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Preclinical Antitumor Activity of a Novel Folate-Targeted Dual Drug Conjugate

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Abstract: We have designed a new type of tumor-targeted agent by tethering two different drug molecules, with distinct biological mechanisms of action, to the same ligand. This compound, named EC0225, represents the "first in class" multidrug, folate receptor (FR)-targeted agent to be disclosed. It was constructed with a single folate molecule, extended by a hydrophilic peptide-based spacer, which was in turn attached to mitomycin and *Vinca* alkaloid units via two separate disulfide-containing linkers. EC0225 produced potent, dose-responsive activity *in vitro*, and curative activity was observed against FR-positive syngeneic and xenograft tumors following the administration of well-tolerated dosing regimens. Multiple complete responses and cures were also noted when EC0225 was used to treat mice initially bearing tumors as large as 750 mm^3 in volume. Overall, EC0225's impressive preclinical activity allowed for its selection as a development candidate and for the start of Phase 1 clinical trials, which began in March of 2007, for the treatment of advanced malignancies.

Keywords: Folate receptor; targeting; endocytosis; chemotherapy; *Vinca* alkaloid; mitomycin

Introduction

Most chemotherapeutic agents function by blocking or interrupting key cellular processes that control cell division. Examples include the perturbation of DNA synthesis, gene expression, or both with various alkylating agents and the disruption of the microtubule network with stabilizing or destabilizing compounds. In addition to cancer cells, many normal, rapidly proliferating cell types are unfortunately also killed during chemotherapy. As a consequence, severe side effects are observed, and clinical doses become limited due to the risk of severe toxicity.

In an effort to enhance a cancer drug's selectivity and simultaneously reduce unwanted toxicity (i.e., improve the therapeutic index), we and others have been developing targeted agents that display enhanced tumor-specific cell killing activity relative to their unconjugated drug counterparts. Our focus has been on the use of the small molecular weight ligand folic acid (or vitamin B9), which is capable of targeting covalently attached bioactive agents quite specifically and with very high affinity to folate receptor (FR)-positive cells and tissues.[1,2] The FR is a well-known tumor-associated protein, and it can actively internalize bound folates and folate–drug conjugates via the natural process of endocytosis.[1,3] This receptor is present at very high levels in most ovarian and other gynecological cancers, as well as at high to moderate levels in brain, lung, kidney, and breast carcinomas.[4–12] But, normal tissues that express the FR are apparently (i) inaccessible to blood-borne

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folates,[11,13] (ii) not adversely affected by folate–drug conjugates (e.g., the kidney),[14–16] or perhaps (iii) lack the critical quantity of FRs needed to elicit a biological response.[6,12,17] Because of this distinctive expression/accessibility pattern, the concept of "folate–drug targeting" has been pursued by many as an alternative method for treating FR-positive cancers; in fact, folate-targeted delivery of drug payloads as diverse as small imaging agents to large DNA-containing formulations have successfully been exemplified at both the preclinical and clinical levels [see ref 18 for a comprehensive overview of this technology].

Recently, our laboratory has published on the synthesis and preclinical pharmacology for novel folate conjugates of mitomycin C (MMC),[15,16] desacetylvinblastine monohydrazide (DAVLBH),[14,19,20] and maytansinoid DM1.[21] Throughout our continued endeavor, we have repeatedly observed pronounced antitumor effect against FR-expressing tumors in mice using well-tolerated treatment regimens (e.g., cures are typically observed under conditions that cause little to no weight loss). Experience has further taught us that antitumor activity with a monodrug conjugate (i.e., single drug moiety) is more often observed when the conjugated drug is intrinsically very potent, such as those with single digit nanomolar IC_{50} values *in vitro*.[14,19,20,22] Yet, we have also envisioned that the power of folate drug targeting could possibly be amplified by virtue of delivering more than one drug molecule per unit of folate. Certainly, when antibodies are exploited for drug targeting purposes, it is very typical to find an average of four to six drug molecules attached per IgG.[23–30] Obviously, there is a distinct therapeutic advantage to facilitate greater drug deposition within the tumor mass, and "multidrug" targeting approaches provide for a sensible solution.

Two distinct approaches for multidrug targeting were initially considered. The first option was to tether two

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molecules of the same drug to a single folate unit, whereas the second option involved the tethering of two different drug molecules having different mechanisms of action. Here, we exemplify the latter option with EC0225. This novel compound consists of a hydrophilic folate derivative anchored to both mitomycin and *Vinca* alkaloid drug units. The choice of these two drugs was based, in part, on our prior experience with their folate monodrug counterparts;[14,15,19,20] however, as described within, there was also reason to believe that these two agents might allow for greater than additive therapeutic responses. Overall, EC0225 represents the "first in class" multidrug folate-targeted agent to be disclosed; and as shown below, this molecule was found to be very active and specific against FR-expressing tumors. Importantly, due to EC0225's impressive preclinical performance, this agent was selected for development, and Phase 1 clinical trials were recently initiated for the treatment of advanced cancers.

Experimental Section

Materials. Pteroic acid and N^{10}-trifluoroacetylpteroic acid were prepared according to Xu et al.[31] Peptide synthesis reagents were purchased from NovaBiochem (La Jolla, CA) and Bachem (San Carlos, CA). Folate-free RPMI media (FFRPMI) and PBS were obtained from Gibco, Grand Island, NY. ^{3}H-thymidine was purchased from Moravek Biochemicals, Brea, CA. Vinblastine sulfate, mitomycin C, and all other common reagents were purchased from Sigma (St. Louis. MO) or other major suppliers.

Test Articles. DAVLBH, EC145, EC0225, folate-γ-ethylenediamine–fluorescein (EC17), and EC20 were produced by Endocyte, Inc. (West Lafayette, IN). Their syntheses, purifications, and analytical characterizations have been described in detail elsewhere.[14,32–35]

Cell Culture. KB cells are FR-positive human nasopharyngeal cells (ATCC). Madison 109 (M109) are FR-positive lung adenocarcinoma cells that are syngeneic to Balb/c mice (gift from Alberto Gabizon). 4T1 is a FR-negative breast carcinoma cell line that is also syngeneic to the Balb/c mouse (gift from former Rhone Poulenc Rorer, Inc.). All cells were maintained in folate-free RPMI (FFRPMI) containing 10% heat-inactivated fetal calf serum (HIFCS) as previously described.[14]

Relative Affinity Assay. The relative affinity of EC0225 was determined according to a previously described method using KB cells as the FR source.[14]

EC0225 *in Vitro* Dose Response. Cells were heavily seeded in 24-well Falcon plates and allowed to form nearly confluent monolayers overnight. Thirty minutes prior to the addition of EC0225, spent medium was aspirated from all wells and replaced with fresh FFRPMI. Note that designated wells received medium containing 100 μM EC17 (a nontoxic FR blocker) and were used to determine the targeting specificity. Following one rinse with 1 mL of fresh FFRPMI/HIFCS, each well received 1 mL of medium containing increasing concentrations of EC0225 (three wells per sample) in the presence or absence of 100 μM EC17, as appropriate. Treated cells were pulsed for 1 h at 37 °C, rinsed 4 times with 0.5 mL of medium, and then chased in 1 mL of fresh media up to 70 h. Spent medium was aspirated from all wells, and cell viability was assessed by a ^{3}H-thymidine incorporation assay, as previously described.[14]

***In Vivo* Antitumor Experiments.** Four- to six-week-old female *nu/nu* mice (Charles River, Wilmington, MA) or six- to seven-week-old female Balb/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were maintained on a standard 12 h light–dark cycle and fed *ad libitum* with Harlan diet no. TD00434 (Harlan Teklad, Madison, WI) for the duration of the experiment.[14] KB, M109, or 4T1 cells (1×10^6 per mouse) in 100 μL were injected in the subcutis of the dorsal medial area. Mice were divided into groups of four or five (as indicated), and test articles were freshly prepared and injected through the lateral tail vein under sterile conditions in a volume of 200 μL of phosphate-buffered saline (PBS). Intravenous (i.v.) treatments were typically

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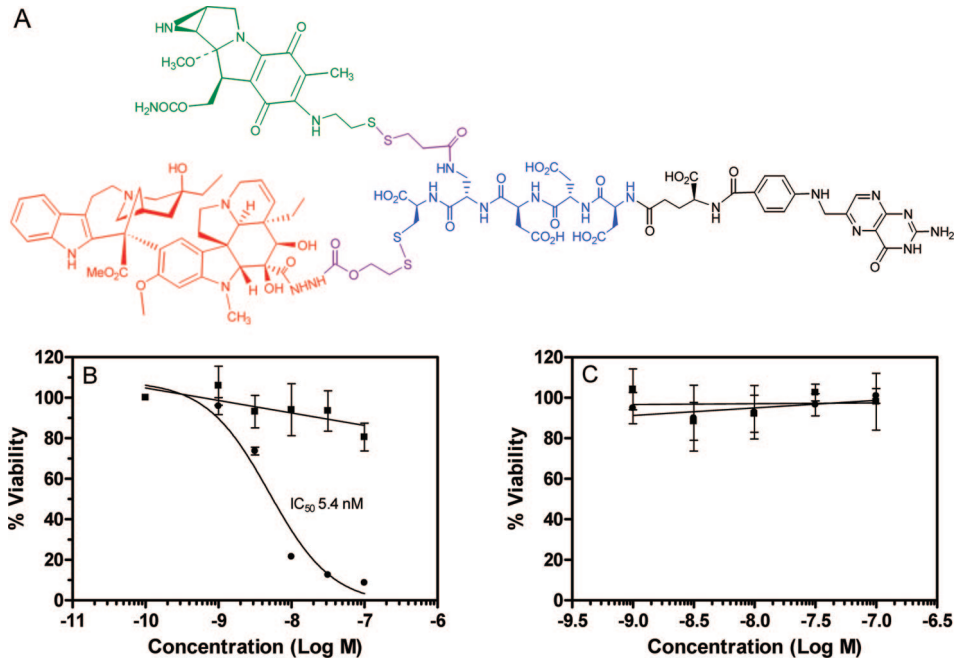

Figure 1. Structure, *in vitro* cytotoxic activity, and FR specificity of EC0225. Panel A shows the chemical structure of EC0225. Folic acid is shown in black, the hydrophilic peptide spacer (-Asp-Asp-Asp-βDpr-Cys-) is shown in blue, the linkers with biologically cleavable bonds are shown in purple, the *Vinca* alkaloid drug moiety (DAVLBH) is shown in red, and the DNA alkylating drug moiety (N^7-me-MMC) is shown in green. In panels B and C, FR-positive KB cells were treated for 1 h (panel B) and FR-negative 4T1 cells for 2 h (panel C) with increasing concentrations of EC0225 in the presence (■) or absence (●) of 0.1 mM EC17 (as a benign competitor). Following a 3-day chase in fresh medium, cells were incubated with ^{3}H-thymidine for the final 2 h in culture and then counted for radiolabel incorporation into newly synthesized DNA. Data represent the average ± 1SD ($n = 3$).

initiated when tumors were approximately 50–110 mm^3. In the "large" KB tumor study, mice were dosed 13 days PTI in the 250 mm^3 group, 20 days PTI in the 500 mm^3 group, and 24 days PTI in the 750 mm^3 group. Mice in all control groups received no treatment. Growth of each s.c. tumor was followed by measuring the tumor three times per week during treatment and twice per week thereafter until a volume of 1500 mm^3 was reached. Tumors were measured in two perpendicular directions using Vernier calipers, and their volumes were calculated as $0.5 \times L \times W^2$, where L = measurement of longest axis in mm and W = measurement of axis perpendicular to L in mm. As a general measure of toxicity, changes in body weights were determined on the same schedule as tumor volume measurements. Survival of animals was monitored daily. Animals that were moribund (or unable to reach food or water) were euthanized by CO_2 asphyxiation. All *in vivo* studies were performed in accordance with the American Accreditation Association of Laboratory Animal Care guidelines. For individual tumors, a complete response (CR) was defined as a disappearance of measurable tumor mass (<2 mm^3) at some point after tumor implantation. Cures were defined as CRs without tumor regrowth by study day 90.

Results

Structure of EC0225. The chemical structure of EC0225 is shown in Figure 1A. This molecule basically consists of five subparts: (1) folic acid is shown in black; (2) the hydrophilic peptide spacer, Asp-Asp-Asp-βDpr-Cys, is shown in blue; (3) biologically cleavable bonds are shown in purple; (4) the *Vinca* alkaloid drug moiety, DAVLBH, is shown in red;[14] and (5) the DNA alkylating drug moiety, N^7-mercaptoethyl-mitomycin C (N^7-me-MMC), is shown in green. Similar to the previously described mono-DAVLBH–folate conjugate, EC145 (a clinically investigated agent),[19] and the mono-N^7-me-MMC conjugate, EC72,[15] both linkers in EC0225 contain disulfide bonds. Importantly, such linkages are well-known to be cleaved within endosomal structures;[22,36] further, EC0225 was designed such that only the intact DAVLBH drug moiety (in red) and intact N^7-me-MMC moiety (in green) get efficiently released following the cleavage and spontaneous degradation of these linkers.[14,32,35]

Relative Affinity Assay. The affinity of EC0225 for the FR was evaluated using an *in vitro* assay that measures a ligand's ability to directly compete with folic acid for binding to its receptor. EC0225 was experimentally determined to have an affinity of 0.31 relative to that of folic acid for human FRs (KB cells; data not shown). Thus, chemical modification of folate with the DAVLBH and mitomycin drug motifs only minimally altered the vitamin's intrinsic affinity for the FR.

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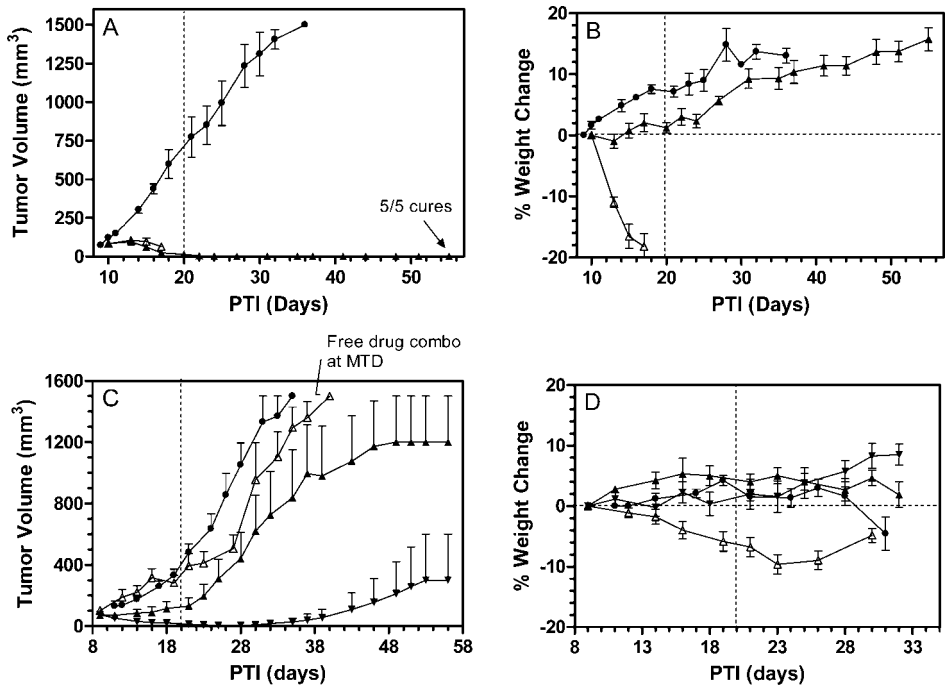

Figure 2. EC0225 activity is dose responsive, more active, and less toxic than the free drug combination. In panel A, nude mice bearing s.c. FR-positive KB tumors (83 ± 20 mm^3) were treated i.v. with (▲) 2 μmol/kg EC0225 or (△) 2 μmol/kg each of DAVLBH and MMC following a TIW, 2 week schedule. Changes in animal weights are shown in panel B. In panel C, Balb/c mice bearing s.c. M109 tumors (72 ± 14 mm^3) were treated i.v. with EC0225 at 1 μmol/kg (▲) or 2 μmol/kg (▼) or a 1 μmol/kg mixture of DAVLBH and MMC (△) following a TIW, 3 week schedule. Changes in animal weights are shown in panel D. Control cohorts (●) for either study were not treated. Dotted vertical lines represent the final day of dosing. $N = 5$ animals per cohort. PTI, post-tumor implantation.

In Vitro **Dose-Responsive Cytotoxicity and Specificity.** EC0225's dose-responsive activity and specificity were next evaluated *in vitro*. As shown in Figure 1B, EC0225's cell killing activity was found to be concentration dependent with an IC_{50} of ~5 nM when KB cells were pulsed for only a 1 h period at 37 °C. Importantly, although such short pulse incubations are not commonly used when evaluating the *in vitro* activity of chemotherapeutic drugs,[37] we feel that this approach is more meaningful for evaluating a targeted agent's activity *in vitro* since (i) a short incubation pulse should be effective for loading the high-affinity cell surface receptors with a targeted agent, (ii) folate–drug conjugates are known to rapidly distribute throughout the body and clear from the bloodstream within the first couple of minutes,[33,38] and (iii) tumors are not persistently bathed in the presence of drug.

Two common controls were used to evaluate EC0225's specificity.[22] The first involved the blocking of EC0225's activity with the use of an excess of free folate (or EC17 as shown), indicating that the observed cytotoxic activity was FR-mediated (see Figure 1B). The second method tested the cytotoxic effect of EC0225 against an FR-negative cell line; thus as shown in Figure 1C, EC0225 was not found to be toxic towards FR-negative 4T1 cells despite the fact that the drug exposure period was twice that for the KB cells. Taken together, the data in Figure 1 indicate that EC0225's potent cell-killing activity is specific for cells that express the FR.

In Vivo **Activity against FR-Positive Tumor Models.** EC0225 was first evaluated for antitumor activity against the well-known FR-positive KB tumor xenograft model.[19,33] As shown in Figure 2A, tumors in untreated animals reached ~1500 mm^3 by approximately day-34 post-tumor implantation (PTI). However, beginning when tumors were well-established (83 ± 20 mm^3), a regimen consisting of 2 μmol/kg EC0225, given intravenously (i.v.) and following a three times per week (TIW) 2 week schedule, was found to produce 5/5 cures (i.e., no measurable tumor growth past day-90 PTI). These remarkable effects also occurred in the apparent absence of weight loss (Figure 2B), and no gross toxicity or adverse events were noted in these animals during and after therapy. In contrast, therapy with 2 μmol/kg each of the unconjugated parent drugs, DAVLBH and MMC (and following the identical schedule used for EC0225), was found to be extremely toxic to the animals; although there was evidence of emerging antitumor activity, this untargeted regimen was clearly not tolerable (see Figure 2B). Subsequent studies confirmed that the maximum tolerated dose

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(MTD) for the co-administration of these two untargeted agents in this and other models was ~1 μmol/kg each. Therefore, the performances of the free drug combination and of EC0225 were next evaluated in a second FR-expressing tumor, the therapeutically challenging M109 model.[39] The M109 tumor is an FR-positive lung adenocarcinoma that is syngeneic to the Balb/c mouse strain. Despite this model being rather chemoresistant, it has previously been used to document *in vivo* activity of various folate-targeted chemotherapeutics.[14,16,40] As shown in the Figure 2C, a TIW, 3-week regimen of EC0225 was found to be highly effective against M109 tumors under conditions where animals once again did not appreciably lose weight. EC0225's activity was found to increase with dose, and four of five mice treated with a 2 μmol/kg dose level experienced complete responses (CRs). In contrast, treatment with the untargeted drug mixture of DAVLBH and MMC at their MTDs (1 μmol/kg each when in combination) was found to be ineffective against the M109 tumor. Notably, animals did continue to lose weight for a period of time after the dosing with the untargeted agents had ended, which is a characteristic trait of all other mitomycin-based therapies.[41] Clearly, EC0225 offered a distinct therapeutic advantage over the unconjugated drugs.

EC0225's Targeted Antitumor Activity Is Specific. One method that can be used to demonstrate an agent's FR-specific activity *in vivo* is to co-dose with an excess of either folic acid or a benign high-affinity folate analog.[16,19] Under such competitive conditions, therapeutic benefit is predicted to be compromised or even ablated. We currently favor using a rhenium chelate of EC20 (pteroyl-γ-D-Glu-βDpr-Asp-Cys)[33] as the competitor simply because this nontoxic agent is more hydrophilic and can be dosed at higher levels than folic acid without precipitating in the kidneys (ref 42 and unpublished observations). As shown in Figure 3A, EC0225 was again found to produce 5/5 CRs against established s.c. KB tumors in *nu/nu* mice in a manner that did not produce weight loss (compare with Figure 2). Importantly, four of these five animals remained tumor-free by day 90 and were therefore considered to be cured (data not shown). In contrast, when co-dosed with a modest 40-fold excess of Re-EC20, EC0225's activity was determined to be compromised, since no CRs or cures resulted in this "competed" cohort. This outcome indicated that EC0225's activity was predominantly dependent on binding to tumor-associated FRs.

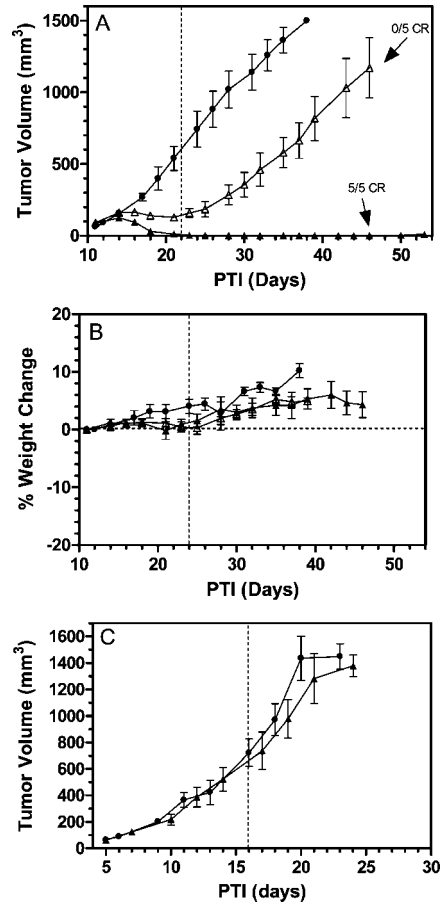

Figure 3. Target-specific *in vivo* activity of EC0225. In panel A, animals bearing s.c. KB tumors (90 ± 23 mm^3) were treated i.v. with 1 μmol/kg of EC0225 alone (▲) or co-dosed with a 40-fold excess of Re[pteroyl-γ-D-Glu-βDpr-Asp-Cys] (△, a nontoxic folate-based peptide ligand) TIW for 2 consecutive weeks. Panel B shows animal weight change due to EC0225 therapy. In panel C, Balb/C mice bearing s.c. 4T1 tumors (63 ± 7 mm^3) were treated i.v. with 2 μmol/kg of EC0225 (▲) following a TIW, 2 week schedule. Control cohorts (●) for either study were not treated. *N* = 5 animals per cohort. Dotted vertical lines represent the final day of dosing. CR, complete response. PTI, post-tumor implantation.

As a second measure for target specificity, EC0225 was tested for activity against a recognized FR-negative tumor model, the murine breast carcinoma 4T1.[14,16] As shown in Figure 3C, despite using an established, therapeutically effective dosing regimen (2 μmol/kg, TIW, 2 weeks; compare with Figure 2A), EC0225 did not display any antiproliferative activity against the s.c. 4T1 tumors. Coupled with the *in vivo* competition studies shown in Figure 3A, these results confirm that EC0225's activity *in vivo* is predominantly specific for FR-expressing tumors.

EC0225 versus the Combination of Its Individual Folate–Monodrug Counterparts. As described above, EC0225 is a folate conjugate containing two distinct drug moieties (DAVLBH and N^7-me-MMC), each having different

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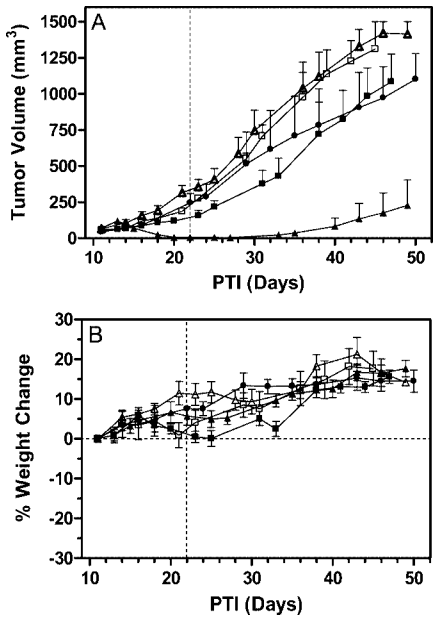

Figure 4. EC0225's activity is superior to the combination of individual folate–drug counterparts. Animals bearing s.c. KB tumors (64 ± 12 mm^3) were treated i.v. starting on day 11 with 1 μmol/kg of test articles following a TIW, 2 week schedule. Panel A shows activity; panel B shows weight loss. EC0225 (▲; $N = 4$), EC145 (■; $N = 5$), EC72 (□; $N = 4$), EC145 + EC72 (△; $N = 5$). Dotted vertical line = day of final dose; (●), untreated.

biological mechanisms of action. These oncolytic agents were evaluated for their possible ability to produce greater than additive effects against the growth of FR-positive tumors (see discussion). Although EC0225 has displayed powerful preclinical antitumor activity against multiple FR-positive tumor models (*vide infra*), we questioned whether a simple mixture of independent folate–"monodrug" conjugates might provide similar *in vivo* activity. Thus, animals bearing s.c. KB tumors were treated with 1 μmol/kg of either (i) EC0225, (ii) a disulfide-linked folate–DAVLBH conjugate (EC145),[19,20] (iii) a disulfide-linked folate–N^7-me-MMC conjugate (EC72),[15,16] or (iv) a 1:1 mixture of EC145 and EC72. Each test article/formulation was evaluated using a TIW, 2 week schedule. Importantly, EC72 was previously shown not to be active against well-established s.c. KB tumors,[16] and EC145's activity was predicted to be marginal when using this low dose and brief treatment schedule (unpublished observations).

As shown in Figure 4, EC0225 was found to provide marked antitumor activity, where KB tumors in 4 of 4 treated animals had completely regressed during therapy; notably, only 1 of the 4 EC0225-treated animals had a recurrent tumor form at approximately 10 days past the cessation of dosing. In contrast, animals treated with EC145 had experienced a modest growth delay, and those treated with EC72 did not receive any therapeutic benefit. Perhaps the most striking result of this study was that animals treated with the equimolar mixture of EC145 and EC72 also failed to receive any therapeutic benefit. The reason for this outcome could possibly be explained by the information provided in Table 1. Two important facts should be considered. First, the affinities of each of the folate-targeted agents for the FR are very similar (refs 15, 19 and this report); thus, it is reasonable to assume that each agent potentially has equal capability for binding to and being retained within the tumors. Second, the administered dose level was close to that required to achieve FR saturation.[43] Therefore, given a fixed level of FRs within the tumor (represented as X in the example of Table 1), EC0225 would be predicted to deliver X molecules each of DAVLBH and N^7-me-MMC. In contrast, EC145 could only deliver X molecules of DAVLBH to the tumor, while EC72 would deliver only X molecules of N^7-me-MMC. Because a simple 1:1 mixture of EC145 and EC72 created an inherent "competitive" situation, one might expect that only about $0.5X$ of each drug component would be bound to the tumor. Obviously, the latter situation would result in compromised activity against the tumor, which is what we observed experimentally. Regardless, it was clear from this study that tethering both DAVLBH and N^7-me-MMC to the same folate molecule was the best of the four different therapeutic strategies.

EC0225 Is Highly Active against Large Tumors. EC0225's activity against s.c. tumors was tested in a separate study where the onset of intravenous therapy was delayed until the volumes of human KB tumor xenografts reached an average of either 250, 500, or 750 mm^3. Therapy consisted of 2 μmol/kg of test article given intravenously according to a TIW, 3 consecutive week schedule. As shown in Figure 5A, KB tumors decreased in volume shortly after each dosing period began. Further, the tumors completely regressed in a manner that was seemingly independent of the initial tumor volume size. Remarkably, animals receiving EC0225 therapy lost very little weight (≤4%) during the 3-week dosing period, and these effects were practically indistinguishable from that in the control animals (Figure 5B).

Preliminary Toxicity Assessment. Aside from minimal weight loss during EC0225 therapy, no other obvious gross toxicities were observed. Euthanasia, when applied, was principally due to the tumors reaching 1500 mm^3 or to end the study. Formal GLP toxicology studies have been completed with EC0225 in both rats and dogs to support the filing of an Investigational New Drug (IND) application. A full report on the findings in those studies will be published elsewhere. However, it may be interesting to note that at all dose levels studied (up to and beyond the MTD), there was no evidence of neuropathy, renal damage, or even delayed myelosuppression. The latter is remarkable because it is a

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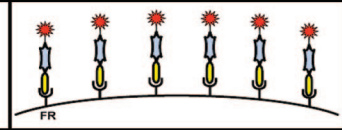
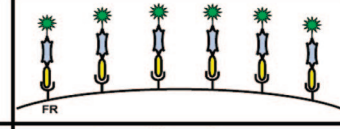
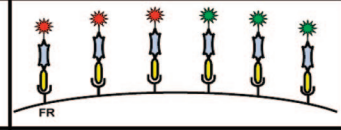
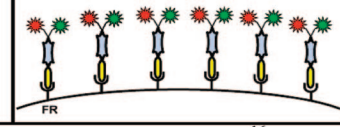
Table 1. Advantage of Multidrug Targeting. EC0225 is capable of delivering 1 molecule each of DAVLBH (red star) and N^7-me-MMC (green star) per FR per tumor cell. At best, an equimolar mixture of EC145 and EC72 can deliver half as much of each drug per tumor cell as compared to EC0225. Yellow oval, folic acid; blue hexagonal-like structure, molecular spacer.

Cohort Number	Test Articles		Cell Membrane Diagram: Cell with X Folate Receptors	Predicted Outcome	
	Name	*Drug Component(s)*		*Drug Load*	*Expected Activity*
1	EC145	DAVLBH		1X DAVLBH	Active
2	EC72	N^7-me-MMC		1X N^7-me-MMC	Inactive[a]
3	EC145 EC72	1 eq. EC145 1 eq. EC72		0.5X DAVLBH 0.5X N^7-me-MMC	Compromised
4	EC0225	DAVLBH and N^7-me-MMC		1X DAVLBH 1X N^7-me-MMC	Maximum

[a] EC72 is not active against well-established, subcutaneous tumors.[16]

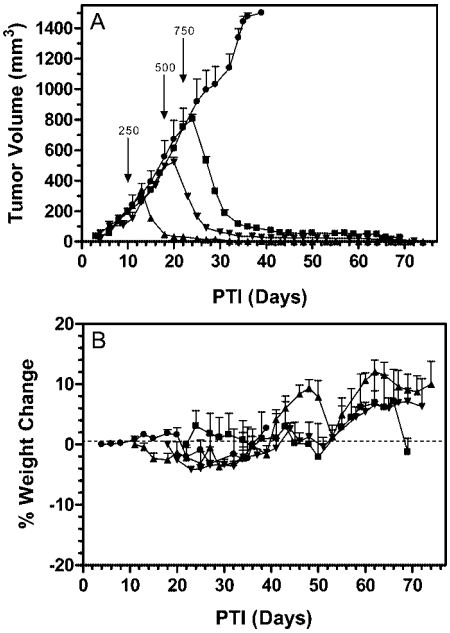


Figure 5. Activity of EC0225 against large subcutaneous human tumor xenografts. Animals bearing large s.c. KB tumors were treated i.v. with 2 μmol/kg of EC0225 following a TIW, 2 week schedule. Panel A shows antitumor activity; panel B shows animal weight change due to EC0225 therapy. Arrows indicate the onset of dosing for distinct cohorts. Untreated (●); EC0225 treated, 250 mm^3 tumors (▲); 500 mm^3 tumors (▼); 750 mm^3 tumors (■).

characteristic trait of mitomycin toxicity.[41] Particular findings that defined the MTD were related to proliferative compartment toxicities, such as the gastrointestinal tract and the hematopoietic system (manuscript in preparation). But, all toxicities were declared to be reversible.

Discussion

Based on a solid history for producing novel folate-targeted chemotherapeutic agents,[14–16,19–22] our laboratory has recognized the probable benefit for not only tethering multiple copies of the same drug to the tumor-targeting ligand folate but also the simultaneous tethering of different drug molecules that function by distinct biological mechanisms of action to this vitamin. EC0225 represents the "first in class" multidrug, folate-targeted agent to be disclosed. It was constructed with a single folate moiety, extended by a hydrophilic peptide-based spacer, which in turn was attached to *Vinca* alkaloid and mitomycin units via two distinct disulfide-containing linkers. Despite its large, bulky size (molecular weight 2327 g/mol), EC0225 was found to retain high affinity for FR-positive cells, and it produced potent dose-responsive activity *in vitro* via an endocytic mechanism. EC0225 also proved to be very active against syngeneic and xenograft *in vivo* models, with curative activity occurring with the administration of well-tolerated regimens.

As previously reported, the monodrug N^7-me-MMC–folate conjugate, EC72, was found to be active against young tumors (i.e., recently implanted), but its therapeutic potential decreased with respect to the increasing size of subcutaneous tumors.[15,16] This property was further substantiated in this new study (see Figure 4) where EC72 again failed to exert activity against well-established tumors. However, EC72 was subsequently found to potentiate the antitumor efficacy of paclitaxel against well-established tumors with no apparent increase in toxicity, thereby suggesting a possible synergistic

relationship with this microtubule inhibitor.[16] Searching for a possible explanation, we accepted the findings of Ihnat et al., which showed that cells exposed to subtoxic dose levels of mitomycins experience a marked decrease in *p*-glycoprotein (pgp) expression with a concomitantly dramatic reduction in multidrug resistance.[44] Thus, it appeared sensible that rational combinations of our targeted mitomycin analog along with any potent chemotherapeutic/pgp substrate (e.g., paclitaxel) might in fact lead to greater antitumor responses. Similar to most taxanes, *Vinca* alkaloids are also substrates for pgp;[45] however, we have reproducibly observed that *Vinca* alkaloids (like DAVLBH) are the more potent of the two cell-killing agents. Knowing that cells have a defined quantity of FRs, and that suprasaturable dose levels will not likely translate into greater tumor cell uptake (because folate conjugates are very hydrophilic species that are not observed to enter cells by simple diffusion), we opted to construct EC0225 with N^7-me-MMC and DAVLBH (instead of paclitaxel). Our choice for these two agents was admittedly also influenced by the fact that combination regimens of mitomycins and *Vinca* alkaloids are currently being used in clinical practice.[46,47]

There are many obvious advantages for the simultaneous targeting of multiple drugs to tumors, including the potential for increasing the antitumor effect in a manner that does not appreciably increase toxicity. We believe that those properties are effectively exemplified with the data shown in Figure 4. However, whether one designs the multidrug conjugate to deliver different drugs (like EC0225) or multiple copies of the same drug (Endocyte, Inc., unpublished data), it is likely that either strategy would be more effective against tumors that (i) express lower levels of the FR such that monodrug conjugates are only moderately effective or (ii) have slower FR recycling rates. Our laboratory favors the approach where different drug moieties are used in the conjugate design because it provides for the potential to develop greater than additive or even synergistic responses. Future challenges will nonetheless be related to the rational choice of agents to use in this "targeted cocktail" setting.

EC0225's prospects are encouraging. Current work is devoted towards understanding the pharmacokinetics and disposition of EC0225 and its metabolites. In addition, it is of interest to determine whether tumor-associated pgp activity plays a role in EC0225's overall activity. Regardless, due to the successful completion of multiple GLP toxicology studies, and together with the recent scale up and production of GMP quality bulk drug substance (Endocyte, Inc.), we are excited to report that EC0225 did successfully enter Phase 1 clinical trials in the first quarter of 2007 for the treatment of refractory solid tumors.

Abbreviations Used

DAVLBH, desacetylvinblastine monohydrazide; FR, folate receptor; GLP, good laboratory practice; GMP, good manufacturing practice; MMC, mitomycin C; N^7-me-MMC, N^7-mercaptoethyl-MMC; s.c., subcutaneous; TIW, three times per week; M109, Madison 109; FFRPMI, folate-free RPMI media; HIFCS, heat-inactivated fetal calf serum; PTI, post-tumor implantation; CR, complete response; MTD, maximum tolerated dose.

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