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Design and regioselective synthesis of a new generation of targeted therapeutics. Part 3: Folate conjugates of aminopterin hydrazide for the treatment of inflammation

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ABSTRACT

Efficient syntheses of folate receptor (FR) targeting conjugates of the anti-inflammatory, aminopterin hydrazide, are described. 2-{4-Benzoylamino}-5-oxo-5-{*N*′-[2-(pyridin-2-yldisulfanyl)-ethoxycarbonyl]-hydrazino}-pentanoic acid is synthesized from commercially available 4-[(2-amino-4-imino-3,4-dihydro-pteridin-6-yl-methyl)-amino]-benzoic acid. Conjugation of this novel, activated aminopterin hydrazide to folic acid through cysteine-terminating (C-terminus), peptide/carbohydrate spacers results in highly water soluble conjugates which allow for the release of free aminopterin hydrazide within the endosomes of targeted cells.

The vitamin folic acid (folate, 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.[2] Consequently, FA can be exploited as a molecular 'Trojan horse' for the targeted delivery of covalently-attached, biologically active molecules.[3] To date, three isoforms of the FR have been identified and cloned: FR-α, FR-β, and FR-γ and its truncated cogener, FR-γ′.[4] In normal tissues, the distribution of measurable levels of FR-α is limited only to the apical membrane surface of certain polarized epithelial cells, in placental trophoblasts, and on the apical side of kidney proximal tubule cells, the latter serving as a salvaging route for folates prior to urinary excretion.[5] In contrast, in many human malignant cells the FR-α is highly overexpressed, especially in aggressively growing cancers.[6] FR-β is present on the cell surface of human activated macrophages but not on resting/quiescent macrophages.[7] In response to pro-inflammatory stimuli, activated macrophages concentrate at areas of inflammation. Excessive and persistent build-up can cause tissue damage, resulting in such inflammatory diseases as: rheumatoid arthritis, psoriasis, Crohn's disease, systemic lupus erythematosus, atherosclerosis, type II diabetes, ulcerative colitis, osteoarthritis, organ transplant rejection, and ischemia/reperfusion injury.[8] In addition, activated macrophages produce and release pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1, promoting and intensifying the disease.[9] Consequently, developing therapies which target FR-β might lead to an elimination of the activated macrophages and the resulting auto-immune/inflammatory disease while diminishing the inherent collateral toxicity to normal cells.

Previously, we reported the design and synthesis of a folate targeted chemotherapeutic conjugate, EC145, which is in phase 2 clinical trials.[10] In EC145, an anticancer drug, desacetyl vinblastine hydrazide (DAVLBH) is attached to folic acid (FA) via a water-soluble peptidic spacer and a reducible disulfide linker system. Recently, we published the synthesis of folate-DAVLBH conjugates using carbohydrate-based peptide spacers designed to decrease undesired hepatobiliary clearance of free DAVLBH without affecting the conjugate's targeted anti-tumor activity.[11]

In this Letter, we utilize a peptidic or a carbohydrate-based spacer along with a disulfide linker system to synthesize targeted folate-aminopterin hydrazide conjugates **1** and **2**, the first conjugates in a novel class of compounds designed to suppress inflammatory disease (Fig. 1). Aminopterin, a highly active antifolate originally developed to treat cancer, was reported in 1951 to produce rapid improvement in patients with rheumatoid arthritis and psoriasis; however, due to manufacturing problems and a poor therapeutic index, the clinical use of aminopterin was discontinued in favor of the safer alternative, methotrexate.[12] In our approach, we use a novel derivative of aminopterin: aminopterin hydrazide.

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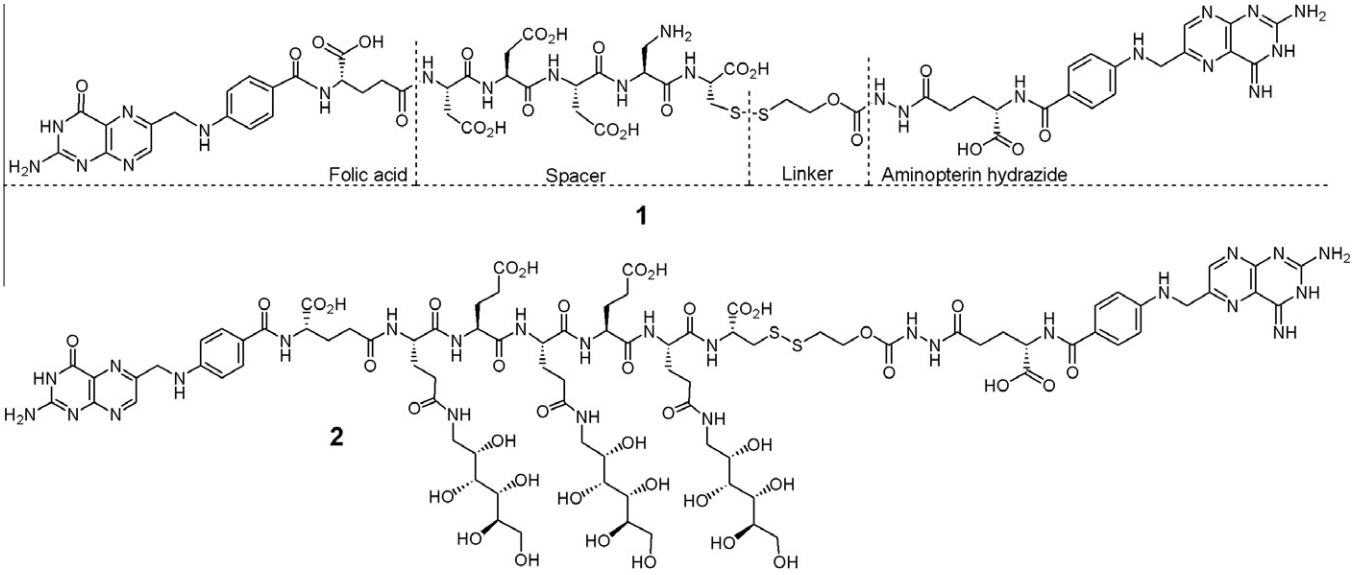


Figure 1. Aminopterin hydrazide-folate conjugates **1** (peptidic spacer region) and **2** (carbohydrate based peptidic spacer region).

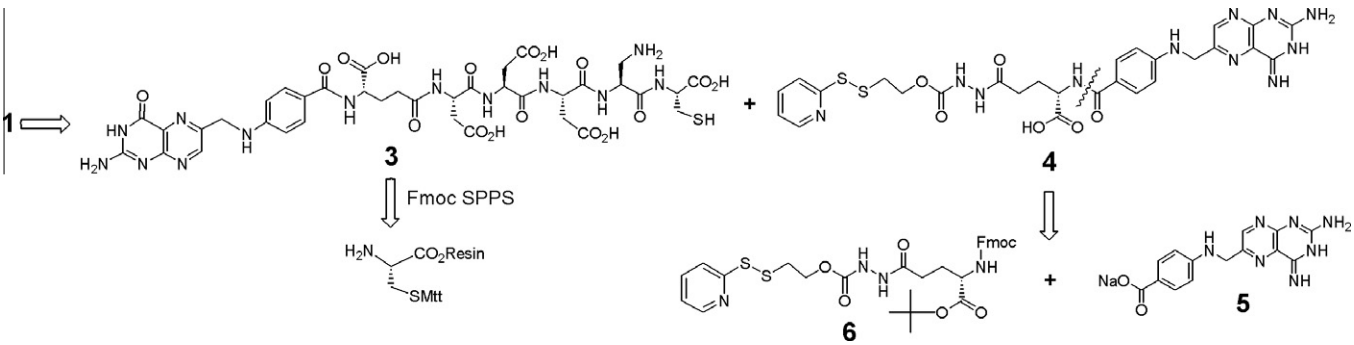
Aminopterin and aminopterin hydrazide both displayed very similar in vitro activities in a series of cells (unpublished results). We selected the hydrazide because base drugs which contain this moiety are readily releasable from their derived conjugates using our linker technology. In contrast, a conjugate derived from aminopterin and linked through an ester moiety would be expected to be readily releasable, but have poor serum stability.

As outlined in the retrosynthetic analysis (Scheme 1), disulfide-linked conjugate **1** can be assembled from the corresponding thiol-containing FA-spacer **3** and thiol-reactive derivative of aminopterin hydrazide **4**. The disulfide bond is readily reduced in the internalized endosome of the targeted cell, of crucial importance for drug delivery applications.[13] Aminopterin derivative **4** can be readily derived from commercially available 4-aminopteroic acid sodium salt **5** and activated glutamic acid **6**. The peptide-based FA-spacer **3** is designed to introduce a discrete number of charged amino acids for better water solubility and was synthesized using standard fluorenylmethyloxycarbonyl-based solid phase peptide synthesis (Fmoc-SPPS).[10]

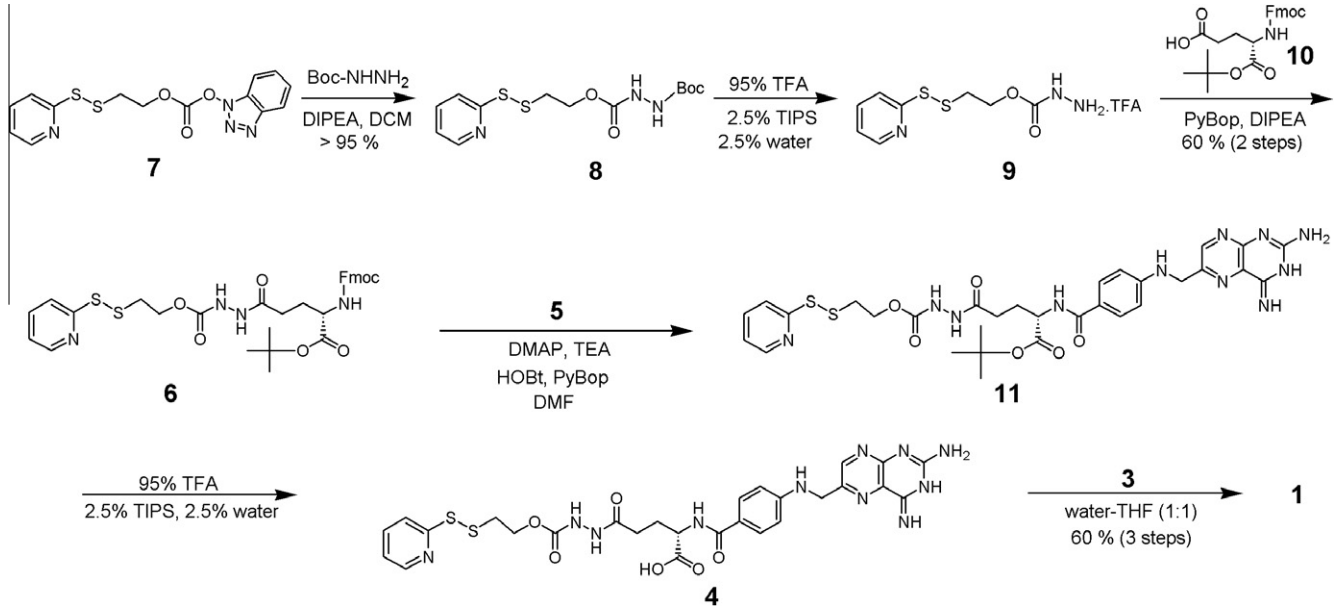
The nucleophile- and thiol-activated heterobifunctional carbonate **7** (Scheme 2) served as an important starting material for the releasable drug conjugate synthesis.[10] Reaction of this mixed carbonate with *t*-butyl-carbazate in the presence of diisopropylethylamine (DIPEA) gave the corresponding *t*-butyl-carbazate **8**. Trifluoroacetic acid (TFA) mediated Boc deprotection of **8** in the presence of triisopropylsilane (TIPS) resulted in **9** as a TFA salt. Coupling of **9** with protected glutamic acid **10**, using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop) and DIPEA, yielded glutamic acid derivative **6**. 4-Dimethylaminopyridine (DMAP) mediated Fmoc deprotection of **6** followed by in situ coupling with commercially available sodium 4-[(2-amino-4-imino-3,4-dihydro-pteridin-6-yl-methyl)-amino]-benzoate **5** using PyBop and hydroxybenzotriazol (HOBt) resulted in fully protected aminopterin hydrazide **11**. Treatment of **11** with TFA expediently removed the *t*-butyl moiety to yield pyridinedisulfanyl-activated aminopterin hydrazide **4**.[14] Finally, treatment of a suspension of FA-spacer **3** in H_2O under Argon with 0.1 N $NaHCO_3$ resulted in a clear yellow solution at pH >6.9. To this mixture was added at once under extensive stirring a solution of **4** in THF.[15] According to the HPLC profile, the reaction was completed in less then 15 min. HPLC purification gave pure conjugate **1**.[16]

Second generation folate-aminopterin conjugate **2** was designed using carbohydrate-based folate-spacer **12** (Scheme 3). FA-spacer unit **12** was synthesized using a standard Fmoc-SPPS protocol as described in our previous publication.[11] Treatment of a suspension of **12** in phosphate buffer and under argon with $NaHCO_3$ resulted in a clear yellow solution. To this mixture was added at once under vigorous stirring a dimethylsulfoxide (DMSO) solution of **4** to yield the final conjugate **2**.[17]

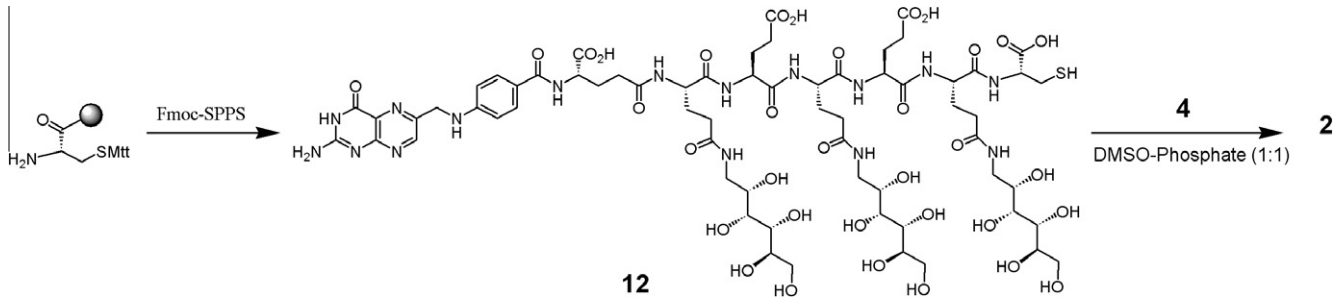
Treatment of conjugate **2** with a reducing agent demonstrates the release of free aminopterin hydrazide (Scheme 4). In brief, a 1 mM solution of **2** in phosphate buffer (pH 7.4) was treated with 40 equiv of dithiothreitol (DTT) at room temperature.[18] The HPLC profile (Fig. 2) showed cleavage of the disulfide bond ($t_{1/2}$ ~22 min.) with



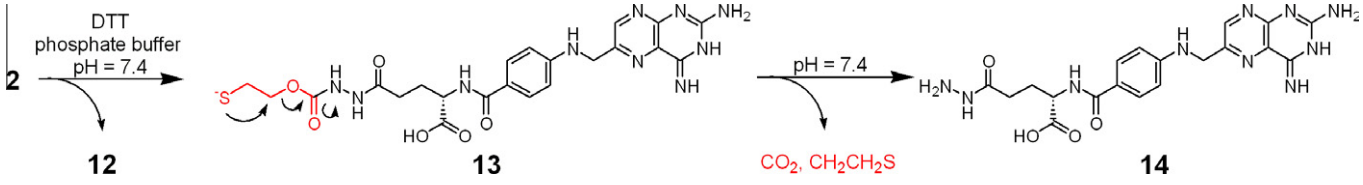
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

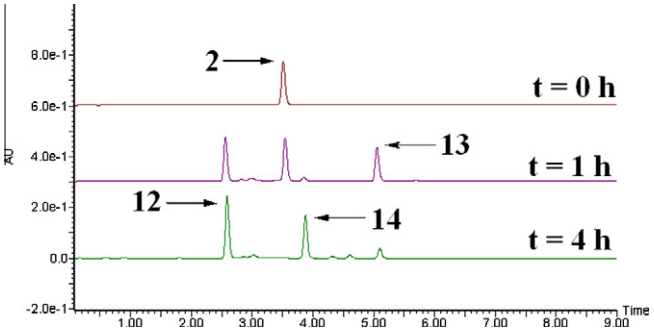


Figure 2. HPLC profile (280 nm) of the treatment of **2** with DTT.

concomitant release of the FA-Spacer **12** and 2-thioethyl carbazate **13.** Subsequently, **13** fragments into free aminopterin hydrazide **14**, thus demonstrating the self-immolative nature of the linker system.[19]

Following positive in vitro and in vivo results and toxicological evaluation, **2** (known also as **EC0746**) was selected as a lead candidate for further (pre)clinical development. Results of the complex biological investigations will be reported soon in the appropriate scientific journals.

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14. *Analytical data for activated aminopterin hydrazide* **4**: ^{1}H NMR (DMSO-d_6 & D_2O) δ 8.82 (s, 1H), 8.44 (d, J = 4.7 Hz, 1H), 7.80 (m, 2H), 7.71 (d, J = 8.8 Hz, 2H), 7.24 (t, J = 4.6 Hz, 1H), 6.74 (d, J = 8.8 Hz, 2H), 4.60 (s, 2H), 4.32 (dd, J = 5.0 Hz, 1H), 4.20 (t, J = 6.0 Hz, 2H), 3.07 (t, J = 6.0 Hz, 2H), 2.22 (t, J = 7.8 Hz, 2H), 2.15 - 1.94 (m, 2H). ESI-MS: $(M+H)^+$ = Calculated 668.2; found 668.2.
15. *General procedure for conjugation*: To H_2O (bubbled with argon for 10 min before use) was added folate linker in a centrifuge tube. To this suspension, while bubbling with argon, was added drop-wise saturated $NaHCO_3$ solution (bubbled with argon for 10 min before use) until the pH of the resulting solution reached 6.9. 1 equiv of **4** in THF was added quickly and the resulting homogenous solution was stirred under argon for 15 min. The reaction progress was checked by analytical HPLC. The mixture was diluted with of phosphate buffer and the THF was removed under vacuum. The cloudy solution was centrifuged and filtered. The yellow filtrate was purified by preparative HPLC.
16. *Analytical data for aminopterin folate* **1**: ^{1}H NMR (DMSO-d_6 & D_2O) δ 8.68 (s, 1H) 8.62 (s, 1H), 7.64 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.8 Hz, 2H), 6.72 (d, J = 8.8 Hz, 2H), 6.60 (d, J = 8.8 Hz, 2H), 4.55 (t, J = 6.5 Hz, 2H), 4.46 (m, 7H), 4.36(t, J = 6.5 Hz, 2H), 4.18 (m, 4H), 3.29 (m, 3H), 3.08 (m, 3H), 2.87 (m, 4H), 2.15 (m, 4H), 2.02 (m, 2H), 1.88 (m, 2H). ESI-MS: $[(M+2H)^{2+}]/2$ = Calculated 767.2; found 767.4.
17. *Analytical data for aminopterin carbohydrate-based folate* **2**: ^{1}H NMR (DMSO-d_6 & D_2O) δ 8.67 (s, 1H) 8.60 (s, 1H), 7.62 (d, J = 9 Hz, 2H), 7.59 (d, J = 9 Hz, 2H), 6.71 (d, J = 8.7 Hz, 2H), 6.62 (d, J = 8.1 Hz, 2H), 4.47 (m, 4H), 4.26–4.04 (m, 10H), 3.70–3.30 (m, 22H), 3.30–3.10 (m, 6H), 3.10–2.76 (m, 9H), 2.40–2.04 (m, 15H), 2.04–1.60 (m, 4H). ESI-MS: $[(M+2H)^{2+}]/2$ = Calculated 1119.09; found 1119.10.
18. The species which reduces the disulfide bond to free thiol in the endosome is not known, but the reduction is unlikely to result from disulfide exchange with small thiol-containing molecules due to the relatively low pH. The use of DTT or another reductant such as glutathione does not accurately mimic the biological system. See Ref. 13.
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