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Improving anticancer activity and selectivity of camptothecin through conjugation with releasable substance P

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ABSTRACT

Substance P, an 11-residue neuropeptide, can be rapidly internalized through specific interaction with the neurokinin-1 receptor. Therefore, we designed and synthesized the substance P targeted camptothecin (CPT) conjugates via a releasable disulfide carbonate linker. All the conjugates exhibited comparable or stronger cytotoxicity to cancer cells that highly over-express neurokinin-1 receptor than free CPT. More importantly, the selectivity of conjugates was significantly improved compared with CPT. Our results indicated that these conjugates can be promising candidates for new chemotherapeutic drugs. In addition, increasing CPT loading or attachment of CPT to the C-terminal hexapeptide of substance P are useful strategies to enhance the therapeutic efficacy of substance P targeted conjugates.

The therapeutic activity of most traditional anticancer drugs in clinical use is limited by their general toxicity to normal cells. Although novel cytotoxic agents were developed with unique mechanisms of action, many of these compounds still lack cancer selectivity and have not been therapeutically useful. Receptor-specific targeting, as one approach, is attracting increased attention because of their ability to improve the efficacy of treatment.[1–4] In the last few years, conjugates of cytotoxic agents with target molecules, addressed to different receptors over-expressed by cancer cells, have been studied as potential selective anticancer agents.[5–8] Among these molecules, substance P (SP), an 11-residue neuropeptide member of the tachykinin family, appears an attractive candidate, because (i) SP is rapidly internalized through specific interaction with the neurokinin-1 receptor (NK1R),[8–10] (ii) NK1R is highly over-expressed in many types of cancers, including breast carcinomas, astrocytomas, glioblastomas.[11–14] More importantly, SP is easily chemically synthesized, allowing the incorporation of various reactive groups to facilitate coupling to myriad biomolecular cargos. Consequently, SP can be a novel target molecule that mediates delivery of cytotoxic agents to cancer cells with high selectivity.

Camptothecin (CPT), originally isolated from the Chinese tree *Camptotheca acuminate*, possesses potent anticancer properties that derive from its inhibition of topoisomerase I.[15,16] However, major limitations of the drug, including poor solubility and instability of its lactone ring under physiological conditions, prevent full clinical utilization.[17–19] A variety of different strategies were adopted to improve its solubility, lactone stability and bioavailability.[20–22]

Ever since conjugation to the 20-OH of CPT was demonstrated to stabilize the lactone ring, conjugates derivatized at this site have been vigorously pursued.[23–25] Simultaneous approaches to improve water solubility are the attachment of CPT to hydrophilic polymers or peptides.[26–28] A useful feature of SP is that only its C-terminal amino acids are essential for binding to the NK1R and subsequent internalization,[29,30] allowing incorporation of reactive groups for cargo attachment at the N-terminus without affecting its function. For a drug deliverable release strategy, we focused on a disulfide linker, because its cleavage would occur only after encountering a high glutathione concentration in cells.[33,34] In this study, we synthesized the conjugate SC by attaching CPT to the N-terminus of SP at the 20-OH position via a disulfide releasable carbonate linker, which has been extensively used in the conjugated drugs.[28,35,36] To improve the anticancer efficacy of the conjugate, S2C was synthesized by simultaneously attaching two molecules of CPT to the N-terminus of SP. The smallest C-terminal fragments of SP with measurable affinity for the NK1R consist of five amino acid residues.[30] Addition of one to three residues at the N-terminus of the C-terminal pentapeptides leads to rather selective compounds.[31] C-terminal hexapeptide of SP was reported

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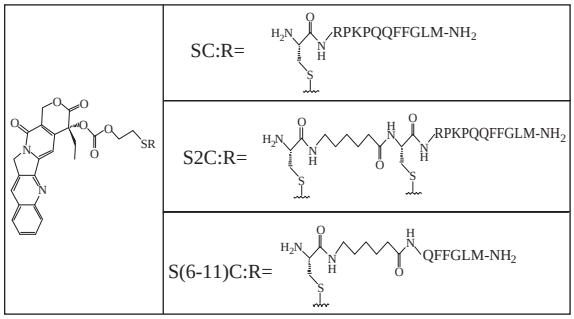
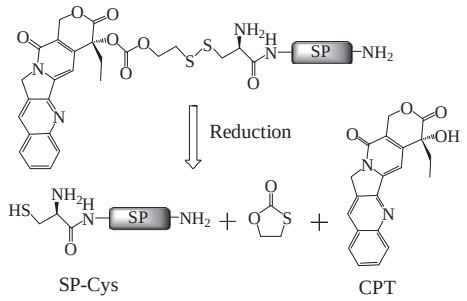


Figure 1. The structure of SP targeted CPT conjugates.



Scheme 1. Disulfide mediated release of camptothecin from SP.

to have high binding affinities to the NK1 receptor.[32] Therefore, to reduce the size of the conjugate, we also synthesized the conjugate S(6–11)C by attaching CPT to the N-terminus of C-terminal hexapeptide of SP. The designed conjugates are presented in Figure 1.

The SP and its C-terminal hexapeptide were synthesized on a MBHA resin using the standard Fmoc-chemistry-based strategy. Cysteine was to serve as N-terminus, where the thiol group was to serve as the attachment site for the cleavable linker. To minimize the potential steric interactions, aminohexanoic acid (AHX) was introduced to the S2C and S(6–11)C as a spacer. Following a procedure reported in previous literature,[28] CPT was attached to the N-terminus of peptides with a disulfide carbonate releasable linker. All crude peptides and conjugates were purified by reversed phase high performance liquid chromatography (RP-HPLC) on a C18 column, and then characterized by electrospray ionization mass spectrometry (ESI-MS).

Solubility of all agents was measured in PBS. Excess CPT and conjugates were suspended in PBS (pH 7.4). After sonification in an ultrasound bath for 10 min at 25 °C, the samples were centrifuged, and the UV absorptions of the supernatants were measured at 370 nm. Finally, the concentrations of the saturated solutions were calculated by comparing the UV absorptions with the calibration curve. The CPT solubility is 2.7 μg/ml (7.8 μM) and in the range of the published value.[17] SC showed more water soluble than CPT with a solubility of 103 μg/ml (54.1 μM). Attachment to the C-terminal hexapeptide only slightly improved the water solubility of CPT with a solubility of 18.6 μg/ml (12.3 μM). However, increased CPT loading to SP could not effectively improve the solubility of CPT with a solubility of 8.4 μg/ml (3.3 μM).

To assess release of CPT from the conjugates, 50 μM conjugates in PBS (pH 7.4) was treated with 10-fold molar excess of dithiothreitol (DTT) for 1 h at 37 °C. The thiol resulting from DTT reduction was expected to cyclize into the proximate carbonyl group of the linker, leading subsequently to the release of free CTP (Scheme 1). The HPLC profile (UV detection at 280 nm) showed complete cleavage of the disulfide bond with concomitant release of CPT from all conjugates within 1 h (Fig. 2).

The anticancer activity of these conjugates was compared with free CPT in different cell lines, two glioblastoma cell lines (U87-MG and U251-MG), and two breast cancer cell lines (MCF-7 and MDA-MB-231), which were reported to over-express NK1R.[8,37–39] Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After incubation with various concentrations of CPT and all conjugates for 1 h, the remaining agents that did not enter cells were washed to remove. Then, the cytotoxicity of the relevant agents was determined by the MTT assay after 72 h. As shown in Figure 3, the SP and its C-terminal hexapeptide S(6–11) had almost no effects on the proliferation of tested cancer cell lines. However, the conjugates and free CPT exhibited significant anticancer activity in a dose-dependent manner. Although attachment to SP can remarkably improve physicochemical properties of CPT, the anticancer activity of SC was not remarkably improved compared with free CPT. However, increasing CPT loading can significantly enhance the cytotoxicity of S2C to cancer cells. Gratifyingly, attachment of CPT to S(6–11), the short fragment of SP, could significantly increased the cytotoxicity of SP targeted conjugate to all tested cancer cells compared with CPT. Compared with SC, the high internalization efficacy of the S(6–11)C may be contributed to its higher cytotoxicity. Our previous study showed that the fragment S(6–11) has higher internalization efficacy than the parent peptide.[40] Overall, these results demonstrated that increasing CPT loading or attachment of CPT to the

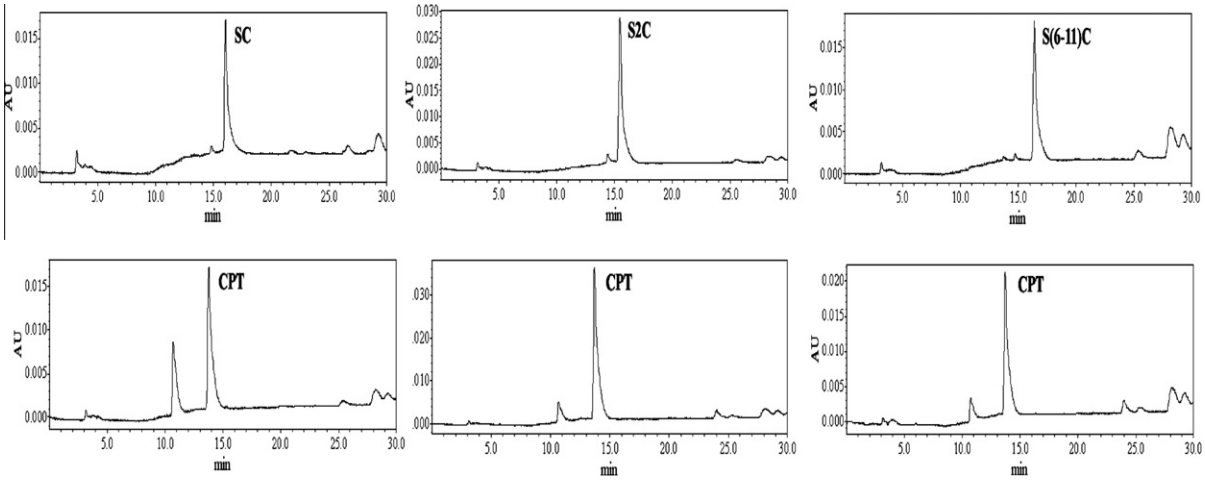


Figure 2. Release of CPT from SP targeted CPT conjugates by disulfide reduction. The conjugates were analyzed using RP-HPLC (Abs = 280 nm) in the absence (top chromatogram) and presence (bottom chromatograms) of a 10-fold excess of dithiothreitol (DTT).

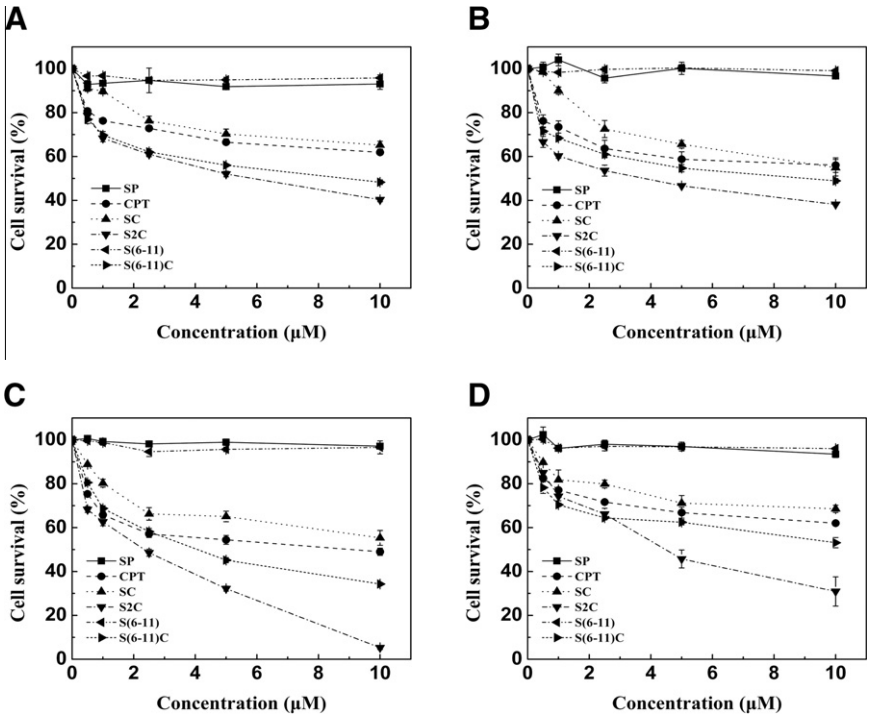


Figure 3. Cytotoxicity of SP targeted CPT conjugates, free CPT and peptides to different cancer cells that highly over-express NK1R. (A) U87 cell line; (B) U251 cell line; (C) MCF-7 cell line; (D) MDA-MB-231 cell line. Cell viability was assessed by MTT assay.

C-terminal hexapeptide of substance P were useful strategies to enhance the cytotoxicity of SP targeted conjugates to cancer cells that highly over-express NK1R.

The majority of clinically approved anticancer drugs have very little or no specificity, which leads to systemic toxicity, causing undesirable severe side effects. To study the selectivity of the SP targeted conjugates, the CHO cells stably expressing NK1R was established. The eukaryotic vector containing HA-tagged human NK1R, pcDNA3.1-3 × HA-NK1, was introduced into CHO cells by Lipofectamine2000 according to the manufacture's instruction. The day after transfection, G418 (200 μg/ml) was added to the medium for two weeks. Then the antibiotic-resistant clones derived from single cell were selected and further characterized by RT-PCR (Fig. 4A). In this study, the selectivity of all conjugates was assessed by comparing their cytotoxicity to CHO cells and CHO cells expressing NK1R. All the conjugates exhibited comparable to or stronger activity than free CPT in CHO cells expressing NK1R (Fig. 4C). In contrast, SC and S(6–11)C have little effect on proliferation of CHO cells, and cytotoxicity of S2C to CHO cells was significantly weaker than that of free CPT (Fig. 4B). These results suggests that attachment to SP or S(6–11) can remarkably enhance the selectivity of CPT to cells that highly over-express NK1R.

To further prove the selectivity of the conjugates, the internalization of the conjugates and free CPT were analyzed by confocal microscopy. CHO cells and CHO cells expressing NK1R were incubated with 10 μM CPT or conjugates for 30 min. After washing with PBS to remove the remaining agents, cells were examined by confocal microscopy for the uptake of CPT and conjugates. Excitation was performed by a 405-nm line, blue fluorescence was observed. As shown in Figure 5, CPT could translocate into CHO cells and CHO cells expressing NK1R at similar fluorescence intensity. The fluorescence intensity of conjugates in CHO cells expressing NK1R was comparable to or stronger than that of free CPT, whereas, the fluorescence intensity of conjugates in CHO cells was remarkably weaker than that of CPT. This result demonstrated that the attachment to SP or S(6–11) can significantly improve selectivity

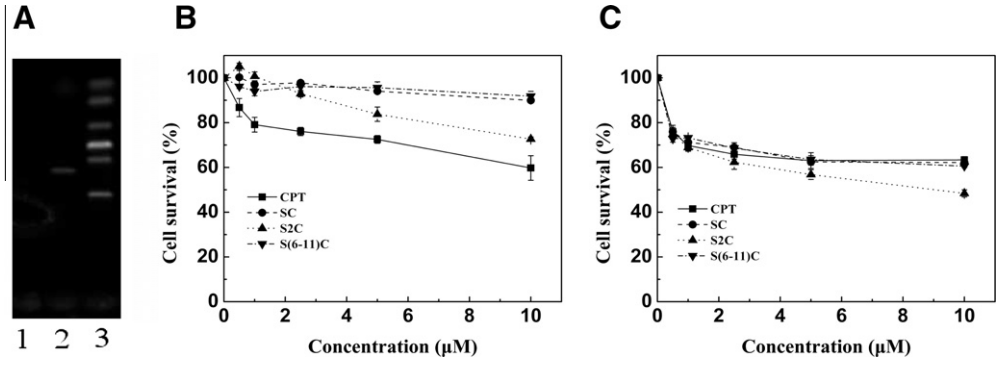


Figure 4. Selectivity of SP targeted CPT conjugates and free CPT to cells that over-express NK1R. (A) Agarose gel electrophoresis of RT-PCR fragments for NK1R. Line 1: CHO cell line, line 2: CHO cell line expressing NK1R, line 3: marker. (B) Cytotoxicity of SP targeted CPT conjugates and free CPT to CHO cells. (C) Cytotoxicity of SP targeted CPT conjugates and free CPT to CHO cells expressing NK1R.

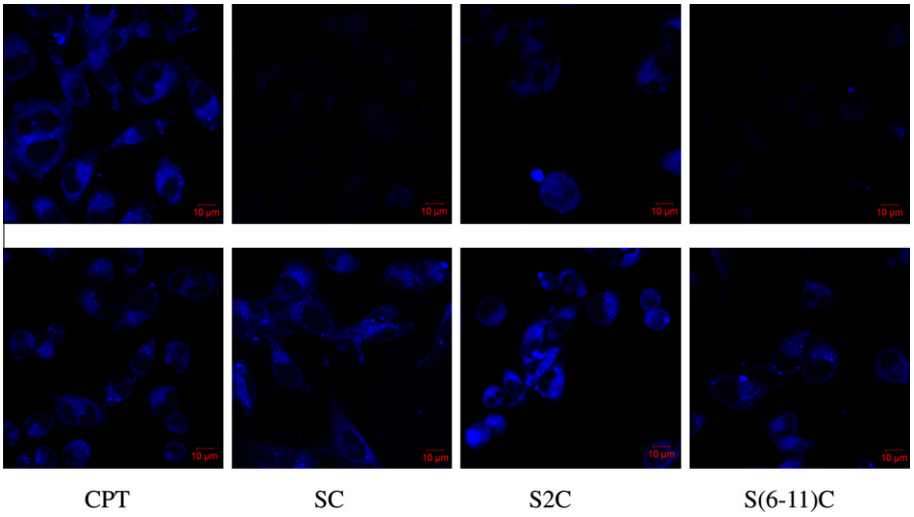


Figure 5. The internalization of SP targeted CPT conjugates and free CPT into CHO cells (top pictures) and CHO cells expressing NK1R (bottom pictures). Cell observation was done with a confocal microscopy.

of CPT to cells that over-express NK1R. Additionally, we found that the conjugates can also translocate into CHO cells. The uptake of conjugates may be mediated by other mechanisms rather than receptor mediated endocytosis, and further work is needed to study this mechanism.

In summary, we have developed SP targeted CPT conjugates with significant cytotoxicity and selectivity to cancer cells that highly over-express NK1R, indicating that these conjugates can be promising candidates for new chemotherapeutic drugs. Increasing drug loading can enhance the cytotoxicity of SP targeted conjugates to cancer cells that over-express NK1R, but concomitant with an increased toxicity to normal cells. Gratifyingly, S(6–11)C had enhanced significant cytotoxicity and selectivity to cancer cells that highly over-express NK1R compared with free CPT. Accordingly, our study demonstrated the C-terminal hexapeptide of SP may be a more efficient target molecule compared with SP. Thus, increasing CPT loading or attachment of CPT to the C-terminal hexapeptide of substance P were useful strategies to enhance the therapeutic efficacy of substance P targeted CPT conjugates.

Acknowledgments

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