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Design and regioselective synthesis of a new generation of targeted chemotherapeutics. Part 1: EC145, a folic acid conjugate of desacetylvinblastine monohydrazide

Iontcho R. Vlahov,* Hari Krishna R. Santhapuram, Paul J. Kleindl, Stephen J. Howard, Katheryn M. Stanford and Christopher P. Leamon

Endocyte Inc., 3000 Kent Ave, Suite A1-100, West Lafayette, IN 47906, USA

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Abstract—An efficient synthesis of the folate receptor (FR) targeting conjugate EC145 is described. EC145 is a water soluble derivative of the vitamin folic acid and the potent cytotoxic agent, desacetylvinblastine monohydrazide. Both molecules are connected in regioselective manner via a hydrophilic peptide spacer and a reductively labile disulfide linker.

The design and synthesis of novel chemotherapeutics continues with the aim of improving upon the treatment of cancers that are refractory to currently available methods. Under ideal circumstances, these new compounds would also diminish the inherent collateral toxicity to normal tissues. Receptor-specific targeting, as one approach, can potentially herald a new era in selective drug delivery to pathologic cells.

The vitamin folic acid (FA) binds with high affinity ($K_D < 10^{-9}$ M)[1] to a glycosylphosphatidylinositol anchored cell-surface glycoprotein called the folate receptor (FR). After binding, FA is transported into the cell via FR-mediated endocytosis.[3] Consequently, FA can be exploited as a molecular 'Trojan horse' for the targeted delivery of covalently attached, biologically active molecules.[4] The physiological events involved in the latter process (i.e., cell uptake) are identical to those for the free FA.[4a,5] Thus, after binding, the plasma membrane surrounding the FA or FA-Drug/FR complex invaginates to form an internal vesicle, called an early endosome, which entraps the FA or FA-Drug moiety. Next, proton pumps located in the endosome membrane lower the pH of the vesicle lumen to ~5, thus causing a conformational change in the FR which subsequently enables the FA (FA-Drug) moiety to be released.[6] The destinies of the FA (or FA-Drug) and the FR are thereafter determined during a sorting process occurring inside the late endosome; currently, it is known that the FR protein recycles back to the plasma membrane where it can participate in another round of internalization/drug delivery, whereas the released ligand (i.e., FA or FA-Drug) remains inside the cell. Since the FR is expressed at relatively high levels in human epithelial cancers, but has limited expression in normal tissues, there has been much interest in exploiting this natural cellular uptake mechanism for the targeted delivery of chemotherapeutic compounds.[2,4]

In this paper, we report our approach for the design and regioselective synthesis of a FA-*vinca* alkaloid conjugate **1** (EC145, Scheme 1). As indicated in the retrosynthetic scheme, **1** can be assembled by tethering a FA-Spacer unit **2** to the highly potent cytotoxic molecule, desacetylvinblastine monohydrazide **3**,[8] via a linker containing a reducible disulfide bond. The latter is important for drug delivery applications since real-time imaging using a fluorescence resonance energy transfer technique has recently demonstrated that reduction-mediated release of the drug cargo from a disulfide linked FA-conjugate efficiently occurs within the endosomes of cancer cells.[7]

The peptide-based derivative **2** was envisioned as a novel molecular spacer unit with the added advantage of introducing a discrete number of polar acidic (Asp)

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* Corresponding author. Tel.: +1 765 463 7175; fax: +1 765 463 9271; e-mail: ivlahov@endocyte.com

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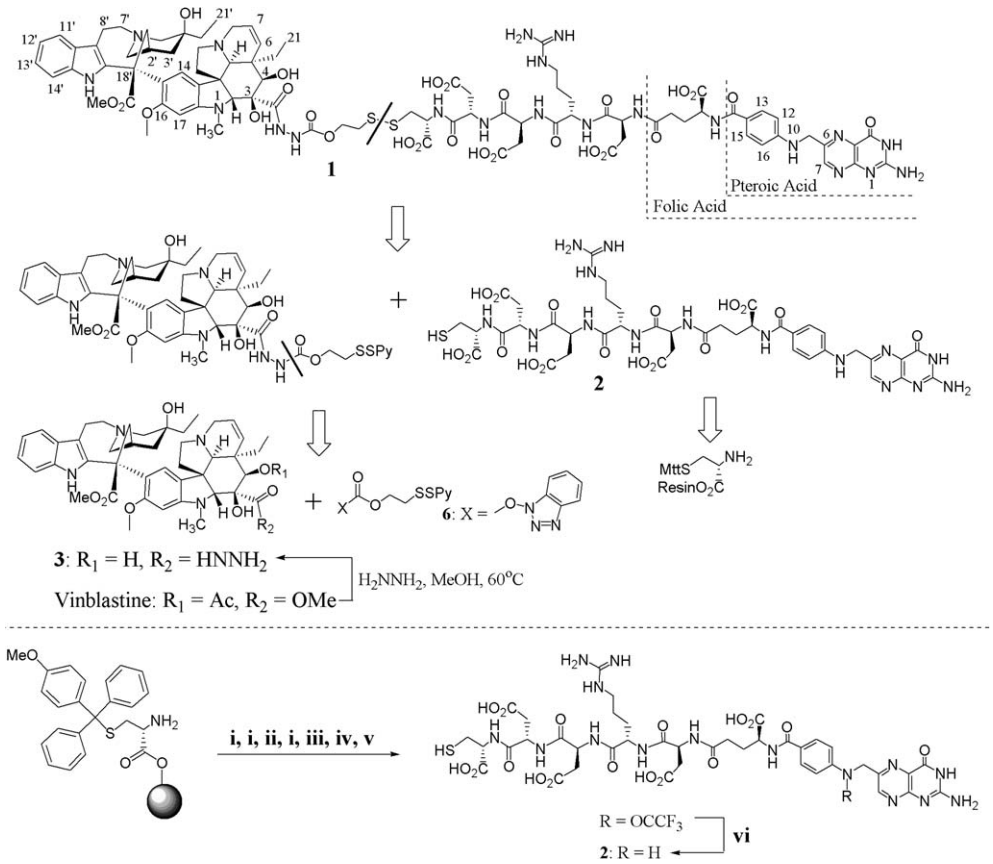


Scheme 1. Reagents and conditions: (i) a—Fmoc-Asp(OtBu)-OH, PyBOP, DIPEA, RT, 1 h; b—20% piperidine/DMF, rt, 10 min; (ii) a—Fmoc-Arg(Pbf)-OH, PyBOP, DIPEA, rt, 1 h; b—20% piperidine/DMF, rt, 10 min; (iii) a—Fmoc-Glu-OtBu, PyBOP, DIPEA, rt, 1 h; b—20% piperidine/DMF, rt, 10 min; (iv) N^{10}-TFA-pteroic acid, PyBOP, DIPEA, rt, 1.5 h; (v) TFA/H_2O/TIPS/EDT (92.5:2.5:2.5:2.5), rt, 1 h; (vi) aq NH_4OH, pH 9.3, rt, 1 h.

and/or basic (Arg) amino acids to tailor the water-solubility of the final drug conjugate. Pteroic acid[9] was to serve as N-terminus, whereas the thiol group of cysteine was to serve as the attachment site for the cleavable linker. These concepts allowed for the assembly of the spacer unit using standard fluorenylmethyloxycarbonyl-based solid phase peptide synthesis (Fmoc SPPS) on a Wang-resin polymeric support. The structure of **2** was confirmed by ^{1}H NMR[†] and LCMS [ESI $(M+H)^+$: 1046] analysis.

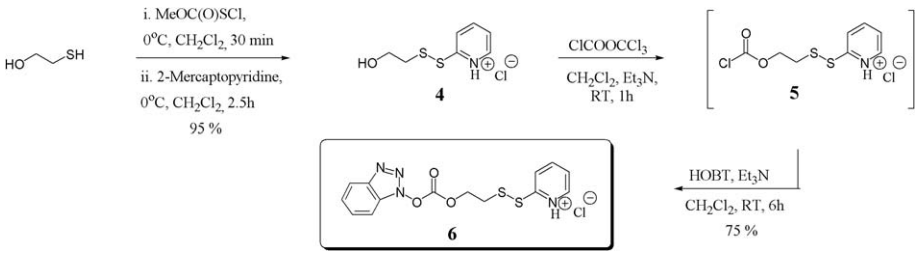
Activated carbonate **6** (Scheme 2) served as an important heterobifunctional crosslinker for conjugate synthesis. Carbonate **6** was readily prepared from 2-mercaptoethanol in two steps. Thus 2-mercaptoethanol was treated with methoxycarbonylsulfenyl chloride in DCM, followed by 2-mercaptopyridine to yield 2-(2-hydroxy-ethyldisulfanyl)-pyridine **4**.[10,11] Compound **4** and one equivalent of triethylamine were then added to a solution of diphosgene in dichloromethane to yield chloroformate derivative **5**. The chloroformate was not isolated, but was subsequently treated with *N*-hydroxybenzotriazole (HOBT) and an additional equivalent of triethylamine to give 2-[benzotriazole-1-yl-(oxycarbonyloxy)-ethyldisulfanyl]-pyridine **6** as a hydrochloride salt.[‡]

The synthesis of **6** has been found to be scaleable, as **6** and intermediate **4** are obtained in good yield and are readily isolated from their respective reaction mixtures by filtration. Activated carbonate **6** has an excellent shelf life (>2 years). More importantly, **6** has been found to react under mild conditions with many *N*- and *O*-nucleophiles, and in our hands has been shown to be a convenient tool for the incorporation of reductively labile disulfide linkages into a wide variety of drug conjugates.[12]

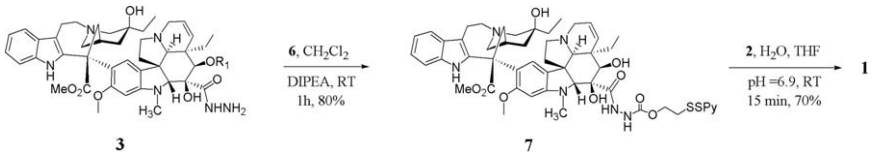
Desacetylvinblastine monohydrazide **3** was prepared from commercially available vinblastine (VLB) sulfate following a literature procedure.[13]

[†] Selected ^{1}H NMR data for **2** (D_2O, 300 MHz): δ 8.68 (s, 1H, FA H-7), 7.57 (d, 2H, J = 8.4 Hz, FA H-12 & 16), 6.67 (d, 2H, J = 9 Hz, FA H-13 & 15), 4.40–4.75 (series of m, 5H), 4.35 (m, 2H), 4.16 (m, 1H), 3.02 (m, 2H), 2.55–2.95 (series of m, 8H), 2.42 (m, 2H), 2.00–2.30 (m, 2H), 1.55–1.90 (m, 2H), 1.48 (m, 2H).

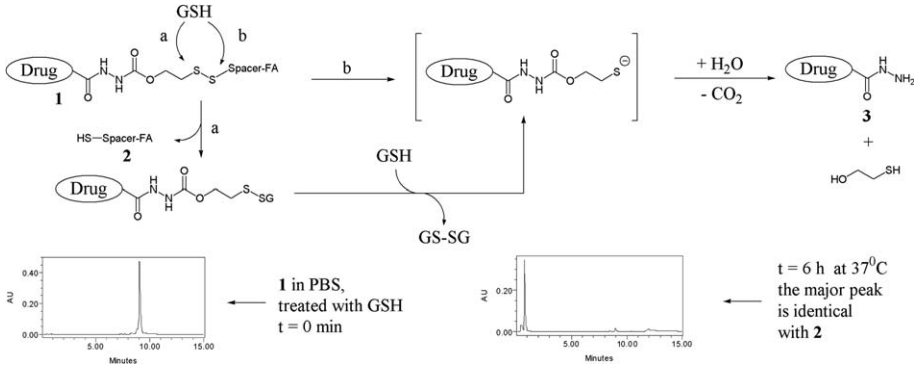
[‡] ^{1}H NMR for compound **6** (DMSO-d_6, 300 MHz): δ 8.38 (m, 1H), 8.16 (dt, 1H, J = 8 Hz, 1 Hz), 8.02 (dt, 1H, J = 8 Hz, 1 Hz), 7.88 (ddd, 1H, J = 8 Hz, 7 Hz, 1 Hz), 7.7 (m, 2H), 7.63 (ddd, 1H, J = 8 Hz, 7 Hz, 1 Hz,), 7.4–7.2 (br, 1H), 7.2 (m, 1H), 4.72 (t, 2H, J = 6 Hz), 3.36 (t, 2H, J = 6 Hz).



Scheme 2.



Scheme 3.



Scheme 4.

As shown in Scheme 3, hydrazide **3** was treated with the activated carbonate **6** and diisopropylethylamine (DIPEA) in dichloromethane to yield 2-(vinblastinyl)hydrazinecarboxylic acid 2-pyridyldithioethyl ester **7**. After chromatographic purification on silica gel, **7** was isolated in 80% yield. Treatment of a suspension of FA-Spacer **2** in H_2O under argon with 0.1 N $NaHCO_3$ resulted in a clear yellow solution at pH > 6.5. To this mixture was added at once under extensive stirring a solution of **7** in THF. According to the HPLC profile, the reaction was completed in 15 min. HPLC purification gave pure conjugate **1**.[14] LCMS [ESI $(M+H)^+$: 1918] and ^1H NMR signals[§] were in agreement with the expected structure. In brief, the ^1H NMR spectrum (300 MHz, D_2O) contained 11 aromatic signals in the range from 6.4 to 8.8 ppm (five from the folate moiety and six from the desacetylvinblastine moiety). The signals for the two olefinic protons in desacetylvinblastine appeared at 5.5 ppm (d) and 5.7 ppm (m).

Release of the free drug from the EC145 conjugate was also studied. Thus, a 20 mM solution of **1** in PBS buffer (pH 7.4) was treated with 1 mM L-glutathione (GSH) at 37 °C. The HPLC profile (UV detection at 280 nm) showed complete cleavage of the disulfide bond with concomitant release of the FA-Spacer **2** within 6 h, ($t_{1/2}$ = 1 h) (see Scheme 4).[15] Free desacetylvinblastine monohydrazide **3** was not observed on HPLC (UV detection) as the result of its extremely low solubility in PBS buffer; however, its formation was confirmed by LCMS.

Following positive in vitro and in vivo results and toxicological evaluation, EC145 was selected as a clinical candidate. Phase 1 clinical trials are currently underway at multiple US sites. The results of the complex biological investigations will be reported soon in appropriate scientific journals.

Acknowledgment

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[§] Selected ^1H NMR data for EC145 (D_2O, 300 MHz): δ 8.67 (s, 1H, FA H-7), 7.50 (br s, 1H, VLB H-11′), 7.30–7.40 (br s, 1H, VLB H-14′), 7.35 (d, 2H, J = 7.8 Hz, FA H-12 & 16), 7.25 (m, 1H, VLB H-13′), 7.05 (br s, 1H, VLB H-12′), 6.51 (d, 2H, J = 8.7 Hz, FA H-13 & 15), 6.4 (s, 2H, VLB H-14 & 17), 5.65 (m, 1H, VLB H-7), 5.5 (m, 1H, VLB H-6), 4.15 (m,1H, VLB H-8′), 3.82 (s, 3H, VLB $C_{18'}$ –CO_2CH_3), 3.69 (s, 3H, VLB C_{16} –OCH_3), 2.8 (s, 3H, VLB N-CH_3), 1.35 (br s, 1H, VLB H-3′), 1.15 (m, 1H, VLB H-2′), 0.9 (t, 3H, J = 7 Hz, VLB H-21′), 0.55 (t, 3H, J = 6.9 Hz, VLB H-21).

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14. Typical procedure: into a 0.1 N $NaHCO_3$ solution and a solution of 302 mg of **2** in 12 ml H_2O was bubbled Ar for 10 min. With continuous Ar bubbling, the bicarbonate solution was added to the solution of **2** so as to adjust the pH to ca. 7 (maintain Ar bubbling in the reaction vessel throughout the procedure). A solution of 258 mg of the **7** in 13 ml THF was then added to the solution of **2**. The reaction mixture was allowed to stir for 15 min. At this point HPLC indicated that reaction was complete. The THF was removed under reduced pressure. The resulting mixture was centrifuged. The aqueous layer was syringe filtered and loaded onto the preparative HPLC. HPLC purification using 10.0 mM sodium phosphate buffer (pH 7) and acetonitrile, followed by a second HPLC run with water and acetonitrile to remove the salt, resulted in the recovery of 350 mg of product.
15. A recent publication has suggested that in the case of phenolic carbonates, release of the linker takes place via formation of a cyclic, five-membered thio-carbonate. Wender, P. A.; Contag, C. H.; Jones, L. R.; Goun, E. A.; Shinde, R.; Rothbard, J. B. *J. Am. Chem. Soc.* **2006**, *128*, 6526.