Human Carboxylesterase-2 Hydrolyzes the Prodrug of Gemcitabine (LY2334737) and Confers Prodrug Sensitivity to Cancer Cells

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Abstract

Purpose: The oral prodrug of gemcitabine LY2334737 is cleaved systemically to gemcitabine; the mechanism responsible for hydrolysis is unknown. LY2334737 cytotoxicity was tested in the NCI-60 panel; mining of microarray expression data identified carboxylesterase (CES) as a top hydrolase candidate. Studies examined whether CES is responsible for hydrolysis and whether cellular CES expression confers prodrug sensitivity.

Experimental Design: Human recombinant CES isozymes were assayed for LY2334737 hydrolysis. Stable CES-overexpressing HCT-116 transfectants and a SK-OV-3 knockdown were prepared. Cell lines were tested for drug sensitivity and CES expression by quantitative real time-PCR (qRT-PCR), Western blotting, and immunohistochemical staining. Bystander cytotoxicity studies were conducted with GFP-tagged PC-3 cells as the reporter cell line. Therapeutic response of the HCT-116 transfectants was evaluated in xenografts.

Results: Of 3 human CES isozymes tested, only CES2 hydrolyzed LY2334737. Five cell lines that express CES2 responded to LY2334737 treatment. LY2334737 was less cytotoxic to a SK-OV-3 CES2 knockdown than parental cells. The drug response of CES2-transfected HCT-116 cells correlated with CES2 expression level. Bystander studies showed statistically greater PC-3–GFP growth inhibition by LY2334737 when cells were cocultured with CES2 and not mock transfectants. Oral treatment of xenograft models with 3.2 mg/kg LY2334737 once a day for 21 days showed greater tumor growth inhibition of CES2 transfectant than the mock transfectant ($P \leq 0.001$).

Conclusions: CES2 is responsible for the slow hydrolysis of LY2334737. Because intact prodrug circulates at high plasma levels after oral LY2334737 administration, improved response rates may be observed by tailoring LY2334737 treatment to patients with CES2 tumor expression. *Clin Cancer Res; 19(5); 1159–68. ©2012 AACR.*

Introduction

Gemzar (gemcitabine hydrochloride) is a nucleoside anticancer agent registered for the treatment of pancreatic, non–small cell lung, breast, and ovarian cancers (Fig. 1). This nucleoside analog is sequentially phosphorylated to mono-, di-, and triphosphates, which ultimately block DNA synthesis (1). Gemcitabine is rapidly deaminated by cytidine deaminase (CDA), prevalent in plasma and liver, resulting in 2′-deoxy-2′,2′-difluorouridine (dFdU), which is noncytotoxic (2). A prodrug of gemcitabine, LY2334737, was prepared that alters the properties of gemcitabine to allow oral administration and improved stability (Fig. 1). The new chemical entity LY2334737 has an amide-linked valproate that blocks deamination by CDA and is noncytotoxic *in vitro* (3). LY2334737 is orally absorbed intact and is hydrolyzed by slow systemic cleavage resulting in prolonged gemcitabine exposure (4). Oral administration permits frequent low dosing, on a "metronomic schedule" that may enhance antitumor efficacy (3, 5). Because the mechanism of prodrug activation was unknown, studies were conducted to elucidate the enzyme responsible for prodrug cleavage.

Cancer cells display varied responses to anticancer drugs and exhibit diverse gene expression as shown with the NCI-60 human cell line panel (6–8). We wondered whether some cells can hydrolyze the prodrug to gemcitabine and consequently respond to the prodrug *in vitro*. Gemcitabine inhibits the growth of the majority of cell lines in the NCI-60 panel and therefore was a good test system to evaluate prodrug response. Screening of LY2334737 in the panel

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Translational Relevance

LY2334737 is an oral prodrug of the clinically efficacious anticancer agent, gemcitabine. Phase I trials of orally dosed LY2334737 showed higher levels of circulating prodrug than gemcitabine in plasma consistent with slow systemic hydrolysis and prolonged gemcitabine exposure. The slow hydrolysis of LY2334737 to gemcitabine is mediated by human carboxylesterase 2 (CES2) expressed at high levels in the liver and the gastrointestinal tract. CES2 expression in cancer cells confers sensitivity to LY2334737 via slow intracellular cleavage to gemcitabine that arrests division as cells traverse the cell cycle. CES2-expressing tumors may respond to oral metronomic dosing of LY2334737 as gemcitabine that is generated from both systemic and intracellular cleavage, thereby further enhancing efficacy. CES2 expression may be a useful tumor biomarker for patient stratification, identifying patients most likely to benefit from LY2334737 treatment.

identified only a few cell lines that showed growth inhibition at concentrations comparable with that of gemcitabine. Subsequent mining of the publicly available gene expression data for the NCI-60 cells revealed carboxylesterase (CES)1 as a top hydrolase candidate.

CESs are members of a family of α/β-fold serine hydrolases that are important in phase I metabolism (9). They mediate the detoxification of xenobiotics and drug metabolism of compounds with ester, thioester, and amide linkages (10) and are known to hydrolyze several drugs including 2 anticancer agents, irinotecan (CPT-11) and capecitabine (11, 12). Humans have 3 CES isozymes that are microsomal membrane-bound and exhibit differential tissue distribution and substrate specificity (12–14). CES1 is highly expressed in the liver, kidney, lung, brain, and macrophages and considerably reduced expression in the

gastrointestinal tract, whereas CES2 is present predominantly in the intestine, kidney, and liver (13). CES3 is expressed in trachea, intestine, and placenta (14, 15). The first 2 isozymes have known polymorphisms that can affect their hydrolytic activity (15–18).

The present study investigates the potential role of CESs in the cleavage of LY2334737 to gemcitabine. Hydrolysis studies with human recombinant CESs identified CES2 as a LY2334737 hydrolase. Subsequently, CES2 expression in several cancer cell lines, HCT-116 transfectants, and a stable SK-OV-3 knockdown was correlated with prodrug antiproliferative response. Moreover, *in vivo* studies comparing the efficacy of LY2334737 treatment of xenografts of mock with CES2 transfectants showed an enhanced growth inhibition of tumors expressing CES2. Taken together, these studies identify CES2 as a hydrolase of LY2334737 and as a potential biomarker for improved tumor response to LY2334737 treatment.

Materials and Methods

Chemicals and supplies

Gemcitabine hydrochloride, LY2334737, and a hemi-P-toluenesulfonic acid hemihydrate salt of LY2334737 were prepared by Eli Lilly and Company (3). [^{3}H] LY2334737 (21 Ci/mmol; purity of 97%) was custom synthesized by GE (Amersham). Perkin Elmer supplied [^{3}H]naltrindole (20–40 Ci/mmol). Merck KGaA supplied tetrahydrouridine (THU). Sigma-Aldrich supplied puromycin, protamine sulfate, loperamide, and 4-methylumbelliferyl butyrate (4-MUB). Dr. Steven Howell (University of California, San Diego, CA) provided the 2008 ovarian cell line; American Type Culture Collection provided the others. All cell culture reagents, Hank's balanced salt solution (HBSS), optiMEM, and Superscript III First-Strand cDNA Synthesis Kit were purchased from Invitrogen. TaqMan reagents were purchased from Applied Biosciences. Monoclonal antibodies to CES1 and CES2 were from Santa Cruz Biotechnology and Novus Biologicals. BD Gentest supplied human liver S9.

Cytotoxicity testing

Cells (2,000–10,000/well) were grown 3 or 5 days in each cell's recommended growth medium with 10% FBS (19). Cell viability was measured with CellTiter 96 AQueous One Solution Cell Proliferation reagent (Promega Corp.); EC_{50} values were determined as reported (14). The ratio of EC_{50} values for LY2334737 treatment divided by gemcitabine treatment was calculated for each line. The bystander studies used PC-3 cells tagged with GFP (PC-3-GFP; ref. 20) grown alone or cocultured in a 1:1 ratio with either mock or CES-2 (subclone 61b1) HCT-116 transfectant. Cells were grown for 3 days in clear bottom Blackwell plates biocoated with poly-D-lysine in Dulbecco's modified Eagle's medium (DMEM) F-12 medium (3:1), 10% FBS (tetracycline free) with nonessential amino acids. To determine PC-3-GFP viability, cell fluorescence was measured using a Cellomics ArrayScan VTi with an excitation of 475 nm and an emission of 535 nm.
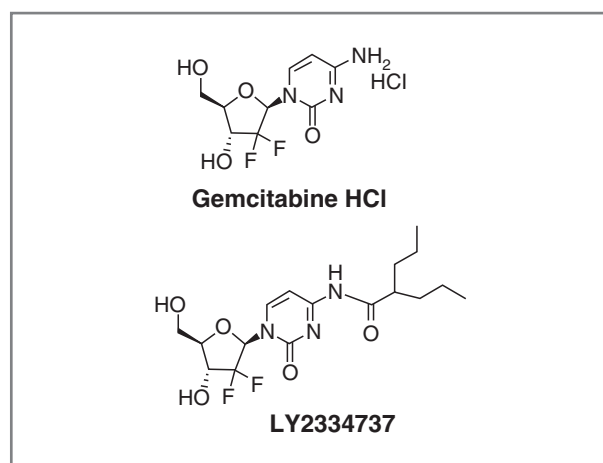

Figure 1. Structures of gemcitabine and the prodrug of gemcitabine, LY2334737.

Data mining

NCI-60 microarray data (U95Av2) were analyzed for known hydrolases using Gene Ontology annotation GO:0016787. A PubGene tool was queried and returned 882 genes. Concordance of gene expression was computed by the concept of vector projection (21).

LY2334737 hydrolysis

Drug hydrolysis assays used high-pressure liquid chromatography (HPLC) that followed the disappearance of LY2334737 and the appearance of gemcitabine and dFdU. Enzyme studies were conducted at 37°C with 100 nmol/L [3H]LY2334737 and 25 to 100 μg/mL of each rhCES preparation. Kinetic studies used recombinant CES2 (22.5 μg/ml), a 30-minute time point, and 2.5 to 250 μmol/L [3H]LY2334737. Cell extracts were prepared by disruption in ice-cold calcium and magnesium-free HBSS, pH 7.5, with a 10-second pulse of a polytron followed by centrifugation. Reactions containing 2 mg/mL cell lysates, 500 nmol/L [3H]LY2334737, and 1 mmol/L THU, a CDA inhibitor, were incubated 18 to 20 hours at 37°C. An internal standard [3H]naltrindole (final concentration, 0.3 mCi/mL) was added before sample processing. Time course studies with recombinant human carboxylesterase (rhCES)2 and cell lysates showed that hydrolysis was completely stopped by the addition of a half volume of acetonitrile; supernatants of samples were prepared for injection with a 2-minute 800 × g centrifugation. Samples were analyzed on an Atlantis C18 column reverse-phase column (Waters 186001342, 3 mm, 4.6 × 150 mm) with a Waters 2795 HPLC system using 50 mmol/L ammonium acetate, pH 5.5 (A), and acetonitrile (B) as resolving solvents. The protocol used a gradient of 5% B to 50% B over 3.5 minutes, 50% B was held for 1.5 minutes, and the gradient returned to 5% B for the final 3 minutes. A Packard 500TR Flow Scintillation analyzer with Ultima Flo M scintillant (Perkin Elmer; 3:1 with sample; flow rate 1 mL/min) was used for analyte detection. The retention times for gemcitabine, dFdU, and LY2334737 were 3.2, 3.5, and 6.4 minutes, respectively. Retention times for the [3H]naltrindole peaks were 4.8 and 5.6 minutes. Percent hydrolysis was calculated on the basis of the peak areas of intact [3H]LY2334737 present in each sample relative to the LY2334737 buffer control and is also a measure of the conversion to gemcitabine and dFdU.

PNB and 4-MUB assays

rhCESs, CES1A1, CES2 and CES3, and transfectant cell lysates were prepared and assayed for hydrolysis of 10 μmol/L p-nitrophenyl butyrate (PNB) as described (22). The 4-MUB hydrolysis assay was designed to provide a rapid method for screening clones of transfectants or knock-downs for activity that did not require the growth of large cell numbers (20). Cells were grown with 6 replicates to confluency in 96-well white plate, clear bottom, and tissue culture plates (Costar). The plates were frozen, thawed, and incubated with 100 μmol/L 4-MUB in HBSS, pH 7.5, at room temperature in the absence or presence of 10 μmol/L loperamide. Fluorescence was measured over 80 seconds at excitation of 365 nm and emission of 420 nm with a PolarStar fluorescent time resolved BMG Labtech plate reader. CES2-mediated cleavage was defined as the hydrolysis that was loperamide inhibitable. HCT-116 parental cell had no loperamide-inhibitable activity. Putative CES2-expressing transfectants showed enhanced activity. Additional studies used an EnVision instrument (Perkin Elmer) to follow the hydrolysis of 100 μmol/L 4-MUB at 340 nm excitation and 460 nm emission wavelengths. Linear hydrolysis rates were calculated from a 5-minute time course using a 4-methylumbelliferone standard curve.

Quantitative real-time PCR analysis

RNA was isolated from cell pellets using TRIzol, and contaminating DNA was removed with DNAse1, (Invitrogen) and purified with the RNA easy kit (Qiagen) according to the manufacturers' protocol. The yield and purity were measured by the ratio of A260/A280. Absolute quantitative real-time PCR (qRT-PCR) was conducted with the ABI 7900 HT Instrument (Applied Biosystems) using the standard curve method. Real-time PCR amplification mixtures contained 25 ng template cDNA, 2X Master Mix (Applied Biosystems), and 300 nmols Inventoried Gene Specific 20X Assay-on-Demand forward and reverse primers (Applied Biosystems) for CES2 (Hs 01077945_mL), CES1 (Hs00275607_mL), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 4326317E). The cycling conditions were as described in the instrument manual. Each experiment included standard curves for each cDNA and a no template control. Data were normalized to GAPDH and analyzed statistically by one-way ANOVA.

Immunoblots

Proteins from supernatants of cell lysates (40 μg/lane) were separated on 4% to 20% SDS-PAGE as described (23) and transferred to a nitrocellulose membrane. The primary monoclonal antibodies were: anti-CES2 (Santa Cruz-100685, G5; 1/200 dilution), CES1 (Santa Cruz-365249, diluted 1/200), or anti-β-actin (Sigma A5441, diluted 1/10,000). Detection used a secondary anti-mouse antibody conjugated with horseradish peroxidase (diluted 1/10,000) and Enhanced chemiluminescence (Perkin Elmer) following the manufacturer's instructions.

Immunohistochemical staining

Approximately 10^8 cells were formalin-fixed 24 for hours, concentrated, pelleted, paraffin-embedded, and 3 μm sectioned with 2 to 4 replicates per slide. The Universal Biocare Decloaker was used for antigen retrieval (125°C, 30 seconds) followed by a prediluted protein block (DAKO catalog number X0909; 37°C, 30 minutes). Staining was completed at ambient temperature using a Ventana autostainer with 3 μg/mL anti-CES2 mouse monoclonal antibody (Abnova, M02, clone 4F12) for 1 hour followed by a 30-minute incubation with a biotinylated goat anti-mouse secondary antibody (DAKO, E0433; 1/300). A prediluted DAB MAP detection system (Ventana, catalog number 760-124) was applied followed by counterstaining with

hematoxylin (24). Quantitative analysis was conducted with Aperio ImageScope (version 9.0.19.1516) positive pixel count analysis tool. CES2 percent-positive area was calculated. Tumor samples used these same methods. A pathologist (K. Heinz-Taheny) assessed all immunolabeled samples.

Knockdown of CES2 expression

SK-OV-3 cells were transduced with Mission short hairpin RNA (shRNA) lentiviral transduction particles to human CES2 (Sigma, TRCN0000046965) in medium containing 8 μg/mL protamine sulfate and were selected for puromycin resistance (2 μg/mL). A stable pool of puromycin-resistant cells was cultured, assayed for CES2 expression by qRT-PCR, and exhibited approximately 60% reduction. This pool was subcloned by limiting dilution; 10 independent clones were isolated, cultured, and assayed for 4-MUB cleavage. Clone E was selected for further study.

HCT-116 transfectants

CES-overexpressing and mock cells were generated by transfection of HCT-116 using 1 μg full-length CES1 (NM_001025195.1)- or CES2 (NM_003869.4)-pCDNA5 (Invitrogen) or empty vector with FuGENE 6 reagent (Roche Applied Sciences) following the manufacturer's protocol. Two days after transfection, resistant clones were selected in DMEM, 10% FBS containing 100 μg/mL hygromycin and 5 μg/mL blasticidin. For CES2 transfection, 144 clones were picked and assayed for 4-MUB cleavage. Of these, 34 clones were identified, grown, and reassayed for CES2-mediated 4-MUB hydrolysis, yielding 12 clones that were subsequently evaluated for CES2 transcript levels. Seven clones continued to grow and were furthered studied by Western blot analysis and drug sensitivity. Single subclones were isolated by fluorescence-activated cell sorting and characterized as described and used in the xenograft studies. For CES1 transfection, 9 clones were identified that expressed elevated CES1 transcript levels; only one continued to grow and express CES1. Transfectants were maintained in selection growth medium and were washed free of drug 24 hours before cytotoxicity assays.

Xenograft studies

A detailed protocol for the HCT-116 xenograft was published previously (25). HCT-116 transfectants (mock and 5 CES2 HCT-116 subclones) were initially evaluated for tumor growth and for stability of CES2 expression when grown *in vivo*. After implantation, tumor growth and mouse weights were measured twice per week; mice were sacrificed once their tumors reached 1,000 mm^3 in volume. CES2 protein expression was assessed by immunohistochemistry in formalin-fixed cell pellets prepared at the time of implantation and in fixed sections of transfectant tumors harvested at sacrifice. Only one clone had a growth rate that matched the mock transfectant and stably expressed CES2 protein. Subsequently, HCT-116 transfectants (mock or CES2 subclone 61F7) were implanted subcutaneously into the left flank of athymic nude mice; treatment began when tumors were 200 mm^3. Mice (20/group) were treated by oral gavage once a day for 21 days with vehicle control (10 mL/kg of 0.1 mol/L Na$_3$P0$_4$, pH 6) or 3.2 mg/kg LY2334737. Another set of mice (10/group) received 80 mg/kg gemcitabine hydrochloride intraperitoneally (i.p) every third day for 4 doses (Q3D × 4). Statistical analysis was conducted by 2-way ANOVA. Tumors were removed at the end of study and analyzed by immunohistochemistry for CES2 expression.

Results

Evaluation of human recombinant carboxylesterases

CES1 was identified as a top hydrolase candidate based on mining of the microarray gene expression data for hydrolases using the LY2334737 screening results from the NCI-60 cell line panel. CES1 was the only CES represented on the microarray, consequently, 3 rhCESs were evaluated. Their activity was initially assessed over a 10-minute time course using PNB, a substrate shared of these isozymes (22). The hydrolysis rates were 38, 69, and 0.22 μmol p-nitrophenol released/min/mg protein for CES1A1, CES2, and CES3, respectively, indicating that these isozymes were enzymatically active. Much longer incubation times were required to observe hydrolysis of [^{3}H]LY2334737 to gemcitabine. CES2 nearly completely hydrolyzed 100 nmol/L [^{3}H]LY2334737 within 2 hours, whereas CES1A1 and CES3 showed no hydrolysis during an 18-hour incubation (data not shown). Subsequently, CES2 hydrolysis was examined at 30 minutes over the concentration range of 2.5 to 250 μmol/L [^{3}H]LY2334737; Michaelis–Menten kinetic parameters were determined to be: K_m of 43 μmol/L and V_{max} of 40 nmol/min/mg protein (data not shown). Thus, CES2 was the only isozyme that showed hydrolytic activity of LY2334737 and therefore, was the CES of primary interest.

Drug response and CES-2 expression

Gemcitabine is cytotoxic to most cell lines in the NCI-60 panel with EC$_{50}$ values of less than 1 μmol/L. When LY2334737 was tested, the majority of these lines had EC$_{50}$ values that were 80-fold more than the values for gemcitabine. Cell lines with ratios (LY2334737 EC$_{50}$/gem EC$_{50}$) of 20 or less were reevaluated and additional cell lines not present in the NCI-60 panel were also tested. This yielded a set of prodrug-responsive cell lines for further analysis (Fig. 2). Figure 2A shows the ratio of drug response to LY2334737 and gemcitabine for 7 cell lines. LY2334737 had little to no effect on the growth of 2 cell lines, HCT-116 and HEK293, with ratios of 300 or more, whereas 5 other cell lines, 2008, HepG-2, Colo-205, A498, SK-OV-3, and MSTO-211H showed LY2334737 growth inhibition with ratios of 15 or less. When CES1 and CES2 expression was assessed by qRT-PCR (Fig. 2B), 2 cell lines, HCT-116 and HEK293, had little CES2 mRNA expression, whereas 5 others exhibited significantly higher transcript levels ($P \leq$ 0.05). CES1 expression in HepG-2 was nearly 20-fold higher than the next highest expressing line, SK-OV-3, and was at least 260-fold higher than all of the other cell lines (Fig. 2B). Western blotting of CES1 and CES2 recombinant proteins
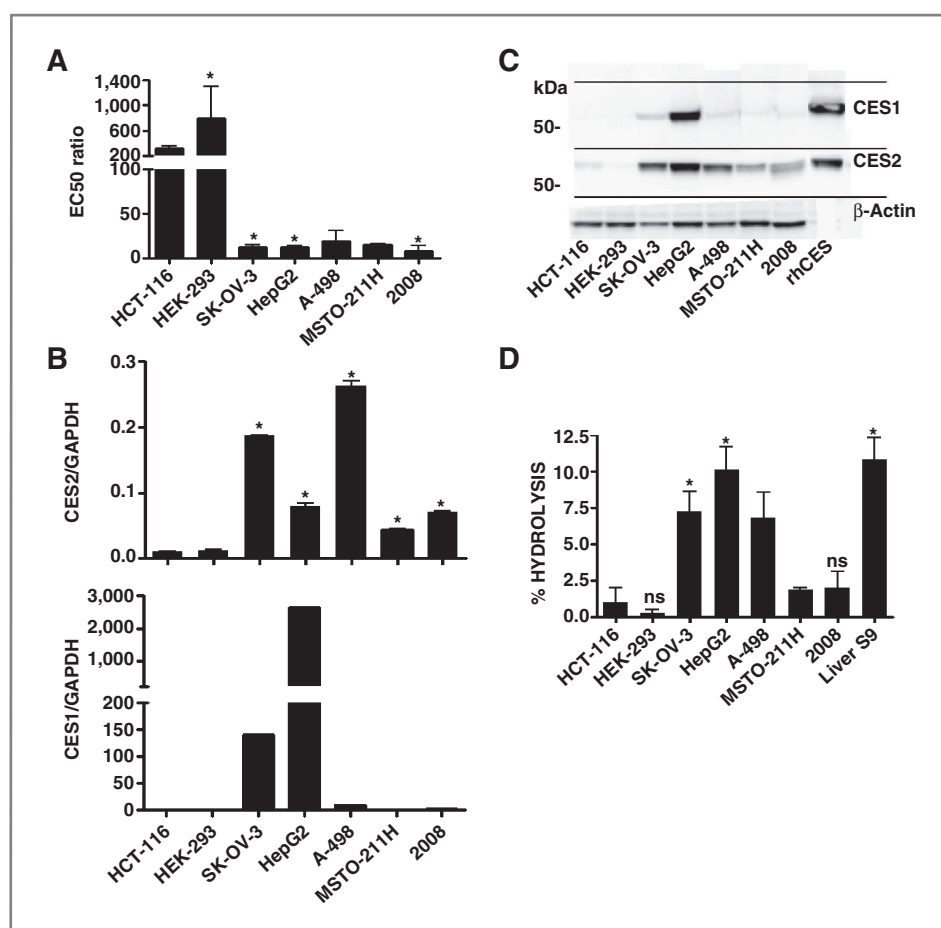
Figure 2. Survey of selected cell lines for drug response, prodrug hydrolysis, and CES2 expression. A, drug sensitivity. The cytotoxicity of LY2334737 and gemcitabine was measured and the ratio of EC$_{50}$ values was calculated as described in Materials and Methods. Data are the mean $\pm$ SEM of 2 or more independent experiments measured in quadruplicate. B, CES transcript levels. Values for CES1 and CES2 were measured by qRT-PCR and normalized to GADPH. CES2 data are the mean $\pm$ SEM of quadruplicates and represent 2 independent experiments, whereas the CES1 data are the mean of quadruplicates from one experiment. C, Western blot analysis. Proteins of cell lysates (40 µg/lane) or rhCES (1 ng/lane) were separated by SDS-PAGE and immunoblotted with anti-CES1, CES2, or β-actin antibody as described in Materials and Methods. Molecular weight markers (kDa) are indicated on the left. D, LY2334737 hydrolysis. Cell lysates were incubated for 18 hours at 37°C with 500 nmol/L [^{3}H]LY2334737 and 1 mmol/L THU to block CDA deamination of gemcitabine as described in Materials and Methods. Human liver S9 lysate served as a control. Data are the mean $\pm$ SEM of 1 to 7 independent determinations measured in duplicate. Statistical analyses were conducted on samples with 3 or more independent measurements. *, significantly different from HCT-116 hydrolysis ($P \leq 0.05$). ns, not significantly different from HCT-116 hydrolysis ($P > 0.05$).

and cell lysates separated by SDS-PAGE (Fig. 2C) confirmed that 60 kDa proteins were immunoreactive with their specific anti-CES2 or anti-CES1 antibodies (Fig. 2C). All 5 of the prodrug-responsive cell lines expressed the CES2 protein although to varying levels. Only 2 of these cell lines, HepG2 and SK-OV-3, expressed appreciable CES1 protein, consistent with mRNA expression. As shown in Fig. 2D, [^{3}H] LY2334737 hydrolysis was examined with cell extracts and a human liver S9 preparation. Two lines, HCT-116 and HEK293, had negligible hydrolytic activity ($\leq$1%), whereas the prodrug-responsive cells HepG2, SK-OV-3, and A-498 along with liver extract showed significantly greater hydrolysis (7.5%–12%; $P \leq 0.05$), consistent with CES2 enzyme expression. The presence of high levels of CES1 in HepG2 did not result in significantly greater prodrug hydrolysis than either SK-OV-3 or A-498, which have low to negligible

levels of CES1. On the basis of these results, CES2 was chosen for further study for its potential role in cellular prodrug response.

HCT-116 transfectants

HCT-116 cells are sensitive to gemcitabine and possess low endogenous basal levels of CES1 and CES2 (Fig. 2C). To test the roles of CES1 and CES2 in drug sensitivity, transfectants of HCT-116 cells were prepared. A response to prodrug treatment would then be attributable to exogenous enzyme expression, as the transfectants would have the same cellular content of enzymes important for gemcitabine response (26–28). Clones of CES transfectants were initially screened for their ability to hydrolyze 4-MUB and positive clones were picked and further characterized. 4-MUB hydrolysis was enhanced 5.5- and 8-fold in the CES

transfectants compared with mock (99, 148, and 18 nmol MU released/min/mg protein for CES1, CES2, and mock transfectants, respectively). Similarly, CES transfection enhanced the ability of HCT-116 cells to hydrolyze PNB by 6- and 12-fold compared with mock transfection (95, 179, and 15 μmol p-nitrophenol released/min/mg protein for CES1, CES2, and mock transfectants, respectively). Western blot analyses of cell extracts showed the presence of a 60 kDa protein in the CES1 transfectant that was immunoreactive with an anti-CES1 antibody (and not with an anti-CES2 antibody) and in CES2 transfectants of a immunoreactive protein with an anti-CES2 antibody (ref. 12; Fig. 3A). As shown in Fig. 3B, the dose–response curves for gemcitabine or LY2334737 treatment were not different between the CES1 and mock transfectants, whereas the CES2 transfectant was 14-fold more sensitive to LY2334737 than the mock transfectant. Cell lysates were prepared from the CES2 and mock transfectants and assayed for hydrolysis of 250 nmol/L [^{3}H]LY2334737 and gave 8.9% and 2.2% hydrolysis, respectively, within 20 hours, consistent with the transfectants' differing prodrug response. As shown in Fig. 3C, analysis of 6 CES2 transfectants and mock revealed a high correlation ($r^2 = 0.94$) between CES2 transcript levels and prodrug response. Thus, growth inhibition by LY2334737 was directly correlated to the CES2 expression level. These data indicate that CES2 and not CES1 are responsible for the cytotoxicity of the prodrug.

Knockdown of CES2 expression

If chemosensitivity is due to CES2 expression, then inhibition of its enzymatic activity should reduce LY2334737 cytotoxicity. To test this, a stable SK-OV-3 knockdown cell line was created with CES2 shRNA (Fig. 4). The SK-OV-3 knockdown had an 80% to 90% reduction in CES2 mRNA transcript compared with parental cells (Fig. 4A). Western

blot analysis of cell extract proteins separated by 4% to 20% SDS-PAGE showed a concomitant reduction in a 60 kDa protein corresponding to CES2 (Fig. 4B; ref. 12). Immuno-histochemical (IHC) staining of the SK-OV-3 knockdown revealed an 89% reduction in the CES2-reactive protein compared with parental cells (Fig. 4C). Cytotoxicity was evaluated in the absence or presence of a noncytotoxic concentration of loperamide (10 μmol/L), a cell permeable inhibitor of CES1 and CES2 that is a 1,000-fold more potent inhibitor of CES2 (K_i of 1.5 nmol/L) than CES1 (29, 30). Figure 4D shows the drug dose–response curves. Parental SK-OV-3 cells were approximately 4-fold more sensitive to LY2334737 cytotoxicity than the SK-OV-3 knockdown (EC$_{50}$ values of 1.1 and 4.5 μmol/L, respectively; $P < 0.001$). Addition of 10 μmol/L loperamide to the medium reduced the response of parental SK-OV-3 cells to LY2334737 by approximately 2.5-fold (EC$_{50}$ of 2.7 μmol/L) and had little effect on the knockdown (EC$_{50}$ of 3.4 μmol/L). In contrast, gemcitabine cytotoxicity was unaffected by CES2 expression or its inhibition. The EC$_{50}$ values ranged from 24 to 34 nmol/L (Fig. 4D). Therefore, inhibition of CES2 activity by knockdown or chemical inhibition resulted in a reduction in LY2334737 cytotoxicity without interference of gemcitabine cytotoxicity.

Bystander effect

Some tumors are reported to express CES2 but not necessarily uniformly throughout the tumor (31–33). To test whether neighboring CES2-expressing cells may alter growth of cells devoid of CES2, HCT-116 transfectants and fluorescent GFP-tagged PC-3 cells were grown individually or cocultured together. In coculture studies, fluorescence was used to monitor growth of only the reporter cell line that lacks CES2 expression. Table 1 gives the EC$_{50}$ values for treatment with gemcitabine, LY2334737, or G418 used for
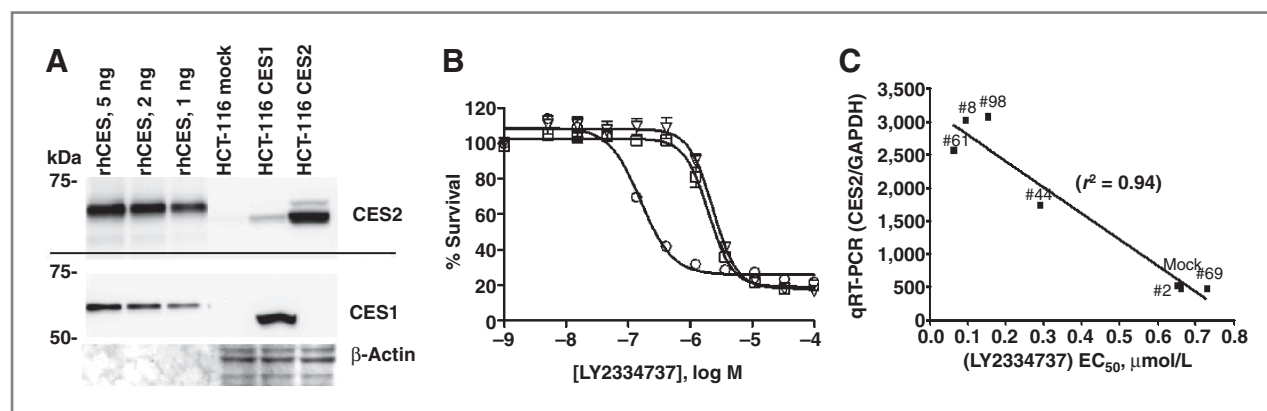
Figure 3. Characterization of CES-transfected HCT-116 cells. A, Western blots ofrhCES and transfectant cell lysates (40 μg/lane) were separated by SDS-PAGE; blots were developed using antibodies to CES1, CES2, or β-actin as described in Materials and Methods. The blot was probed with the anti-CES antibody corresponding to the isoform that was loaded and is indicated on the right. Molecular weight markers on the left. B, LY2334737 dose response of transfectants. Cells were grown for 3 days in the presence of drug. Data are the mean ± SEM of quadruplicates from one representative experiment. Symbols for the transfectants with their EC$_{50}$ are: (▽) mock transfectant (2.33 μmol/L), (□) CES1 transfectant (1.98 μmol/L), and (o) CES2 transfectant (0.16 μmol/L). Gemcitabine EC$_{50}$ values were 0.012 to 0.016 μmol/L (data not shown). C, correlation of CES2 transcript with LY2334737 cytotoxicity of HCT-116 transfectants. EC$_{50}$ values for LY2334737 and gemcitabine were determined in 5-day cytotoxicity assays and their ratios calculated. Expression was measured by qRT-PCR. Values are the mean ± SEM of quadruplicates and representative of 2 independent experiments. The line was fitted by linear regression ($r^2 = 0.94$).
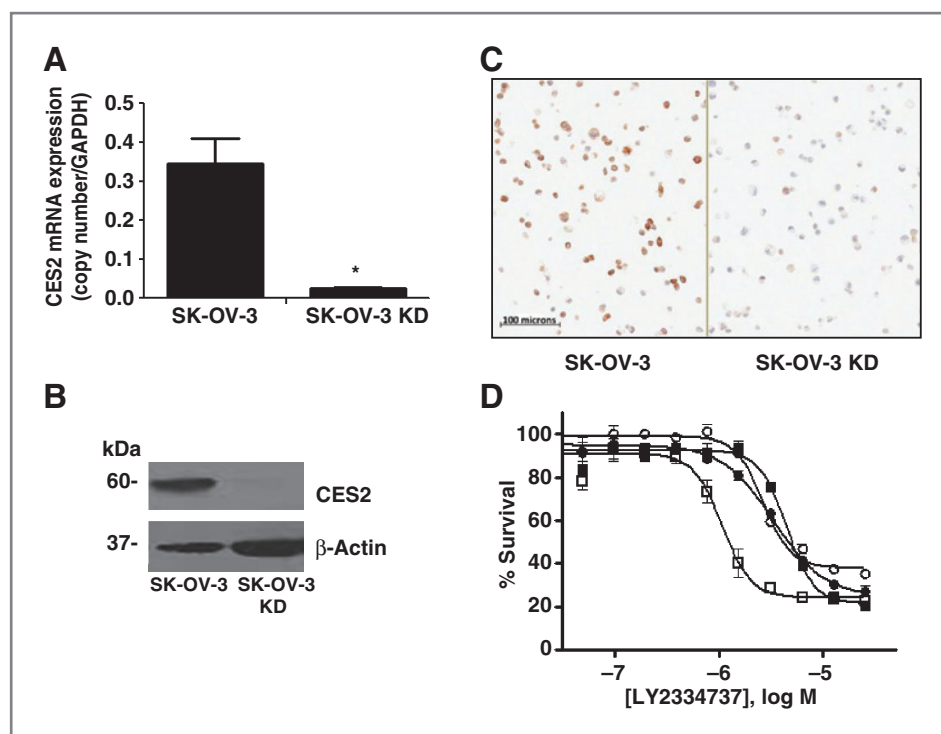
Figure 4. Properties of SK-OV-3 CES2 knockdown. A, CES2 expression in SK-OV-3 parent and CES2 knockdown (KD). qRT-PCR measurements are the mean ± SEM of quadruplicate determinations and represent 2 independent experiments. B, Western blot analysis. Proteins in cell lysates were separated by SDS-PAGE electrophoresis and stained with anti-CES2 and anti-β-actin antibodies as described in Materials and Methods. C, IHC labeling. Expression was quantified by positive pixel count. CES2 protein immunolabeling levels were 66.8% in parental SK-OV-3 and 6% in the CES2 knockdown. D, effect of CES2 inhibition by shRNA knockdown or loperamide on cytotoxicity of LY2334737 and gemcitabine. SK-OV-3 parent (■; □) and knockdown (●; ○) cells were grown 5 days in the absence (□; ○) or presence (■; ●) of a noncytotoxic concentration of loperamide (10 μmol/L), a CES2 inhibitor. Data are the average ± range of duplicate determinations from one experiment and are representative of 4 independent experiments. For LY2334737 cytotoxicity, the drug response of SK-OV-3 cells with CES2 neutralization, either by knockdown or chemical inhibition or both, were not significantly different from one another but were significantly different than the drug response of CES2-expressing parental SK-OV-3 cells ($P \leq 0.001$). For gemcitabine, the EC_{50} values ranged between 24 to 34 nmol/L.

the selection of the PC-3-GFP cells. When grown individually, the 3 cell lines were equally responsive to gemcitabine (EC_{50} values of 0.006 μmol/L); PC-3-GFP sensitivity to gemcitabine was unaffected by coculturing with either transfectant. The CES2 transfectant was 11-fold more sensitive to LY2334737 treatment than either the mock transfectant or PC-3-GFP cells. LY2334737 cytotoxicity to PC-3-GFP cells (EC_{50} of 1.35 μmol/L) was enhanced 7.5-fold by coculturing with the CES2-transfected HCT-116 and was unchanged with the mock transfectant (EC_{50} values of 0.18 μmol/L vs. 1.53 μmol/L, respectively). To rule out that this result was an indirect effect of neighboring cell death, G418 cytotoxicity was examined. When grown alone, PC-3-GFP cells were approximately 10-fold resistant to G418 compared with HCT-116 transfectants similar to the 15-fold difference observed with LY2334737 treatment. When cocultured with a G418 concentration that had no effect on PC-3-GFP but was lethal to HCT-116, PC-3-GFP was unaffected by G418-induced death of the neighboring HCT-116 cells. Thus, the bystander effect seen with LY2334737 treatment of cocultured PC-3-GFP and CES2 transfectant was due to CES2 hydrolysis of LY2334737 to gemcitabine and not the result of the necrosis of adjacent dying cells.

Xenograft studies

Previous pharmacokinetic studies in mice showed slow systemic hydrolysis of LY2334737 and prolonged exposure to gemcitabine after oral dosing of LY2334737 (3). Because prodrug levels persist in the blood stream due to slow hydrolysis, we wondered whether tumor CES2 expression would enhance tumor growth inhibition with LY2334737 treatment *in vivo*. Previous evaluation of metronomic prodrug dosing in the HCT-116 xenograft model showed that oral dosing of 7.5 mg/kg LY2334737 once-a-day for 14 days resulted in tumor growth inhibition that was comparable with treatment with 160 mg/kg gemcitabine HCl i.p every third day for 4 doses (Q3D × 4), even though the total gemcitabine exposure by metronomic prodrug dosing was roughly 10-fold lower than intraperitoneal gemcitabine dosing (3). To examine the effect of CES2 tumor expression, xenografts bearing CES2- or mock-HCT-116 transfectants were treated once-a-day for 21 days by oral gavage with a suboptimum dose of LY2334737 (3.2 mg/kg). This low prodrug dose gave a minimal tumor response in preliminary xenograft studies (data not shown) and would readily allow the measurement of a CES2-mediated differential growth response. As shown in Fig. 5, tumor growth of both vehicle-treated HCT-116 transfectants and LY2334737-

Table 1. Bystander effect of drug response of GFP-tagged PC-3 reporter cell line when grown alone or cocultured with HCT-116 transfectants

Cell line	Gemcitabine	LY2334737	G418
Cytotoxicity (EC$_{50}$, µmol/L)			
PC-3-GFP alone	0.006 ± 0.001 [1]	0.512 ± 0.016 [1]	6311 ± 693 [1]
HCT-116 mock transfectant	0.006 ± 0.001[a] [1]	0.402 ± 0.018[b] [1.3]	659 ± 48[b] [9.6]
HCT-116 CES2 transfectant	0.006 ± 0.001[a] [1]	0.034 ± 0.002[b] [15]	551 ± 30[b] [11.5]
Fluorescence (EC$_{50}$, µmol/L)			
PC-3-GFP alone	0.013 ± 0.001 [1]	1.35 ± 0.08 [1]	6310 ± 0.0 [1]
PC-3-GFP + mock transfectant	0.016 ± 0.001[a] [0.81]	1.53 ± 0.05[a] [0.88]	4765 ± 1265[a] [0.76]
PC-3-GFP + CES2 transfectant	0.015 ± 0.001[a] [0.87]	0.18 ± 0.02[b] [7.5]	6384 ± 204[a] [1.01]

NOTE: Cells were grown for 3 days with the indicated drug as described in Materials and Methods. Viability was determined by dye reduction or fluorescence of the GFP-tagged PC-3 cells. Data are the mean ± SEM of quadruplicate samples and are representative of 2 independent experiments. Values in brackets are the fold-shift in EC$_{50}$ value compared with PC-3-GFP cells grown under the same experimental conditions.
[a]Not significantly different from PC-3-GFP when compared with the same viability detection method ($P > 0.05$).
[b]Significantly different from PC-3-GFP when compared with the same viability detection method ($P \leq 0.001$).

treated mock transfectant were indistinguishable ($P > 0.05$). Both transfectant tumors responded equally well ($P > 0.5$) to 80 mg/kg gemcitabine hydrochloride i.p Q3D × 4, showing that CES2 expression did not alter the tumor's response to gemcitabine. In contrast, growth of the CES2 transfectant was inhibited 25% by LY2334737 treatment and was significantly different from the LY2334737-treated mock transfectant ($P \leq 0.001$) and the vehicle-treated CES2 transfectant ($P \leq 0.01$). IHC tumor staining confirmed that the CES2 transfectant maintained high CES2 protein expression levels (85% positive area) throughout the course of treatment; the mock tumors did not express CES2 (data not

Figure 5. Efficacy of LY2334737 in a xenograft model bearing mock or CES2- HCT-116 transfectants. Mice (20/group) were treated by oral gavage QD × 21 with vehicle (■ mock; □ CES2) or 3.2 mg/kg LY2334737 (▲ mock; Δ CES2). Mice (10/group) received 80 mg/kg gemcitabine HCl i.p Q3D × 4 (● mock; o CES2). Dosing began on day 20. Tumor growth was significantly different (***, $P \leq 0.001$) between LY2334737-treated CES2 transfectant versus mock transfectant, and was significantly different than vehicle-treated CES2 transfectant ($P \leq 0.01$). Tumor growth of mock and CES2 transfectants was not significantly different from each other when treated with vehicle ($P > 0.05$) or gemcitabine ($P > 0.5$).

shown). Thus, tumors that expressed CES2 had a greater response to LY2334737 treatment due to their ability to hydrolyze the prodrug intracellularly.

Discussion

The present studies identify CES2 as a hydrolase of LY2334737. Screening of the NCI-60 panel for growth inhibition by LY2334737 and comparison with inhibition by gemcitabine indicated that only a few cell lines in the panel were sensitive to the prodrug (consistent with this prodrug requiring hydrolytic activation). Initially, CES1 was found as a candidate hydrolase by datamining the transcript profile; however, it was the only CES represented on the microarray. When 3 human recombinant CESs (CES1, CES2, and CES3) were evaluated in biochemical assays, all quickly hydrolyzed a well-characterized ester substrate, PNB, whereas only CES2 hydrolyzed the prodrug to gemcitabine and required long incubation periods of 0.5 to 2 hours (22). Kinetic studies determined that CES2 has an affinity (K_m) for LY2334737 of 43 µmol/L and a V_{max} of only 40 nmol/min/mg protein. The affinity of CES2 for LY2334737 is in the same range as PNB; however, the CES2 turnover rate is 8000-fold lower for LY2334737 than PNB (22). This slow cleavage rate is likely due to the prodrug containing an amide linkage to valproic acid rather than an ester linkage. The prodrug is absorbed intact with oral dosing and systemic formation of gemcitabine is rate limiting (3, 4). Phase I trials showed higher plasma levels of circulating prodrug than gemcitabine (4) consistent with slow CES2 hydrolysis in both the liver and the intestine (13, 15, 34). If prodrug absorption is sufficiently rapid, limiting mucosal residence time, LY2334737 may escape hydrolysis in the intestinal mucosa thereby reducing cytotoxicity to this epithelium. Thus, the slow hydrolysis of LY2334737 by CES2 is consistent with its observed pharmacokinetic properties.
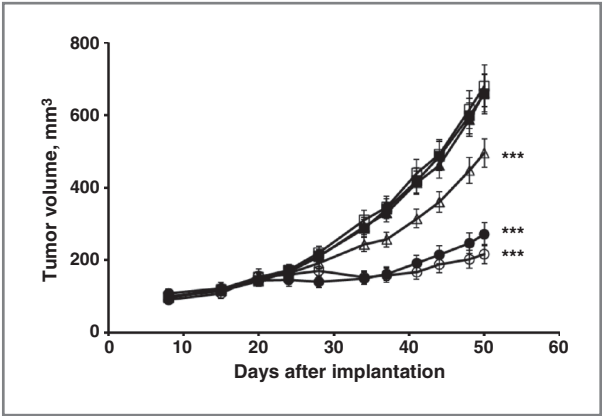
Of particular interest is the finding that cellular CES2 expression confers sensitivity to prodrug cytotoxicity. This gene–drug response relationship was examined in 4 ways. First, 5 prodrug-responsive cell lines expressed CES2, whereas 2 nonresponsive lines had basal levels, suggesting a role for CES2 in intracellular activation. Cell extracts of prodrug-responsive lines with high CES2 protein expression, HepG2, SK-OV-3, and A-498 showed greater hydrolysis of LY2334737 to gemcitabine than HCT-116, a nonresponsive line that lacked CES2 expression ($P \leq 0.05$). Even though CES2 expression and hydrolysis rates varied in the 5 prodrug-responsive cell lines, the cytotoxicity EC_{50} ratios for LY2334737 were similar in these lines due to the 5-day growth period that permitted continuous CES2-mediated intracellular prodrug hydrolysis thereby prolonging gemcitabine cellular exposure. Second, inhibition of CES2 activity by approximately 90% knockdown of mRNA and protein expression in LY2334737-sensitive SK-OV-3 cells, or by addition of the CES2 inhibitor loperamide to growth medium, reduced prodrug cytotoxicity by 4- and 2.5-fold, respectively, without interfering with gemcitabine cytotoxicity. Third, a strong correlation ($r^2 = 0.94$) was observed between CES2 transcript levels and the transfectants' prodrug response using a panel of 7 CES2 transfectants. Fourth, studies of mice-bearing mock- or CES2–transfected HCT-116 tumors showed an enhanced growth inhibition of CES2-expressing tumors to LY2334737 treatment ($P < 0.01$). Thus, CES2-expressing tumors respond directly to gemcitabine released systemically and to plasma LY2334737 by virtue of intracellular CES2-mediated prodrug cleavage. A potential advantage to intracellular hydrolysis is the slow, consistent release of gemcitabine and the likelihood of arresting growth of more cells as they enter cell division. Taken together, these findings show a role for CES2 activation of the prodrug intracellularly and for the cell's response to the cytotoxic effects of LY2334737 via gemcitabine resulting in augmentation of prodrug efficacy by metronomic dosing for CES2-expressing tumors.

Two other anticancer prodrugs, capecitabine and irinotecan, are hydrolyzed by CESs. Capecitabine requires a 3-step activation process resulting in the formation of 5-fluorouracil, where the first step is the cleavage of an ester bond by either CES1 or CES2 in the liver with similar kinetics (30, 35). Irinotecan is also an ester-containing drug that is cleaved by both CESs; however, CES2 catalysis is roughly 70 times more efficient than that of CES1 (36). LY2334737 differs from these 2 prodrugs in that hydrolysis is at an amide bond, and CES1 exhibited no hydrolytic activity. rhCES1 did not hydrolyze LY2334737 and CES1 transfectants of HCT-116 cells were not sensitive to prodrug treatment in cytotoxicity assays. In addition, HepG-2 cells, with high expression of CES1 and CES2, had comparable hydrolytic prodrug activity to 2 other lines SK-OV-3 and A-498 that had similar CES2 protein levels and little to no detectable CES1 protein. Thus, CES2 is the sole CES responsible for the cleavage of LY2334737, and this may also contribute to the prolonged exposure to the intact prodrug.

Tumor expression of CES2 has been previously examined due to this enzyme's pivotal role in the activation of irinotecan (31, 37). Appreciable CES2 levels are expressed in certain tumors (colon, esophageal, liver, cervix, ovarian, renal, thyroid, multiple myeloma, and non–small cell lung cancer) although CES2 may not be uniformly expressed throughout the tumor (31–33, 38, 39). To test whether CES2-expressing cells may have a bystander effect on the growth of nonexpressing cells treated with LY2334737, coculture experiments were carried out. The cytotoxicity of LY2334737 to the non-CES2–expressing reporter cell line PC-3-GFP was enhanced 7-fold when grown together with CES2–expressing HCT-116 transfectant and was unaltered when grown with the mock transfectant. No bystander effect was seen with gemcitabine treatment. A similar bystander effect was reported for CES activation of irinotecan in transfected A549 cells (30, 40). Taken together, these studies suggest enhanced prodrug efficacy even for tumors with heterogeneous CES2 expression.

In summary, the prodrug of gemcitabine, LY2334737, is cleaved by CES2 and not CES1. Slow LY2334737 hydrolysis is consistent with pharmacokinetic properties and higher plasma levels of intact prodrug than gemcitabine observed in mice and humans (3, 4). CES2-expressing cancer cells respond to LY2334737 cytotoxicity via intracellular cleavage to gemcitabine. Oral metronomic dosing coupled with intracellular prodrug cleavage may permit growth inhibition of more cancer cells as they go through cell division. Therefore, CES2 expression may be a useful tumor biomarker for patient stratification for the identification of patients with cancer most likely to benefit from LY2334737 treatment.

Authors' Contributions

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