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Folate–Vinca Alkaloid Conjugates for Cancer Therapy: A Structure–Activity Relationship

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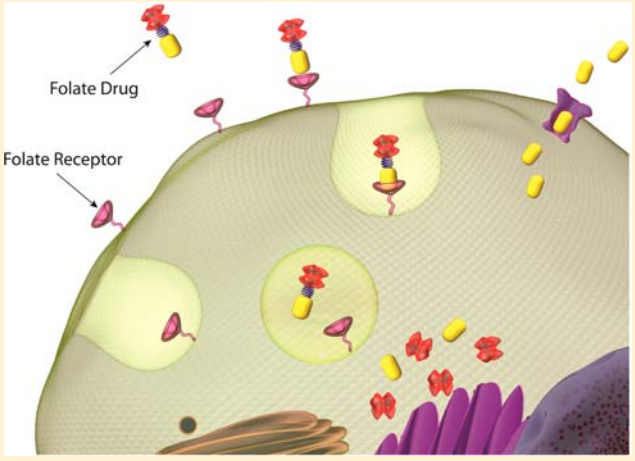
ABSTRACT: Vintafolide is a potent folate-targeted vinca alkaloid small molecule drug conjugate (SMDC) that has shown promising results in multiple clinical oncology studies. Structurally, vintafolide consists of 4 essential modules: (1) folic acid, (2) a hydrophilic peptide spacer, (3) a disulfide-containing, self-immolative linker, and (4) the cytotoxic drug, desacetylvinblastine hydrazide (DAVLBH). Here, we report a structure–activity study evaluating the biological impact of (i) substituting DAVLBH within the vintafolide molecule with other vinca alkaloid analogues such as vincristine, vindesine, vinflunine, or vinorelbine; (ii) substituting the naturally (*S*)-configured Asp-Arg-Asp-Asp-Cys peptide with alternative hydrophilic spacers of varied composition; and (iii) varying the composition of the linker module. A series of vinca alkaloid-containing SMDCs were synthesized and purified by HPLC and LCMS. The SMDCs were screened *in vitro* against folate receptor (FR)-positive cells, and anti-tumor activity was tested against well-established subcutaneous FR-positive tumor xenografts. The cytotoxic and anti-tumor activity was directly compared to that produced by vintafolide. Among all the folate vinca alkaloid SMDCs tested, DAVLBH-containing SMDCs were active, while those constructed with vincristine, vindesine, or vinorelbine analogues failed to produce meaningful biological activity. Within the DAVLBH series, having a bioreleasable, self-immolative linker system was found to be critical for activity since multiple analogues constructed with thioether-based linkers all failed to produce meaningful activity both *in vitro* and *in vivo*. Substitutions of some or all of the natural amino acids within vintafolide's hydrophilic spacer module did not significantly change the *in vitro* or *in vivo* potency of the SMDCs. Vintafolide remains one of the most potent folate–vinca alkaloid SMDCs produced to date, and continued clinical development is warranted.



INTRODUCTION

Tumor-targeted agents are being developed in an attempt to increase the selectivity of anticancer drugs while reducing the risk of unwanted off-target tissue toxicity. One such agent, vintafolide (formerly EC145), is a potent folate-targeted vinca alkaloid small molecule drug conjugate (SMDC) that selectively delivers a powerful microtubule-destabilizing agent (desacetylvinblastine hydrazide, DAVLBH) to folate receptor (FR)-positive tumors (Figure 1).[1−3] The FR is a high affinity membrane protein (K_d ∼ 0.1 to 1 nM for folic acid) that is functionally expressed in high quantities in many primary and metastatic cancers, with very limited expression in normal tissues.[4−7]

Previous *in vivo* studies have demonstrated that vintafolide (Figure 2) is curative against FR-positive tumor xenografts, and it is specific and well-tolerated in contrast with the untargeted DAVLBH molecule.[1,2] In clinical studies, vintafolide has demonstrated promising activity as a single agent in two Phase 2 trials (ovarian and nonsmall cell lung cancers)[8,9] as well as in combination with doxorubicin in a randomized open-label Phase 2 study in women with platinum-resistant ovarian cancer.[10]

Structurally, vintafolide consists of four essential modules: (1) folic acid, (2) a hydrophilic peptide spacer, (3) a disulfide-containing, self-immolative linker, and (4) the drug DAVLBH. Due to vintafolide's potential for success, recent efforts have focused on determining the impact of modifying the spacer, linker, or drug payload on the anti-tumor activity. As such, preclinical studies were conducted to determine the impact of (i) substituting the DAVLBH vinca alkaloid unit in vintafolide with analogues of vincristine, vindesine, vinorelbine, or vinflunine; (ii) substituting the naturally (*S*)-configured Asp-Arg-Asp-Asp-Cys peptide spacer in vintafolide with other hydrophilic spacers; and (iii) varying the composition of the

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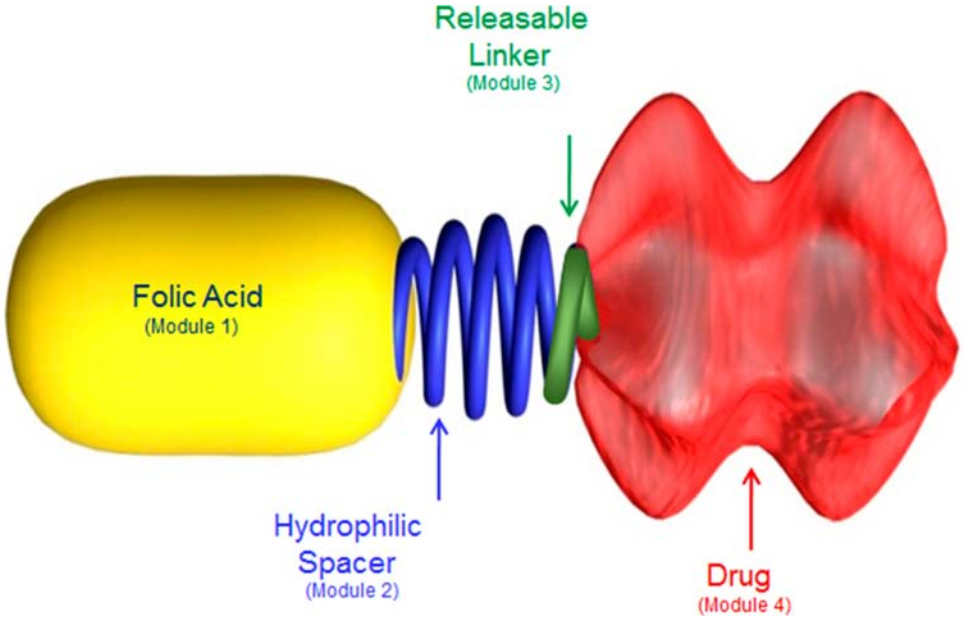

Figure 1. Modular design of folate-drug SMDCs (upper) and corresponding structure of vintafolide (lower). A folate-drug SMDC consists of four basic modules: (1) folic acid, (2) a hydrophilic spacer, (3) a releasable linker, and (4) a potent drug moiety.

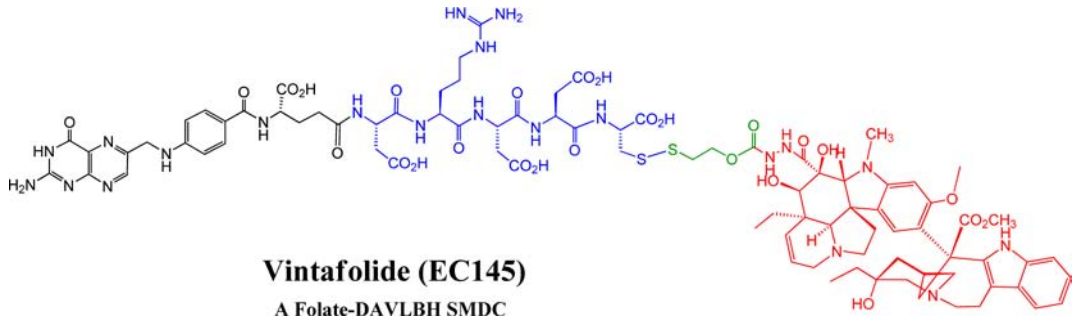

Figure 2. Structure of Vintafolide.

linker module to evaluate the impact of having a readily releasable, self-immolative linker system (e.g., disulfide-based) versus a more stable linker system (e.g., a thioether). New SMDCs were screened *in vitro* against FR-positive cells; those showing activity were then subsequently tested *in vivo*. The cytotoxic and anti-tumor activities of all SMDCs were directly compared to vintafolide, the most clinically advanced vinca alkaloid-based SMDC.

RESULTS

Folate-DAVLBH SMDCs with Various Spacer Modifications Remain Highly Active. Vintafolide is a folate-DAVLBH SMDC constructed with an Asp-Arg-Asp-Asp-Cys peptide spacer with each residue in the "L" configuration.[3] To determine the effect on biological activity by modifying the spacer within vintafolide, four additional SMDCs were synthesized with the following modifications: EC0260, which contains the same Asp-Arg-Asp-Asp-Cys peptide spacer but with each residue in the "D" configuration; EC0396 and EC0409 which contain the non-amino acid based spacers EDTA and PEG, respectively; and EC0489, which contains alternating L-amino acid (Glu) and carbohydrate (1-amino-1-deoxy-glucitolyl-γ-glutamate) segments. As shown in Table 1, all of the folate-DAVLBH SMDCs, including vintafolide, were found to have single digit nanomolar IC_{50} activities *in vitro,* and all produced curative anti-tumor activity *in vivo*. From these results we concluded that the potency of a folate-DAVLBH SMDC was not significantly altered by substitutions within the hydrophilic spacer module, which is in line with the role of the spacer serving as a hydrophilic separator of the SMDC's ligand and drug modules.

Folate-DAVLBH SMDCs Require a Cleavable Linker for Biological Activity. Vintafolide contains a bioreducible disulfide bond located between the folic acid and DAVLBH moieties. Following vintafolide's FR-mediated internalization, this disulfide is cleaved within the endosome to release the active DAVLBH molecule which, in turn, diffuses to the cytoplasmic space to disrupt microtubules.[11] To better define the importance of this linker system on cytotoxic activity, analogues of vintafolide were synthesized which contain various linker modifications, including EC140 with an acid-cleavable acyl hydrazone linker,[17] as well as EC1142 and EC1177 which contain noncleavable thioether-based linkers. As shown in Table 2, potency was found to be significantly impacted by linker composition. Hence, substitution of the disulfide with a more stable thioether bond proved to be counterproductive, since both EC1142 and EC1177 were found to be inactive both *in vitro* and *in vivo*. Similar to previous findings,[1] substituting vintafolide's disulfide with an acid-sensitive acyl hydrazone-based linker system did yield specific targeted cell kill both *in*

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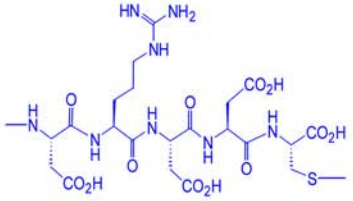
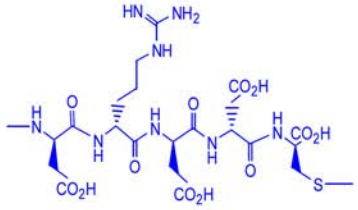
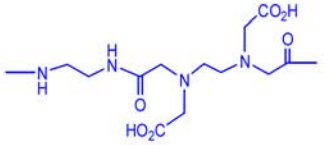
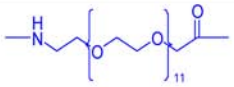
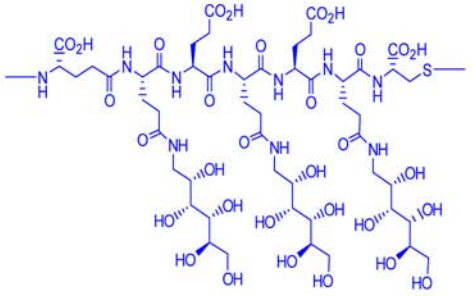
Table 1. Hydrophilic Spacer Modifications Have Little Impact on Targeted Activity of Folate-DAVLBH SMDCs

EC#	MW	Spacer		SMDC Activity	
		Structure	Composition	IC_{50} +/- s.d.; KB Cells (nM)	Best In Vivo Response
EC145 (Vintafolide)	1919		Asp-Arg-Asp-Asp-Cys	8 .44 +/- 1.46	80% cures, 20% CR's; Ref 2
EC0260	1919		D-Asp-D-Arg-D-Asp-D-Asp-D-Cys	8.86 +/- 1.95	16% cures, 33% CR's, 50% PR's; Ref 2
EC0396	1732		EDTA	7.74 +/- 1.25	100% cures
EC0409	2015		PEG_{12}	7.46 +/- 1.78	100% cures
EC0489	2551		Glu-Glu(adg)-Glu-(adg)-Glu-(adg)-Cys adg = 1-amino-1deoxy-D-gluitol	9.24 +/- 2.09	100% cures; Ref 12

vitro and *in vivo,* presumably due to linker cleavage inside the mildly acidic endosome; however, anti-tumor activity was found to be highest with the disulfide linker-containing vintafolide SMDC. These results confirmed that bioreleasable linkers are critical for cytotoxic activity of folate-based DAVLBH-containing SMDCs.

DAVLBH-Based SMDCs Are the Most Potent Folate–Vinca Alkaloid Constructs. A series of folate vinca-alkaloid SMDCs containing the same cleavable linker system was constructed with DAVLBH (vintafolide), vincristine (EC0275), vindesine (EC192), vinorelbine (EC1041), or vinflunine (EC1044). Three of these SMDCs contained the Asp-Arg-Asp-Asp-Cys spacer (Table 3; same spacer as in vintafolide), whereas the other three contained an advanced saccharopeptidic spacer (Table 4).[16,12] *In vitro* activity against FR-expressing KB cells of the unconjugated vinca alkaloid drugs was also determined. As shown in Tables 3 and 4, when tested against KB cells for 72 h *in vitro*, unconjugated vindesine was found to be highly active (IC_{50}: 5 nM) while the corresponding desacetylhydrazido derivatives of vinorelbine (IC_{50}: 149 nM) and vinflunine (IC_{50}: 202 nM) were less potent. Unconjugated DAVLBH and vincristine showed comparable *in vitro* activity (IC_{50}: 24 nM and 39 nM, respectively).

Folate-SMDCs of each of these base drugs were then prepared. Of the five tested, only vintafolide, (i.e., the DAVLBH-containing SMDC) was found to be active against the KB cells (IC_{50} = 8 nM). Surprisingly, the remaining folate–vinca SMDCs were found to be inactive, even when the cells were exposed to concentrations as high as 1 μM (Tables 3 and 4). *In vivo* experiments were subsequently conducted in athymic nude mice bearing s.c. implanted, FR-expressing KB xenografts (70–150 mm^3). Activity of the SMDCs was assessed by administering the agents beginning 9 or 11 days post tumor implant (PTI) using a 2 μmol/kg dose level and following a TIW, 2-week schedule. Similar to the aforementioned *in vitro* activity results, vintafolide was found to be the only folate–vinca alkaloid SMDC to produce strong anti-tumor activity against the KB tumors.

DISCUSSION

The goal of this study was to evaluate the impact of independently varying three of the four functional modules

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Table 2. Bioreleasable Linkers Are Critical for Activity

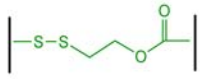
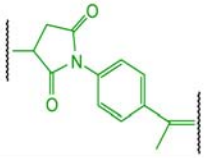
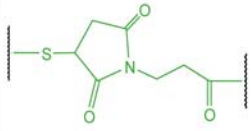
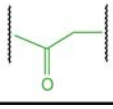
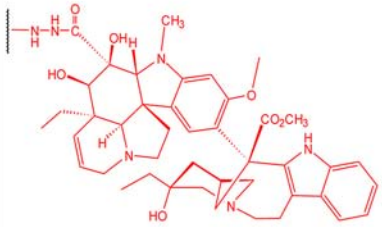
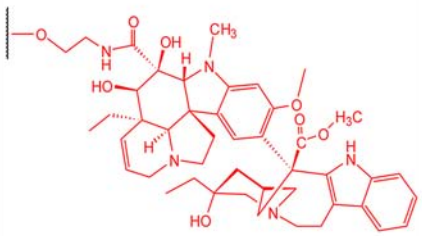
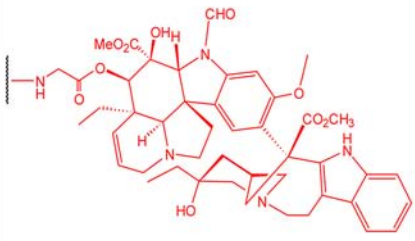
EC#	MW	Linker		SMDC Activity	
		Structure	*Cleavage Site*	*IC_{50} +/- s.d.; KB Cells (nM)*	*Best In Vivo Response*
EC145 (Vintafolide)	1919		Disulfide	8 .44 +/- 1.46	80% cures, 20% CR's; Ref 2
EC140	2014		Acyl Hydrazone	11.06 +/- 1.49	60% CR's, 40% PR's; Ref 13
EC1142	1966		Non-cleavable	Inactive (> 1 μM)	0% PR's
EC1177	1885		Non-cleavable	Inactive (> 1 μM)	0% PR's

Table 3. DAVLBH SMDCs Are the Most Potent Folate–Vinca Alkaloid Agents[a]

EC#	MW	Drug Payload		SMDC Activity	
		Structure	*Name*	*IC_{50} +/- s.d.; KB Cells (nM)*	*IC_{50} +/- s.d.; KB Cells (nM)*	*Best In Vivo Response*
EC145 (Vintafolide)	1919		DAVLBH	24.15 +/- 1.44	8 .44 +/- 1.46	80% cures, 20% CR's; Ref 2
EC192	1946		Hydroxyethyl-Vindesine	4.82 +/- 1.19	Inactive (> 100 nM)	Not tested
EC0275	1988		Vincristine	39.36 +/- 5.95	Inactive (> 100 nM)	0% PR's

[a]Each SMDC contains the Asp-Arg-Asp-Asp-Cys spacer.

within vintafolide while leaving the folic acid targeting module intact. This project included varying (i) the hydrophilic peptide spacer module, (ii) the self-immolative linker module, and (iii) the vinca alkaloid drug module (i.e., replacing DAVLBH).

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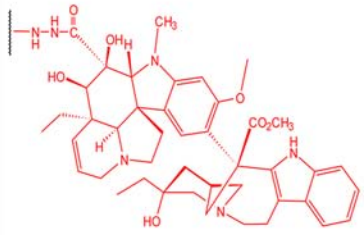
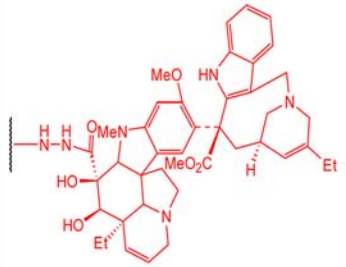
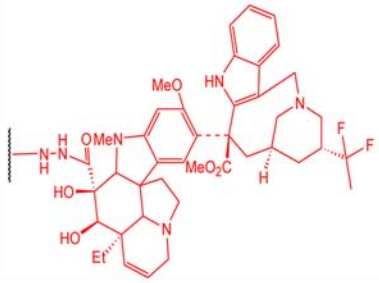
Table 4. DAVLBH SMDCs Are the Most Potent Folate−Vinca Alkaloid Agents[a]

EC#	MW	Drug Payload			SMDC Activity	
		Structure	Name	IC_{50} +/- s.d.; KB Cells (nM)	IC_{50} +/- s.d.; KB Cells (nM)	Best In Vivo Response
EC0489	2551		DAVLBH	24.15 +/- 1.44	9.24 +/- 2.09	100% cures; Ref 12
EC1041	2519		Desacetylhydrazido-Vinorelbine	148.55 +/- 27.24	Inactive (> 100 nM)	Not tested
EC1044	2557		Desacetylhydrazido-Vinflunine	202.49 +/- 18.06	Inactive (> 100 nM)	Not tested

[a]Each SMDC contains the Glu-Glu(adg)-Glu(adg)-Glu-(adg)-Cys spacer.

The effect of spacer chemistry on DAVLBH activity was evaluated by replacing the all-L-amino acid Asp-Arg-Asp-Asp-Cys peptide contained in vintafolide with (i) an all-D-amino acid Asp-Arg-Asp-Asp-Cys peptide segment, (ii) an alternating L-amino acid (Glu)-carbohydrate segment, or (iii) non-amino acid based spacers including ethylenediaminetetraacetic acid and polyethylene glycol. *In vitro* results confirmed that all 4 test articles display similar single-digit nM IC_{50} potency to vintafolide when tested against FR-positive human cancer cells. Furthermore, like vintafolide, all four spacer-modified SMDCs produced curative anti-tumor efficacy when tested against established FR-positive human tumor xenografts. These data confirm that composition of the spacer can vary widely without compromising efficacy as long as the spacer affords water high hydrophilic character and enables strong binding potential by physically separating the folic acid targeting moiety from the DAVLBH payload.

The composition of the self-immolative linker module was also evaluated by replacing the disulfide linker in vintafolide with an acid-cleavable acyl hydrazone linker, or stable noncleavable thioether linkers. Having a releasable linker system (such as a disulfide or acyl hydrazone) was found to be critical for activity since both thioether-based linkers failed to produce meaningful activity both *in vitro* and *in vivo*. These results are consistent with previous observations with folate−protein toxin conjugates, whereby those constructed with releasable linkers (disulfides) were pharmacologically active against FR-positive cells, but those constructed with stable linkers (e.g., amide bonds) were not.[13] What is not yet known, but is currently under investigation, is whether or not the observed trend will also be observed for folate-SMDCs constructed with drug payloads other than DAVLBH.

Surprisingly, when DAVLBH was substituted with other vinca alkaloid forms, such as vincristine, vindesine, vinorelbine, or vinflunine, none of the resulting SMDCs were found to be biologically active when tested *in vitro* or *in vivo*. The inactivity of the vinorelbine and vinflunine SMDCs can likely be explained by the high IC_{50} values of the corresponding unconjugated base drugs, because successful targeted cell kill of an FR-positive cell/tumor does require the delivery of a highly potent drug.[11,14] In contrast, the inactivity of the vindesine- and vincristine-based SMDCs remains puzzling. One possible explanation for this lack of activity is that the actual vinca alkaloid forms being released following disulfide reduction and linker release (shown in Table 3) are slightly altered from their original chemical forms. Another consideration is that unconjugated vinca alkaloids quickly diffuse through the plasma membrane to access microtubules inside the exposed cell; this results in low nM IC_{50} potencies. In contrast, SDMC constructs of these same base drugs must rely on endocytosis for cellular entry. Therefore, what is not known is to what extent these drug molecules get metabolized/

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deactivated while being transiently entrapped within the acidic endosomes. Further investigation is obviously warranted. Nonetheless, our data confirm that substitution of the DAVLBH drug payload with other clinically approved vinca alkaloids was not found to yield active folate-targeted SMDCs.

CONCLUSIONS

These data confirm that vintafolide is among the most potent folate−vinca alkaloid SMDCs produced to date. For the class of folic acid-targeted, vinca alkaloid-based SMDCs, the drug payloads must be separated from folic acid to be biologically active. DAVLBH is amenable to various self-immolative linker chemistries, and it is clearly the most effective vinca alkaloid tested to date for targeted drug delivery applications.

METHODS

Materials. Pteroic acid (Pte) and N^{10}-trifluoroacetyl-Pte were prepared according to Xu et al.[15] Peptide synthesis reagents were purchased from NovaBiochem (La Jolla, CA) and Bachem (San Carlos, CA). Vintafolide was synthesized as previously described.[3] All other common reagents were purchased from Sigma (St. Louis, MO) or other major suppliers.

Drug Payload Modifications. EC192, EC0275, EC1041, and EC1044 were synthesized in an identical fashion to vintafolide[3] with the exception that the DAVLBH drug payload was substituted with hydroxyethyl-vindesine (EC192), vincristine (EC0275), desacetylhydrazido-vinorelbine (EC1041), or desacetylhydrazido-vinflunine (EC1044).

EC192. Crude DAVLBH (330 mg, 0.43 mmol) was added to a 1 N HCl solution (19 mL). After the solid dissolved, the solution was cooled to 0 °C and $NaNO_2$ (30 mg) was added. The reaction was allowed to stir for 8 min, at which point saturated $NaHCO_3$ solution was added to raise the pH to 8.5. The reaction was extracted with CH_2Cl_2. The organic layer was washed with water, brine, dried over $MgSO_4$, and concentrated to 2 mL. To this solution was added 2-aminoethanol (0.3 mL, 5.0 mmol) and the reaction was stirred in the dark for 2.5 h. Thin layer chromatography (TLC; 25% MeOH in EtOAc) confirmed reaction completion. The reaction was washed with water, brine, dried over $MgSO_4$, and concentrated. 300 mg of crude product was recovered. A portion of this aminoethanol adduct (37 mg, 0.046 mmol) was dissolved in 1 mL CH_2Cl_2 and 25 mg (0.060 mmol) of *O*-(2-pyridyldisulfanylethoxycarbonyl) oxybenzotriazole (referred to in the rest of this experimental as "activated carbonate"),[3] and DMAP (11 mg, 0.090 mmol) was added. After 2 h, TLC (10% MeOH in CH_2Cl_2) indicated that the reaction was not complete. Additional activated carbonate (11 mg) and DMAP (6 mg) were then added and the reaction was allowed to stir for an additional 2 h. TLC confirmed reaction completion. The reaction was loaded directly onto a SiO_2 column (gradient: 2% to 10% MeOH in CH_2Cl_2) and purified. Ten milligrams of purified product was recovered (21% yield). LCMS (ESI) $[M+H]^+$ = 1011.2. Thirteen milligrams of EC119 (0.012 mmol), adjusted to pH 3.0, was dissolved in 300 mL of DMSO. To this solution was added DIPEA (35 mL, 20 equiv) followed by the vindesine derivative (10 mg; 10 mmol) in 400 mL of DMSO. The solution was allowed to stir at room temperature for 1 h. The reaction was loaded directly onto the preparative HPLC (Waters Xterra, 19 × 300 mm, eluents: 2 mM sodium phosphate, pH = 7 (A), acetonitrile (B), gradient: 1% to 50% B in 30 min). Four milligrams of clean product was recovered (20% yield). LCMS (ESI) $[M+H]^+$ = 1946.7, $[M+2H]^{2+}$ = 973.7.

EC0275. Vincristine free base (60 mg, 0.073 mmol) was dissolved in 3 mL anhydrous MeOH plus several drops of DBU. The reaction was stirred for 2 days at room temperature. TLC (20% MeOH in CH_2Cl_2) indicated that all of the starting material had been consumed. The solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and the resulting solution was loaded onto a SiO_2 column (gradient: 5% to 20% MeOH in CH_2Cl_2). 40 mg of purified desacetylvincristine was recovered (70% yield). LCMS (ESI) $[M-H]^-$ = 781.4. Boc-Gly-OH (33.6 mg) was dissolved in 600 mL dry EtOAc and then cooled to −15 °C. To this solution was added TEA (26.7 mL), followed by isobutyl chloroformate (25.1 mL). This solution was allowed to stir for 30 min. At this point desacetylvincristine (50 mg, 0.064 mmol) was added, and the reaction was allowed to slowly warm to room temperature and stirring continued overnight. TLC (10% MeOH in CH_2Cl_2) indicated that the reaction was complete. The reaction was concentrated and then treated with MeOH. The reaction solution was concentrated once again, dissolved in CH_2Cl_2, and then loaded onto a SiO_2 column (gradient: 2% MeOH to 15% MeOH in CH_2Cl_2) and purified. 35 mg of vincristine glycinate, 4-(Boc-Gly)-vincristine, was recovered (58% yield). LCMS (ESI) $[M+H]^+$ = 940.5. The protected vincristine glycinate (32 mg, 0.034 mmol) was dissolved in 300 mL of MeOH and then cooled to 0 °C. To this cool solution was added 100 mL of AcCl. The reaction was allowed to warm to room temperature and stirred for 1 h. TLC (10% MeOH in CH_2Cl_2) indicated that the reaction was complete. The solvent was evaporated under reduced pressure. The resulting residue was dissolved in CH_2Cl_2 (500 mL) and cooled to 0 °C. DIPEA (47 mL, 7 equiv) and activated carbonate (13 mg, 1 equiv) were added. The reaction was allowed to stir at 0 °C for 1 h. TLC (10% MeOH in CH_2Cl_2) indicated that the reaction was complete. The reaction mixture was loaded onto a SiO_2 column (gradient 5% to 10% MeOH in CH_2Cl_2) and purified. 23 mg of desformylvincristine, 4-(2-pyridyldisulfanylethoxycarbonyl)-Gly)-desformylvincristine was recovered (65% yield). LCMS (ESI) $[M+H]^+$ = 1025.7. Acetic anhydride (11 mL) and formic acid (5 mL) were mixed together and heated to 50 °C for 45 min and then cooled to 0 °C. A portion of the resulting mixed anhydride (300 mL) was added to the activated desformylvincristine (12 mg, 0.012 mmol). This reaction was allowed to stir for 1.5 h. The reaction was diluted with CH_2Cl_2 and 1 mL of a 20% ammonium hydroxide solution was added. The layers were quickly separated and the combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. HPLC showed mono- and bisformylation products. The products were separated by preparative HPLC. Seven milligrams of purified vincristine glycinate, 4-(2-pyridyldisulfanylethoxycarbonyl)-Gly)-vincristine, was recovered (51% yield). After separation it was found that the bis product could be easily hydrolyzed to the mono adduct in aqueous solution. LCMS (ESI) $[M+H]^+$ = 1053.7. EC119 (3.5 mg, 3.3 mmol) suspended in 1 mL of water, under bubbling argon, and saturated $NaHCO_3$ solution was added to adjust the pH to 7. To the resulting solution was added the activated vincristine adduct (3.5 mg, 3.33 mmol) in 1 mL THF. The reaction was allowed to proceed for 40 min. HPLC indicated that the reaction was complete. The reaction was purified by preparative HPLC (Waters Xterra, 19 × 300 mm, eluents: 2 mM sodium phosphate, pH = 7 (A), acetonitrile

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(B), gradient: 1% to 50% B in 30 min). 6.7 mg of product was recovered (48% yield). LCMS (ESI) $[M+H]^+$ = 1989.8, $[M+2H]^{2+}$ = 995.4.

EC1041. Vinorelbine free base (56 mg, 0.072 mmol) was dissolved in a 1:1 solution of hydrazine and MeOH (2 mL). The resulting solution was heated at 60 °C overnight. The reaction was cooled to room temperature. TLC (15% MeOH in CH_2Cl_2) showed that all of the starting vinorelbine had been consumed. The reaction was diluted with EtOAc and water, and the layers were separated. The aqueous layer was washed with EtOAc. The combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated to give crude desacetylvinorelbine hydrazide (40 mg). This material was used in the next reaction without further purification. Crude desacetylvinorelbine hydrazide (40 mg, 0.050 mmol) was dissolved in CH_2Cl_2 (1 mL). Activated carbonate (30 mg, 1.5 equiv) and DIPEA (22 mL, 2.5 equiv) were then added. After 2 h, TLC (15% MeOH in CH_2Cl_2) indicated that all of the starting material had been consumed. The reaction was loaded onto a SiO_2 column (gradient elution 0% MeOH to 15% MeOH in CH_2Cl_2) and purified. 23 mg of purified material (2-pyridyldisulfanylethoxycarbonyl activated vinorelbine hydrazide) was recovered (50% yield). LCMS (ESI) $[M+H]^+$ = 950.5. EC0488 (39.5 mg, 0.95 equiv) was dissolved in an argon purged, pH = 7.4, 50 mM phosphate buffer (2 mL). To this argon bubbled solution was immediately added the activated vinorelbine (23.5 mg, 0.0247 mmol) in 2 mL of THF. The reaction was allowed to proceed for 1 h. HPLC indicated that the reaction was complete. The THF was removed under reduced pressure, and the reaction filtered and then purified by preparative HPLC (Waters XBridge, 19 × 200 mm, eluents: 50 mM ammonium bicarbonate, pH = 7 (A), acetonitrile (B), gradient: 0% to 50% B in 30 min). 22 mg of purified product was recovered (37% yield). LCMS (ESI) $[M+2H]^{2+}$ = 1260.3, $[M+3H]^{3+}$ = 840.6.

EC1044. Vinflunine free base (17.8 mg, 0.022 mmol) was dissolved in a 1:1 solution of hydrazine and MeOH (1 mL). The resulting solution was heated at 60 °C overnight. The reaction was cooled to room temperature. TLC (10% MeOH in CH_2Cl_2) showed that all of the starting vinflunine had been consumed. The reaction was diluted with EtOAc and water, and the layers were separated. The aqueous layer was washed with EtOAc. The combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated to give crude desacetylvinflunine hydrazide (17 mg). This material was used in the next reaction without further purification. LCMS (ESI) $[M+H]^+$ = 775.5. Crude desacetylvinflunine hydrazide (17 mg, 0.022 mmol) was dissolved in 0.8 mL of CH_2Cl_2 and cooled to 0 °C. Activated carbonate (11.3 mg, 1.4 equiv) and DIPEA (9 mL, 2 equiv) were then added. After 2 h, the reaction was loaded onto a SiO_2 column and purified. Five milligrams of purified material (2-pyridyldisulfanylethoxycarbonyl activated vinflunine hydrazide; EC1043) was recovered (23% yield). LCMS (ESI) $[M+H]^+$ = 988.5. EC0488 (11 mg, 6.5 mmol), adjusted to pH 2, was dissolved in 0.7 mL DMSO. To this solution was added DIPEA (16 mL, 20 equiv) followed by EC1043 (4.5 mg, 4.5 mmol) in 0.7 mL DMSO. The reaction was allowed to stir for 1 h at room temperature. HPLC showed the reaction was complete. The reaction was purified by preparative HPLC (Waters XBridge, 19 × 200 mm, eluents: 50 mM ammonium bicarbonate, pH = 7 (A), acetonitrile (B), gradient: 0% to 50% B in 30 min). Four milligrams of purified compound was recovered (33% yield). LCMS (ESI) $[M+2H]^{2+}$ = 1279.3.

Spacer Modifications. EC0260 is analogous to vintafolide, except D-amino acids were used for its synthesis.[2] EC0396, EC0409, and EC0489 are identical to vintafolide, with the exception that the folate-containing spacer unit (Asp-Arg-Asp-Asp-Cys) was substituted with either EDTA (EC0396), PEG_{12} (EC0409), or Glu-Glu-(1-amino-1-deoxy-D-gluitol)-Glu-Glu-(1-amino-1deoxy-D-gluitol)-Glu-Glu-(1-amino-1deoxy-D-gluitol)-Cys, (EC0489).[16]

ECO0396. EDTA dianhydride (70 mg, 0.273 mmol), H-Cys (Trt)-OH (90 mg, 0.248 mmol), and DIPEA (0.2 mL) were dissolved in 2 mL DMSO at 25 °C. Folate-EDA[15] (120 mg, 0.248 mmol) with TMG (trimethylglycine, 1 equiv) in 2 mL of DMSO was sonicated for 1 h to yield a clear solution, which was then added to the above solution and stirred overnight. LC/MS confirmed product formation. The mixture was purified by preparative HPLC (Waters Xterra, 19 × 300 mm, eluents: 10 mM ammonium acetate, pH = 7 (A), acetonitrile (B)). 30 milligrams of folate-EDA-EDTA-Cys (Trt)-OH (EC0394) was recovered (11% yield). LCMS (ESI) $[M+H]^+$ = 1103.7. 24 mg, (0.0218 mmol) of EC0394 was dissolved in TFA/EDT/TIPS/H_2O (92.5:25:2.5:2.5) and stirred for 1 h. The mixture was purified by preparative HPLC purification HPLC (Waters Xterra, 19 × 300 mm, eluents: 10 mM ammonium acetate, pH = 5 (A), acetonitrile (B)). 17 mg (91%) of folate-EDA-EDTA-Cys-OH (EC0395) was recovered (yield 91%). LCMS (ESI) $[M+H]^+$ = 861.5. The 17 mg (0.0198 mmol) of EC0395 was dissolved in 1 mL of H_2O. The pH of the solution was adjusted to 7.5 with saturated $NaHCO_3$ while being purged with argon. Pyridyldisulfanyl-activated vinblastine hydrazide (25 mg, 0.0255 mmol) was dissolved in tetrahydrofuran (THF; 1 mL) and purged with argon. The EC0395 aqueous solution was then added to the vinblastine hydrazide solution with continued argon purging. After 10 min, high-performance liquid chromatography (HPLC) showed complete consumption of EC0395. The reaction was purified by preparative HPLC [Waters Xterra, 19 × 300 mm, eluents: 1 mM sodium phosphate, pH = 7 (A), acetonitrile (B)]. 16.7 mg of desired product was recovered (49% yield). Liquid chromatography–mass spectrometry (LCMS) (ESI) $[M+H]^+$ = 1732.9.

EC0409. The PEG folate spacer, EC0399 (20 mg, 0.017 mmol), was synthesized as previously described for the spacer in vintafolide by substituting *N*-Fmoc-amido-dPEG$_{12}$-acid (Quanta Biodesign, Ltd.) for the Asp-Arg-Asp moieties.[3] EC0399 powder was dissolved in 1 mL of water under bubbling argon, and saturated $NaHCO_3$ solution was added until the pH reached 7. To this solution was added the pyridyldisulfanyl-activated DAVLBH (17.2 mg, 0.017 mmol) in 1 mL of THF. The reaction was allowed to stir for 1 h. HPLC indicated that the reaction was complete. The reaction was purified by preparative HPLC (Waters Xterra, 19 × 300 mm, eluents: 2 mM sodium phosphate, pH = 7 (A), acetonitrile (B), gradient: 1% to 50% B in 30 min). Seventeen milligrams of product was recovered (50% yield). LCMS (ESI) $[M+H]^+$ = 2016.0, $[M+2H]^{2+}$ = 1008.8.

Linker Modifications. EC140, EC1142, and EC1177 are similar in structure to vintafolide with the exception that the disulfide-containing linker module was substituted with an acyl hydrazone (EC140),[17] a maleimidyl-based thioether (EC1142), or an alkyl-based thioether (EC1177).

EC1142. To a solution of DAVLBH (48.5 mg, 0.063 mmol), in 2 mL CH_2Cl_2, was added *N*-(*β*-maleimidopropyloxy)-succinimide ester (BMPS, 21 mg, 1.25 equiv) and DIPEA (22 μL, 2 equiv). The reaction was allowed to stir at room temperature for 2 h. TLC (10% MeOH in CH_2Cl_2) showed complete consumption of the DAVLBH. The reaction was loaded directly onto a SiO_2 column (gradient elution 0% MeOH to 20% MeOH in CH_2Cl_2). 32 mg of the maleimido adduct (EC1141) was recovered (54% yield). LCMS (ESI) $[M+H]^+$ = 920.8. EC119 (36 mg, 0.034 mmol), adjusted to pH 2, was added to 2 mL of DMSO. After stirring for 30 min, DIPEA (121 mL, 20 equiv) was added followed by the 32 mg (0.035 mmol) of EC1141 in 700 mL of DMSO. The reaction was allowed to stir for 1 h and was then loaded onto a preparative HPLC column (Waters XBridge, 19 × 200 mm, eluents: 50 mM ammonium bicarbonate, pH = 7 (A), acetonitrile (B), gradient: 0% to 50% B in 30 min). Nine milligrams of product was recovered (13% yield). LCMS (ESI) $[M+2H]^{2+}$ = 984.2, $[M+3H]^{3+}$ = 656.5.

EC1177. To a solution of DAVLBH (35 mg, 0.0455 mmol) in 2 mL of THF was added succinimidylbromoacetate (14 mg, 1.3 equiv) and DIPEA (20.5 mL, 2.6 equiv). The reaction was allowed to stir at 0 °C for 30 min and then allowed to warm to room temperature. LC-MS showed the formation of the desired bromoacetate adduct ($(M+H)^+$ = 889 *m*/*z*) and the "reverse" addition product, the substituted acetyl succinimidyl ester adduct (M+H)+ = 924 *m*/*z*) in roughly a 1:1 ratio. This crude reaction mixture was added to a solution of EC119 (92 mg, 0.088 mmol) in H_2O, under argon bubbling, adjusted to a pH = 9 by a saturated $NaHCO_3$ solution. The resulting solution was stirred for 45 min. LCMS showed conversion to the desired product. The reaction was purified by preparative HPLC (Waters XBridge, 19 × 200 mm, eluents: 50 mM ammonium bicarbonate, pH = 7 (A), acetonitrile (B), gradient: 0% to 50% B in 30 min). Thirteen milligrams of product was recovered (16% yield). LCMS (ESI) $[M+2H]^{2+}$ = 928.6, $[M+3H]^{3+}$ = 619.5.

***In Vitro* Dose Response.** FR-positive human nasopharyngeal carcinoma KB cells (now reported to be derived from cervical cancer HeLa contamination)[18] were grown in 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. Exponentially growing cells were seeded in 24-well plates 24 h before treatment with drugs. Thirty minutes prior to the addition of the test article, spent medium was aspirated from all wells and replaced with fresh FFRPMI. Note that designated wells received medium containing 100 μM EC17 (folate-fluorescein; a nontoxic FR blocker) and were used to confirm the targeting specificity of each folate-based SMDC, similar to published procedures (data not shown).[16] Following one rinse with 1 mL of fresh FFRPMI/HIFCS, each well received 1 mL of medium containing increasing concentrations (0.1 nM to 1000 nM) of the test article (3 wells per sample) in the presence or absence of 100 μM EC17, as appropriate. Treated 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 media up to 70 h. Different from SMDC treatment, cells treated with unconjugated base drugs were incubated continuously for the 72 h period. Spent medium was aspirated from all wells, and cell viability was assessed by a ^{3}H-thymidine incorporation assay, as previously described.[17] The IC_{50} values were calculated by nonlinear regression analysis with the equation of a sigmoidal dose response (*GraphPad Prism* software). Average IC_{50} values and the corresponding standard deviations were then determined from two independent experiments.

***In Vivo* Anti-Tumor 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 low folate chow (Harlan diet TD00434; Harlan Teklad, Madison, WI) for the duration of the experiment.[19] FR-positive KB cells were grown continuously as a monolayer or in suspension using folate-free RPMI containing 10% HIFCS at 37 °C in a 5% CO_2/95% air-humidified atmosphere with no antibiotics. Note that the HIFCS contains endogenous folates at concentrations sufficient for FR-expressing cells to survive and proliferate in this medium,[20] which consequently is more physiologically relevant than typical cell culture media, which contain 100- to 1000-fold higher levels of folates than that found in human serum. KB cells (1×10^6 per *nu/nu* mouse) in 100 μL were injected in the subcutis of the dorsal medial area. Mice were divided into groups of five. 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 initiated on days 9 or 11 post-tumor cell implantation (PTI) when the KB tumors were ∼70 to 150 mm^3 in volume. Test articles were administered at 2 μmol/kg following a three times per week (TIW), two-week schedule. The mice in the control groups received no treatment. Growth of each s.c. tumor was followed by measuring the tumor thrice per week (TIW) 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 millimeters and W = measurement of axis perpendicular to L in millimeters. 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) or had tumors greater than 1500 mm^3 were euthanized by CO_2 asphyxiation. Individual tumor response end points were reported in terms of tumor volume change. 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. All *in vivo* studies were approved by the Purdue Animal Care and Use Committee.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Leamon, C. P., Reddy, J. A., Vlahov, I. R., Westrick, E., Parker, N., Nicoson, J. S., and Vetzel, M. (2007) Comparative preclinical activity of the folate-targeted Vinca alkaloid conjugates EC140 and EC145. *Int. J. Cancer 121*, 1585–92.

(2) Reddy, J. A., Dorton, R., Westrick, E., Dawson, A., Smith, T., Xu, L. C., Vetzel, M., Kleindl, P., Vlahov, I. R., and Leamon, C. P. (2007) Preclinical evaluation of EC145, a folate-vinca alkaloid conjugate. *Cancer Res. 67*, 4434−42.
(3) Vlahov, I. R., Santhapuram, H. K., Kleindl, P. J., Howard, S. J., Stanford, K. M., and Leamon, C. P. (2006) Design and regioselective synthesis of a new generation of targeted chemotherapeutics. Part 1: EC145, a folic acid conjugate of desacetylvinblastine monohydrazide. *Bioorg. Med. Chem. Lett. 16*, 5093−6.
(4) Parker, N., Turk, M. J., Westrick, E., Lewis, J. D., Low, P. S., and Leamon, C. P. (2005) Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal. Biochem. 338*, 284−93.
(5) Ross, J. F., Chaudhuri, P. K., and Ratnam, M. (1994) Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer 73*, 2432−43.
(6) Toffoli, G., Cernigoi, C., Russo, A., Gallo, A., Bagnoli, M., and Boiocchi, M. (1997) Overexpression of folate binding protein in ovarian cancers. *Int. J. Cancer 74*, 193−198.
(7) Weitman, S. D., Lark, R. H., Coney, L. R., Fort, D. W., Frasca, V., Zurawski, V. R., and Kamen, B. A. (1992) Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res. 52*, 3396−3401.
(8) Edelman, M. J., Harb, W. A., Pal, S. E., Boccia, R. V., Kraut, M. J., Bonomi, P., Conley, B. A., Rogers, J. S., Messmann, R. A., and Garon, E. B. (2012) Multicenter trial of EC145 in advanced, folate-receptor positive adenocarcinoma of the lung. *Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer 7*, 1618−21.
(9) Naumann, R. W., Morris, R., Harb, W., Micha, J., Sutton, G., Dinh, T., Mcintyre, J., Patel, R., Davidson, J., Hu, E., and Messmann, R. (2009) Abstract 1181 In *16th International Meeting of the European Society for Gynecological Oncology*.
(10) Naumann, R. W., Coleman, R. L., Burger, R. A., Herzog, T. J., Morris, R., Sausville, E. A., Kutarska, E., Ghamande, S. A., Gabrail, N. Y., De Pasquale, S., Nowara, E., Gilbert, L., Caton, J. R., Gersh, R. H., Teneriello, M. G., Harb, W. A., Konstantinopoulos, P., Symanowski, J. T., Lovejoy, C., and Messmann, R. A. (2011) *J. Clin. Oncol. 29*, suppl; abstr 5045.
(11) Leamon, C. P. (2008) Folate-targeted drug strategies for the treatment of cancer. *Curr. Opin. Investig. Drugs 9*, 1277−86.
(12) Vlahov, I. R., Santhapuram, H. K., You, F., Wang, Y., Kleindl, P. J., Hahn, S. J., Vaughn, J. F., Reno, D. S., and Leamon, C. P. (2010) Carbohydrate-based synthetic approach to control toxicity profiles of folate-drug conjugates. *J. Org. Chem. 75*, 3685−91.
(13) Leamon, C. P., Pastan, I., and Low, P. S. (1993) Cytotoxicity of folate-Pseudomonas exotoxin conjugates toward tumor cells. Contribution of translocation domain. *J. Biol. Chem. 268*, 24847−54.
(14) Vlahov, I. R., and Leamon, C. P. (2012) Engineering folate-drug conjugates to target cancer: from chemistry to clinic. *Bioconjugate Chem 23*, 1357−69.
(15) Xu, L., Vlahov, I. R., Leamon, C. P., Santhapuram, H. K. R., and Li, C. H. (2005) Synthesis, purification, and uses of pteroic acid and derivatives and conjugates thereof. US Patent application number PCT/US2006/009153, filed March 14, 2006.
(16) Leamon, C. P., Reddy, J. A., Klein, P. J., Vlahov, I. R., Dorton, R., Bloomfield, A., Nelson, M., Westrick, E., Parker, N., Bruna, K., Vetzel, M., Gehrke, M., Nicoson, J. S., Messmann, R. A., LoRusso, P. M., and Sausville, E. A. (2011) Reducing undesirable hepatic clearance of a tumor-targeted vinca alkaloid via novel saccharopeptidic modifications. *J. Pharmacol. Exp. Ther. 336*, 336−43.
(17) Leamon, C. P., Reddy, J. A., Vlahov, I. R., Kleindl, P. J., Vetzel, M., and Westrick, E. (2006) Synthesis and biological evaluation of EC140: A novel folate-targeted vinca alkaloid conjugate. *Bioconjugate Chem. 17*, 1226−32.
(18) Jiang, L., Zeng, X., Wang, Z., and Chen, Q. (2009) Cell line cross-contamination: KB is not an oral squamous cell carcinoma cell line. *European Journal of Oral Sciences 117*, 90−1.
(19) Leamon, C. P., Reddy, J. A., Dorton, R., Bloomfield, A., Emsweller, K., Parker, N., and Westrick, E. (2008) Impact of high and low folate diets on tissue folate receptor levels and antitumor responses toward folate-drug conjugates. *J. Pharmacol. Exp. Ther. 327*, 918−25.
(20) Leamon, C. P., and Low, P. S. (1991) Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis. *Proc. Natl. Acad. Sci. U.S.A. 88*, 5572−6.