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An Adjustable Release Rate Linking Strategy for Cytotoxin–Peptide Conjugates

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Abstract—Peptide hormones are often rapidly internalized after binding to and activation of their receptors which are sometimes over-expressed on tumor cells. Thus, peptide ligands are increasingly being utilized for specific tumor cell targeting and internalization of radioactive isotopes for tumor imaging and for specifically delivering and internalizing cytotoxic moieties. Here, we describe a new carbamate linker system containing a series of built-in nucleophile assisted releasing (BINAR) groups which enable the 'fine-tuning' of intracellular cleavage rates of free cytotoxic agents containing reactive OH groups. Release rates were found to fit well with the chemical model and several conjugates of camptothecin and one of combretastatin were shown to have potent cytotoxic effects on cultures of human neuroblastoma IMR-32 cells which over-express somatostatin receptors.

Over the past several years, there has been an increased interest in using neuroendocrine peptides as vectors for both radioisotopes[1] and cytotoxic compounds.[2] One example of a vector peptide currently marketed is Pentetreotide™ which has the structure DTPA-D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Throl, a somatostatin analogue bearing DTPA on the N-terminus which can be complexed with ^{111}In and used for diagnosis of somatostatin receptor positive tumors.[3] By increasing the radiation dose, Pentetreotide™ has also been used to actually treat carcinoid and other tumor types, it exhibits little toxicity, and is well tolerated by patients.[4] More recently, we utilized[5] a newer type of somatostatin analogue containing an N-terminal linking motif which allowed long peptide nucleic acid sequences to be attached to its N-terminus with retention of high affinity. Nucleic acid sequences anti-sense to the n-myc oncogene mRNA were shown to be more effective in killing somatostatin-receptor positive cells when attached to the somatostatin vector via a normal peptide bond.

These results provided an impetus for us to explore the possiblilty of creating and developing new peptide-cytotoxin conjugates as an alternative or adjunct to radiation therapy. The 'prodrug' concept has traditionally been used to enhance or promote more desirable qualities of a drug such as increased chemical stability, bioavailability, and decreased side effects. Much work on prodrug conjugates has been done utilizing hydrophilic polymers, steroid hormones, antibodies, and to a lesser extent, peptide hormones and peptide sequences subject to specific cleavage enzymes.[6] All try to achieve the same goal, that is to deliver the therapeutic or cytotoxic agent selectively to the tissue of interest at which time the linker will ideally unmask the cytotoxic agent thus producing a lethal response. For peptide prodrugs, it is necessary to mask the cytotoxic agent until binding and internalization of the complex and at the same time retain the binding affinity of the peptide complex to its receptor. An additional design requirement in this laboratory was the ability to do all peptide chemistry by rapid solid phase synthesis using acid-cleavable supports. After reviewing the chemistry of most of the described methods of cytotoxin attachment, we chose carbamates as a potential linker since they are known to be reasonably stable in plasma[7] compared, for instance, to commonly used ester-type linkages. We then designed a new intramolecular cyclization approach[8] which allows adjustment of the release rates of the alcoholic component of the carbamate culminating in the release of an attached alcohol derivative and formation of the

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cyclic urea which we hypothesized could be utilized to control release rates (Fig. 1) of suitable cytotoxins. It is known that the pK_a value of the leaving alcohol of a carbamate is important for the stability of the prodrug[9] and we chose two different chemically suitable cytotoxic agents each containing one hydroxyl group with significantly different pK_a values. One cytotoxic agent was the topoisomerase I inhibitor, camptothecin[10] which bears a tertiary hydroxyl group in ring position 20 (Fig. 1) with a pK_a of approximately 18, and the other was the tubulin-binding agent, combretastatin[11] which has one phenolic hydroxyl group attached to an aromatic ring thus exhibiting a much lower pK_a value of approximately 10.3.

All peptide conjugates shown in Table 1 were synthesized totally on Rink amide resin support using the standard FMOC (9-fluorenylmethoxycarbonyl) protection/deprotection strategy employing simple DIC couplings and HBTU activation as a second step only if a Kaiser test was positive. Once the peptide portion of the conjugate was complete, bromoacetic acid was coupled to the N-terminus using DIC/DCM. After washing the resin, a 5 M excess of N-BOC-ethylenediamine (or the appropriate protected diamine) in NMP were added and mixed for 1 h. Seperately, camptothecin (200 mg) (Aldrich) and DMAP (400 mg) in anhydrous DCM (40 mL) were mixed and cooled to 0 °C. To this suspension was added 20% phosgene in toluene (600 uL) and the mixture was allowed to react for about 45 min. After solvent evaporation, the powder was suspended in DCM (40 mL) and added to the peptidyl resin bearing the free secondary amine and allowed to react overnight followed by washing several times with DMF, DCM and methanol.

The peptide was then cleaved from the resin for 2 h using the standard acid mixture TFA:$H_2$0:EDT:TIS, 95:2:2:1. After cleavage, the conjugate was precipitated 4 times in ethyl ether, dissolved in 60% HOAC, cyclized with iodine in methanol. It was then subjected to preparative chromatography and characterized by MS and amino acid analysis. All peptides were obtained in at least 90 + % purity and yields were around 35% theoretical.

Full retention of conujugate agonists potencies relative to somatostatin itself was demonstrated by their ability to inhibit GHRH-stimulated GH release from monolayer cultures of rat pituitary cells, an assay system that has been shown[12] to correlate well with binding affinity to the human type 2 somatostatin receptor. Inhibitory potencies (IC_{50}'s) of the conjugates shown in Table 2 ranged from 0.26 to 0.49 nM compared to 0.63 nM for somatostatin-14 itself. This retention of full affinity is due to the employment of the N-terminal Nle-D-Tyr-D-Ser tripeptide extension of the cyclic somaostatin portion of the conjugate which we have found generally allows large groups to be attached with little to no loss of affinity.

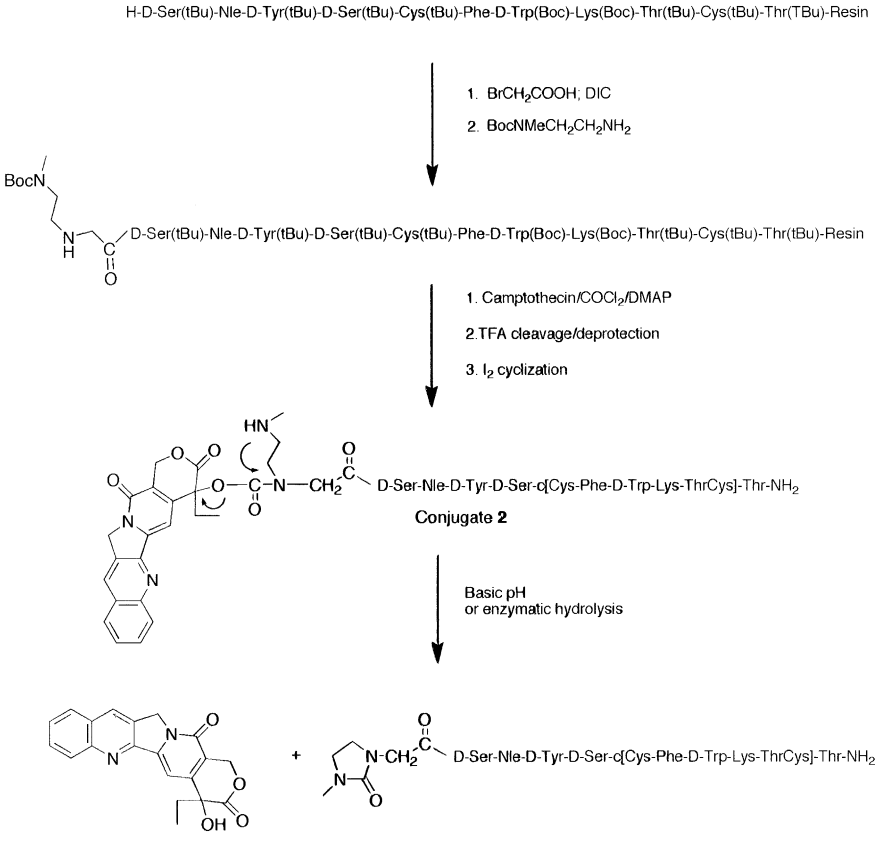
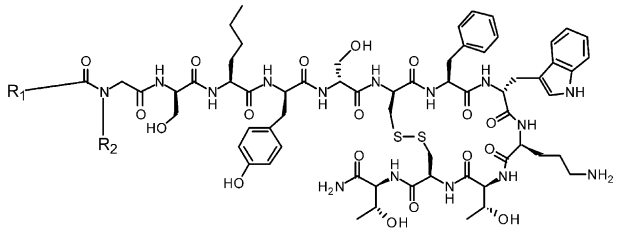
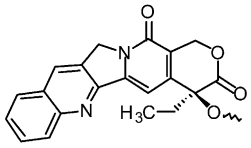
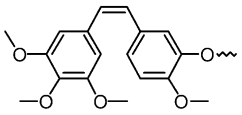


Figure 1. On-resin synthesis of a BINAR group-containing somatostatin analogue and attachment of camptothecin via a carbamate linking group followed by removal from the resin to give compound **2** (Table 1). Basic conditions or enzymatic attack results in nucleophile assisted release of free camptothecin.

Table 1. Structures of camptothecin and combretastatin conjugates containing various BINAR linking groups



R_1	R_2	Compd
		**1**
		**2**
		**3**
		**4**
		**5**
		**6**

Each peptide conjugate was also subjected to phosphate buffer and rat serum stability studies. The analogues were incubated in either 0.1 M phosphate buffer or fresh rat serum at 37 °C and aliquots were taken at different time points and examined by HPLC. Compounds **1**, **3**, and **4** showed (Table 2) complete buffer stability for the length of the experiment (50 h). Compound **2** was less stable in buffer with a half-life of around 120 h, while compound **6** was the least stable with a half-life of only 30 h in buffer. In rat serum, this same trend was evident, except that there was clearly a catabolic effect of serum on the stability of the compounds. Compounds **1**, **3**, and **4** were again the most stable, while compound **2**'s half-life was 18 h and combretastatin compound **6**, which contains the phenolic hydroxyl, was least stable with a 4 h half-life. It is important to point out that if the conjugate breaks apart while measuring its cytotoxic effect, then the peptide loses its vectorizing ability and the test effectively reverts to measuring the cytotoxin activity alone.

The cytotoxic activities of these conjugates were measured using a standard MTT assay kit (Promega Corporation, Madison, WI). Each compound was incubated for 3 days with human neuroblastoma IMR32 cells (somatostatin receptor over-expression) at different concentrations in quadruplate and IC_{50}'s were generated from the dose-response curves (Fig. 2). Although the combretastatin-ethlylenediamine BINAR compound **6** was the most cytotoxic, after reviewing the stability data in buffer, it is evident that 50% of this conjugate is free cytoxin after 3 days and perhaps more since cell media will contain some enzymes. The N-Me-ethylenediamine camptothecin conjugate **2** was the second most potent. After 3 days, more than 80% of this conjugate would still be intact. The next most potent was the ethlylenediamine compound **1**. This analogue demonstrated complete stability in phosphate buffer but was seven times less potent than compound **2** and slightly more active than compound **3** even though it was more stable in serum. This property could be due to compound **1**'s ability to form a cyclic urea while compound **3** is unable to form this same species. In preliminary tumor bearing animal studies, compounds **1** and **2** appear to be equally effective after repeated ip administration. Compounds based on N,N-dimethye-thylenediamine, diaminopropyl, and 2-hydroxyethylamine BINAR groups (Fig. 2; Table 2) were essentially inactive presumably due to lack of release of any free cytotoxic agent intra- or extracellularly.

The value of the BINAR linking strategy lies both in its ability to be adjusted to accommodate ideal release rates for various types of alcohol-containing groups and its ease of incorporation onto a peptide free amino group by simple solid-phase chemistries. The chemistry for introducing the groups is well suited for solid phase synthesis and is easily adapted for many different peptides, therapeutic agents, and cytotoxins. Although this strategy is

Table 2. Agonist activity, cytotoxicity, and buffer and serum half-life of **1**–**6** and control compounds

Compd	Cytotoxicity IC_{50}, nM[a] IMR-32 Cells	Half-life phosphate buffer (h)	Half-life rat serum (h)	Growth hormone inhibition IC_{50}, nM[a]
1	374±18	Stable[a]	106	0.32±0.02
2	54.2±6	123	18	0.27±0.02
3	571±48	Stable	59	0.30±0.04
4	>1000	Stable	72	0.26±0.03
5	>1000	na	na	0.49±0.1
6	2.79±0.33	31	4	0.31±0.1
Camptothecin	2.21±0.44	na	na	na
Combretastatin	4.36	na	na	na
SRIF (1-14)	na[b]	na	2–5 min	0.63±0.029

[a]Experiment carried out for 50 h.
[b]Somtostatin analogues were non-toxic to IMR32 cells at up to 10^{-5} M concentrations.

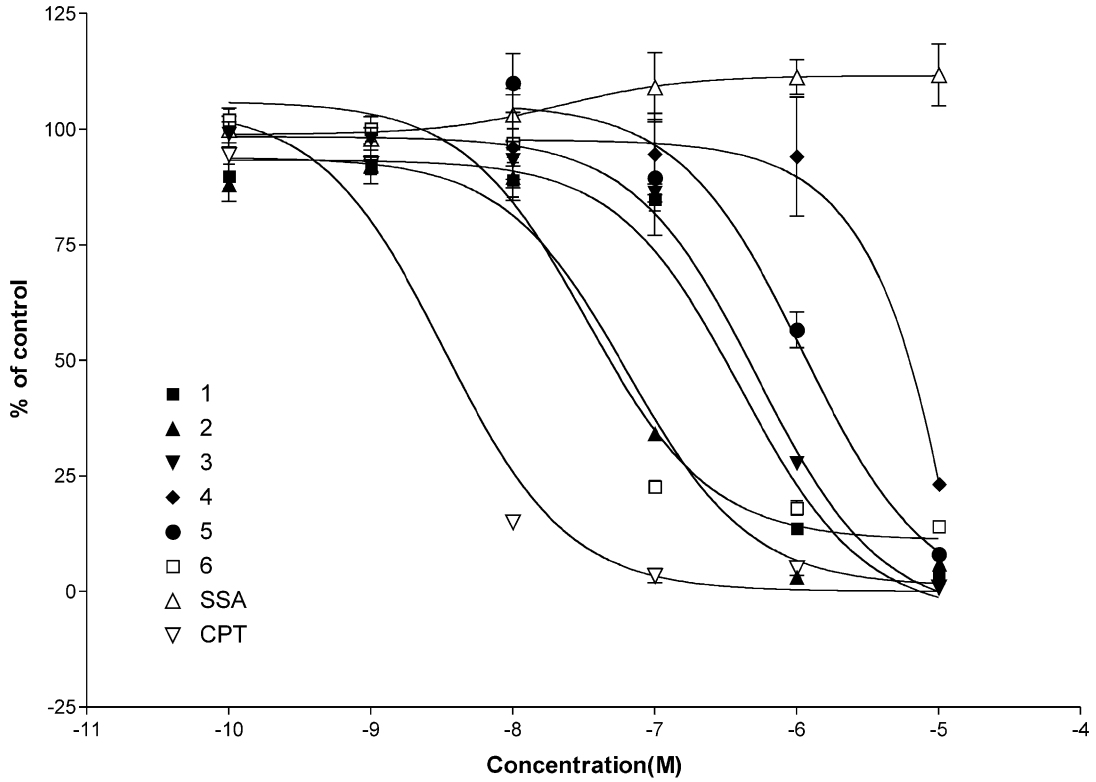


Figure 2. Dose–response curves for conjugates 1–6 abilities to kill SSTR2 positive IMR-32 cells after incubation for 3 days uisng a cell viability MTT assay.

readily applied to the N-terminus, it can also be applied to an orthogonally protected side chain as well should this be necessary for preservation of peptide binding affinity.

With the present camptothecin conjugates, high cytotoxicity was achieved with compounds **1** and **2** (Fig. 2; Table 2) even though they were very stable to cell media incubation conditions. Although higher breakdown rates will occur after systemic administration, it appears likely that high concentrations of intact peptide conjugate will be specifically bound to tumor cells whereupon internalization would take place. Furthermore, the conjugates are highly soluble and tissue clearance rates and routes can be readily further adjusted by altering the hydrophilicity of the peptide component. If the peptide conjugate remains reasonably intact during clearance, then toxic side effects should be much reduced. In preliminary studies, the lead camptothecin conjugate **2** was able to significantly stabilize the growh of transplanted NCI-H69 small cell lung carcinomas (expression of SSTR2)[13] in nude mice at doses far below the maximum tolerated equivalent doses of camptothecin alone. The combretastatin conjugate **6** attached through the phenolic OH group appears to be too unstable to be of much use with respect to tumor treatment and work is in progress to synthesize combretastatin analogues with more suitable attachment points for peptide vectors. The data suggest not only a chemical component to the release rate of the alcohol but also some enzymatic component. Since carbamates are known to be metabolized by P450,[14] and P450[15] is also know to be up-regulated by human cancers, P450 may be one enzyme responsible for activation/catabolism of the BINAR linker.

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