules.

Efficacy in human lung tumor xenograft models

Gemzar is registered for the treatment of non–small cell lung cancer (NSCLC). Consequently, studies were conducted comparing LY2334737 and gemcitabine in xenografts of patient-derived lung tumors. Mice were treated by oral gavage daily for 21 days with either vehicle or 6.0 mg/kg LY2334737, or treated intravenously once a week for 3 weeks with 240 mg/kg (or in 1 study 160 mg/kg) gemcitabine.HCl (Fig. 3). Both LY2334737 and gemcitabine gave significant tumor growth inhibition (P \leq 0.001) in mice bearing the human pleural mesothelioma tumor PXF 1118 or the human squamous NSCLC tumor LXFE 937 (Fig. 3A and B) and drug efficacy did not significantly differ. In contrast, LY2334737 antitumor activity was not significantly different from the vehicle treatment (P > 0.05)
in the LXFE 1422 xenograft model (Fig. 3C), whereas gemcitabine·HCl (160 mg/kg) showed strong antitumor activity. In the third NSCLC tumor LXFE 397 model (Fig. 3D), LY2334737 and gemcitabine·HCl had equivalent tumor growth inhibition of approximately 65% during the 21-day treatment period; however, significantly greater tumor growth inhibition was seen with the prodrug relative to gemcitabine·HCl (P ≤ 0.001) during the posttreatment period. Across all 4 studies, weight loss was less than 6% and only 2 deaths occurred, one in the LY2334737-treated LXFE 937 group and one in vehicle-treated LXFE 1422 animal. Thus, metronomic low-dose oral LY2334737 was well tolerated, and efficacy was dependent on the individual tumor being treated.

Gene expression in NSCLC tumors
To gain insight into response mechanisms, expression of enzymes, and/or transporters important for gemcitabine activity and prodrug cleavage (11) was examined (Fig. 4). Transcripts for genes of interest CES2, RRM1, and the gemcitabine transporters, ENT1, ENT2, CNT1, and CNT3 were measured in tumors from vehicle-treated mice. LXFE 937 expressed significantly higher CES2 levels than the other tumors (P ≤ 0.001) and LXFE 397 expressed higher transcript levels of RRM1 (P ≤ 0.001), important in gemcitabine resistance (29–31), and ENT1 (P ≤ 0.001), a high-affinity gemcitabine transporter. The other transcripts did not differ (data not shown); CNT1 was not detected. Thus, expression differed significantly in these tumors for RRM1, ENT1, and CES2 important for gemcitabine and/or LY2334737 responsiveness.

Efficacy of LY2334737 combined with capecitabine
Both LY2334737 and capecitabine are oral metronomically dosed prodrugs and were evaluated for benefits of dual therapy in colon cancer xenografts (Fig. 5). Mice bearing HCT-116 tumors were treated once a day for 14 days with vehicle, 4 mg/kg LY2334737, the maximum tolerated dose (MTD) of 175 mg/kg capecitabine, or both together. As shown in Fig. 5A, monotherapy of LY2334737 or capecitabine, each gave significant overall tumor inhibition (P ≤ 0.001 and P ≤ 0.05, respectively) with maximum tumor growth inhibition of 50% to 55% at the end of the study compared with vehicle control group. Dual therapy gave significantly greater tumor growth inhibition than treatment with either monotherapy (P ≤ 0.01) with a maximum inhibition of 77% by the end of the study. The treatment was well tolerated and no animal deaths occurred (Supplementary Fig. S2). To determine which genes might be responsible for the improved response, transcript levels of genes of interest were measured on tumor samples removed at 2 different treatment times (Fig. 5B). Significant CES2 mRNA induction was observed 24 hours after the seventh day dose of LY2334737 and also 24 hours after both seventh and fourteenth day doses of combination therapy (P ≤ 0.05). In addition, induction of the nucleoside transporter CNT3 transcript was seen with LY2334737 and dual treatments after 1 week and with capecitabine after 2 weeks. No changes were detected in the transcripts of ENT1, ENT2, or thymidine phosphorylase (data not shown).

Combination treatment was also tested in human colon xenograft models HT-29 and patient-derived C676 676 tumors (Fig. 5C and D). Mice were treated by oral gavage once a day for 14 days with 150 mg/kg capecitabine (MTD), 6 mg/kg LY2334737, or dual therapy. Treatment with these monotherapies (Fig. 5C) gave significant antitumor activity in mice bearing HT-29 (P ≤ 0.001) compared with the vehicle and LY2334737 had greater antitumor activity than capecitabine (P ≤ 0.001). Overall tumor inhibition with dual therapy was significantly greater than capecitabine alone (P ≤ 0.001) or LY2334737 alone (P ≤ 0.05). The C676 tumor xenograft (Fig. 5D) showed the same response pattern. Overall tumor inhibition was significantly greater with monotherapy therapies of LY2334737 (P ≤ 0.01) or capecitabine (P ≤ 0.001) than the vehicle; dual therapy was significantly better than either monotherapy (P ≤ 0.01). No deaths and no significant weight loss occurred in the HT-29 or C676 676 studies (Supplementary Fig. S2). Taken together, these xenograft studies indicate that combination therapy was generally well tolerated and provided greater antitumor activity than either drug alone in colon cancer models.

Valproic acid
VPA has antitumor activity and inhibits several histone deacetylases (HDAC) 1 through 7 and 10 at concentrations of 0.5 mmol/L or more (32). VPA released by LY2334737 cleavage could be partially responsible for activity observed in vivo. Cytotoxicity assays were conducted with HL-60, a VPA-sensitive cell line, and varying VPA concentrations alone or in combination with a minimally cytotoxic gemcitabine concentration (10 nmol/L; ref. 33). VPA was cytotoxic at concentrations of 1 mmol/L and potentiated gemcitabine activity when present at 1,000-fold higher concentration (0.1 mmol/L; data not shown). Because LY2334737 cleavage releases VPA and gemcitabine in a one-to-one stoichiometric ratio, sufficient VPA
levels would not be present in vivo for synergy with gemcitabine.

**ENT1**

To determine whether the prodrug is a substrate of the sodium-independent ENT1 transporter, uptake studies were conducted over a wide concentration range (2–1,000 μmol/L). Transport of [3H]LY2334737 was measured in sodium-free buffer in the presence or absence of a potent ENT1 inhibitor, NBMPR, and compared with the uptake of cytidine, a well-characterized ENT1 substrate. The kinetic parameters for [3H]cytidine uptake were: V\text{max} of 613 ± 308 pmol/min/mg protein and K\text{m} of 125 ± 32 μmol/L, close to the published value (34). In contrast, LY2334737 uptake was uninhibitable and nonsaturable consistent with simple diffusion (data not shown).

**Discussion**

The oral prodrug of gemcitabine, LY2334737, was developed to permit more frequent dosing that may enhance efficacy (9). Preclinical and clinical studies comparing standard high-dose short-duration gemcitabine infusions with low-dose long-administrations show similar or improved responses to the latter schedule with similar toxicity (5, 6, 35, 36). To evaluate the effect of metronomic dosing on gemcitabine potency, in vitro cytotoxicity studies treating cells with a 2-hour exposure once-

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**Figure 5.** Efficacy of LY2334737, capecitabine and dual therapy in human colon cancer xenograft models and gene expression in HCT-116 tumors. All therapies were given once a day for 14 days by oral gavage. Group comparisons were made to the vehicle (○); capecitabine (■); and LY2334737 (●); significant inhibition: *, P < 0.05; **, P < 0.01; ***, P < 0.001. A, HCT-116 xenograft model. Mice (8/group) were treated with vehicle (○), 4 mg/kg LY2334737 (●), 175 mg/kg capecitabine (MTD, O), or both (■). The combination treatment resulted in a greater tumor volume reduction than either monotherapy (P < 0.01). B, HCT-116 tumor transcript expression. Tumors were removed from 3 mice per group 24 hours after the 7th and the 14th doses as described in Materials and Methods. Values are the mean ± SEM. Symbols: *, significantly different from vehicle group (P < 0.05). C, HT-29 xenograft model. Mice (7/group) were treated with vehicle (○), 4 mg/kg LY2334737 (●), 150 mg/kg capecitabine (MTD, o), or dual therapy (■). Maximum tumor inhibition was 89% for LY2334737, 68% for capecitabine, and 93% for dual therapy. D, efficacy in patient-derived colon tumor CXF 676 xenograft. Mice (7/group) were treated with vehicle (○), 4 mg/kg LY2334737 (●), 150 mg/kg capecitabine (MTD, o), or dual therapy (■). Maximum tumor inhibition was 51% for LY2334737, 60% for capecitabine, and 78% for dual therapy.
a-day for 3 to 4 days were compared with a single 2-hour exposure, simulating daily metronomic dosing and a bolus infusion, respectively. Gemcitabine activity was enhanced by approximately 10-fold by metronomic dosing daily compared with a single drug exposure and was also enhanced 3- to 5-fold by every other-day dosing. Thus, more frequent gemcitabine exposure would be expected to reduce the drug dose required for efficacy. Prodrug hydrolysis also releases VPA, a weak HDAC inhibitor that possesses anticancer activity. Sufficient VPA concentrations (in the mmol/L range) necessary for synergistic antitumor activity, however, are not liberated during LY2334737 treatment (32). Therefore, the efficacy of the prodrug is due to more frequent dosing and prolonged exposure and unrelated to the release of VPA.

After oral administration to mice, the prodrug is rapidly absorbed intact and the systemic LY2334737 concentration is approximately twice that of gemcitabine (9). The resulting half-life of the prodrug is approximately 1.1 hours extending the half-life of gemcitabine to 1.2 hours, nearly 4 times longer than intravenous gemcitabine (~0.3 hour; ref. 37). Thus, release of gemcitabine following hepatic first-pass prodrug hydrolysis is rate limited, resulting in prolonged gemcitabine exposure that may further augment the benefits of metronomic dosing of the prodrug.

Gemcitabine activity is schedule dependent and therefore the schedule dependency of metronomically dosed LY2334737 was evaluated in vivo. Three metronomic dosing schedules were examined in the HCT-116 xenograft model: (i) once-a-day dosing for 14 days, (ii) every other day for 7 doses, or (iii) once a day for 7 days, 1 week of rest, and once-a-day dosing for another 7 days and delivered similar total doses of LY2334737 (91–106 mg/kg). All were efficacious, generally well tolerated, and showed that LY2334737 exhibits good and similar antitumor activity on several schedules. The greatest margin of safety for the single agent was 2 and was obtained on the every-other-day schedule that permitted a day of rest between treatments.

Carboxylesterases, CES1 and CES2, are responsible for the activation of 2 anticancer prodrugs, capecitabine and irinotecan (38, 39). LY2334737 is cleaved only by the CES2 isozyme (11). Certain tumor types express elevated CES2 levels; these include colon tumors, NSCLC, pleural mesothelioma tumors, hepatic, renal, and ovarian cancers (40–42). We chose 3 different tumor types, NSCLC, mesothelioma, and colon to evaluate the prodrug efficacy in xenograft models.

Gemzar is registered for the treatment of NSCLC, which has 3 subtypes, adenocarcinomas, squamous carcinomas, and large cell cancers. The histology of the tumor has been successfully used to tailor chemotherapy (43). Only squamous NSCLC carcinoma showed elevated CES2 expression that might be useful for tailoring prodrug treatment (42). Thus, we compared the response of xenograft models bearing squamous NSCLC patient-derived tumors to treatment with oral LY2334737 (6 mg/kg, once a day for 21 days) and intravenous gemcitabine.HCl (160 or 200 mg/kg, once a week for 3 weeks), a schedule used clinically. The total gemcitabine dose administered differed considerably between these 2 regimens: LY2334737 released 57 mg/kg of gemcitabine, which is approximately 10% of the total dose of circulating gemcitabine delivered by gemcitabine.HCl (421 or 632 mg/kg). The xenografts exhibited different treatment response patterns and had differential expression of genes responsible for drug uptake and metabolism: (i) one of the tumors, LXFE 1422, responded well to gemcitabine.HCl but not LY2334737. A possible mechanism for this lack of prodrug response was not identified; (ii) the LXFE 937 xenograft exhibited equivalent, strong responses to both the prodrug and gemcitabine. This tumor had high CES2 expression, which was similar to the high levels seen in CES2 transfectants that exhibited an enhanced tumor response to LY2334737 treatment (11). The efficacy of the prodrug was likely enhanced by intracellular hydrolysis and continued localized release of gemcitabine; and (iii) the most interesting response was observed with LXFE 397 where efficacy was significantly greater with LY2334737 treatment. The expression of ENT1 of this tumor was elevated compared with the other 2 tumors. ENT1 is a key transporter responsible for the uptake of gemcitabine but not LY2334737, and is a biomarker for the response of patients with pancreatic cancer to Gemzar treatment (44–46). Low-dose LY2334737 treatment provided only approximately 10% of the total gemcitabine dose delivered by the gemcitabine.HCl therapy; however, elevated tumor expression of the high-affinity ENT1 transporter would enhance gemcitabine uptake, thereby arresting more cells progressing through S-phase due to prolonged systemic gemcitabine exposure. Thus, NSCLC tumors with elevated ENT1 and/or elevated CES2 expression may respond better to metronomic dosing of LY2334737 (11). Improved treatment outcomes may be obtained by patient tailoring.

The oral prodrugs LY2334737 and capecitabine were tested alone and in combination. Previous xenograft studies showed that HCT-116 and HT-29 tumors are capecitabine responsive and express moderate levels of thymidine phosphorylase necessary for the last step of conversion of capecitabine to 5-FU (47). In the present study, dual oral therapy of metronomically dosed LY2334737 and a maximally tolerated dose of capecitabine in 3 human colon xenografts was well tolerated and significantly more efficacious than either monotherapy. To gain insight into the potential mechanisms for enhanced activity, transcripts of genes of interest were examined in tumors removed during treatment. Two transcripts, CES2 and CNT3, were significantly upregulated during dual therapy. In addition to LY2334737 hydrolysis, CES2 catalyzes the first step in the metabolism of capecitabine and therefore is essential for the activation
of both prodrugs (11, 17). CES2 was induced significantly, 2- to 3-fold, by LY2334737 after 7 days of treatment, and by dual therapy after 7 and 14 days (P ≤ 0.05). CNT1 and CNT3 transport gemcitabine and capcitabine’s metabolite 5’-deoxy-5-fluorouridine and are important for cellular drug response (17, 47). Significant CNT3 induction was also observed at the early 7-day timepoint with LY2334737 and dual therapy, and later with capcitabine (P ≤ 0.05). ENT1 expression remained unchanged despite earlier reports of upregulation by treatment with thymidylylate synthase inhibitors (48, 49). Upregulation of thymidine phosphorylase seen in gemcitabine-treated breast MX-1 tumors was not observed in HCT-116 tumors (50). Thus, induction of CNT3 and CES2 may be responsible, in part, for the improved colon tumor responses obtained with dual therapy.

These studies show that the oral prodrug LY2334737 can be administered by metronomic dosing on more than one schedule. As a single agent, low-dose LY2334737 was efficacious and well tolerated in several cell line- and patient-derived human tumor xenograft models. In xenograft models bearing patient-derived NSCLC tumors, growth inhibition by LY2334737 treatment was enhanced compared with gemcitabine treatment for tumors with elevated levels of ENT1 or CES2. After oral prodrug dosing, tumor gemcitabine exposure would be expected to be enhanced by both ENT1 and CES2 due to the high systemic plasma level of intact prodrug and the long exposure time of gemcitabine. ENT1 can facilitate increased tumor cellular gemcitabine uptake, and CES2 permits intratumoral prodrug hydrolysis to gemcitabine. In human colon xenografts, dual oral therapy of the prodrug with a maximally tolerated dose of capcitabine was well tolerated and significantly more efficacious than monotherapies, and may have resulted from induction of CNT3 and CES2 expression in the colon tumor. Interestingly, elevated CES2 levels were seen in both NSCLC and colon tumor types that had improved treatment responses with the prodrug relative to gemcitabine treatment. This is consistent with an earlier study where xenografts bearing tumors expressing the CES2 transgene had significantly greater tumor growth inhibition than non-CES2 expressing tumors with LY2334737 treatment (11). Taken together, these studies indicate that low-dose metronomic dosing of the oral prodrug of gemcitabine LY2334737 is efficacious, well tolerated, and easily combined with capcitabine. Patient tailoring may further enhance tumor response.

Disclosure of Potential Conflicts of Interest

J. Starling is employed as a chief scientific officer in Eli Lilly and Co. E.J. Perkins has ownership interest (including patents) in Eli Lilly and Co. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: S.E. Pratt

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.E. Pratt, S. Durland-Busbee, R.L. Shepard, G.P. Donoho, E.J. Perkins

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Efficacy of Low-Dose Oral Metronomic Dosing of the Prodrug of Gemcitabine, LY2334737, in Human Tumor Xenografts

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