Reducing Undesirable Hepatic Clearance of a Tumor-Targeted Vinca Alkaloid via Novel Saccharopeptidic Modifications


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ABSTRACT

During a phase I trial of EC145 (a folate-targeted vinca alkaloid conjugate), constipation was identified as the dose-limiting toxicity, probably from a nonfolate receptor-related liver clearance process capable of releasing unconjugated vinca alkaloid from EC145 and shuttling it to the bile. Here, we report on the selective placement of novel carbohydrate segments (1-amino-1-deoxy-glucitolyl-glutamate) spaced in-between the folate and vinca alkaloid moieties of EC145, which yielded a new agent (EC0489) that is equipotent but less toxic than EC145. Whereas both compounds could cure tumor-bearing mice reproducibly, EC0489 differed from EC145 with i) a shorter elimination half-life, ii) approximately 70% decrease in bile clearance, iii) a 4-fold increase in urinary excretion, and iv) improved tolerability in rodents. This combination of improvements justified the clinical evaluation of EC0489 where currently administered dose levels have exceeded the maximal tolerated dose of EC145 by approximately 70%, thereby reflecting the translational benefits to this new approach.

Introduction

Selectively targeting chemotherapeutic agents to tumors can be realized through the use of covalently attached, high-affinity ligands (monoclonal antibodies, peptides, vitamins, etc.) that bind to cell surface receptors (Gilbert et al., 2003; Henry et al., 2004; Tassone et al., 2004; Cao et al., 2008; Leamon, 2008). A benefit of this approach is having the ability to generate anti-tumor responses without causing much of the associated off-target toxicities that typically limit a drug’s utility. For more than 2 decades, we have focused on the use of the vitamin folic acid (FA) for targeting a variety of bioactive agents to pathological tissues that abnormally express high levels of the folate receptor (FR) (Leamon, 2008). To date, four folate-based therapeutics and two companion radiodiagnostic imaging agents have been brought forward into clinical testing. The most advanced of these targeted cytotoxic agents is called EC145.

EC145 represents a novel water-soluble FA conjugate of the potent microtubule-destabilizing agent, desacetylvinblastine monohydrazide (DAVLBH; a derivative of the natural product, vinblastine) (Leamon et al., 2007b; Reddy et al., 2007a). EC145 can produce marked anti-tumor effect against well established, subcutaneous FR-positive tumor xenografts using well tolerated regimens. Hence, brief treatment schedules were found to produce 100% tumor remissions (i.e., cures) in mice under conditions that did not produce significant weight loss or normal tissue degeneration (Reddy et al., 2007a). Based on its preclinical activity and safety profiles, EC145 was evaluated in a phase I dose escalation safety study in refractory cancer patients. When administered as an intravenous bolus dose on days 1, 3, and 5 (week 1) and 15, 17, and 19 (week 3) of a 4-week cycle, EC145 was generally found to be well tolerated at dose levels up to 2.5 mg (flat dose) and for as many as 12 cycles (Sausville et al., 2007). Evidence of anti-tumor activity was noted among patients with ovarian or head-neck carcinoma, which prompted the continuation of this agent’s development to phase II. It is noteworthy that constipation was identified as the dose-lim-
iting toxicity at levels of >2.5 mg (Sausville et al., 2007). Constipation is a common side effect associated with the vinca alkaloid class of agents due to their affinity for axonal tubulin, and it is believed to be predicated by either systemic autonomic neuropathy and/or enteric neuropathy following hepatobiliary clearance, as vinca alkaloids are primarily excreted into the bile.

Based on the aforementioned findings, we reasoned that the clinically observed constipation at high EC145 dose levels could have resulted from hepatic clearance with concomitant metabolism to cause the release of the active vinca alkaloid agent into the bile and subsequent drug exposure to the intestinal tract and the myenteric plexus. In the absence of general autonomic dysfunction, this direct exposure seems to be a plausible explanation. It is noteworthy that normal human liver is not known to express appreciable levels of the FR (Parker et al., 2005). However, one cannot exclude the possibility that EC145 may be cleared by the liver via promiscuous organic anion transporters, especially because the hepatic clearance of a related folate conjugate was found to be significantly lowered (5-fold) upon coadministration of the organic anion transporter inhibitor bromosulfophthalein (C. Leamon, J. Reddy, and N. Parker, unpublished data). Based on these observations, we rationalized that increasing the steric hydrophilic bulk of the structure of EC145 could possibly alter the distribution characteristics of the molecule by limiting hepatic clearance without adversely changing its targeted anti-tumor potential or increasing its systemic toxicity through reduced clearance.

After extensive structure-activity efforts around the “spacer” region of EC145 (see diagram in Fig. 1), a new and superior chemical entity, herein called EC0489, was identified; this compound contains novel carbohydrate segments (1-amino-1-deoxy-glucitolyl-glutamate) spaced in-between the folate and vinca alkaloid moieties. The preclinical pharmacology and toxicology results for EC0489 are disclosed below. Evidence is provided to show that the clearance, elimination route, and tolerability for this molecule are substantially different from EC145, whereas its targeted anti-tumor activity remains unchanged. Furthermore, these improvements seem to be translational, because molar dose levels greater than that achievable with EC145 are currently being administered clinically (with minimal constipation issues) in an open phase I trial with EC0489. To the best of our knowledge, this is the first report of reducing undesirable hepatic clearance and/or metabolism effects of a cancer-targeted cytotoxic molecule through the use of saccharopeptidic chemical modifications.

**Materials and Methods**

**Test Articles.** EC145 was prepared as described previously (Vlahov et al., 2006). EC0489 was prepared using a semi-solid phase approach (Vlahov et al., 2010), with the exception that 9-fluorenyl-
methoxy carbonyl-γ-3,4,5,6-diisopropylidene-glucamino)Glu-OH and 9-fluorenylethoxycarbonyl-γ-Glu-O-Tbu were used to construct its hydrophilic spacer (see Fig. 1), as detailed elsewhere (Vlahov et al., 2010).

**Relative Affinity Assay.** The relative affinity of EC0489 was determined according to a procedure published previously (Leamon et al., 2009). Data (n = 3 per point) were analyzed using a One Site Competition algorithm provided by Prism version 4.00 software (GraphPad Software, Inc., San Diego, CA).

**Dose-Dependent FR-Specific Activity of EC0489.** Cells were seeded in individual 12-well Falcon plates and allowed to form nearly confluent monolayers overnight in folate-deficient RPMI 1640 medium supplemented with 10% fetal bovine serum. Following a detailed published procedure (Leamon et al., 2008b), a 2-hour pulse, 70-h chase assay format was used to evaluate the cytotoxic effects of increasing concentrations of EC0489. Viability was assessed by measuring [3H]thymidine incorporation into trichloroacetic acid-precipitable material (Leamon et al., 2008b). Final results were expressed as the percentage of [3H]thymidine incorporation relative to untreated controls.

**Pharmacokinetic Evaluation of EC145 and EC0489.** Female Sprague-Dawley rats (150–200 g) with surgically implanted jugular vein catheters (Harlan, Indianapolis, IN) were used for rodent pharmacokinetic studies. Rats were randomized and then dosed intravenously via the tail vein with 2 μmol/kg (n = 3) of either EC145 or EC0489. Kinetic sampling was done at 7, 15, 30, 60, 120, and 240 min after injection. Blood samples were collected via a jugular vein catheter into a 1-ml syringe and then added into glass tubes containing 50 μl of an anticoagulant and test-article stabilizing solution of K2EDTA (68 mg/ml), N-maleoyl β-alanine (17 mg/ml), mannitol (40 mg/ml), and acetic acid (0.15%). The tubes were then centrifuged for 15 min at 2000g. Pharmacokinetic sampling was also conducted in female beagle dogs treated with EC145 or EC0489 at doses of 0.24 and 0.31 mg/kg, respectively. Whole-venous blood samples of approximately 1.0 ml were collected from a peripheral vein of all animals for determination of plasma concentrations of EC145 or EC0489. Samples were collected at the following target time points after the first (day 1) and last (day 19) doses: predose and immediately after dosing and 0.25, 0.5, 1, 4, and 24 h after dosing. Samples were placed in tubes containing K2EDTA and stored on ice until centrifugation under refrigeration for at least 10 min at 3000 rpm. After centrifugation, plasma was removed and stored frozen at −70°C. EC0489 or EC145 was extracted from the plasma matrix by protein precipitation with acetonitrile. After sample filtration and dilution, the samples, along with standard calibrators and quality-control samples, were transferred to the LC-MS/MS for analysis. In brief, a 35-μl plasma aliquot was combined with the stable label internal standards of EC145, EC0489, and DAVLBH and combined with an ammonium buffer. The mixture was loaded onto a preconditioned Oasis HLB 96-well plate (Waters, Milford, MA) and washed with water followed by 10% methanol in water. The analytes of interest were eluted from the solid phase matrix with a 4:1 acetonitrile/water mixture followed by sonication to derivatize DAVLBH with acetonitrile. The acetonitrile was removed under nitrogen stream at room temperature leaving the analytes in water. The extract was chromatographed with an XBridge BEH Shield RPC18 1.7 μm, 2.1 × 30 column (Waters) and detected with electrospray positive ion MS/MS. PK Solutions pharmacokinetic software (Summit Research Services, Montrose, CO) was used for further analysis of the data.

**Urinary Clearance of EC145 and EC0489.** Male Sprague-Dawley rats (Harlan) weighing approximately 250 g were randomized to receive either EC145 or EC0489 as a single intravenous dose of 2 μmol/kg (n = 2). Immediately after dosing, each animal was placed into an individual metabolism cage where urine was collected in a 50-ml tube and then kept on ice for 24 h. The collected urine was assayed for EC145, EC0489, and DAVLBH (liberated drug) by LC-MS/MS. A 25-μl urine sample was diluted with stable label internal standards of EC145, EC0489, and DAVLBH and passed through a Phenomenex 96-well sample filter plate. A portion of the filtered sample was chromatographed with a Waters XBridge BEH C18, 1.7 μm, 2.1 × 50-mm column and detected with electrospray positive ion MS/MS.

**Hepatobiliary Clearance of EC145 and EC0489.** Male bile-duct cannulated Sprague-Dawley rats (200–225 g) were randomized to receive a single 1 μmol/kg dose of EC145, EC0489, or DAVLBH (n = 3 per cohort). Before dosing, the animals were anesthetized using ketamine/xylazine and then dosed intravenously with one of the three test articles. Bile from the free-flowing catheter was collected into microcentrifuge tubes at regular intervals beginning just before dosing and at 15-min intervals for 2 h and 15 min. The concentration of EC145, EC0489, and DAVLBH was determined following the same procedure as plasma samples utilizing solid-phase extraction followed by LC-MS/MS analysis.

**Comparative Repeat Dose Toxicity Studies of EC145 and EC0489.** Repeat dose toxicity studies were conducted with EC145 and EC0489 at BASI Evansville Laboratory (Evansville, IN), a facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and registered with and inspected by the United States Department of Agriculture. Female Sprague-Dawley rats between 6 and 10 weeks of age were purchased from Harlan. Upon arrival, the rats were housed in stainless-steel cages suspended over flush pans and provided standard rat diet (2018C; Harlan Teklad, Madison, WI) and municipal water ad libitum. At the start of the treatment period for each study, 15 female rats in each group received intravenous doses of EC145 or EC0489 in phosphate-buffered saline (pH 7.4) following a dosing schedule of days 1, 3, and 5 and 15, 17, and 19 (EC145) or on days 1, 3, 5, 8, 10, 12 (EC0489) followed by a 2-week recovery period. The doses examined were 0.64, 1.28, and 2.56 mg/kg for EC145 and 0.86, 1.71, 2.58, and 3.42 mg/kg for EC4089. Rats were monitored daily for clinical signs, and body weights were measured before dosing and every other day thereafter.

**Tumor Models and Therapy.** Six- to eight-week-old female BALB/c or nu/nu (BALB/c-derived) mice (Charles River, Wilmington, MA) were maintained on a standard 12-h light/dark cycle for the duration of the experiment. Because normal rodent chow contains a high concentration of FA (6 mg/kg chow), mice used in these studies were fed a folate-free diet (Harlan diet TD00434; Harlan Teklad) beginning 2 weeks before tumor implantation and maintained throughout the experiment to achieve serum folate concentrations closer to the range of normal human serum (Mathias et al., 1996; Leamon et al., 2008a). FR-positive KB or M109 cells (1 × 10⁶ per mouse) in 100 μl of folate-free RPMI 1640 medium containing 1% BALB/c serum were injected in the subcutis of the dorsal medial area. Tumors were measured in two perpendicular directions every 2 to 3 days using a caliper, and their volumes were calculated as 0.5 × L × W², where L = measurement of longest axis in millimeters and W = measurement of axis perpendicular to L in millimeters. Dosing solutions were prepared fresh each day in phosphate-buffered saline and administered through the lateral tail vein of the mice (n = 5 per cohort). It is noteworthy that dosing was initiated when the KB tumors were 141 ± 25 mm³ and the M109 tumors were 90 ± 3 mm³ in volume. Body weights were measured every 2 to 3 days to assess gross toxicity. Survival of animals was monitored daily. Animals that were moribund (or unable to reach food or water) due to larger tumors in the control; 0.5 and 1 μmol/kg EC0489 dose groups were euthanized by CO₂ asphyxiation.

**Results**

EC0489 Is a Saccharopeptidic Spacer-Modified Form of EC145. All FA-drug conjugates reported to date contain a modular design (Fig. 1) (Leamon and Jackman, 2008). The color-coded modularity of the EC145 and EC0489 structures is shown in Fig. 1, b and c, respectively. The principal differ-
ences between these two related molecules lie solely within the blue spacer region (module 2). Whereas EC145 contains a hydrophilic pentapeptide spacer (Asp-Arg-Asp-Asp-Cys), EC0489 contains multiple polar carbohydrate segments constructed with novel L-amino-L-deoxy-glucitolyl-γ-glutamate residues, each separated from the other with L-Glu residues and then terminating with L-Cys. Selection of this saccharopeptidic spacer in EC0489 was found to be highly sensitive to EC0489 with an IC50 of 5 nM, next in vitro. As shown in Fig. 2b, FR-positive KB cells were identical to that reported for EC145 (Leamon et al., 2007b). The half of that of FA (relative affinity EC0489 toward the FR was determined to be approximately (Leamon et al., 2009). As shown in Fig. 2a, the affinity of FA, was evaluated using an in vitro competition assay on data to be presented below, EC0489 was selected as a leading second generation conjugate.

**FRs Are Required for the Dose-Dependent Cytotoxicity of EC0489.** The binding affinity of EC0489, relative to FA, was evaluated using an in vitro competition assay (Leamon et al., 2009). As shown in Fig. 2a, the affinity of EC0489 toward the FR was determined to be approximately half of that of FA (relative affinity = 0.50), which is nearly identical to that reported for EC145 (Leamon et al., 2007b). The cytotoxicity and specificity of EC0489 were evaluated next in vitro. As shown in Fig. 2b, FR-positive KB cells were found to be highly sensitive to EC0489 with an IC50 of 5 nM, a value that is also nearly identical to that measured for its non-SPS counterpart, EC145 (Leamon et al., 2007b). This result was important because it confirmed that EC0489's unique SPS moiety had no impact on the ability of target cell to cleave the intermolecular disulfide bond and release the cytotoxic DAVLBH drug. The activity of EC0489 was confirmed next to be dependent on FR expression because i) an excess of folate-γ-ethylenediamine-fluorescein (used as a benign competitor ligand) (Leamon et al., 2008b) could completely abrogate the cytotoxicity of EC0489 (Fig. 2b) and ii) no cytotoxicity was observed against the FR-negative cell lines A549 and 4T1 (Fig. 2c). It is clear that the saccharopeptidic spacer in EC0489 does not impede the reductive cleavage of the disulfide linker or subsequent release of the vinca alkaloid (Vlahov et al., 2010), and this new targeted agent seems to be quite active and selective for FR-expressing cells.

**Clearance of EC0489 Is Faster and Different Than EC145.** EC145 and EC0489 both share a biphasic pharmacokinetic profile distinguished by rapid distribution and elimination (Fig. 3). A comparison of the key pharmacokinetic parameters as outlined in Table 1 showed similar distribution half-lives for both drugs in the rat and dog; however, EC0489 demonstrated a >4-fold reduction in the elimination half-life compared with EC145, a trend observed in both species. A notable increase in the clearance rate for EC0489 compared with EC145 was also evident in both species.

**The Distribution Experiments Indicate That EC0489 Favors Urinary Rather Than Hepatobiliary Excretion.** Excretion measurements in rat revealed that 4-fold more EC0489 was eliminated via the urine compared with EC145 (Fig. 4a). As a consequence, hepatobiliary clearance of...
EC0489 was 3-fold less than EC145 (Fig. 4b). From these observations, it was apparent that the SPS modification in EC0489 allows for more drugs to be cleared from circulation and eliminated by renal filtration, which carries the additional benefit of allowing the drug to remain intact.

**EC0489 Has a More Favorable Toxicology Profile Than EC145 in Rats.** When evaluated in toxicology studies using female Sprague-Dawley rats, EC0489 was found to be more tolerable compared with EC145. In repeated-dose toxicology studies, female Sprague-Dawley rats were dosed with either agent following a dosing schedule of days 1, 3, 5, and 15, 17, and 19 (EC145) or on days 1, 3, 5, 8, 10, and 12 (EC0489) followed by a 2-week recovery period. The more dose-dense regimen was explored with EC0489 to support the potential utility of this schedule in future clinical trials.

The results of these studies showed clear differences and some similarities in the toxicity profiles between the two drugs. A clear difference in tolerability was evident at the highest (equimolar) dose levels tested in each group wherein two rats in the high-dose EC145 group (1.34 μmol/kg) were found dead, whereas all rats in the high-dose EC0489 group (1.34 μmol/kg) survived. This equated to a 2-fold increase in the maximal tolerated dose (0.67 μmol/kg EC145 versus 1.34 μmol/kg EC0489). Furthermore, as evident in Fig. 5, the body weight changes in rats treated with the maximal tolerated EC0489 dose were equivalent to rats treated with 2-fold less EC145. It is important that, whereas EC145 caused shortened villi and erosions in the intestines of some treated animals, gastrointestinal toxicity was notably absent from the EC0489 cohort (i.e., no clinical signs and no histopathologic evidence of injury), which are findings in support for a decrease in the hepatobiliary elimination of EC0489 compared with EC145 (see Fig. 4b). Both EC145 and EC0489 caused dose-dependent myelosuppressive effects in treated rats; however, the lack of comorbidities associated with EC0489 such as gastrointestinal toxicity resulted in greatly improved tolerability. The reduction in toxicity is even more significant when considering again that the EC0489-treated rats were challenged with a more dose-intensive schedule by removing the mid-cycle recovery week.

Neither EC145 nor EC0489 was found to be toxic to the FR-rich kidney. This latter observation becomes even more significant when one considers that, at least in the rat, more EC0489 is being excreted through the kidneys compared with EC145 (see Fig. 4a). Of note is the large fraction of intact conjugate found in the urine from EC0489-treated rats, which is in contrast to mostly free drug (DAVLBH) that is found in bile.

**The Targeted Therapy of EC0489 Is Dose-Responsive and Curative using Well Tolerated Regimens.** The anti-tumor activity and specificity of EC0489 were evaluated against nu/nu mice bearing well established (approximately 140 mm³) subcutaneous KB tumors. As shown in Fig. 6a, tumors in the untreated animals rapidly proliferated and reached approximately 1500 mm³ by approximately day 42, whereas tumors in the EC0489-treated cohorts quickly regressed at dose levels ≥1 μmol/kg. Specific targeting was confirmed because a 40-fold excess of cojected competitor was found to completely block the anti-tumor effect of EC0489. All of the animals in the three highest cohorts (2, 3, and 4 μmol/kg) were found to be tumor-free by day 27, and that response persisted until the end of this 95-day study. This apparent curative activity at the higher dose levels is

### TABLE 1
Pharmacokinetic parameters of EC145 and EC0489

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>Sprague Dawley Rats</th>
<th>Beagle Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC145 (3.8 mg/kg)</td>
<td>EC0489 (5.1 mg/kg)</td>
</tr>
<tr>
<td>t\textsubscript{1/2} (h)</td>
<td>0.23</td>
<td>0.14</td>
</tr>
<tr>
<td>t\textsubscript{1/2} (h)</td>
<td>1.89</td>
<td>0.42</td>
</tr>
<tr>
<td>C\textsubscript{0} (ng/ml)</td>
<td>4371.76</td>
<td>48034.7</td>
</tr>
<tr>
<td>AUC\textsubscript{0} ° (ng h⁻¹ mL⁻¹)</td>
<td>6140</td>
<td>6298</td>
</tr>
<tr>
<td>V\textsubscript{d} (ml)</td>
<td>119.7</td>
<td>117.9</td>
</tr>
<tr>
<td>V\textsubscript{c} (ml)</td>
<td>1041.4</td>
<td>529.1</td>
</tr>
<tr>
<td>Cl (ml/h)</td>
<td>601</td>
<td>814</td>
</tr>
</tbody>
</table>

For rats, pharmacokinetic parameters were calculated from EC145 or EC0489 plasma concentrations from three animals at each of eight time points. For dogs, individual pharmacokinetic parameters were calculated from EC145 or EC0489 plasma concentrations from five animals and are depicted as the mean ± S.D.
mean % body weight change for the treatment group from the pre-study control; n control rats (comparative representation from two separate studies, the untreated 1, 3, 5, 8, 10, and 12 (i.e., more aggressive schedule). Because this is a

EC145 control; n control rats (comparative representation from two separate studies, the untreated

1, 3, 5, 15, 17, and 19 or with EC0489 (F; 1.34 mol/kg) on days 1, 3, and 5 and 15, 17, and 19 of a 28-day cycle. As of September 2010, EC0489 had been safely dosed up to 2.5 mg/mg through 14-month-long cycles (ongoing). It is noteworthy that, although patients treated at this high dose level are experiencing toxicities that are more characteristic of vinca alkaloids (e.g., mild neuropathy), they have not experienced significant constipation or gastrointestinal-related toxicity. This latter observation is important in that, on a comparative molar basis, the 2.5 mg/mg dose level represents as much as a 70% increase in systemic delivery of vinca alkaloid equivalents relative to the EC145 human MTD. Although the phase I trial is ongoing, it seems that the preclinical prediction of greater vinca alkaloid delivery with less gastrointestinal toxicity has been realized.

Clinical Translation. On the basis of encouraging preclinical data, a phase I clinical study was implemented to assess the safety and pharmacokinetics of escalating intravenous bolus doses of EC0489. Eligible patients with adequate performance status and organ function that had previously failed standard therapy were treated with EC0489 on days 1, 3, and 5 and 15, 17, and 19 of a 28-day cycle. As of September 2010, EC0489 had been safely dosed up to 2.5 mg/mg through 14-month-long cycles (ongoing). It is noteworthy that, although patients treated at this high dose level are experiencing toxicities that are more characteristic of vinca alkaloids (e.g., mild neuropathy), they have not experienced significant constipation or gastrointestinal-related toxicity. This latter observation is important in that, on a comparative molar basis, the 2.5 mg/mg dose level represents as much as a 70% increase in systemic delivery of vinca alkaloid equivalents relative to the EC145 human MTD. Although the phase I trial is ongoing, it seems that the preclinical prediction of greater vinca alkaloid delivery with less gastrointestinal toxicity has been realized.

Discussion

Drug development nearly always focuses on the forward-looking translation of a preclinical finding into something clinically relevant. However, perhaps just as important to the clinical success of a drug development program is the capacity to translate an undesirable clinical observation backward into preclinical development to provide a solution for future therapeutic approaches. To that end, EC0489 was designed to potentially increase the tolerability of a targeted vinca alkaloid warhead while circumventing or reducing the dose-limiting constipation that was observed in a phase I clinical trial with EC145 (Sausville et al., 2007). This observed toxicity appeared likely to be the result of hepatobiliary elimination and duodenal deposition of EC145 and/or unconjugated DAVLBH, and it was consistent with the nature of the vinca alkaloid moiety.

Although EC145 has demonstrated the tolerability of a dose-dense administration of a potent vinca alkaloid warhead without precipitating myelosuppression, there is room for improvement. In an effort to alter the toxicity profile of EC145 without compromising its anti-tumor potency, numerous chemical analogs of this targeted agent were produced, each differing from the other only within the “spacer” unit (refer to Fig. 1). It is noteworthy that various forms of peptides, polymers (including short polyoxymethylene), carbohydrates, organic species, and heterogeneous combinations thereof were substituted for the Asp-Arg-Asp-Asp-Cys spacer of EC145. This library consisted of >50 compounds (a comprehensive report on the structural analogs of EC145 will be published elsewhere), and following a progressive screening process, EC0489 with its three novel glucitolyl-γ-glutamate residues emerged as a potential lead. Similar to EC145, EC0489 displayed high selective cytotoxicity for FR-express-
ing cells as well as curative activity against FR-expressing tumor xenografts, with regimens that caused no noticeable toxicity. It is important that, although not presented here, the water solubility of EC0489 analogs containing fewer (i.e., 1 or 2) glucitolyl-$\gamma$-glutamate residues was not conducive for parenteral administration, whereas four or more glucitolyl-$\gamma$-glutamate residues did not significantly improve the biological performance over EC0489. Therefore, EC0489 was selected for further testing.

When the pharmacokinetics of each drug were compared head-to-head, the distribution phase for both EC145 and EC0489 was virtually superimposable in both rats and dogs (see Fig. 3, a and b). This initial distribution phase probably contributes to folate receptor saturation; therefore, such similarity was expected because EC0489 was determined to be equipotent to EC145 against well established tumor xenograft models (see Fig. 6). The real difference appeared to be in the elimination half-life where EC0489 was found to clear faster than EC145 (confirmed in rat and dog). EC0489 displayed a significant reduction in biliary elimination, which at first glimpse did not correlate with the more rapid elimination rate for EC0489. In most cases, a reduction in liver clearance is associated with a reduction in elimination. However, with EC0489, we also observed a concomitant increase in urinary clearance, collectively suggesting that non-FR-bound compound was more favorably and more quickly eliminated by renal filtration. These beneficial changes were supported by toxicology studies where EC0489 was universally found to be less toxic to test animals. Because of these desirable improvements and the fact that folate-drug conjugates (including EC0489; Fig. 6) generally display steep dose-related preclinical anti-tumor responses (Reddy et al., 2007a; Leamon et al., 2008a,b), EC0489 was selected for clinical development. A phase I dose-escalation study was initiated for this agent in 2009. Although that trial is still in its preliminary stages, evidence has already been obtained showing that at least 70% more vinca alkaloid equivalents can be administered to patients in the form of EC0489 compared with EC145. Therefore, early observations seem to support the notion that the preclinical data presented within this report have translated clinically. Furthermore, it will be exciting to learn whether patient responses improve with EC0489 therapy because greater amounts of active drug may now be administered.

It is inevitable that the body clears parenterally administered pharmaceuticals, mostly via the liver and kidney. As exemplified here, certain agents may cause unwanted toxicity to tissues during the excretion process. Although the exact mechanism for hepatic clearance of folate-drug conjugates has yet to be identified, it is clear from the gastrointestinal toxicities in both preclinical and clinical studies that this route of elimination may contribute to undesirable off-target effects and a subsequent reduction in tolerability. The spacer (i.e., glucitolyl-$\gamma$-glutamate) of EC0489 has been integrated successfully into nearly all subsequently produced folate-drug conjugates and preclinical leads within our targeted drug programs for the treatment of FR-positive cancer and inflammatory diseases. The successful incorporation of this chemical modification may provide an enhancement to the benefits of targeted therapy, resulting in a more effective and tolerable treatment strategy for patients with life-threatening and difficult to treat diseases.

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Authorship Contributions

Participated in research design: Leamon, Reddy, Klein, Dorton, Messmann, LoRusso, and Sausville.

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Performed data analysis: Leamon, Reddy, Gehrke, Klein, and Nicoson.

Wrote or contributed to the writing of the manuscript: Leamon, Reddy, Klein, Messmann, and Vlahov.

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