Research Article

Folate Targeting Enables Durable and Specific Antitumor Responses from a Therapeutically Null Tubulysin B Analogue

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Abstract

The membrane-bound high-affinity folate receptor (FR) is highly expressed on a wide range of primary and metastatic human cancers, such as those originating in ovary, lung, breast, endometrium, kidney, and brain. Because folate-linked conjugates bind to and become internalized within FR-expressing cells (similar to that of free folic acid), we explored the possibility of using the folate ligand to target a potent, semisynthetic analogue of the microtubule inhibitor tubulysin B to FR-enriched tumors. When tested *in vitro*, a novel folate conjugate, herein referred to as EC0305, was found to specifically inhibit the growth of a panel of FR-positive cell lines (IC_{50} range, 1–10 nmol/L) in a dose-dependent manner, whereas cells lacking FR expression were unaffected. The potency of EC0305 was also confirmed against a human KB xenograft-*nu/nu* mouse cancer model. Here, a brief three times per week, 2-week regimen yielded remarkable antitumor activity (100% tumor-free animals) without causing significant weight loss or major organ tissue degeneration. In contrast, antitumor activity was completely abolished in EC0305-treated animals that were co-dosed with an excess of a nontoxic folate-containing analogue, thereby confirming that the antitumor effect of this agent was mediated by FRs. The advantage provided by folate conjugation was further proved by the untargeted free drug, which was found to be completely inactive at both tolerable and highly toxic dose levels. Collectively, these results show that this potent antiproliferative tubulysin compound can be specifically delivered to FR-positive tumors to provide substantial therapeutic benefit using well-tolerable dosing regimens. [Cancer Res 2008;68(23):9839–44]

Introduction

Targeting chemotherapeutic agents more selectively to tumors can be realized through the use of covalently attached, high-affinity ligands that bind to cell surface receptors. Such approaches are expected to produce antitumor responses without much of the associated off-target toxicities that commonly limit the utility of any drugs. Thus, ligands such as monoclonal antibodies, peptides, and vitamins have been evaluated for their ability to enhance the activity of certain pharmacologic agents; further, the activities of these ligand-drug "conjugates" are sometimes found to be enhanced to levels much above that produced by their untargeted drug counterparts (1–7).

Our laboratory has focused on the development of small molecule targeting systems based on folic acid (FA or folate) to deliver covalently linked therapeutic or imaging agents to cells that express the folate receptor (FR; refs. 8–11). FA has high affinity (K_d ~0.1 to 1 nmol/L) for the FR, which consequently is a biomarker protein that is expressed in high quantities by many primary and metastatic cancers (12–14), but not on most normal cells (15). FA has been used for the delivery of a wide range of drugs, and promising preclinical activity has repeatedly been observed (2, 8, 9, 11, 16–24). Because FA-linked drugs will efficiently bind to FRs and become internalized by receptor-mediated endocytosis, we have explored the ability of FA to target the highly potent antitubulin agent tubulysin B (Tub-B).

Tubulysins are natural peptidic-like compounds produced by certain myxobacteria (25). Unlike taxanes and epothilones, tubulysins mimic the tubulysin-destabilizing ability of the *Vinca* class of agents; however, their *in vitro* GI_{50}s are typically 20- to 1,000-fold greater (26). Unfortunately, tubulysins display extraordinarily high toxicity when dosed in the untargeted form, a problem that has (*a*) delayed further development of this class of compounds (27) and (*b*) supported our efforts toward producing a safer and more tumor-specific form of tubulysin. As shown below, we describe the biological performance of the first ligand-tubulysin conjugate to be reported. This compound, herein called EC0305, represents a water-soluble, highly potent, and tolerable form of tubulysin that preferentially targets cells expressing the FR.

Materials and Methods

Materials. Tub-B was purchased from R&D Biopharma. Tubulysin B-hydrazide (Tub-B-H) and EC0305 were synthesized as previously described.[1] Re[pteroyl-γ-D-Glu-βDpr-Asp-Cys] is a nontoxic folate-based peptide ligand prepared according to Leamon and colleagues (28). Folate-γ-ethylenediamine-fluorescein (EC17) was prepared according to Lu and Low (19). All other chemicals were of reagent grade and obtained from major suppliers.

Cell culture. All cell lines used for this study were obtained from American Type Culture Collection, the Purdue Cancer Center (West Lafayette, IN), or research collaborators. Cells were grown continuously as a monolayer using folate-free RPMI medium (FFRPMI) containing 10% heat-inactivated FCS (HIFCS) at 37°C in a 5% CO_2/95% air-humidified atmosphere with no antibiotics. The HIFCS contains endogenous folates at concentrations sufficient for FR-expressing cells to survive and proliferate in this medium (16), which consequently is more physiologically relevant than typical cell culture media, which contain 100- to 1,000-fold higher levels of folates. All cell culture experiments were done using FFRPMI containing 10% HIFCS (FFRPMI/HIFCS) as the growth medium.

Relative affinity assay. The relative affinity of EC0305 was determined according to a previously published procedure (29).

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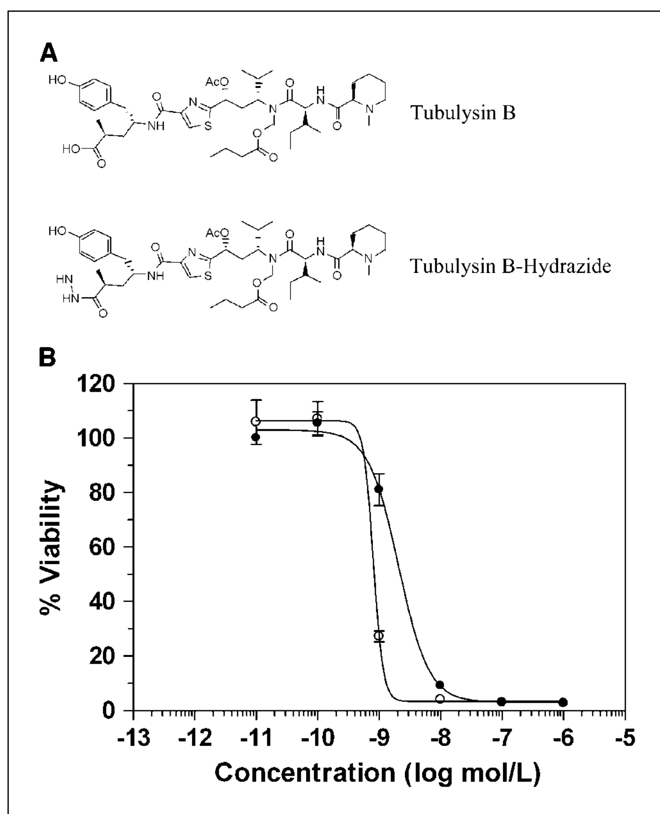

Figure 1. Potency of Tub-B and its analogue Tub-B-H. *A,* structures of natural Tub-B and the semisynthetic analogue Tub-B-H. *B,* 72-h dose responsive activity of tubulysins against KB cells. ○, Tub-B; ●, Tub-B-H. *Bars,* SD ($n = 3$).

FR quantitation. FR levels for each cell line tested were quantitated according to a previously reported procedure (15).

Dose-dependent, FR-specific activity of EC0305. A panel of FR-positive and FR-negative cells were seeded in individual 12-well Falcon plates and allowed to form nearly confluent monolayers overnight in FFRPMI/HIFCS. Thirty minutes before the addition of EC0305, spent medium was aspirated from all wells and replaced with either fresh FFRPMI or FFRPMI supplemented with 100 mol/L FA or 100 μmol/L EC17. At this concentration, free FA (or EC17) completely blocked the binding of EC0305 to FR on the cell surface, thus revealing the extent of nontargeted FR-independent cytotoxicity of EC0305. Each well then received 1 mL of medium containing increasing concentrations of EC0305 (three wells per sample). Cells were pulsed for 2 h at 37°C, rinsed four times with 0.5 mL of medium, and then chased in 1 mL of fresh medium up to 72 h. Spent medium was aspirated from all wells and replaced with fresh medium containing 5 μCi/mL of [^{3}H]thymidine. Following a 2-h incubation at 37°C, cells were washed thrice with 0.5 mL PBS and then treated with 0.5 mL of ice-cold 5% trichloroacetic acid per well. After 15 min, the trichloroacetic acid was aspirated and the cells solubilized by the addition of 0.5 mL of 0.25 N sodium hydroxide for 15 min at room temperature. Four hundred fifty microliters of each solubilized sample were transferred to scintillation vials containing 3 mL of Ecolume scintillation cocktail and counted in a liquid scintillation counter. Final results were expressed as the percentage of [^{3}H]thymidine incorporation relative to untreated controls.

Tumor model and therapy. Six- to eight-week-old female *nu/nu* mice (Charles River) were maintained on a standard 12-h light/12-h 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 wk before tumor implantation and maintained throughout the experiment to achieve serum folate concentrations closer to the range of normal human serum (30). FR-positive KB cells (1×10^6 per *nu/nu* mouse) in 100 μL of FFRPMI containing 1% BALB/c serum were injected in the subcutis of the dorsal medial area. Tumors were measured in two perpendicular directions

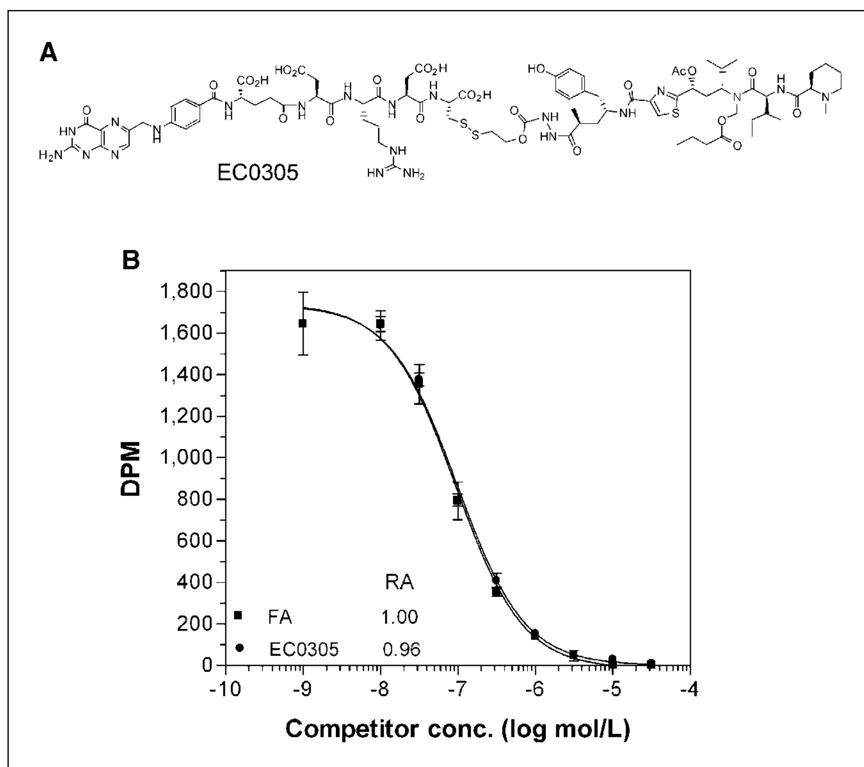

Figure 2. Relative FR binding affinity of EC0305. *A,* structure of EC0305. *B,* relative affinity. KB cells were incubated for 1 h at 37°C with 100 nmol/L ^{3}H-FA in the presence and absence of increasing competitor concentrations. ■, FA; ●, EC0305. *Bars,* SD ($n = 3$). *DPM*, disintegrations per minute.

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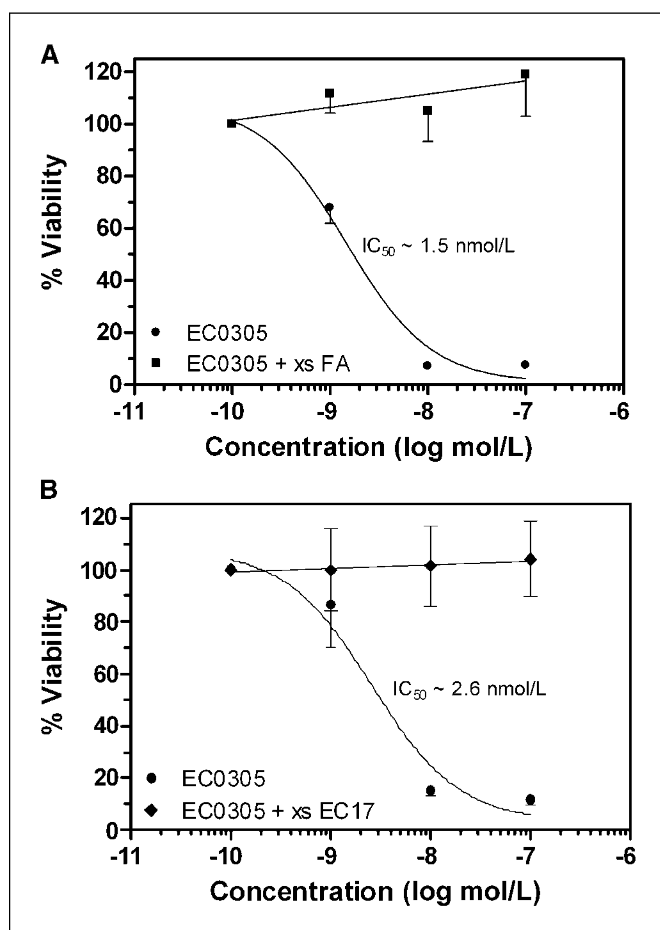

Figure 3. EC0305 *in vitro* activity and specificity. Cells were pulsed for 2 h with increasing concentrations of EC0305 in the absence (●) or presence of either 100 mol/L FA (■) or EC17 (◆; as benign competitors). Following a 70-h chase in fresh media, cells were incubated with [^{3}H]thymidine for 2 h and then counted for radiolabel incorporation into newly synthesized DNA, as described in Materials and Methods. *A,* KB cells. *B,* RAW 264.7 cells. *Points,* average (n = 3); *bars,* SD.

every 2 to 3 d with a caliper, and their volumes calculated as $0.5 \times L \times W^2$, where L is the longest axis (in millimeters) and W is the axis perpendicular to L (in millimeters). Dosing solutions were prepared fresh each day in PBS and administered through the lateral tail vein of the mice. Importantly, dosing was initiated when the s.c. tumors were in the range of 74 ± 14 mm^3 in volume. Drug toxicity was assessed by collecting blood via cardiac puncture and submitting the sera for independent analysis of blood urea nitrogen, creatinine, the liver enzymes aspartate aminotransferase (AST-SGOT) and alanine aminotransferase (ALTSGPT), as well as whole blood samples for hematologic changes to Ani-Lytics, Inc. In addition, histopathologic evaluation of formalin-fixed heart, lungs, liver, spleen, kidney, intestine, skeletal muscle, and bone (tibia/fibula) was done at Animal Reference Pathology Laboratories (ARUP).

Results

Tub-B-H is a potent cytotoxic molecule *in vitro*. The IC_{50} values for the natural Tub-B compound and its semisynthetic hydrazide counterpart (Fig. 1*A*) were assessed *in vitro* by measuring the extent of [^{3}H]thymidine incorporation into the DNA of KB cells continually exposed to the drugs for 72 h. As shown in Fig. 1*B*, both agents displayed dose-dependent cytotoxicity, with Tub-B having an IC_{50} 0f 0.8 nmol/L and Tub-B-H at 2.5 nmol/L. Thus, addition of the hydrazine moiety to Tub-B-H (and presumably the introduction of a positive charge under physiologic pH) only reduced the intrinsic potency of this tubulysin by 3-fold.

EC0305 displays high binding affinity for the FR. A folate conjugate of Tub-B-H was constructed with the identical folate-spacer linker modules found in EC145, a folate-desacetylvinblastine conjugate that is currently in phase 2 clinical trials for the treatment of ovarian and non–small-cell lung carcinomas (1, 31, 32). The structure of this new agent (EC0305) is shown in Fig. 2*A*. It represents the first disclosed, highly water-soluble folate conjugate of tubulysin.

The binding affinity of EC0305, relative to FA, was first evaluated using an *in vitro* competition assay (33). As shown in Fig. 2*B*, the affinity of EC0305 toward the FR was determined to be nearly identical to that of FA (0.96 relative to FA). Thus, conjugation of the Tub-B-H molecule did not alter the intrinsic binding affinity of the vitamin to its native biological receptor.

EC0305 cytotoxicity is dose dependent and FR specific. The cytotoxicity and specificity of EC0305 were evaluated against a small panel of cell lines. As shown in Fig. 3*A* and *B*, FR-positive KB and RAW 267.4 cells were determined to be highly sensitive to EC0305, with IC_{50} values of 1.5 and 2.6 nmol/L, respectively. Importantly, this level of activity is $\sim$3- to 10-fold greater than that observed for previously reported folate-drug conjugates against FR-positive KB cells (2, 29, 31, 33). Interestingly, these values are

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Table 1. Correlation between FR expression levels and EC0305 activity

Cell model	Species	Cancer type	FR status		IC_{50} (nmol/L)	Competable (FR specific)
			Level (pmol/mg)	Status		
KB	Human	Nasophyngeal CA	146	Strong	1.5	Yes
4T1-cl2	Mouse	Breast CA	90	Strong	10	Yes
RAW 264.7	Mouse	CML	58	Moderate	2.6	Yes
IGROV	Human	Ovarian CA	45	Moderate	2	Yes
4T1 parent	Mouse	Breast	1.6	Negligible	>1,000	N/A
A549	Human	Lung	1.3	Negligible	>1,000	N/A

NOTE: Data are presented as average ± SD (n = 3).
Abbreviations: CA, cancer; CML, chronic myelogenous leukemia.

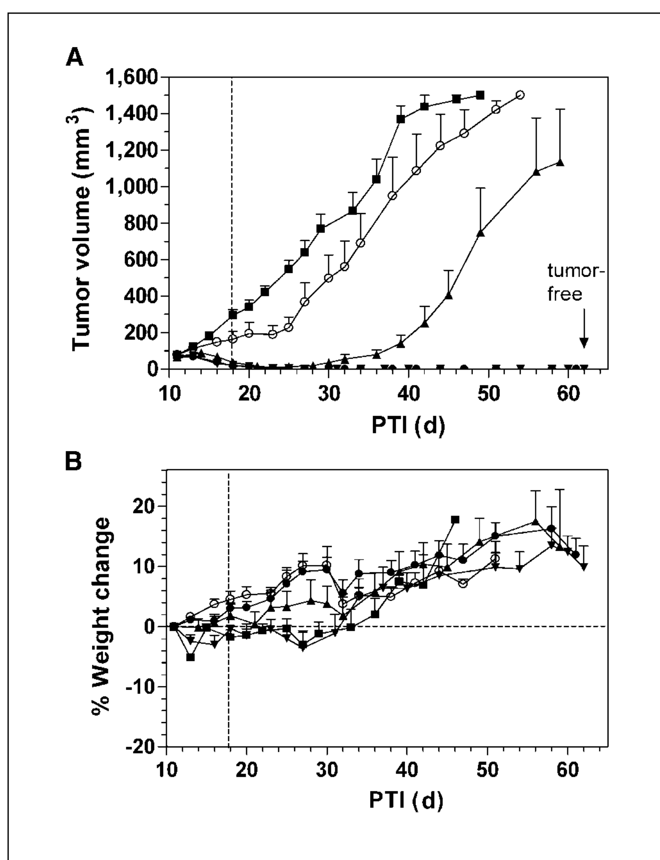

Figure 4. Effect of EC0305 on the growth of s.c. FR-positive KB tumors. KB tumor cells (1×10^6) were implanted s.c. into *nu/nu* mice, and 11 d later, mice were randomized and treated following a three times per week, 2-wk schedule. ■, untreated mice; ▲, ●, ▼, groups treated with EC0305 at 0.5, 1 and 2 μmol/kg, respectively; ○, cohort treated with 1 μmol/kg EC0305 plus 40 μmol/kg of Re[pteroyl-γ-D-Glu-βDpr-Asp-Cys] (a nontoxic folate-based peptide ligand). *Points,* average tumor volume from five animals; *bars,* SD. *Dotted vertical line,* day of final dosing (day 22). *PTI*, post tumor innoculation.

also nearly identical to that produced by the free drug when incubated with cells for 70 straight hours (see Fig. 1*B*). Excess folic acid or folate-γ-ethylenediamine-fluorescein (EC17) were both found to completely abrogate EC0305 cytotoxicity (Fig. 3*A* and *B*), thus indicating that the observed activity was specifically mediated by FRs. Table 1 contains a summary of EC0305 performance against a panel of both FR-positive and FR-negative cell lines. Clearly, this new agent seems to be quite active and selective for those cells expressing the FR.

Folate targeting enables durable and specific antitumor responses for a drug with no therapeutic window. EC0305 antitumor activity and specificity were next evaluated against *nu/nu* mice bearing well-established (~ 80 mm^3) s.c. KB tumors. Animals were treated i.v. with increasing dose levels of EC0305 following a brief three times per week, 2-week regimen. As shown in Fig. 4*A*, tumors in the untreated animals rapidly proliferated and reached ~1,500 mm^3 by approximately day 42, whereas tumors in the EC0305-treated cohorts quickly regressed at all tested dose levels. Animals in the lowest-treated cohort (0.5 μmol/kg) displayed strong antitumor effect, with two mice experiencing complete responses and the other three having partial responses. In fact, tumor volumes in the remaining three animals averaged only 15 mm^3 by day 23. However, all of the animals in the two highest cohorts (1 and 2 μmol/kg) were found to be tumor-free by day 27, and that response persisted until the end of the study (day 90; data not shown). As shown in Fig. 4*B*, no appreciable weight loss was observed during and after each of these regimens (the latter being a common observation for folate-targeted drug therapies refs. 1, 2, 24, 29). Importantly, EC0305 activity was confirmed to be FR specific because a coinjected 40-fold excess of Re[pteroyl-γ-D-Glu-βDpr-Asp-Cys] was found to completely block any antitumor effect (Fig. 4*A*, compare *filled circles* to *open circles*).

Free drug (as Tub-B-H) was given to mice at dose levels spanning 0.5 to 1 μmol/kg following the same three times per week, 2-week schedule. As shown in Fig. 5*A* and *B*, untargeted Tub-B-H produced no significant antitumor effect over this dose range; instead, animals experienced dose-dependent toxicity as measured by progressive weight loss and near-moribund behavior (at the highest dose level). Importantly, animals in the 1 μmol/kg Tub-B-H cohort could only tolerate two consecutive doses, further reflecting the toxicity of this untargeted drug. Because no antitumor effect was observed, and toxicity was manifested only at the higher dose levels, Tub-B-H was declared to be an agent with no therapeutic margin against this model.

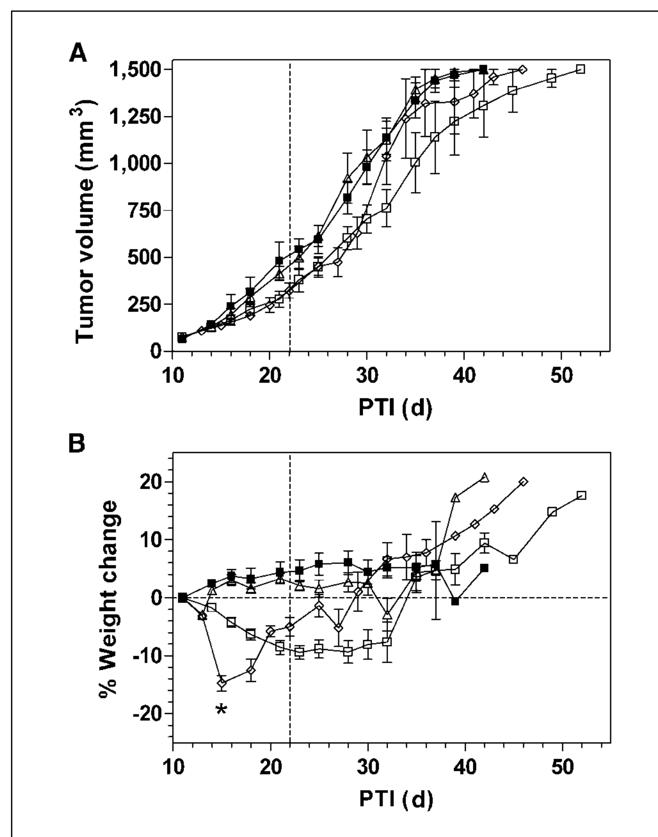

Figure 5. Effect of Tub-B-H on the growth of s.c. FR-positive KB tumors. KB tumor cells (1×10^6) were implanted s.c. into *nu/nu* mice, and 11 d later, mice were randomized and treated following a three times per week, 2-wk schedule. ■, untreated mice; △, □, ◇, groups treated with Tub-B-H at 0.5, 0.75, and 1 μmol/kg, respectively. *Points,* average tumor volume from five animals; *bars,* SD. *Dotted vertical line,* day of final dosing (day 22). The star in *B* indicates that therapy was ended after only two doses in this cohort because the limit of tolerability was reached at day 15 (i.e., an average of 15% weight loss, some motor skill loss, and pilo-erect hair). *PTI*, post tumor innoculation.

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Treatment-related adverse events. Whole blood, sera, and tissue specimens were taken at sacrifice or at euthanasia from animals participating in the aforementioned efficacy study. Samples were then analyzed for treatment-related changes relative to control animals. Sera analyses indicated that the amounts of alanine aminotransferase, creatinine, and total protein were within normal levels in all of the EC0305 treated *nu/nu* mice. The liver enzyme aspartate aminotransferase (<1.5-fold of the upper reference limit) and the kidney marker blood urea nitrogen (<1.4-fold of the upper reference limit) were modestly elevated in a couple of mice. In contrast, the Tub-B-H–treated mice displayed up to a 3.3-fold elevation of aspartate aminotransferase and 1.8-fold elevation of blood urea nitrogen. Blood analyses confirmed that EC0305 did not cause notable changes in any of the hematologic parameters measured. Finally, histopathologic evaluation of the *nu/nu* mice treated with EC0305 showed that the lung, liver, spleen, intestine, bone, muscle, brain, and kidneys were all normal.

Discussion

For all previously reported folate-drug conjugates, the base drug (when administered as an untargeted agent) displayed at least some degree of antitumor activity in the tested animal models (1, 2, 24, 29, 33). Attaching folate to those drugs clearly improved their therapeutic indices by (*a*) enabling a greater amount of total drug to be administered and (*b*) allowing more aggressive dosing regimens to be followed. For the first time, we provide evidence that folate not only can improve the potency of a drug through tumor targeting but can also completely enable the safe dosing of a drug that has no apparent therapeutic window. The tubulysins represent one such class of cytotoxic agents. For example, although Tub-B and its semisynthetic analogue Tub-B-H were found to be highly cytotoxic to cells in culture (Fig. 3), both agents yielded dose-limiting toxicities in mice at levels that did not produce measurable antitumor effect (Fig. 5).[2] In contrast, attachment of folate to Tub-B-H, along with a hydrophilic spacer and biologically releasable linker, yielded a conjugate that could produce durable antitumor responses without significant toxicity to mice bearing well-established human tumor xenografts (Fig. 4). These results clearly highlight one of the more compelling benefits for targeting drugs to the site of disease (i.e., tissue-specific ligands may enable the dosing of certain toxic molecules that cannot otherwise be safely administered to afford therapeutic responses).

EC0305 represents the first "targeted" tubulysin to be disclosed, and it is also the most potent single-agent folate conjugate reported to date. For example, the IC_{50} of EC0305 against the "gold standard" FR-expressing KB cell line is >3-fold lower than any previously reported folate-drug conjugate (29, 31, 33, 34). Further, we present evidence for strong antitumor effect when EC0305 was administered at the low 500 nmol/kg level (Fig. 4*A*), which is a dose ∼6-fold lower than that required to saturate tumor FRs with a single i.v. injection (1). Although not tested in this study, it is likely that even lower dose levels may provide significant antitumor effect, especially when administered on a more frequent schedule (1).

In recent years, our lab and research collaborators have reported on the preclinical pharmacology results for a variety of folate-drug conjugates, including those constructed with mitomycin C, desacetylvinblastine monohydrazide, epothilone B, and the maytansinoid DM1 (1, 2, 24, 29, 31, 33–36). In each case, strong evidence was presented for FR-mediated cytotoxicity and *in vivo* antitumor effect. Three related agents, EC145, EC0225, and BMS-753493, are currently being evaluated for safety and activity in multiple phase 1 and 2 clinical trials. Although EC0305 has not been officially declared a preclinical candidate, structural activity effects and pharmacology/toxicology studies are ongoing in our laboratory with the hope of selecting a new clinical lead in the near future.

[2] Unpublished data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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