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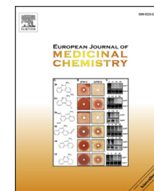

Original article

A new delivery system for auristatin in STxB-drug conjugate therapy

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A key challenge in anticancer therapy is to gain control over the biodistribution of cytotoxic drugs. The most promising strategy consists in conjugating drugs to tumor-targeting carriers, thereby combining high cytotoxic activity and specific delivery. To target Gb3-positive cancer cells, we exploit the non-toxic B-subunit of Shiga toxin (STxB). Here, we have conjugated STxB to highly potent auristatin derivatives (MMA). A former linker was optimized to ensure proper drug-release upon reaching reducing environments in target cells, followed by a self-immolation step. Two conjugates were successfully obtained, and *in vitro* assays demonstrated the potential of this targeting system for the selective elimination of Gb3-positive tumors.

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1. Introduction

For the development of new anti-cancer treatment modalities, the selective delivery of highly cytotoxic drugs to tumor cells while sparing normal tissues is a continuous challenge. Among the strategies that can be addressed to achieve this goal, the coupling of cytotoxic drugs to tumor-targeting carriers appears to be at first sight the most promising. Current drug targeting strategies exploit selective ligands of membrane receptors as carrier for a toxic-payload [1,2]. Indeed, increasing knowledge on cell surface molecules that are overexpressed by cancer cells, termed tumor-associated receptors or antigens [3], allows the use of specific ligands. After conjugation to cytotoxic agents, it results in the cytotoxic drug targeting and accumulating in the tumor with minimal accumulation in normal tissues, thereby increasing the effectiveness and reducing the toxicity of these drugs. Various carriers were developed, including small molecules, polymer and proteins. At this stage, monoclonal antibodies (mAb) are the most widely used carrier moieties for the tumor-specific targeting of cytotoxic drugs [4], with two antibody-drug conjugates (ADC), brentuximab vedotin and ado-trastuzumab emtansyne [5,6], having reached the market. Based on information available as of November 2014, more than forty ADCs are currently being investigated in clinical studies as treatments for a variety of solid and liquid tumors. The ADCs in the clinical pipeline [7,8] are directed against a plethora of different antigenic targets, but are based on a limited number of highly potent drugs, such as calicheamycins, auristatins, maytansinoids and more recently duocarmycins and pyrolobenzodiazepines, and a limited number of linker strategies. The use of highly potent drugs is needed, for one because of limited overexpression of tumor-associated receptors, and second because only limited amounts of cytotoxic payload can be coupled onto mAbs to prevent a loss of antigen binding capacity [9]. Moreover, the linker between the cytotoxic drug and the carrier is a critical piece in the design of an ideal carrier-drug conjugate [10–12]. It must be relatively stable in the circulation and prevent unspecific drug-release, and yet release the drug when it reaches the tumor.

Here we exploit a carrier derived from the non-toxic B-subunit of Shiga toxin (STxB), termed STxB/Cys [13], a homopentameric protein. Each monomer has a molecular mass of 7.7 kDa. STxB binds specifically to the glycosphingolipid globotriaosylceramide (Gb3 or CD77), with an apparent binding constant in the order of 10^9 M^{-1} that results from the capacity to interact with up to 15 Gb3 molecules per homopentamer. After binding to Gb3 at the surface of target cells, STxB is internalized by endocytosis to reach early and recycling endosomes. STxB bypasses the late endocytic pathway

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and avoids the degrading environment of lysosomes. Then STxB directly reaches the trans-Golgi network (TGN), the stacks of the Golgi apparatus and eventually the endoplasmic reticulum (ER). This unconventional intracellular trafficking is termed the retrograde route. Aberrant glycosylation is a general feature of carcinogenesis [14], and Gb3 overexpression has been described for various cancer cell lines and human cancers [15]. Strikingly, cancer cells have up to 10^8 STxB binding sites, whereas antibodies have usually at most 10^6 binding sites per target cells. Therefore STxB would be expected to be more efficient than antibodies as carrier for tumor-specific targeting of cytotoxic drugs. STxB might be a carrier of choice to target Gb3-positive cancers.

STxB/Cys is a genetically engineered STxB possessing a cysteine residue to the C-terminus of each monomer, enabling five defined chemical coupling site. STxB/Cys was previously used as a Gb3-targeting carrier of photosensitizer [16], mild cytotoxic drugs [17,18], or various antigens that were delivered to dendritic cells in immunotherapeutic strategies [19].

We have previously described the use of STxB/Cys as carrier of a camptothecin drug [17], with the synthesis of a STxB-SN38 conjugate that showed a Gb3-dependant cytotoxic activity on cells in culture. The linker that was used in this study was designed on a 2-methylaminoethanethiol core, enabling drug-release due to disulfide bond reduction, followed by an intramolecular self-immolative 5-ring cyclization. The conjugate was completely stable in several media including pure fetal calf serum and the intracellular cleavage to release the free SN38 was shown. The Gb3-specific cytotoxic activity of the STxB-SN38 conjugate was also demonstrated *in vitro*. However, the conjugate showed a moderate therapeutic potency in a xenograft tumor model in mice, even at the maximum tolerated dose of STxB (unpublished data). To optimize the use of STxB as a therapeutic carrier, we choose to generate STxB conjugates with highly potent cytotoxic drugs and to optimize the linker strategy used (Fig. 1).

Monomethylauristatin E and F are potent derivatives of the natural product dolastatin 10 that inhibits tubulin polymerization in dividing cells and thereby induces apoptosis [20,21]. Dose-limiting toxicities of MMAE have been reported [22] and this drug may be more useful when selectively directed to cancer cells [23]. An example of such targeted MMAE delivery is provided by brentuximab vedotin, a marketed anti-CD30 based ADC. Monomethylauristatin F is another auristatin derivative with impaired membrane translocation capabilities due to a negatively charged C-terminal domain at physiological pH. Previous studies have shown that the activity of MMAE is greatly potentiated through active delivery via an antibody-drug conjugate (ADC), suggesting that MMAF mild activity is due to its inability to cross cellular membranes [10]. Here, we describe the synthesis of STxB-based conjugates with two highly potent MMA derivatives [24], MMAE and MMAF pentapeptides (Fig. 3), that differ only in the last amino acid, respectively ephedrine and phenylalanine, thus displaying a different hydrophily. A rational optimization of both structure and synthesis route was carried out to improve the conjugate efficacy.

2. Results and discussion

2.1. Chemistry

Three self-immolative linkers that includes disulfide bond were synthesized to build five MMA-linker intermediates, either via alcohol terminus (MMAE) or the methylamine terminus function (both MMAE and MMAF).

The heterobifunctional linker **6** synthesis was optimized starting from a procedure previously described [17]. Commercial aminoalcohol **1** was first N-monoprotected as a *tert*-butoxycarbonyl (Boc) derivative. The hydroxyl function was then substituted in a thioacetate through Mitsunobu reaction to afford **3**. The sulfhydryl function was activated *in situ* by dithiodipyridine, leading to a disulfide bond (**4**). Deprotection of the amine was performed in acidic medium and the formed chlorohydrate was kept as a salt (**5**). The linker was then reacted with phosgene and triethylamine to give the carbamoyl chloride **6** (Fig. 2). The hydroxyl function of N-Boc-protected MMAE **12** was then coupled to the bifunctional linker **6** via the carbamoyl chloride in the presence of stochiometric amount of 4-dimethylaminopyridine (DMPA) (Fig. 3). Thus we have successfully synthesized a MMAE-linker intermediate **14** including a carbamate involving hydroxyl function of MMAE. The number of steps needed for the synthesis was reduced compared to the one previously described. However the procedure remains long and could limit the scale up for further development.

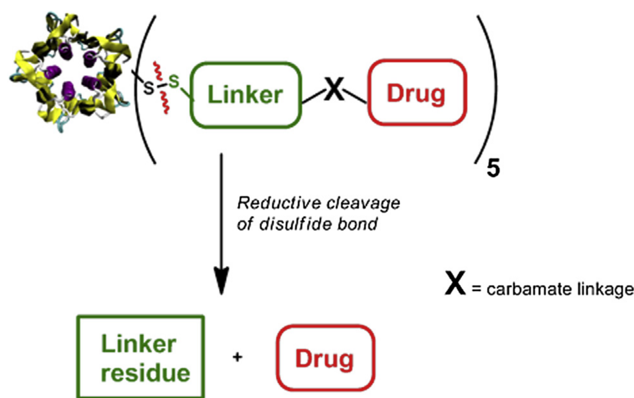

Fig. 1. General design of STxB-drug conjugates.

We designed and synthesized two new linkers **9a** and **9b** around a mercaptoethanol core, as a structure optimization of previous linker **6**. Briefly, commercial mercaptoethanol **7** was first protected by either 2,2-dipyridyl-disulfide (**8a**) or 3-nitro-2-pyridinesulfenyl chloride (**8b**) leading to a disulfide bond. The synthesis of **8a** was performed in acidic medium and allow the formation of the same leaving group thiopyridine (X = H) than the one of the linker **6**. On the other hand, considering the instability of 3-nitro-2-pyridinesulfenyl chloride (Npys-Cl), several solvents were evaluated for the reaction with the mercaptoethanol. The cyclohexane, a non-dissolving solvent was selected to avoid premature degradation of reactant. A leaving group including a nitro function (X = NO_2) was synthesized (**8b**). The free hydroxyl function of these two protected disulfenyl ethanols were then reacted with 4-4-nitrophenylchloroformate (PNP–Cl) to give the heterobifunctional linkers **9a** and **9b** (Fig. 2). Obviously this synthesis way eliminated the use of highly toxic phosgene and allows the easy isolation of the activated linkers **9a** and **9b**. The hydroxyl function of N-Boc-protected MMAE **12** was coupled to the linker **9b** via the PNP carbonate in the presence of stochiometric amount of hydroxybenzotriazole (HOBt) allowing the formation of the MMAE-linker intermediate **19** including a carbonate bond (Fig. 3). In another hand the amine function of commercial MMAE was coupled to the linkers **9a** and **9b**, allowing the formation of two MMAE-linker intermediates **16a** and **16b** including a “reverse” carbamate bond and two different leaving group (**16a** X = H, and **16b** X = NO_2) (Fig. 3). Thus we have successfully synthesized two heterobifunctional linkers allowing the generation of three MMAE-linker intermediates. These linkers allowed the covalent linking of the MMAE via either its hydroxyl or its amine function, including either a carbonate (**19**) or a carbamate bond (**16a** and **16b**). The procedure used reduces significantly the number of steps needed to synthesize the MMAE-linker

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