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Design, synthesis and biological evaluation of a highly-potent and cancer cell selective folate–taxoid conjugate

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ABSTRACT

The folate receptor (FR) has been widely recognized as an excellent target for the tumor-selective delivery of cytotoxic agents, and four folate–drug conjugates have entered clinical evaluations for the treatment of solid tumors to date. However, most of these conjugates required structural modification of the cytotoxic warheads in order to achieve efficient drug release from the linkers. We designed and constructed a novel folate conjugate of a highly-potent next-generation taxoid, SB-T-1214, by exploiting bioorthogonal Cu-free 'click' chemistry. The synthesis was highly convergent and required no HPLC purification to obtain the final folate–taxoid conjugate **1**. Conjugate **1** demonstrated highly FR-specific potency (IC_{50} 2.1–3.5 nM) against a panel of cancer cell lines, with a >1000-fold decrease in cytotoxicity against normal human cells (IC_{50} >5000 nM). The remarkable potency and selectivity of conjugate **1** can be attributed to highly FR-specific receptor-mediated endocytosis as well as efficient release of the unmodified cytotoxic warhead using a mechanism-based self-immolative linker.

1. Introduction

Cancer is a group of over 200 diseases characterized by the uncontrolled growth and spread of abnormal cells. Left untreated, it results in organ failure and death. Despite the recent advances in our understanding of the molecular processes that lead to its development and progression, cancer remains the second most common cause of death in the U.S. and a leading cause of death worldwide.[1] Traditionally, treatment options include the use of chemotherapy, which typically entails the administration of cytotoxic agents that act on the processes of cell division. The rationale for this treatment modality is that rapidly dividing cancer cells will be more susceptible to the cytotoxic effects of the drugs than healthy bystander cells. However, in reality, this is not the case. Commonly used therapies, such as doxorubicin, paclitaxel and cisplatin, cause severe dose-limiting side effects due to their adverse effects on the highly-proliferating cells in certain tissues, including the bone marrow, heart and GI tract. Therefore, there is an urgent need to develop safe and effective alternatives to the existing chemotherapy options.

One strategy that has gained significant attention in recent decades is the use of tumor-targeted drug delivery systems (TTDDS's). By exploiting the molecular and physiological differences between healthy and cancerous tissues, it is possible to rationally develop a TTDDS that has the capability to deliver cytotoxic agents selectively to cancer cells thereby reducing the off-target side-effects observed with traditional chemotherapy. An effective TTDDS is typically composed of three basic components, that is, (i) a highly potent cytotoxic drug, (ii) a tumor-recognition moiety that directs the drug conjugate to cancer specific receptors and promotes efficient internalization into cancer cells via receptor-mediated endocytosis (RME), and (iii) a smart linker, that is, stable in blood plasma yet efficiently releases the drug upon internalization.

Abbreviations: ATCC, American Type Culture Collection; CFM, confocal fluorescence microscopy; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMEM, Dulbecco's modified eagle's medium; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EU, European Union; FBS, fetal bovine serum; ESI, electrospray ionization; FIA, flow injection analysis; Fmoc, 9-fluorenylmethoxycarbonyl; FR, folate receptor; GI, gastrointestinal; GSH, L-glutathione reduced; GSH-OEt, L-glutathione reduced ethyl ester; HBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole hydrate; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MS, mass spectrometry; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PBS, phosphate-buffered saline; PEG, polyethylene glycol; RME, receptor-mediated endocytosis; RPMI, Roswell Park Memorial Institute medium; SPPS, solid phase peptide synthesis; TFA, trifluoroacetate/trifluoroacetic acid; TIPS, triisopropylsilane; TLC, thin-layer chromatography; TOF, time-of-flight; TTDDS, tumor-targeted drug delivery system.

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The next-generation taxoids developed in our laboratory have demonstrated remarkably enhanced potency against drug-sensitive and drug-resistant cancer cells, as well as tumor xenografts, as compared to paclitaxel and docetaxel.[2] With subnanomolar IC_{50} values against a broad panel of cancer cell lines, these compounds are ideally suited to be used as high-potency warheads for TTDDS's.[3] Many of these taxoids retain their remarkable potency against multidrug resistant cell lines derived from colon, pancreatic and renal cancers that overexpress the P-glycoprotein efflux pump.[2] For example, one such next-generation taxoid, SB-T-1214, has demonstrated profound activity against 3D-spheroids derived from highly metastatic colon cancer stem cells, causing the down-regulation of stem cell-related genes and eventually leading to apoptosis.[4] Accordingly, this next-generation taxoid has been incorporated into various TTDDS's, bearing biotin and omega-3-fatty acid as tumor-targeting modules (TTMs),[5–7] as well as single-walled carbon nanotubes and dendrimers as nano-scale vehicles.[8–10]

It is essential to use a well-designed mechanism-based linker system for efficacious TTDD. The self-immolative disulfide linkers, which we have been developing, are rapidly cleaved following internalization of drug conjugates, resulting in a cascade drug release via thiolactonization to release the unmodified original drug (Fig. 1).[3] As the concentration of glutathione is three orders of magnitude greater in tumor cells compared to that in the blood, disulfide linkers are stable in circulation, yet break down during receptor mediated endocytosis (RME).[11] This mechanism-based drug release was demonstrated using a fluorinated model system by ^{19}F NMR spectroscopy,[3,12,13] and has been validated in vitro by confocal fluorescence microscopy (CFM) and flow cytometry using fluorescent probes.[5] Thus, this 'smart-linker system' has been successfully integrated into macromolecule- and small molecule-based TTDDS's.[3,5,8]

Folic acid, also known as vitamin B_9, is the precursor for tetrahydrofolate and is necessary for many biological processes required in cell division, such as DNA synthesis and repair.[14] Consequently, many tumors exhibit greatly enhanced folate uptake and overexpress the transporters required for its internalization.[15] Folic acid–drug conjugates are internalized via the folate receptor (FR), which is highly expressed in some tumors and virtually absent in most healthy tissues.[16] Folate-mediated tumor targeting has been demonstrated to be highly effective both in vitro and in vivo.[17] To date, four folate–drug conjugates have been evaluated in clinical trials for the treatment of solid tumors and the leading candidate, Vintafolide, has advanced to the phase III clinical trials in patients with platinum-resistant ovarian cancer, and recently obtained expedited approval by EU.[18–21] Thus, the FR is a widely accepted and validated target for TTDD.

We report here the design, synthesis and biological evaluation of a novel and highly potent folate–taxoid conjugate with extremely high specificity to FR-overexpressing cancer cell lines.

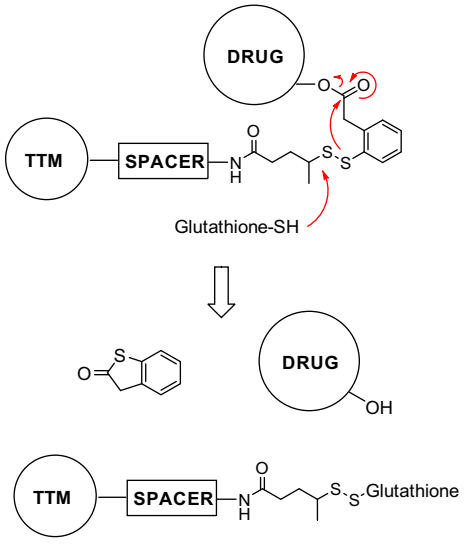

Figure 1. Mechanism of the self-immolative disulfide linker.

2. Results and discussion

2.1. Design of folate–taxoid conjugate

As Figure 2 illustrates, conjugate **1** was designed to include a folic acid moiety for tumor targeting, a PEGylated dipeptide spacer and a self-immolative linker bearing a highly-potent next-generation taxoid, SB-T-1214. Due to the finite number of FRs ($1–3 \times 10^6$)[15,22] on a given tumor cell and the 8–12 h receptor recycling rate,[23] the use of potent warheads with IC_{50} values of low nM or below is necessary to achieve meaningful activity in solid tumors.[24] Furthermore, the cytotoxic drug should be released inside the cancer cell unmodified to fully retain its activity. Early drug conjugates that did not possess these qualities were found to possess insufficient potency and/or specificity.[25–27] Thus, our TTDDS bearing a self-immolative disulfide linker and a highly potent next-generation taxoid is ideally suited for the development of FR-targeted drug conjugates.

We developed a convergent and scalable route to the novel folic acid–taxoid conjugate **1**. As Figure 2 shows, we incorporated a chemically modified Glu-Arg dipeptide unit as a handle for bioorthogonal chemistry, that is, click reaction, and used solid phase peptide synthesis (SPPS) to construct a water-soluble folyl-dipeptide component **2**, bearing an azidoethyl triethylene glycol moiety in the Glu residue. Charged amino acid residues were incorporated into the conjugate to reduce passive diffusion to cells[28] and to increase the solubility of the folic acid moiety to facilitate chemical transformations. The use of a cyclooctyne group in the taxoid-linker component **3** enables a Cu-free click reaction[29] with component **2** to assemble the conjugate **1** in minimal linear synthetic steps without need for elimination of residual Cu-related impurities.

2.2. Synthesis of conjugate 1

The synthesis of component **2** by means of SPPS is illustrated in Scheme 1. First, the EDC coupling of Fmoc-(*S*)-Glu-OBu^t with 11-azido-3,6,9-trioxaundecan-1-amine gave **4** in 83% yield, and the subsequent deprotection of *tert*-butyl ester gave Fmoc-Glu(ω-N_3)-OH (**5**) in nearly quantitative yield.

Next, the peptide couplings, starting with Fmoc-(*S*)-Arg(Pbf)-Wang resin (**6**), were performed sequentially using HOBt and HBTU as coupling agents and piperidine for Fmoc deprotection. The sequence of couplings was as follows (Fmoc of the Wang-resin bound amino acid or peptide was removed before coupling in each case): (i) **6** with **5** to form Fmoc-Glu(ω-N_3)-Arg-O-Wang; (ii) H-Glu(ω-N_3)-Arg-O-Wang with Fmoc-(*S*)-Glu(γ-OH)-α-OBu^t to afford Fmoc-Glu(α-OBu^t)-γ-Glu(ω-N_3)-Arg-O-Wang. Finally, H-Glu(α-OBu^t)-γ-Glu(ω-N_3)-Arg-O-Wang was coupled with N^{10}-trifluoroacetylpteroic acid to afford γ-(N^{10}-trifluoroacetyl-α-OBu^t-folyl)-Glu(ω-N_3)-Arg-O-Wang, which was treated with TFA/TIPS/H_2O to cleave the peptide from the Wang resin to give γ-(N^{10}-trifluoroacetylfolyl)-Glu(ω-N_3)-Arg-OH (**7**) in 56% overall yield from **6**. The trifluoroacetyl group of **7** was removed by ammonium hydroxide to afford γ-folyl-Glu(ω-N_3)-Arg-OH (**2**) in quantitative yield.