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Synthesis of an Immunoconjugate of Camptothecin

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Abstract—The first immunoconjugate of camptothecin has been synthesized wherein the drug is attached to the tumor-recognizing antibody BR96 via a Cathepsin B cleavable linker. Endocytosis of the immunoconjugate upon binding to the tumor cell followed by enzymatic cleavage of the linker inside the endosome ensures tumor-specific release of the drug. In this way, it is hoped that the dose-limiting side effects associated with camptothecin can be eliminated while the antitumor activity is preserved.

The isolation and characterization of camptothecin (**1**, CPT) as the active component contained in extracts from the tree *Camptotheca acuminata* by Wall had offered hope, in 1966, of an antitumor therapeutic agent.[1] Activity has been demonstrated in vivo against leukemia, colon, mammary and ovarian tumor models. Unfortunately, phase I clinical evaluations of CPT uncovered dose-limiting toxicities, myelosuppression, severe and unpredictable hemorrhagic cystitis and diarrhea, halting clinical development.[2] Later studies pointed to the insolubility of CPT, which required that the drug be formulated as the ring-opened carboxylate salt (**2**), as a key aspect in its clinical failure. The pharmacological profile of the carboxylate is different than the lactone and may be responsible in part for the toxicities associated with this drug. Furthermore, as the ring-opened form of the drug is preferred at physiological pH (pH 7.4) delivery of the parent lactone is likely to have resulted in a similar toxicity profile (Fig. 1).[3] Renewed interest in the CPT class occurred when it was shown that CPT is an inhibitor of topoisomerase I and camptothecin derivatives having improved physiochemical properties were evaluated and later approved for therapeutic use.[4,5]

We became interested in camptothecin in the course of our efforts to develop antitumor immunoconjugates. CPT is an ideal candidate for our approach since we have discovered a drug-targeting method which not only ensures delivery of the antitumor agent to the tumor (positive targeting) but also minimizes exposure to normal tissues (negative targeting). The cornerstone to our approach involves, first, the use of the monoclonal antibody BR96 which selectively recognizes a tumor associated antigen (Le^y-antigen) and is taken up by endocytosis upon binding, and second, a cathepsin B hydrolyzable linker to attach the drug to the antibody which selectively releases the drug inside lysosomes.[6] Cathepsin B is a lysosomal enzyme found only in mammalian lysosomes ensuring that the drug is released intracellularly.[7] In addition, the pH inside the lysosome is acidic (pH 5.0) as such only the lactone form of CPT is delivered.

Synthesis of the cathepsin B cleavable linker is illustrated in Scheme 1. The dipeptide Phe-Lys was selected for its ability to be recognized and rapidly cleaved by the enzyme.[8] Furthermore, the linker was designed such that the enzyme cleaves the amide bond which attaches the lysine residue to *p*-aminobenzyl alcohol (PABA).

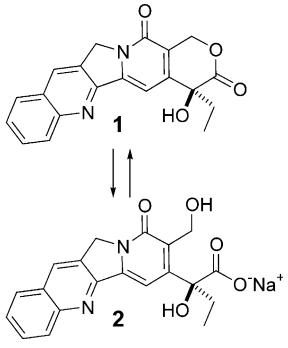

Figure 1. Camptothecin (**1**) and ring-opened camptothecin (**2**).

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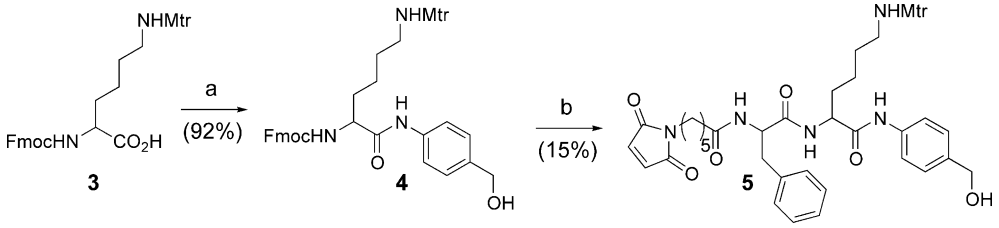
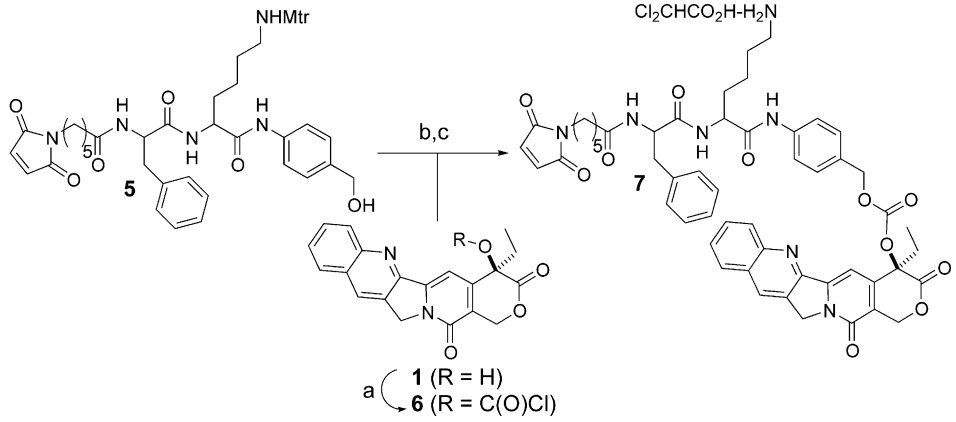
The PABA group serves as a spacer between the dipeptide linker and the drug and ensures that the scissile bond is sterically unencumbered.[9] A maleimide group was employed as a handle for conjugation to the Mab via a reduced cysteine residue. Standard peptide synthetic chemistry was used to assemble the linker. The methoxytrityl (Mtr) protecting group was specifically utilized to ensure that deprotection would be carried out under mildly acidic conditions to avoid nucleophilic reactions with the N-terminal maleimide and, as we discovered later, acid catalyzed hydrolysis of the drug-spacer moiety.

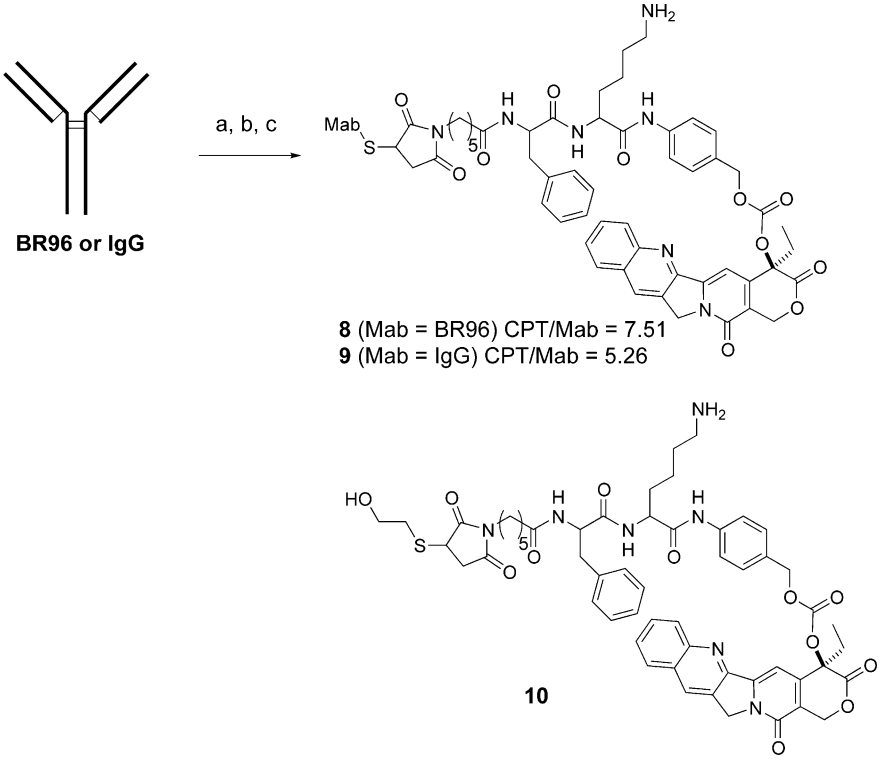
Key to coupling camptothecin to the dipeptide linker was the discovery that treating the drug with Cl_2CO (in



Scheme 1. (a) 0.95 equiv *p*-aminobenzyl alcohol, Boc_2O, pyridine; (b) (i) 1/2 CH_2Cl_2/Et_2NH; (ii) 1.0 equiv iPr_2NEt, 2.0 equiv FmocPheNHS, CH_2Cl_2; (iii) 1/1 CH_2Cl_2/Et_2NH; (iv) 1.5 equiv Mal-$(CH)_5$C(O)NHS, 1.0 equiv iPr_2NEt.



Scheme 2. (a) 4 equiv Cl_2CO (20% in toluene), 1.0 equiv pyridine, CH_2Cl_2 48 h; (b) 1.0 equiv pyridine, 2.0 equiv iPr_2NEt, CH_2Cl_2; (c) 100 equiv anisole, 5.0 equiv Cl_2CHCO_2H.



Scheme 3. (a) 8.0 equiv DTT, pH 7–7.4, 37 °C, 3 h; (b) diafiltration; (c) 8 equiv **7**.

Table 1. Stability and enzymatic cleavage of dipeptide linker

Compd	Conditions	$t_{1/2}$
10	Cat B (pH 5, 37 °C)	$<<$ 2 min
10	pH 5, 37 °C	5–6 h
10	pH 7, 37 °C	5–6 h
9	pH 7, 37 °C	$>>$ 50 h

Table 2. In vitro activity of immunoconjugates **8** and **9** against the L2987 cell line

Compd	Mab	Mole ratio[a]	IC_{50} (μM) [CPT]
CPT	—	—	0.4
8	BR96	7.51	0.1
9	IgG	5.26	3

[a]Mole ratio = [bound CPT]/[Mab].

toluene) and pyridine (Scheme 2) could readily prepare the C20-chloroformate of camptothecin.[10] The product of this reaction was reacted with peptide **5** to yield the completely assembled drug dipeptide linker. Removal of the Mtr protecting group was carried out using a limited amount of Cl_2CHCO_2H. When TFA was used in this step hydrolysis of the C20 carbonate occurred to a large extent. Nonetheless, after removal of the Mtr protecting group the stage was set for conjugating the drug-linker intermediate to BR96.

As illustrated in Scheme 3, BR96 contains four interchain disulfide bonds which can be reductively cleaved under mild conditions to provide four cysteine groups available for conjugating up to 8 equivalents of **7**. The application of this method to synthesize BR96 immunoconjugates has previously been described.[11] Reduction using a stoichiometric amount of DTT followed by reaction with the linker–drug intermediate **7** yields immunoconjugate **8** having close to the theoretical maximum number of drug molecules bound to the antibody (CPT/Mab ratio = 7.51). Using similar chemistry an immunconjugate was constructed with a nontumor binding antibody to be used as a control having CPT/Mab ratio = 5.26. Finally, we synthesized **10** using mercaptoethanol as the nucleophile in order to evaluate the cathepsin B cleavage reaction.

Prior to evaluating the potential utility of **8** as an antitumor agent, we set out to determine the stability of the peptide–drug linker and the corresponding cathepsin B cleavage rate. The results from these studies are shown in Table 1. Enzymatic release of CPT from the model linker **10** was extremely fast ($t_{1/2} << 2$ min).[12] Under the same conditions without enzyme present hydrolysis of the linker occurred at a much slower rate ($t_{1/2} = 5.6$ h). Surprisingly, this slow non-cathepsin B hydrolysis also occurs at the same rate at pH 7 suggesting that H_2O is acting by itself to cleave the linker. The instability appears to be centered around the spacer carbonate group and although the rate of hydrolysis is slow we felt that it was potentially detrimental to the development of this conjugate. Fortunately, we discovered that the peptide in the context of the immunoconjugate is much more stable to hydrolysis. As shown in the table when attached to IgG as in **9**, no CPT was released after 50 h when incubated at physiological pH and temperature.

The antigen specific activity of immunoconjugates **8** and **9** were measured in vitro with the results given in Table 2. Two important points are apparent. First, the BR96 immunoconjugate is 30 times more active than the nontumor cell-binding IgG conjugate. This is expected since, without antigen binding, there is very little, if any, cellular uptake of the conjugate. Thus, we expect the immunoconjugate to be less toxic to non-Le^y bearing tissue in vivo. The second observation is that the immunoconjugate is at least as potent as the free drug. This is significant since our previous observations with a hydrazone linked compound have shown that the BR96-immunoconjugate is almost always less active than the parent drug in vitro.[11] This more than likely reflects the fact that CPT is released from the linker more quickly than our previous conjugate which relied on an acid catalyzed cleavage mechanism.[13]

In conclusion, we present the first synthesis of an immunoconjugate of camptothecin. This immunoconjugate was found to be equipotent to the parent drug in vitro. In addition, binding of the antibody to the cell was required for activity suggesting that the immunoconjugate will be less toxic than the parent drug to normal tissue in vivo.

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