JOC Article

An Assembly Concept for the Consecutive Introduction of Unsymmetrical Disulfide Bonds: Synthesis of a Releasable Multidrug Conjugate of Folic Acid

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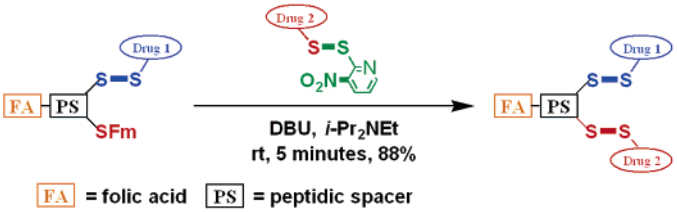

We describe the development of methodology which allows for the introduction of a second disulfide bond into a molecular framework with a pre-existing disulfide linker system. Compounds which contain an *S*-9-fluorenylmethyl-protected thiol and an additional disulfide linkage are deprotected *in situ* and trapped with an activated thiophile. This methodology allowed for the synthesis of the first molecule possessing two different biologically active agents covalently attached to a folate receptor targeting ligand unit *via* two disulfide-based release systems.

Herein, we describe a novel synthetic strategy which allows for the introduction of a second reductively labile disulfide linker system (or simply a disulfide bond) into a molecular framework with a pre-existing disulfide linker system. Such methodology allows for the delivery and release of multiple cargos (parent drugs, reporter molecules, etc.) in targeted cells.

Previously we reported[1] a disulfide-based folate conjugate of the potent antimitotic agent desacetyl vinblastine monohydrazide (dAc-VLB-mH) which is composed of a single cytotoxic molecule attached through a spacer and one releasable disulfide linker to folic acid (Drug−Linker-Spacer-FA). This conjugate exploits the folate receptor (FR), which is overexpressed in several forms of cancer, as a molecular "Trojan horse" for targeted delivery.[2] To improve upon the encouraging biological activity we saw with this compound,[3] we wished to synthesize a new folate conjugate with two releasable drug cargos with disparate mechanisms of action. Our efforts have resulted in the synthesis of **1** (Figure 1). **1** is the first molecule possessing two different biologically active agents: dAc-VLB-mH and the highly chemically sensitive alkylating agent mitomycin C (MMC). Both cargos are covalently attached to a FA−Spacer unit *via* two disulfide-based release systems.

The selection of synthons with suitable protecting and leaving groups, orthogonally stable toward multiple functional groups, and their application in an environment of highly charged molecules was of crucial importance to the synthetic design. As indicated in the retrosynthetic analysis (Scheme 1), the key precursor to the final construct **I** (simplified representation of dual drug conjugate **1**) is the thiol-containing disulfide conjugate **III**. Molecules such as **III**, which possess a disulfide bond as well as a free thiol functionality, are highly reactive, unstable, and prone to rearrangement (**VI**) and intramolecular cyclization (**VII**), thereby producing multiple side products. An elegant solution to this challenging problem involves a synthetic route in which **III** is generated *in situ* from the stable *S*-protected monoconjugate **IV**. Trapping the thiol group of **III** with a **Drug 2**-containing, disulfide-forming synthon **II** should result in the desired dual drug conjugate **I**. Monoconjugate **IV** would be synthesized using conjugation techniques which we have previously described.[1]

Proper selection of the protecting group for the thiol functionality is key to the successful implementation of this synthetic strategy. An ideal protecting group should be (i) easy

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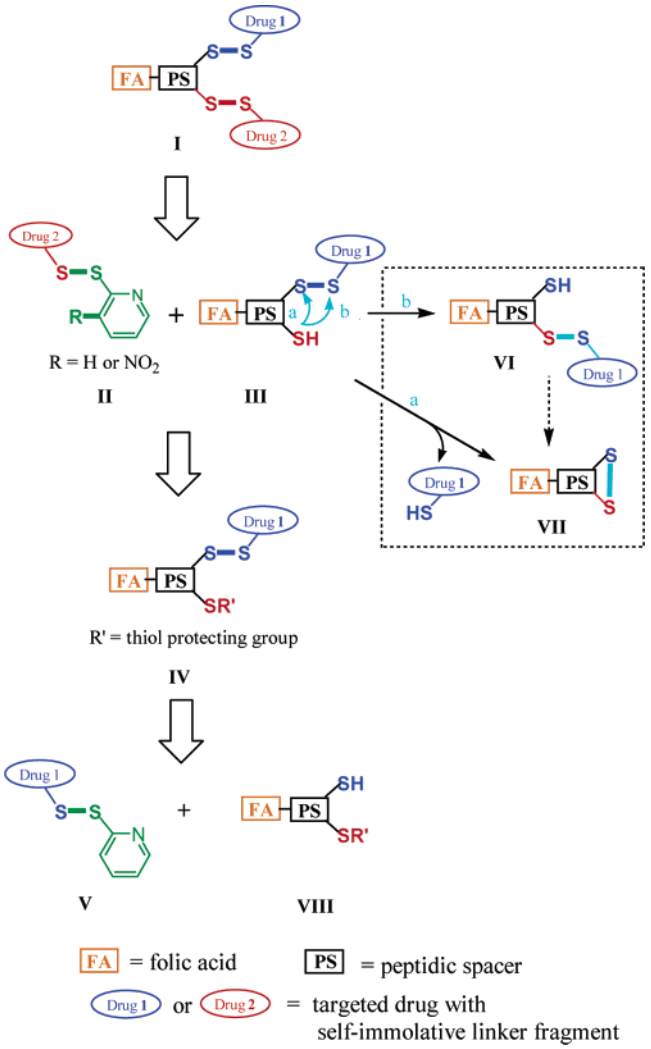
SCHEME 1



to introduce in a solid-phase peptide synthesis (SPPS) protocol on a suitable resin, (ii) stable under the strong acidic conditions applied for the removal of the peptidic moiety from the resin with simultaneous removal of all other protecting groups, and (iii) possessing sufficient chemoselectivity to ensure a fast and regioselective *in situ* generation and concomitant trapping of the thiol group by an external thiophile in the presence of a existing disulfide moiety. The rarely used *S*-9-fluorenylmethyl thioether (Fm-SR)[4] satisfies all of these criteria.

The synthesis of **1** began with the construction of monoconjugate **5** from peptide-based derivative **3** (Scheme 2). Peptide-based derivative **3** is a novel FA−Spacer unit designed to introduce a discrete number of charged amino acids (Asp). As such, **3** tailors the water-solubility and suppresses the passive diffusion of the FA−Spacer−Linker−Drug system across the lipophilic cell membrane, thereby decreasing undesirable delivery to cells not expressing the folate receptor. Pteroic acid[5] served as the N-terminus, whereas the thiol group of cysteine provided attachment site for one of the cleavable linkers. A second attachment site was achieved by the addition of *S*-Fm-3-thiopropionic acid to the β-amino functionality of the diaminopropionic acid moiety prior to resin cleavage. Assembly of monoconjugate **5** was performed by the addition of a solution of the activated vinblastine disulfide **4** in THF to a pH-adjusted (~6.9) solution of the FA−Spacer **3** in water.[1] **5** was isolated in excellent yield (75%) after preparative HPLC.

With monoconjugate **5** in hand, we attempted to remove the *S*-Fm group in the presence of the known disulfide forming MMC derivative **6**.[6] Our attempts to utilize the two common deprotection protocols were of only limited success. First, the removal of the Fm moiety using piperidine[7] was slow and generated multiple side products. Second, applying 2-*tert*-butyl-1,1,3,3-tetramethylguanidine[8] (TBTMG) in lieu of piperidine, as suggested by the work of Corey et al., resulted in a complex mixture of the desired conjugate **1**, cyclic disulfide **8**, and 2-pyridyl-disulfide **9** (Scheme 3 and Figure 2). One of our original ideas for the deprotection of the Fm group was to use DBU as a strong non-nucleophilic base. In the presence of **6**, these novel reaction conditions provided in less than 5 min a mixture of **1** and **9** as a major side product. We concluded that after the formation of the desired bis-disulfide **1**, the released 2-thiopyridine acted as a thiophile and attacked the newly generated disulfide bond in **1** to form **9**. A concise solution to this synthetic problem was developed using the known MMC derivative **7**.[6] The presence of the strong electron-withdrawing nitro group on the C-3-atom in the 2-thiopyridine moiety in **7** dramatically reduced the thiophilic and/or nucleophilic power of the leaving group. While fine-tuning the reaction conditions of the final conjugation step, we found that the addition of *i*-Pr_2-NEt to a DMF solution of the fully protonated form of **5** and **7**, followed by DBU, resulted in a clean and fast conversion to the desired product. According to the HPLC profile (Figure 3), the reaction was complete in 5 min. HPLC purification gave the dual drug conjugate **1** in 88% yield.

We have found this methodology for introducing a second disulfide bond to be superior to methods in which the second disulfide linker−drug moiety is constructed separately and then appended to the peptidic backbone of a monoconjugate. For example, acylation of the *C*-3-amino group in the 2,3-diaminopropionic acid moiety in **10** using MMC-$(CH_2)_2SS(CH_2)_2$-CO_2H was hampered by poor, irreproducible yields (Scheme 4, first reaction). The extreme instability of this MMC derivative and its tendency, after activation of the carboxy group, toward self-condensation through the aziridine group was the likely cause of this difficulty. We have also attempted to apply the Huisgen reaction in a "click" chemistry protocol as described by Sharpless et al.[9] to synthesize a number of other dual drug conjugates (Scheme 4, second reaction). A propargylglycine moiety was incorporated into the peptidic backbone of a monoconjugate (**11**), and a $(O)CO(CH_2)_2SS(CH_2)_2N_3$ releasable linker moiety was added to an appropriate nucleophilic site of a second drug (**12**), to provide the alkyne and azide for the dipolar [2 + 3] cycloaddition reaction. Unfortunately, only moderate yields of 1,2,3-triazole regioisomeric dual drug conjugates were obtained.[10]

The biological activity of **1** was evaluated by treating mice bearing established, subcutaneous human KB tumor xenografts

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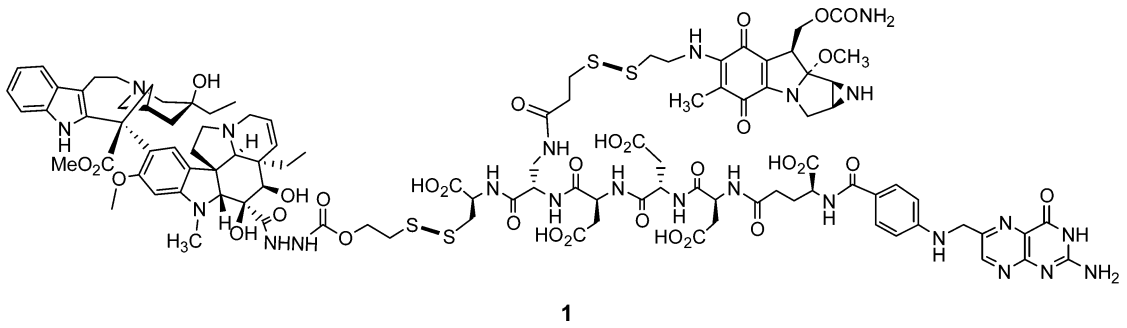

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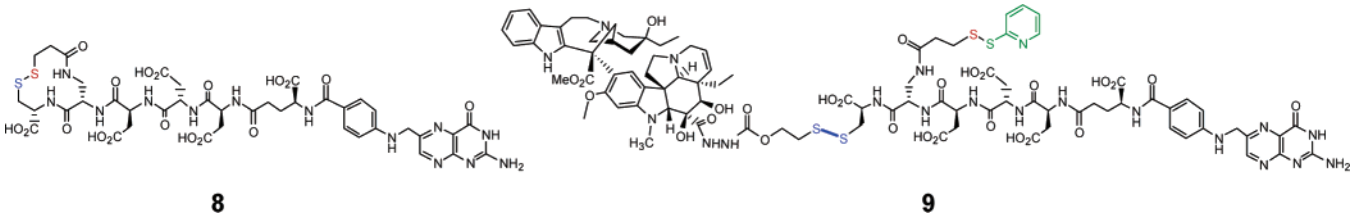
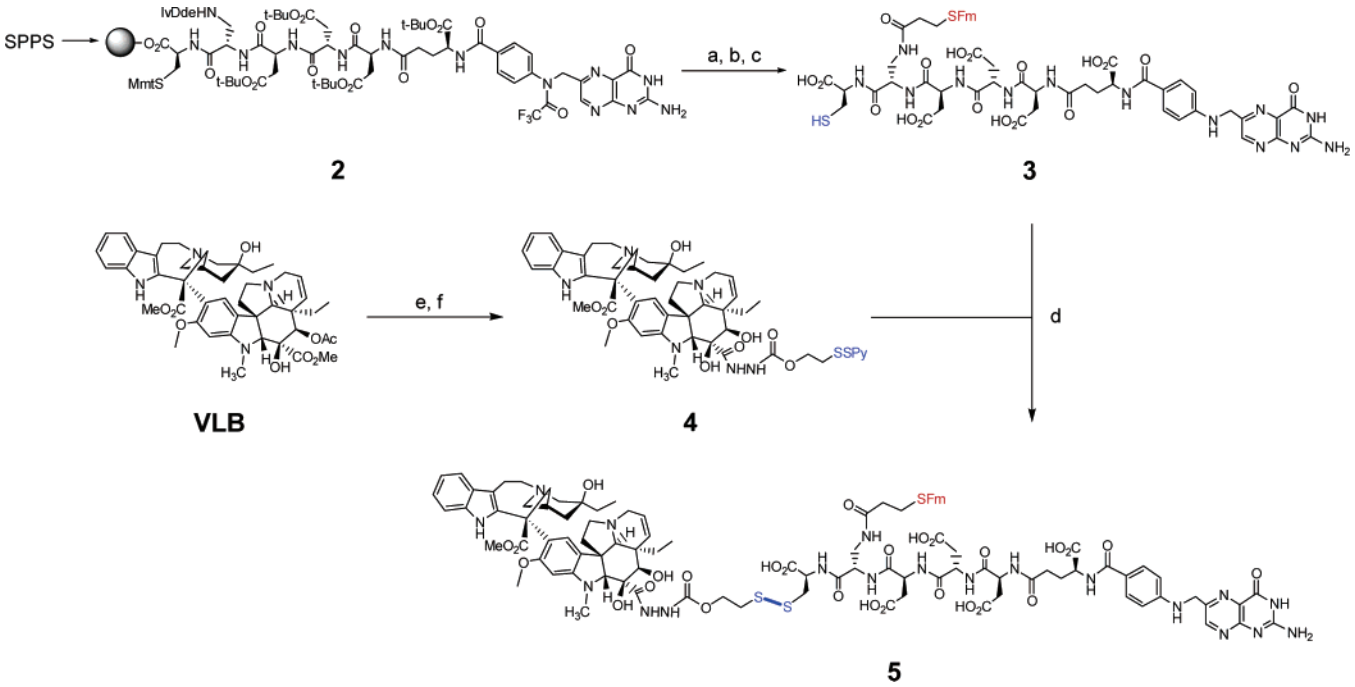
FIGURE 1. The dAc−VLB−mH- and MMC-containing folate conjugate.



8 9

FIGURE 2. Major byproducts resulting from the addition of MMC derivative **6** to monoconjugate **5**.

SCHEME 2



(a) 2% NH_2NH_2 in DMF, 5 min, rt. (b) $FmS(CH_2)_2CO_2H$, PyBoP, *i*-Pr_2NEt, DMF. (c) TFA, H_2O, TIPS, EDT, 65% overall yield from step a. (d) THF/H_2O (1:1), 75%. (e) NH_2NH_2, MeOH, Δ, 15 h, 78%. (f) $PySS(CH_2)_2OC(O)OBt$, TEA, DCM, 83%.

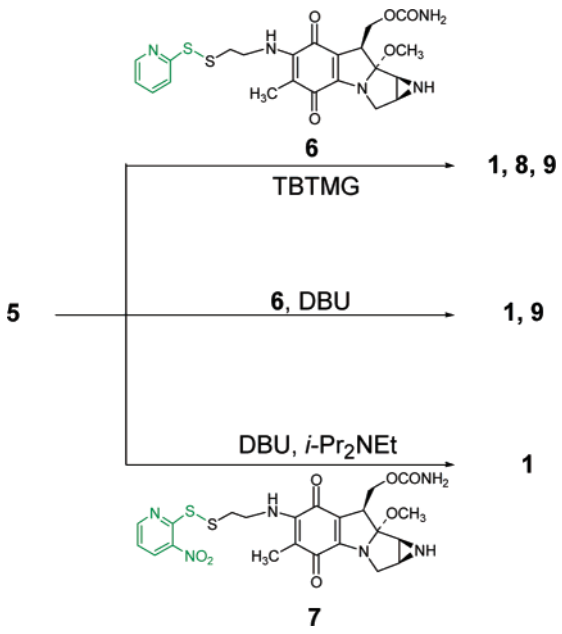
intravenously with **1** following the schedule indicated (Figure 4). **1** convincingly displayed potent antitumor activity, where five out of five of treated animals were determined to be tumor free by Day-26. Importantly, animals did not lose any weight at any time during or after therapy, demonstrating a significant reduction in toxicity to normal cells. The activity of **1** is superior to that of its monodrug−folate counterparts (folate−MMC and folate−dAc−VLB−mH) at equimolar concentrations and when the monodrug−folate conjugates are used in combination, as all of these treatment protocols failed to produce any complete responses ($n = 5$ in each study). These results, as well as others which will be reported simultaneously in an appropriate scientific journal, have led to the advancement of **1** to Phase 1 clinical trials.

(10) Unpublished results.

Experimental Section

Synthesis of Folate−Peptide Linker. Pte-γGlu-Asp−Asp-Asp-Dap(*S*-Fm-3-thiopropionyl)-Cys-OH 3. In a peptide synthesis vessel H-Cys(4-methoxytrityl)-2-chlorotrityl-resin (1.0 g, 0.56 mM) was loaded and washed with *i*-PrOH (3 × 10 mL) followed by DMF (3 × 10 mL). To the vessel were then introduced the amino acid solution (2.0 equiv) in DMF, *i*-Pr_2NEt (4.0 equiv), and PyBOP

SCHEME 3



(2.0 equiv). Argon was bubbled for 1 h, the coupling solution was drained, and the resin was washed with DMF (3 × 10 mL) and *i*-PrOH (3 × 10 mL). Kaiser tests were performed to assess reaction completion. Fmoc deprotection was carried out using 20% piperidine in DMF (3 × 10 mL), before each amino acid coupling. The above sequence was repeated to complete six coupling steps. At the end, the resin was washed with 2% hydrazine in DMF 3 × 10 mL (5 min) to cleave the trifluoroacetyl protecting group on pteroic acid and the IvDde protecting group on diaminopropionic acid. Finally, *S*-Fm-3-thiopropionic acid (1.25 equiv) in DMF using *i*-Pr_2-NEt (2.5 equiv) and PyBop (1.25 equiv) was coupled. Argon was bubbled for 1 h, the coupling solution was drained, and the resin was washed with DMF (3 × 10 mL) and *i*-PrOH (3 × 10 mL). The resin was dried under argon for 30 min. Folate−peptide was cleaved from the resin using a cleavage mixture consisting of 92.5% CF_3CO_2H, 2.5% H_2O, 2.5% triisopropylsilane, and 2.5% ethanedithiol. A 25 mL amount of the cleavage mixture was introduced, and argon was bubbled for 1.5 h. The cleavage mixture was drained into a clean flask. The resin was washed three times with more cleavage mixture. The combined mixture was concentrated under reduced pressure to a smaller volume (∼ 5 mL) and precipitated in ethyl ether. The precipitate was collected by centrifugation, washed with ethyl ether (3 times), and dried under high vacuum. The crude product was purified by preparative HPLC (mobile phase A = 10 mM ammonium acetate, pH = 5; organic phase B = acetonitrile; method: 1% B to 20% B in 40 min at 15 mL/min) and furnished folate−peptide spacer **3** (∼450 mg, 65%). ^{1}H NMR (300 MHz, DMSO-d_6 with D_2O): δ 8.60 (s, 1H), 7.78 (d, J = 7.5 Hz, 2H), 7.71−7.67 (m, 2H), 7.56 (d, J = 8.7 Hz, 2H), 7.31−7.25 (m, 4H), 6.61 (d, J = 8.7 Hz, 2H), 4.51−4.38 (m, 5H), 4.35 (t, J = 6.9 Hz, 1H), 4.19−4.09 (m, 3H), 3.28−3.04 (m, 4H), 2.84−2.44 (m, 12H), 2.27−1.94 (m, 4H). ^{13}C NMR (75.5 MHz, DMSO-d_6): 173.8, 172.0 (4C), 171.5, 171.1 (2C), 170.9, 170.3, 169.6, 166.4, 161.0, 156.5, 153.8, 150.8, 148.6 (2C), 146.0 (2C), 140.5 (2C), 129.1 (2C), 128.0, 127.4 (2C), 127.0 (2C), 125.0 (2C), 121.3, 119.9 (2C), 111.2 (2C), 54.6, 52.7, 52.2, 49.9, 49.8, 49.6, 46.3, 45.9, 36.1, 36.0, 35.9, 35.7, 35.1 (2C), 32.0, 27.9, 26.6, 25.4. HRMS (MALDI): $(M + H)^+$ = Calculated for $C_{54}H_{60}N_{13}O_{18}S_2$,1242.3518; found 1242.3319.

3-(4-Desacetylvinblastinyl)hydrazinecarboxylic Acid (2′-Pyridyldithio)ethyl Ester 4. To a solution of 4-desacetyl-3-vinblastine monohydrazide (200 mg, 0.260 mmol) and 2-[benzotriazole-1-yl-

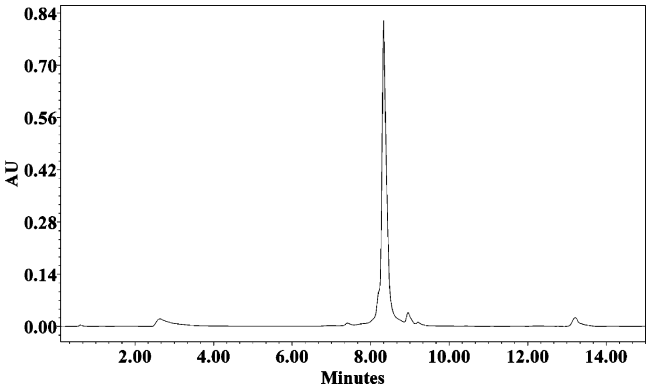


FIGURE 3. HPLC profile of the addition of **5** and **7** under DBU conditions at t = 5 min. The major peak is the desired product **1**.

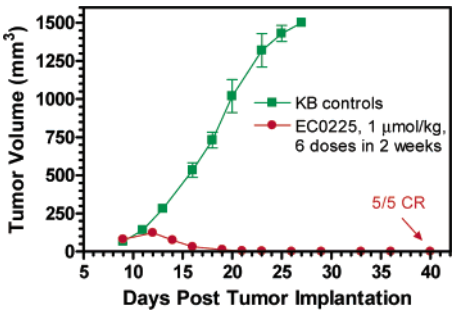
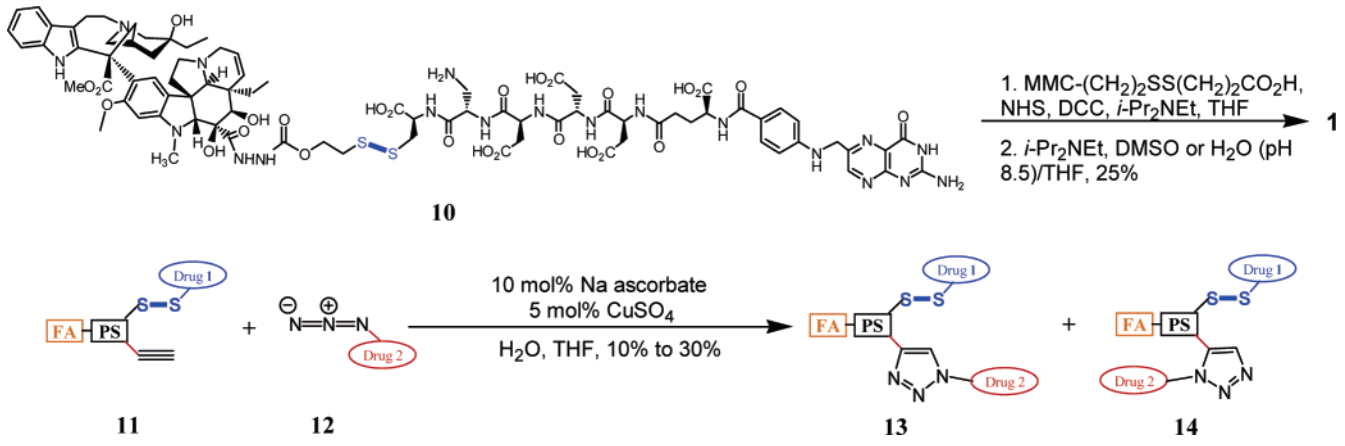


FIGURE 4. Effect of **1** on KB tumor volume in mice. Treatment began 9 days after tumor implantation.

SCHEME 4



(oxycarbonyloxy)-ethyldisulfanyl]-pyridine (110 mg, 0.286 mmol) in CH_2Cl_2 (5 mL) was added TEA (0.087 mL, 0.62 mmol). The reaction was allowed to stir for 2 h. TLC (15% MeOH in CH_2Cl_2) indicated that the reaction was complete. The reaction mixture was diluted with CH_2Cl_2 and washed with 10% aqueous K_2CO_3. The organic layer was dried over $MgSO_4$ and concentrated to a small volume. This material was then loaded onto a SiO_2 column and chromatographed (10% MeOH in CH_2Cl_2) to yield pure 3-(4-desacetylvinblastinyl)hydrazinecarboxylic acid (2′-pyridyldithio)-ethyl ester **4** (204 mg, 83%). ^{1}H NMR (300 MHz, CD_2Cl_2): δ 8.44 (d, J = 5.0 Hz, 1H), 8.15 (s, br, 1H), 7.78−7.70 (m, overlapped, 2H), 7.49 (d, J = 7.6 Hz, 1H), 7.14 (m, 2H), 7.06 (m, 1H), 6.61 (s, 1H), 6.14 (s, 1H), 5.84 (dd, J = 10.5, 3.8 Hz, 1H), 5.70 (d, J = 10.5 Hz, 1H), 4.37 (t, J = 6.3 Hz, 2H), 4.01 (s, 1H), 3.93 (d, J = 12.3 Hz, 1H), 3.79 (s, 3H), 3.60 (s, 3H), 3.55 (s, 1H), 3.41 (d, J = 13.0 Hz, 1H), 3.33−3.12 (m, 2H), 3.08 (t, J = 6.4 Hz, 2H), 2.84−2.80 (overlapped, $\underline{CH_3}$ + 2H), 2.65 (s, 1H), 2.47−2.39 (m, 2H), 2.22 (dd, J = 15.5, 3.2 Hz, 1H), 2.08−1.98 (m, 1H), 1.81−1.62 (m, 2H), 1.52−1.22 (m, 4H), 0.95−0.87 (overlapped, $\underline{CH_3}$+ $\underline{CH_3}$). LCMS (ESI): $(M + H)^+$ = Calculated for $C_{51}H_{64}N_7O_9S_2$, 982.41; found 982.20

Pte-γGlu-Asp−Asp-Asp-Dap(*S*-Fm-3-thiopropionyl)-Cys(*S*-ethyl-3-(4-desacetylvinblastinyl)hydrazinecarboxylate) 5. In a polypropylene centrifuge bottle, Pte-γGlu-Asp−Asp-Asp-Dap(*S*-Fm-3-thiopropionyl)-Cys-OH **3** (112 mg, 0.09 mmol) was dissolved in 7.5 mL of water and bubbled with argon for 10 min. In another flask, a 0.1 N $NaHCO_3$ solution was bubbled with argon for 10 min, and the pH of the folate linker solution was carefully adjusted to 6.9 using the 0.1 N $NaHCO_3$ solution. The 3-(4-desacetylvinblastinyl)hydrazinecarboxylic acid 2-pyridyldithioethyl ester **4** (88 mg, 0.09 mM) in 7.5 mL of tetrahydrofuran (THF) was added to the above solution. The resulting clear solution was stirred under argon for 15 min to 1 h. Progress of the reaction was monitored by analytical HPLC (10 mM ammonium acetate, pH = 7.0 and acetonitrile). THF was removed under reduced pressure, and the aqueous solution was filtered and injected on a prep-HPLC column. Elution with 1 mM sodium phosphate (pH = 7.0) buffer A and acetonitrile B (method: 1% B to 80% B in 25 min at 25 mL/min) resulted in pure fractions containing the product. Pure fractions were pooled, acetonitrile was removed under reduced pressure at ambient temperature, and pH was adjusted to 4.0 using 0.1 N HCl. Vinblastine−folate conjugate **5** was isolated after freeze-drying for 48 h (157 mg, 75%). ^{1}H NMR (300 MHz, DMSO-d_6 with D_2O) δ 8.60 (s, 1H), 7.78 (d, J = 6.9 Hz, 2H), 7.68−7.65 (m, 2H), 7.58 (d, J = 8.7 Hz, 2H), 7.46−7.22 (m, 6H), 7.07−6.95 (m, 2H), 6.61 (d, J = 8.4 Hz, 2H), 6.36 (s, 1H), 6.19 (s, 1H), 5.68 (m, 1H), 5.58 (d, J = 10.2 Hz, 1H), 4.56−4.09 (m, 11H), 3.83−1.85 (m, 50H), 1.56 (br s, 2H), 1.38−1.26 (m, 5H), 0.79 (t, J = 6.9 Hz, 3H), 0.70 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75.5 MHz, DMSO-d_6): 174.5, 173.9, 172.9, 172.3, 172.1, 172.0, 171.8, 171.4, 171.2, 171.0, 170.8, 170.0, 169.7, 166.4, 161.3, 157.4, 155.78, 155.77, 154.2, 152.5, 150.8, 148.7, 148.5, 146.0 (2C), 140.5 (2C), 135.5, 131.9, 131.6, 129.0 (2C), 128.4, 127.8, 127.3 (2C), 127.0 (2C), 125.0 (2C), 123.4, 122.6, 122.5, 121.5, 121.4, 119.9 (2C), 118.8, 118.4, 117.8, 114.1, 111.7, 111.3 (2C), 92.9, 82.8, 80.5, 73.5, 67.0, 64.6, 62.4, 62.0, 56.1 (2C), 54.9 (2C), 52.8 (2C), 52.5 (2C), 52.2 (2C), 52.1 (2C), 50.0, 49.8, 49.4, 48.9, 46.2 (2C), 45.9, 45.5, 45.0, 42.0, 37.8, 37.0, 36.4, 35.9 (2C), 35.7, 35.1 (2C), 34.2, 31.9, 31.8, 27.8 (2C), 26.7, 8.1, 7.0. HRMS (MALDI): $(M + H)^+$ = Calculated for $C_{100}H_{118}N_{19}O_{27}S_3$, 2112.7500; found 2112.7701.

Pte-γGlu-Asp−Asp-Asp-Dap{*S*-[7-*N*-(2-mercaptoethyl)mitomycin C]-3-thiopropionyl}-Cys(*S*-ethyl-3-(4-desacetylvinblastinyl)hydrazinecarboxylate) 1. Anhydrous DMF (4.5 mL) was syringed into a mixture of Pte-γGlu-Asp-Asp-Asp-Dap(*S*-Fm-3-thiopropionyl)-Cys(*S*-ethyl-3-(4-desacetylvinblastinyl)hydrazine-carboxylate) **5** (103 mg, 48.7 μmol) and N^7-((3′-nitropyridyl-2′-yl)dithioethyl)mitomycin C **7** (33.4 mg, 1.25 equiv) at room temperature under argon. To the resulting solution were syringed *i*-Pr_2NEt (84.9 μL, 10 equiv) and DBU (72.9 μL, 10 equiv.) in tandem. The reaction mixture was stirred at room temperature under argon for 20 min. Analytical HPLC (mobile phase A = 1.0 mM phosphate buffer, pH 7.0; organic phase B = acetonitrile; method: 5% B to 50% B in 10 min at 1 mL/min) of the reaction mixture confirms the completion of the reaction. The reaction mixture was transferred into stirring diethyl ether (50 mL). The resulting suspension was centrifuged, and the precipitate was washed with diethyl ether (15 mL × 2), dissolved in phosphate buffer (9 mL, 1.25 mM, pH 6.8), and subjected to preparative HPLC (mobile phase A = 1.0 mM phosphate buffer, pH 7.0; organic phase B = acetonitrile; method: 10% B to 40% B in 25 min at 25 mL/min). Pure fractions were collected, and acetonitrile was removed under reduced pressure and freeze-dried to afford 99.2 mg (88%) of dual drug conjugate **1**. ^{1}H NMR (300 MHz, DMSO-d_6 with D_2O) δ 8.61 (s, 1H), 7.51 (d, J = 8.7 Hz, 2H), 7.35 (d, J = 7.8 Hz, 1H), 7.22 (d, J = 8.1 Hz, 1H), 7.00 (t, J = 7.8 Hz, 1 H), 6.95 (t, J = 8.1 Hz, 1H), 6.61 (d, J = 9.0 Hz, 2H), 6.38 (s, 1H), 6.18 (s, 1H), 5.71 (m, 1H), 5.57 (d, J = 10.2 Hz, 1H), 4.54−4.39 (m, 5H), 4.30−4.19 (m, 5H), 4.06−3.96 (m, 4H), 3.77 (br s, 3H), 3.51 (s, 3H), 3.39−3.34 (m, 4H), 3.24−3.20 (m, 3H), 3.08 (s, 6H), 2.96−2.83 (m, 8H), 2.74 (s, 3H), 2.70−2.28 (m, 21H), 2.08−1.69 (m, 10H), 1.54 (br s, 2H), 1.32−1.15 (m, 5H), 0.77 (t, J = 7.2 Hz, 3H), 0.71 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125.71 MHz, DMSO-d_6/D_2O): 178.7, 176.9, 176.5 (4C), 176.3, 175.1, 174.4, 173.5, 173.1 (2C), 173.0, 170.7, 167.5, 164.1, 158.5 (2C), 158.3, 157.4, 156.4, 155.9, 155.5, 153.5, 151.5, 150.3, 149.7, 148.7, 136.0, 131.9, 131.6, 129.8 (2C), 129.3, 128.4, 124.8, 124.2, 123.5, 123.2, 122.5, 119.9, 119.0, 116.1, 112.7 (2C), 112.3, 109.9, 106.8, 104.1, 94.1, 83.4, 81.3, 74.5, 73.0, 68.4, 65.9, 64.0, 63.5, 62.7, 62.5, 62.2, 57.0 (3C), 55.9, 55.3, 54.9, 54.1, 53.7 (2C), 53.4, 52.2 (3C), 51.5, 50.8, 50.5, 50.4 (3C), 50.0, 46.5, 45.7, 44.1 (2C), 43.7, 42.9, 42.2, 41.1 (2C), 38.3, 36.4, 35.9, 35.1, 34.4, 32.9 (3C), 29.3 (2C), 10.5, 9.1, 7.8. LCMS (ESI): $(M + H)^+$ = Calculated for $C_{103}H_{128}N_{23}O_{32}S_4$, 2327.80; found 2327.93

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Supporting Information Available: Experimental procedures and spectral data. This material is available free of charge via Internet at http://pubs.acs.org.

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