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In Vivo Structural Activity and Optimization Studies of Folate−Tubulysin Conjugates

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Abstract: Herein we report on the potencies of 4 related folate-conjugated tubulysins constructed with either tubulysin B hydrazide (EC0305), tubulysin A hydrazide (EC0510), the *N*,*O*-acetal derivative of natural tubulysins (EC0317) or a tubulysin B ester (EC0302). Our results confirmed that EC0305 is the most favorable conjugate of the group due to its potent antitumor activity [100% cures at 1 μmol/kg, three times a week (TIW) for 2 weeks] and its favorably low toxicity profile. In contrast, the natural tubulysin B drug proved to be inactive against a human nasopharyngeal tumor model when administered at doses near to or greater than the maximum tolerated dose (MTD). When tested against more chemoresistant folate receptor expressing M109 and 4T1-cl2 tumors, EC0305 displayed superior antitumor activity over a previously disclosed folate conjugate of desacetylvinblastine monohydrazide (EC145). These studies demonstrate that EC0305 has significant antiproliferative activity against FR expressing tumors, including those which are generally more chemoresistant, and that EC0305 should be considered for development as a candidate for the treatment of advanced FR-expressing human cancers.

Keywords: Folate receptor; targeted chemotherapy; cancer; tumor; tubulysin

Introduction

Our group has been developing folate targeted oncology agents in hope of improving cancer patient outcomes while simultaneously reducing therapy-related toxicity. The vitamin folate has been successfully used to deliver numerous therapeutic- and imaging-based agents to tumor cells that express the folate receptor (FR) protein.[1−5] The FR is a high affinity membrane protein ($K_d \sim 0.1$ to 1 nM for folic acid) that is functionally expressed in high quantities by many primary and metastatic cancers,[6−8] and it has been successfully exploited for imaging and drug delivery purposes using a wide range of functionalities.[9−19]

We have recently described the biological activity of EC0305, a novel folate conjugate of tubulysin B.[20] The

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tubulysin family of secondary metabolites (species A−I), were originally isolated from the myxobacteria *Archangium geophyra* and *Angiococcus disciformis.* A number of groups have reported the synthesis of various tubulysin analogues[21−24] and their activity toward cancers[25,26] These compounds are powerful microtubule destabilizing agents which show high cytostatic activity against cancer cell lines[27,28] (with IC_{50} values in the picomolar range), which was retained against multidrug resistant cell lines.[29] Despite the potent in vitro activity of tubulysins against cancer cell lines, they are generally limited therapeutically due to severe toxicity. Hence, they are ideal candidates for targeted delivery approaches, such as that directed toward the FR. Here we report on a more elaborate in vivo investigation of EC0305 along with other structurally related folate−tubulysin conjugates.

Experimental Section

Materials. Pteroic acid (Pte) and N^{10}-trifluoroacetyl-Pte were prepared according to Xu et al.[30] Peptide synthesis reagents were purchased from NovaBiochem (La Jolla, CA) and Bachem (San Carlos, CA). EC0302, EC0305 and EC0510 were synthesized as previously described.[31] The synthesis of methoxy-tubulysin has been described in detail previously.[32] Its folate-conjugate counterpart, EC0317, was prepared following the same published procedure.[31] All other common reagents were purchased from Sigma (St. Louis, MO) or other major suppliers.

Generation of FR Positive 4T1-cl2 Cells. The 4T1 cell line, derived from a Balb/c mammary gland tumor, was obtained from ATCC and grown in complete RPMI 1640 medium (containing 2.3 μM folic acid) supplemented with 10% fetal bovine serum (Hyclone). The expression vector, pKJ-FR-α, encoding the full-length murine FR-α cDNA, was

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a gift from Dr. Philip Low.[33] pCMV-Script (Stratagene) containing the gene for neomycin resistance was cotransfected with pKJ-FR-α using Invitrogen's LipofectaminePlus reagent following the supplied protocol. Transformants were selected by culturing in folate deficient RPMI 1640 (Gibco Cat. #27016) with 10% fetal bovine serum and 1 mg/mL G418. Colonies were transferred to 24 well plates in duplicate, using sterile cloning cylinders (Fisher Cat. #07−907−10) and grown to 50% confluency. Each duplicate was then incubated with calcein encapsulated folate-PEG-PE/PC/cholesterol (60:40) liposomes for 1 h, washed with PBS, and then observed with a Diavert fluorescence microscope. The most highly fluorescent clone, 4T1-Cl2, was selected for further growth.

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 folate-deficient chow (Harlan diet #TD00434, Harlan Teklad, Madison, WI) for the duration of the experiment. Since normal rodent chow contains a high concentration of folic acid (6 mg/kg chow), mice used in our studies are maintained on the folate-free diet for 2 weeks before tumor implantation to reduce serum folate concentrations from an initial average of 720 nM to approximately 25 nM, which is close to the range of normal human serum (9−14 nM).[20,34] FR-positive human nasopharyngeal KB cells (∼75 pmol of FR/mg of protein), syngeneic FR-positive Madison 109 (M109) lung carcinoma cells (∼23 pmol of FR/mg of protein) and syngeneic 4T1-cl2 breast cancer cells (∼22 pmol of FR/mg of protein) were grown as a monolayer, using folate-free RPMI medium (FFRPMI) containing 10% heat-inactivated fetal calf serum (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, which consequently is more physiologically relevant than typical cell culture media which contain 100- to 1000-fold higher levels of folates. KB cells (1×10^6 per *nu/nu* mouse) or M109 (1×10^6 per Balb/c mouse) and 4T1-cl2 cells (2×10^5 per Balb/c mouse) in 100 μL were injected in the subcutis of the dorsal medial area. Mice were divided into groups of five, 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 (iv) treatments were typically initiated on day 9 post-tumor cell implantation (PTI) when the KB tumors were approximately 70−110 mm^3 in volume, on day 9 when the M109 tumors were approximately 50−100 mm^3 in volume, and on day 19 PTI when the 4T1-cl2 tumors were approximately 50−100 mm^3 in volume. The mice in the control groups received no treatment. Growth of each sc 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 gross 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. Drug toxicity was assessed by collecting blood via cardiac puncture and submitting the sera and whole blood for independent analysis of serum chemistry and hematological parameters at Ani-Lytics, Inc. (Gaithersburg, MD). 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; Salt Lake City, UT).

Tumor Response Criteria. For individual tumors, a partial response (PR) was defined as volume regression >50% but with measurable tumor (>2 mm^3) remaining at all times. Complete response (CR) was defined as a disappearance of measurable tumor mass (<2 mm^3) at some point within 90 days after tumor implantation. Cures were defined as CRs without tumor regrowth within the 90 day study time frame.

Protein Binding Assay. The percent serum protein binding was determined on a Waters Alliance 2695 HPLC with a 2996 photodiode array detector at 280 nm. A reversed-phase gradient from 10% to 90% acetonitrile was employed to separate EC0305 from the residual serum matrix on a 3.0 × 100 mm, 3.5 μm Waters Sunfire C18 column at 0.5 mL/min. The aqueous mobile phase was buffered at pH 7.0 with 10 mM ammonium acetate. Peak areas from 40 μL injections of test article-spiked, ultrafiltered serum samples were compared to identically treated PBS control samples to determine the percent serum binding. Unspiked serum samples were similarly analyzed to correct for any matrix-related, interfering peaks. The reported results represent the average ± 1 SD from three independent runs.

Results

Tubulysin B Conjugates Are Better Tolerated Than Tubulysin A Conjugates. When tested against KB cells in vitro, a statistical difference in potency between the two base drugs, tubulysin A and tubulysin B, was not observed. In fact, the IC_{50}s of both agents were ∼1 nM (data not published). Further, the potencies of their respective folate conjugate counterparts, EC0510 (folate−tubulysin A hydrazide; Figure 1B) and EC0305 (folate−tubulysin B hydrazide; Figure 1A) were also found to be similar (IC_{50}s ∼ 7 nM; data not shown). But, to better compare their therapeutic potential, both EC0510 and EC0305 were

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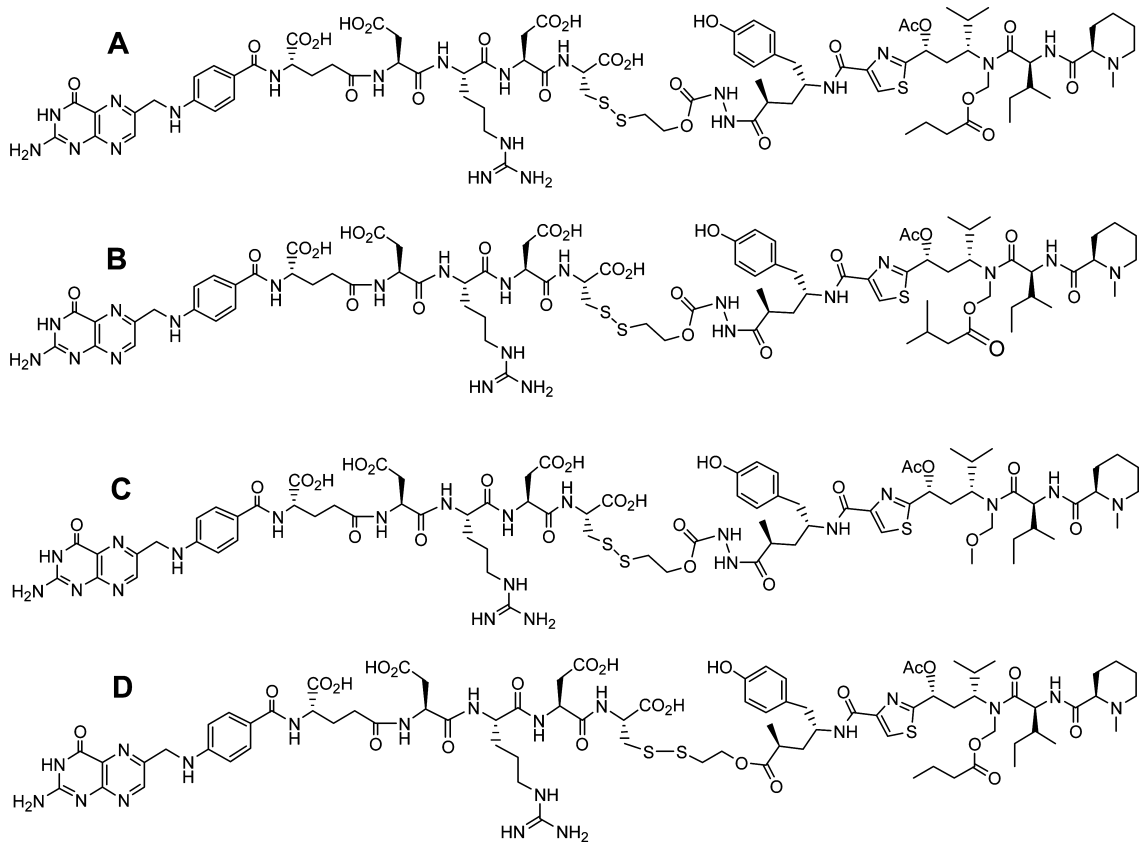

Figure 1. Chemical structures of (A) EC0305, (B) EC0510 (tubulysin A analogue of EC0305), (C) EC0317 (methyl ether analogue of tubulysin B) and (D) EC0302 (ester linker analogue of EC0305).

administered intravenously to KB tumor-bearing mice 10 days post tumor cell inoculation (PTI) at a low 0.5 μmol/kg dose level, three times per week for 2 consecutive weeks. As shown in Figure 2A, EC0510 generated significant antitumor activity where 4/5 animals displayed a complete response (CR) of which 3 of the animals maintained the complete response (i.e., cures) throughout the 80-day duration of the study, and 1/5 animals showed a partial response (PR). In comparison, EC0305 produced less activity when tested at the 0.5 μmol/kg dose level, where only moderate tumor regression or disease stabilization was observed (i.e., no cures; log cell kill (LCK) $\sim$ 0.8). Interestingly, doubling the EC0305 dose to 1 μmol/kg was found to generate remarkable antitumor effect, where 5/5 animals were deemed tumor-free (i.e., cures) at the end of study; this observation confirmed that EC0305 has a steep dose response. Although none of the treated animals lost any significant weight throughout the dosing period, 2 of the 5 animals from the 0.5 μmol/kg EC0510 cohort had to be euthanized due to observed swollen abdomens and sluggish behavior, evident on days 32 and 41 PTI. Due to this finding, EC0510 was not further tested.

Structure−Activity Study of Folate−Tubulysin B Conjugates. A novel methyl ether derivative at the N,O-acetal of the tubuvaline moiety of tubulysin B was synthesized and then conjugated to folate using the same spacer and linker parts as that found in EC0305. The resulting conjugate, EC0317 (Figure 1C), was found to be less active than EC0305 against KB tumors. Even with a higher 2 μmol/kg, TIW 2 week regimen, this conjugate only produced 3/5 cures (Figure 2C). Actually, such results were in accordance with the in vitro activity results of the corresponding base drugs in which EC0313 (base drug to EC0317) was $\sim$4-fold less potent than tubulysin B (base drug to EC0305) (data not published), a finding that could potentially be explained by EC0313's decreased permeability across cellular membranes as compared to tubulysin B.

To confirm the importance of the linker moiety, we next replaced the hydrazide within the EC0305 linker with an ester to create EC0302 (Figure 1D). When tested at both 0.5 μmol/kg (two doses) and 2 μmol/kg (one dose) dose levels, EC0302 was found to be very toxic, causing an average weight loss of 14% and 18% respectively. Based on these findings, we elected to further evaluate the therapeutic and toxicologic properties of EC0305.

The Natural Tubulysin B Parent Drug of EC0305 Has No Therapeutic Window. To conclusively establish the advantage of folate targeting, we attempted to find the therapeutic range of the natural tubulysin B drug. Here *nu/nu* mice bearing well-established KB tumors were treated with three different dose levels (0.1, 0.2, and 0.5 μmol/kg) of this agent. Although the intended regimen was to follow a TIW, 2 week schedule, we found that mice could only be

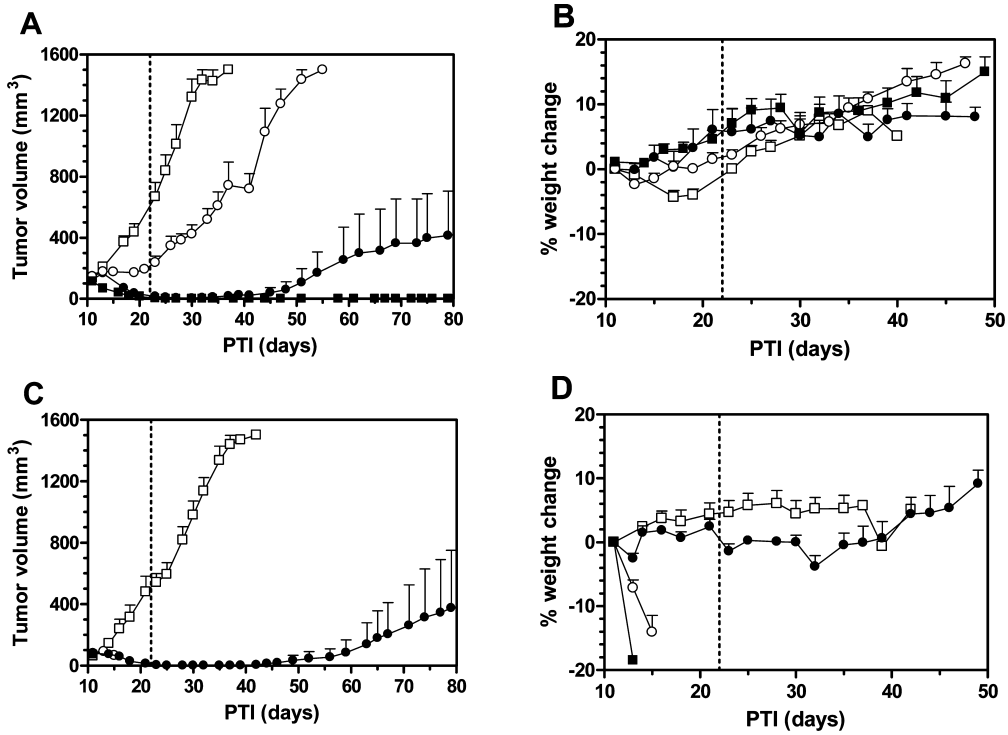

Figure 2. Comparison of the therapeutic efficacy and gross toxicity of EC0305 with EC0510, EC0317 and EC0302 against subcutaneous FR-positive KB tumor growth in *nu/nu* mice. KB tumor cells (1×10^6) were inoculated sc into nude mice and therapy started on randomized mice with tumors in the 70−150 mm^3 range. The therapeutic regimen consisted of iv doses following a TIW schedule for 2 weeks, when feasible. Each curve shows the average (±SD) volume of five tumors (A, C) and weights (B, D) of five mice. A, B: □, control; ○, 0.5 μmol/kg EC0305; ●, 0.5 μmol/kg EC0510; ■, 1 μmol/kg EC0305. C, D: □, control; ●, 2 μmol/kg EC0317; ○, 0.5 μmol/kg EC0302; ■, 2 μmol/kg EC0302. Dotted vertical lines represent the final day of dosing. $N = 5$ animals per cohort. PTI, post-tumor implantation.

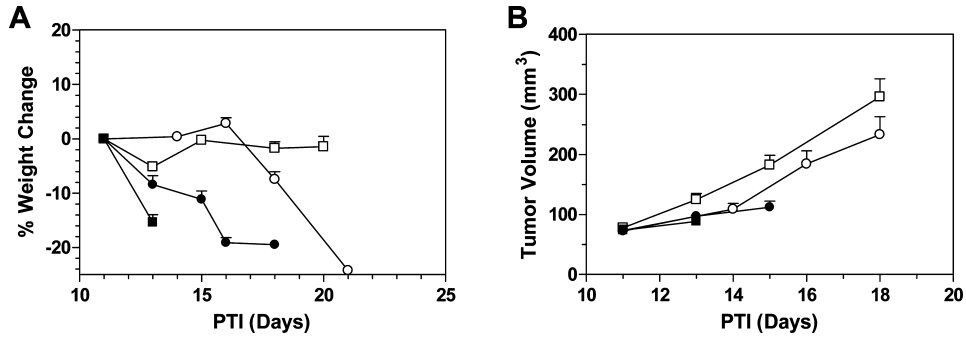

Figure 3. Antitumor effects of nontargeted tubulysin B and its toxicity in *nu/nu* mice. KB tumor cells (1×10^6) were inoculated sc into nude mice, and therapy was started on randomized mice with tumors in the 50−100 mm^3 range. Therapy was given iv following a TIW schedule for up to 2 weeks, as tolerated. Each curve shows the average (±SD) volume of five tumors (A) and weights (B) of five mice: □, control; ■, 0.5 μmol/kg; ●, 0.2 μmol/kg; ○, 0.1 μmol/kg. $N = 5$ animals per cohort. PTI, post-tumor implantation.

dosed 4 times with 0.1 μmol/kg, 3 times with 0.2 μmol/kg, and just once with 0.5 μmol/kg of tubulysin B before losing as much as 20% of their body weight (Figure 3A). As shown in Figure 3B, antitumor activity was also visibly absent with the natural tubulysin B agent at all three dose levels tested. Thus, unlike EC0305, but similar to its hydrazide counterpart,[20] tubulysin B appears to be therapeutically null.

EC0305 Is More Effective Than EC145 against Challenging Tumor Models. EC0305 was next evaluated for activity against FR-positive M109 and 4T1-cl2 tumors, both of which were found to be difficult to treat with the previously disclosed folate-desacetylvinblastine monohydrazide conjugate, EC145.[11,14] Here, Balb/c mice bearing well-established M109 tumors were treated with EC145 at two different doses and schedules: 2 μmol/kg TIW for two weeks, and 4 μmol/kg TIW for three weeks. As shown in Figures 4B and 4C, the 2 μmol/kg cohort yielded 1 CR and 1 cure, while the 4 μmol/kg dose resulted in a modest 2 of 5 cures. In contrast, when mice were treated with EC0305 at 2 μmol/kg, TIW for two weeks (i.e., one-third the total

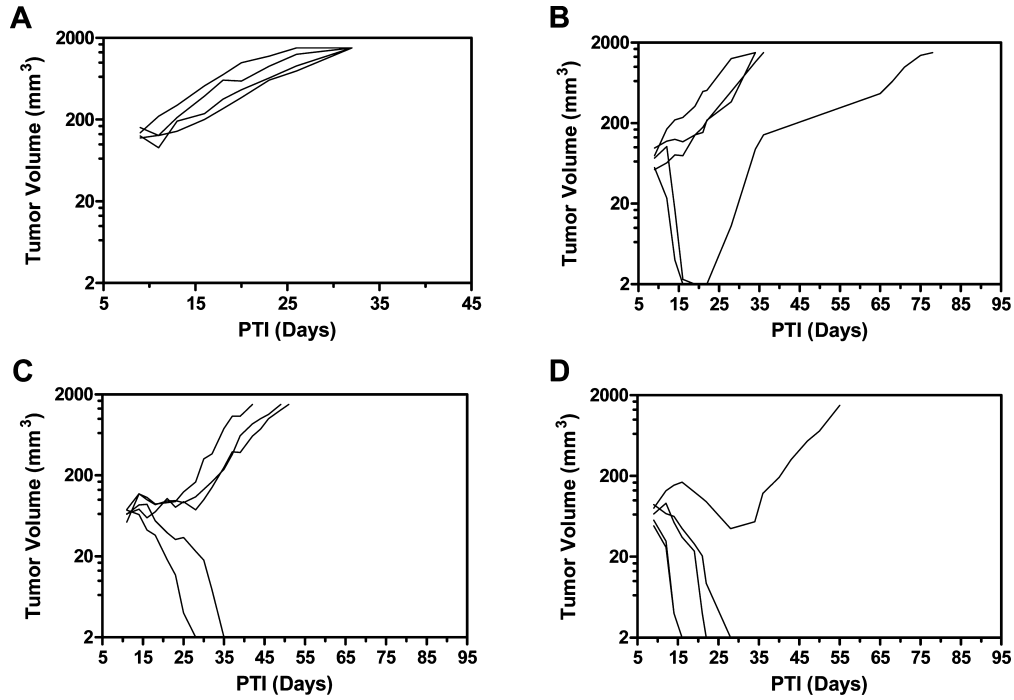

Figure 4. Therapeutic comparison of EC0305 and EC145 against subcutaneous FR-positive M109 tumor growth. M109 tumor cells (1×10^6) were inoculated sc into Balb/c mice, and therapy was started on randomized mice with tumors in the 50−100 mm^3 range. The therapy regimen consisted of iv doses following a TIW schedule: A, M109 controls; B, 2 μmol/kg EC145 for 2 weeks; C, 4 μmol/kg EC145 for 3 weeks; D, 2 μmol/kg EC0305 for 2 weeks. Each curve represents the growth of a single tumor in an individual mouse. PTI, post-tumor implantation.

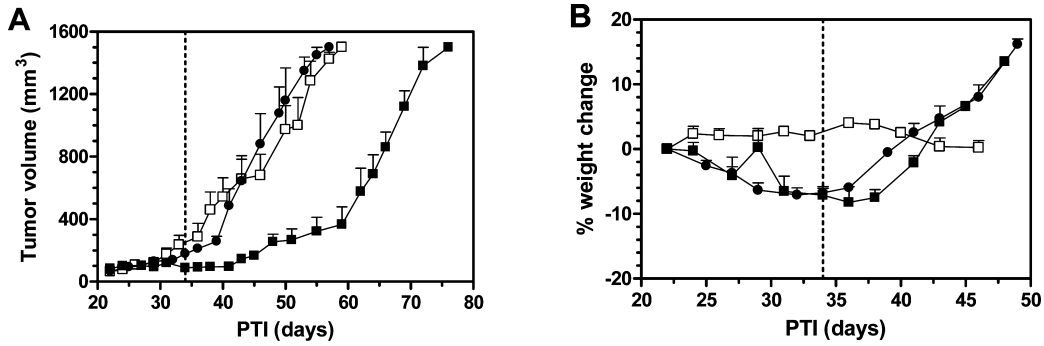

Figure 5. Therapeutic comparison of EC0305 and EC145 against subcutaneous FR-positive 4T1-cl2 tumor growth. 4T1-cl2 tumor cells (2×10^5) were inoculated sc into Balb/c mice, and therapy was started on randomized mice with tumors in the 50−100 mm^3 range. The therapy regimen consisted of iv doses following a TIW schedule for 2 weeks. Each curve shows the average (±SD) volume of five tumors (A) and weights (B) of five mice: □, control; ●, 4 μmol/kg EC145; ■, 2 μmol/kg EC305. Dotted vertical lines represent the final day of dosing. $N = 5$ animals per cohort. PTI, post-tumor implantation.

dose of the 4 μmol/kg EC145 cohort), tumors in all the five treated mice quickly regressed with 4/5 cures and only one relapse (PR) by the end of the study (Figure 4D). Furthermore, in the more drug-resistant 4T1-cl2 tumor model, EC145 given at 4 μmol/kg, TIW for two weeks did not produce any antitumor effect (0.06 LCK), while EC0305 at half that total dose (2 μmol/kg, TIW for two weeks) produced significant tumor regressions or stabilizations to yield an LCK of ~0.6 (Figure 5A). Importantly, EC0305's superior antitumor activity was not at the cost of an associated increase in toxicity, since the maximum average weight loss in the EC145 cohort (~9%) was similar to that of the EC0305 cohort (~10%) (Figure 5B).

EC0305 Moderately Binds to Serum Protein. The pharmacokinetic and pharmacodynamic properties of a compound are profoundly affected by the extent of its binding to plasma proteins. Thus, the determination of a compound's plasma protein-binding properties can be essential during the lead prioritization phase of drug development. In our study, sera collected from human, dog, mouse, rat, rabbit and calf were used to evaluate the in vitro serum protein binding properties of EC0305. As shown in Figure 6, EC0305 was found to be approximately 67% protein bound in human serum; the highest protein binding was observed in rabbit sera at 89%, while the lowest binding was found in dog sera

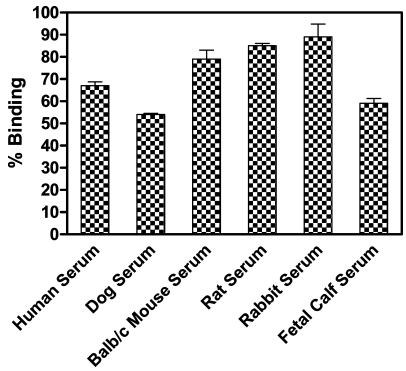

Figure 6. EC0305 binding to serum protein. One minute after 10 μL of EC0305 was mixed with 190 μL of serum (50 μM final concentration) at room temperature, the serum solution was transferred to a clean MicroconYM-30 centrifugal filter (30,000 NMWL). The device was spun at 10000*g* for 30 min at 20 °C. Forty microliters of filtrate was analyzed by HPLC−UV to quantitate the amount of freely soluble test article. Values represent the average (±SD) from three separate determinations from each species.

Table 1. Effect of Single Dose EC0305 on Balb/c Mouse Weights

dose (μmol/kg)	maximum weight loss (%)
2	0
3	7.7
4	5.9
5	9.7
6	24.4

at 54%. For comparison, folic acid is known to bind to human serum proteins to ~ 60% (data not shown). These results suggest that a large fraction of EC0305 should be available to extravasate from the circulation to bind to tumor-associated folate receptors.

Single Dose Toxicology. Pilot toxicity studies were conducted with EC0305 in both mice and rats to gain an initial understanding of the maximum tolerated dose (MTD) levels. Single dose tolerance was studied in naïve Balb/c mice using iv-administered EC0305. As shown in Table 1, mice were found to tolerate single EC0305 doses at levels ≤6 μmol/kg. Except for the highest dose cohort, weights of all other cohorts had recovered by the fifth day after dosing. Male Sprague−Dawley rats were also used to assess the single dose toxicity. As reported in Table 2, the single dose MTD level of EC0305 in rats is greater than 2 μmol/kg. Again, all animals in these cohorts had recovered by day 5 post administration. Organ weights were also measured on day 16 post dosing, and most of the organs (e.g., heart, liver, spleen, kidney and brain) did not seem to be negatively affected, except for the testes which weighed 67% less than those in the control animals.

Discussion

Conjugating anticancer agents to tumor homing ligands, like folic acid, is a promising method to improve efficacy toward tumor cells and to circumvent the toxic side effects to normal cells. In our quest to construct a folate-targeted tubulysin-based conjugate suitable for potential clinical development, we prepared a series of FR-targeted agents that differed in composition only within their tubulysin payloads (see Figure 1). The activity of EC0305 (tubulysin B-hydrazide) was compared to that of its tubulysin A counterpart, EC0510, and to its methyl ether counterpart, EC0317. Finally, an ester-linked tubulysin B analogue of EC0305 was also evaluated. Compared to EC0305, EC0317 was less active while EC0510 was comparably active (although more toxic) when evaluated for antitumor activity. Surprisingly, EC0302 was found to be very toxic to the animals, perhaps because the ester-based linkage was too labile in vivo, causing a premature release of the toxic warhead. Based on these findings, EC0305 was declared the best folate−tubulysin conjugate of the 4 tested against human tumor xenografts in mice.

Table 2. Effect of Single Dose EC0305 on Sprague Dawley Rat Weights

dose (μmol/kg)	maximum weight loss (%)
0.125	2.69
0.250	2.96
0.500	2.80
1.000	6.80
2.000	8.92

The effect of free tubulysin B was also explored in KB xenografts to assess its antitumor activity and its toxicity, and to calculate its therapeutic range for future comparison to EC0305. Although tubulysin B was found to be toxic at all tested dose levels, the 0.1 μmol/kg dose could be administered 4 times before inducing >15% weight loss in the test animals. But regardless of the dose, untargeted tubulysin B was not found to produce any antitumor effect in the KB tumor model (i.e., it has no therapeutic window). In contrast, conjugating folate to this drug created a new molecule (EC0305) with a large therapeutic window and the power to cure animals without associated toxicities.

Tubulysins have shown impressive activity against multidrug resistant cell lines,[29] while the *Vinca* alkaloids as a class have not.[35] In our study, the antitumor activity of EC0305 (folate−tubulysin) was indeed found to be more active than EC145 (folate-*Vinca*) when evaluated against two distinct drug-resistant, FR-expressing tumor models (see Figures 4 and 5). These results are important, because they suggest that EC0305 may be useful in treating tumors that have developed resistance toward EC145 (an agent currently in multiple phase 2 clinical trials).

The disulfide linker in EC0305 is susceptible to endosomal reduction and self-immolative release of tubulysin B-hydrazide.[20] However, stability of such bonds/compounds

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in serum is of concern, because premature release of tubulysin B-hydrazide prior to tumor cell uptake could lead to unwanted nontarget tissue toxicity. Since water-soluble folate conjugates, like EC0305, display rapid blood clearance and concomitantly rapid tumor saturation (i.e., between 1−4 min post iv injection),[36,37] little to no serum-mediated release of tubulysin B-hydrazide is expected following iv administration of EC0305.

In conclusion, these studies demonstrate that, as a class, tubulysins can be effectively targeted to FR-positive tumors to elicit a significant antitumor effect without causing observable toxicity. EC0305 has been found to be perhaps the most potent and specific folate-targeted chemotherapeutic reported to date. Since EC0305 also displays significant efficacy against drug-resistant FR-positive tumor models, it should be considered for clinical development.

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Supporting Information Available: NMR spectra of methoxy-tubulysin. LC−MS data for methoxy-tubulysin. This material is available free of charge via the Internet at http://pubs.acs.org.

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