Bioorganic & Medicinal Chemistry Letters 18 (2008) 4558–4561

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design and regioselective synthesis of a new generation of targeted chemotherapeutics. Part II: Folic acid conjugates of tubulysins and their hydrazides

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ARTICLE INFO

Article history:
Received 13 June 2008
Revised 8 July 2008
Accepted 10 July 2008
Available online 15 July 2008

Keywords:
Tubulysin
Targeted therapeutics
Folate
Anti-cancer
Disulfide

ABSTRACT

Efficient syntheses of folate conjugates of tubulysins and their hydrazides **1a–d** are described. These water soluble folate receptor (FR) targeted conjugates are derivatives of folic acid and the potent cytotoxic agents: tubulysin A, B, or their respective hydrazides, connected in regioselective manner via a hydrophilic peptide spacer and a reducible disulfide linker.

The tubulysins are members of a new class of natural products isolated from a myxobacterial species.[1] As cytoskeleton interacting agents, the tubulysins inhibit tubulin polymerization, leading to cell cycle arrest and apoptosis.[2] Tubulysins are exceptionally potent cytotoxic molecules, exceeding the cell growth inhibition observed with epothilones, paclitaxel, and vinblastine by 20- to 1000-fold. Furthermore, they are potent against multidrug resistant cell lines.[3] Nevertheless, in vivo studies suggest that the natural tubulysins alone are unsuitable for clinical development because of an extremely tight therapeutic window.[4]

On the other hand, receptor-targeted chemotherapy has emerged as one of the major approaches in modern drug discovery as it can potentially satisfy the selective delivery criteria for toxic agents to pathologic cells. In a previous publication,[5] we reported the design and synthesis of a folate-targeted desacetylvinblastine conjugate, EC145, which is currently in phase 2 clinical trials. In EC145, the anti-cancer drug vinblastine was modified and attached to folic acid (FA) via a water soluble peptide spacer and a reducible disulfide linker. Once administered, the conjugate targets folate receptor (FR) over-expressing cancer cells and releases the base drug after internalization. This receptor-targeted delivery allows reduced collateral toxicity and hence improves efficacy.

In this paper we report the design and regioselective synthesis of FA–tubulysin conjugates **1a–d** (Scheme 1). As indicated in the retrosynthetic scheme, **1** can be assembled by tethering a FA-Spacer unit **2** to tubulysins A and B (**3a–b**) or their hydrazides via a self-immolative linker containing a reducible disulfide bond, which is important for drug delivery applications. A recent study involving real-time imaging using a fluorescence resonance energy transfer technique has demonstrated that reduction-mediated release of the drug cargo from a disulfide linked FA-conjugate efficiently occurs within the endosomes of cancer cells.[6]

The peptide-based spacer **2** was designed to be bifunctional containing both acidic (Asp) and basic (Arg) amino acids to provide the best potential for water solubility of the final drug conjugate under physiological conditions. This unit was assembled using standard fluorenylmethyloxycarbonyl-based solid phase peptide synthesis (Fmoc SPPS).[5] The structure of **2** was confirmed by ^{1}H NMR† and LC/MS [ESI $(M+H)^+$: 931.2] analysis.

Our initial attempt at the regioselective conjugation of tubulysin involved the use of the phenolic oxygen as a nucleophile and reacting it with the activated carbonate **5** (Scheme 2). However, the activated tubulysin carbonate **6** proved to be sensitive to water, indicating that its folate conjugate counterpart might have limited stability under physiological conditions, and hence an alternate approach was pursued. Alternatively, the carboxylic group of the tubulysin was selected to connect to the folate-spacer unit **2** via a self-immolative disulfanylethyl ester. A scalable and simple to

† Selected ^{1}H NMR data for **2** (DMSO-d^6 with D_2O exchange, 300 MHz): δ 8.64 (s, 1H), 7.63 (d, J = 8.7 Hz, 2H), 6.65 (d, J = 9.0 Hz, 2H), 4.54–4.48 (m, 4H), 4.25 (m, 2H), 4.03 (t, J = 4.2, 1H), 3.03 (br, 2H), 2.93–2.46 (m, 6H), 2.20 (br, 2H), 1.98–1.80 (m, 3H), 1.61 (m, 1H), 1.50 (m, 2H).

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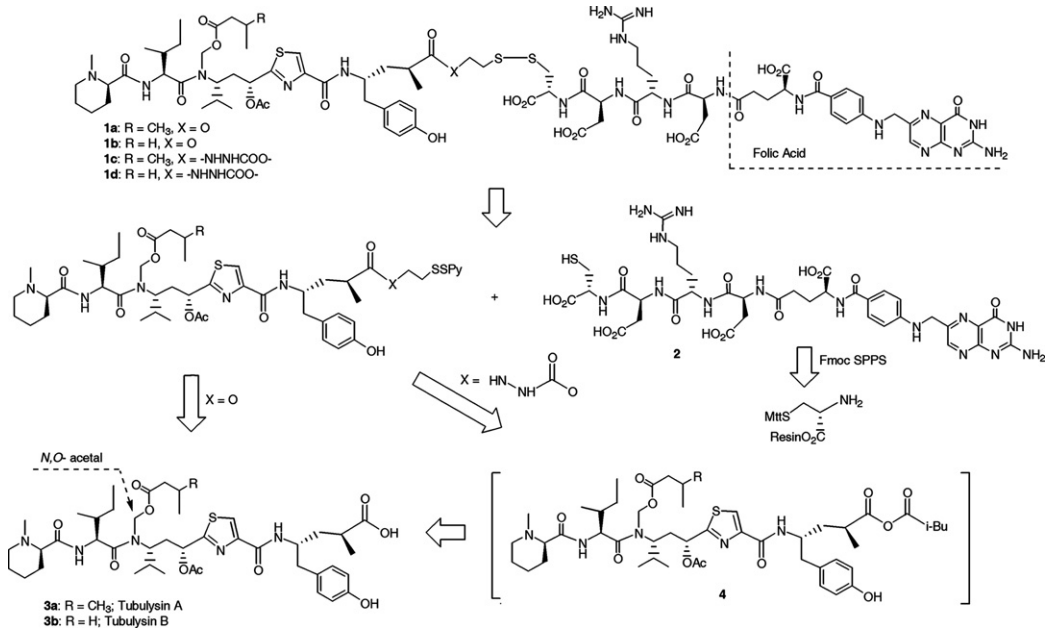
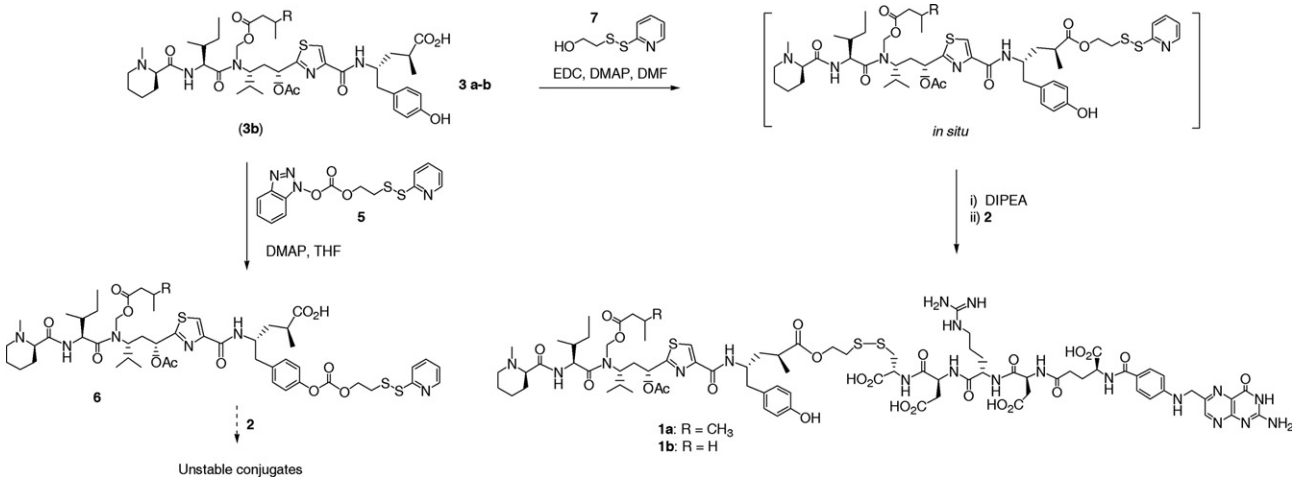
doi:10.1016/j.bmcl.2008.07.041



Scheme 1.

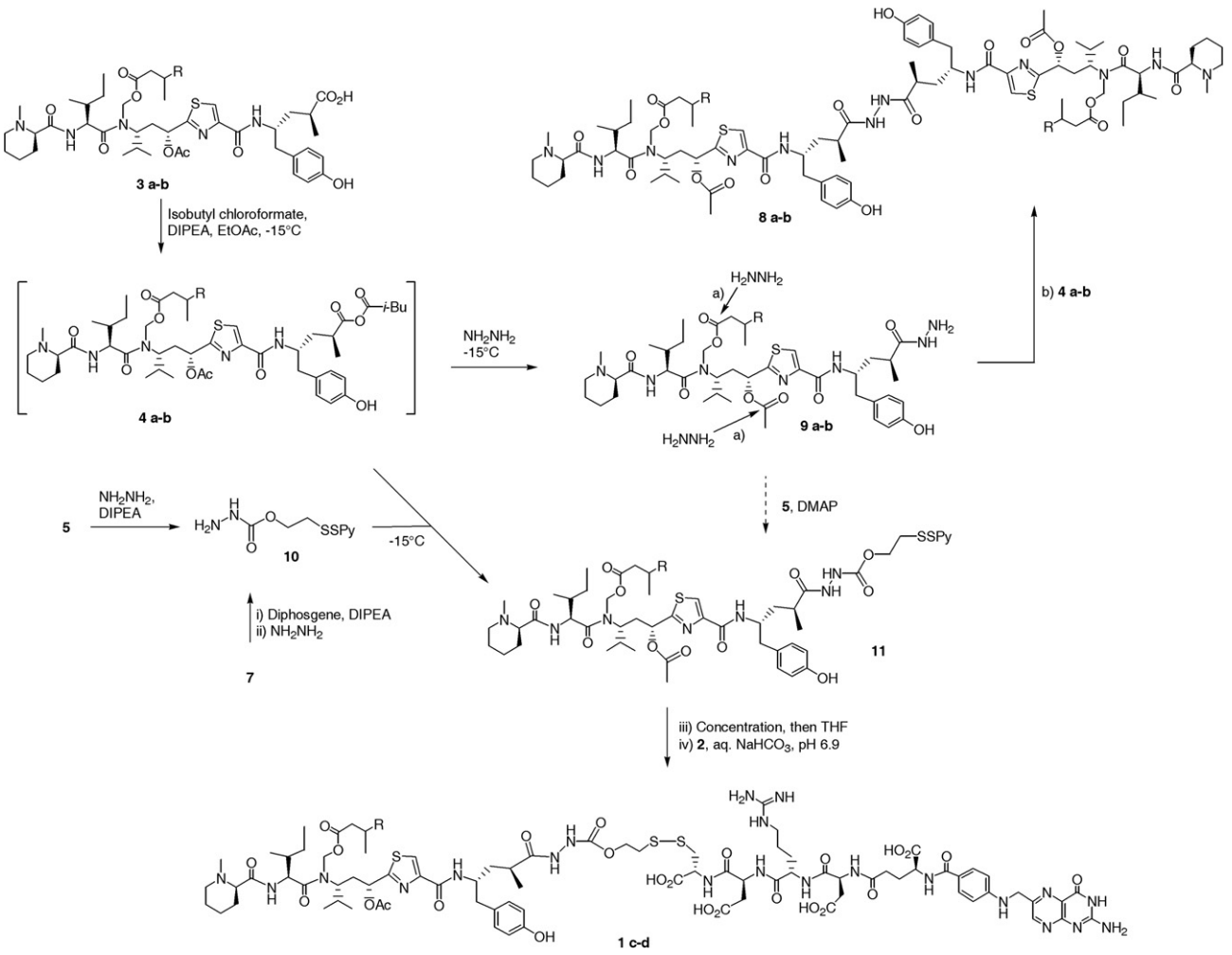


Scheme 2.

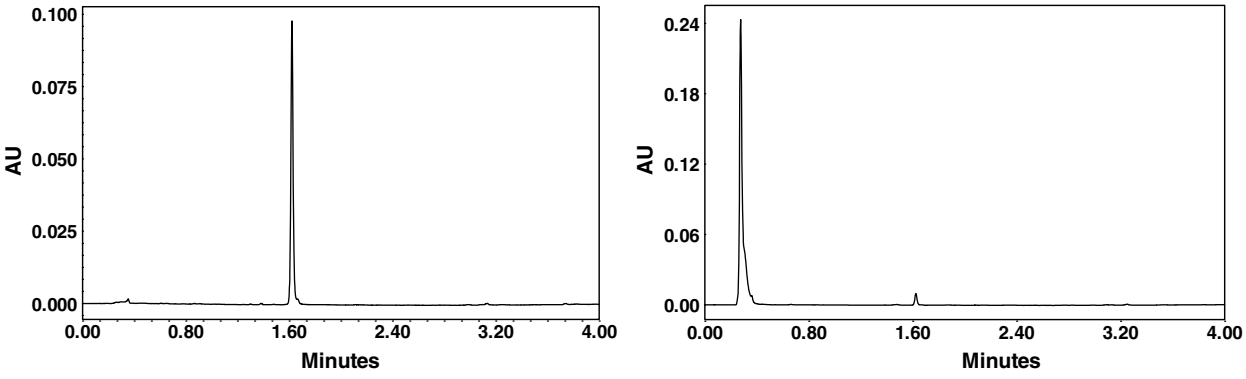
perform one-pot protocol was designed, in which tubulysin **3a** or **3b** was activated by *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) with in situ addition of **7**,[5] followed by treatment with *N*,*N*-diisopropylethylamine (DIPEA) and solution of **2** in DMSO. Subsequent preparative HPLC purification provided the product (**1 a**–**b**) in ca. 30% overall yield.

Next, another class of folate–tubulysin conjugates **1c**–**d** was designed. Based on our biological data[4,7] and previous success with a hydrazide-based cytotoxic drug,[5] the endosomal release of a tubulysin hydrazide (**9a**–**b**)[‡] was envisioned (Scheme 3). This approach might provide potentially different interactions between the base drug and microtubules, and hence lead to a potentially larger therapeutic window for the hydrazide-based drug in contrast to an amino acid form of the natural tubulysin drug. The base drugs **9a**–**b** were prepared directly from the corresponding natural tubulysins for use in biological control experiments.[8] The synthetic yields were low due to (a) sensitivity of the acyl groups in tubulysin skeleton to hydrazine and (b) the further reaction of **9** with the activated intermediate **4**. To minimize undesired side reactions for the synthesis of the target conjugates, a heterobifunctional crosslinker **10** was employed.[9a] This molecule contains an oxycarbonylhydrazine moiety with mono-nucleophilic nitrogen atoms and a 2-mercaptopyridyl leaving group as a specific conjugation site. Compound **10** was prepared in 70–80% yield from **5** or **7** following simple to perform synthetic routes.[9] Following our one-pot protocol, the final folate conjugates **1c**–**d** were synthesized in excellent yields (55–60%) from tubulysins **3a**–**b**.[10] The synthesis involved activation of the tubulysin with isobutyl chloroformate and in situ treatment of the intermediate at low temperature with **10**. The crude reaction mixture was concentrated to remove the solvent and re-dissolved in a water miscible organic solvent, THF, followed by mixing with an aqueous solution of folate-peptide spacer **2** at neutral pH. HPLC purification gave pure

‡ Selected ^{1}H NMR data for **7b** (CD_2Cl_2, 300 MHz): δ 8.52 (br, 1H), 7.94 (s, 1H), 7.10 (d, J = 8.7 Hz, 1H), 6.99 (d, J = 8.7 Hz, 4H), 6.77 (d, J = 7.8 Hz, 2H), 5.95 (d, J = 12.0 Hz, 1H), 5.65 (m, 1H), 5.34 (t, J = 12.0 Hz, 1H), 4.51 (t, J = 9.3 Hz, 1H), 4.14 (m, 1H), 3.83 (br, 2H), 3.36 (q, J = 5.7 Hz, 1H), 2.80–2.67 (m, 3H), 2.34–2.04 (m, 13H), 1.91–1.79 (m, 4H), 1.55–1.18 (m, 11H), 1.06 (t, J = 6.9 Hz, 3H), 0.96–0.75 (m, 15H).



Scheme 3.



Scheme 4. Conjugate **1d** in PBS buffer treated with DTT. Left: $t = 0$ min; right: $t = 2$ h.

conjugate **1c**–**d**. LC/MS [ESI $(M+H)^+$: 1878 and 1892, respectively] and ^{1}H NMR data[§] were in agreement with the expected structure.

In brief, the ^{1}H NMR spectrum (300 MHz, D_2O) contained ten aromatic signals in the range from 6.5 to 8.7 ppm (five from the folate moiety and five from the tubulysin moiety). The signals for the two protons of the intact *N*,*O*-acetal appeared at 6.1 ppm and 5.2 ppm.

When **1d** was incubated as a 1 mM solution with 10 equivalents of DTT in PBS buffer at 37 °C, LC/MS studies indicated a full release of **9b** within 2 h (Scheme 4).

[§] Selected ^{1}H NMR data for **1d** (D_2O, 300 MHz): δ 8.45 (s, 1H), 7.90 (s, 1H), 7.44 (d, J = 8.7 Hz, 2H), 6.74 (d, J = 8.1 Hz, 2H), 6.45 (m, 2H), 5.93 (d, J = 12.0 Hz, 1H), 5.58 (d, J = 11.4 Hz, 1H), 5.08 (d, J = 12.0 Hz, 1H), 4.53 (m, 2H), 4.39 (m, 2H), 4.29 (br, 2H), 4.14 (m, 5H), 3.89 (br, 1H), 3.64 (d, J = 9.9 Hz, 1H), 3.38 (d, J = 12.0 Hz, 1H), 3.06–2.74 (m, 6H), 2.62–2.22 (m, 14H), 1.99 (s, 3H), 1.95–1.28 (m, 20H), 1.14 (q, J = 7.5 Hz, 2H), 1.01–0.86 (m, 4H), 0.79 (d, J = 6.3 Hz, 3H), 0.71–0.63 (m, 6H), 0.51–0.44 (m, 6H).

Conjugates **1a–d** are being tested against a variety of FR positive cell lines as well as in animal models. The results will be reported in an appropriate scientific journal.[7]

Acknowledgments

We thank the analytical group at Endocyte Inc., for LC/MS support and R&D Biopharmaceuticals GmbH for providing the natural tubulysins. We are also thankful to Drs. G. Höfle, W. Richter, and A. Dömling for their insightful discussions.

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8. Typical procedure: DIPEA (6.1 μL) and isobutyl chloroformate (3.0 μL) were added to a solution of **3b** (14 mg) in anhydrous EtOAc (150 μL) in tandem at −15 °C under argon. After stirring for 45 min at −15 °C, to the reaction mixture was added anhydrous hydrazine (5 μL) and the reaction mixture was stirred at −15 °C for an additional 30 min, quenched with sodium phosphate buffer (1 mL, 2 mM, pH 7), and purified by a preparative HPLC to afford **9b** (3.1 mg) as a white powder.
9. (a) Kaneko, T.; Willner, D.; Monkovic, I.; Knipe, J. O.; Braslawsky, G. R.; Greenfield, R. S.; Vyas, D. M. *Bioconjug. Chem.* **1991**, *2*, 133; (b) Procedure: DIPEA (0.60 mL) was added to a suspension of **5** (HCl salt, 91%, 685 mg) in anhydrous DCM (5.0 mL) at 0 °C under argon, stirred for 2 min, and to which was added anhydrous hydrazine (0.10 mL). The reaction mixture was stirred at 0 °C under argon for 10 min and at room temperature for an additional 30 min, filtered, and the filtrate was purified by flash chromatography (silica gel, 2% MeOH in DCM) to provide **10** as a clear thick oil (321 mg), solidified upon standing. Alternative procedure: diphosgene (8.9 μL) was added to a solution of **7** (HCl salt, 30 mg) and DIPEA (49 μL) in anhydrous DCM (1 mL) at 0 °C under argon, stirred for 15 min, and to which was added anhydrous hydrazine (8.4 μL). The reaction mixture was stirred at 0 °C under argon for 10 min and at room temperature for an additional 30 min, filtered, and the filtrate was purified by flash chromatography (silica gel, 2% MeOH in DCM) to provide **10** as a clear thick oil (23 mg), solidified upon standing.
10. Typical procedure: DIPEA (7.8 μL) and isobutyl chloroformate (3.1 μL) were added to a solution of **3a** (18 mg) in anhydrous EtOAc (0.50 mL) at −15 °C. After stirring for 35 min at −15 °C under argon, to the reaction mixture was added a solution of **10** (5.8 mg) in anhydrous EtOAc (0.50 mL). The cooling was stopped and the reaction mixture was stirred under argon for an additional 45 min, concentrated, vacuumed, and the residue was dissolved in THF (2.0 mL). Meanwhile, **2** (20 mg) was taken in deionized water (bubbled with argon for 10 min prior to use) and the pH of the yellow suspension was adjusted to 6.9 by saturated $NaHCO_3$ (bubbled with argon for 10 min prior to use). Additional deionized water was added to the solution of **2** to make a total volume of 2.0 mL and to which was added immediately the THF solution containing the activated tubulysin. The reaction mixture, which became homogeneous quickly, was stirred under argon for 50 min and quenched with sodium phosphate buffer (2 mM, pH 7.0, 15 mL). The resulting cloudy solution was filtered and the filtrate was injected into a preparative HPLC for purification. Fractions containing **1c** were collected and freeze-dried to produce the product (23 mg) as a pale yellow fluffy solid.