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Co-Delivery of Small Interfering Rna Using A Camptothecin Prodrug As the Carrier

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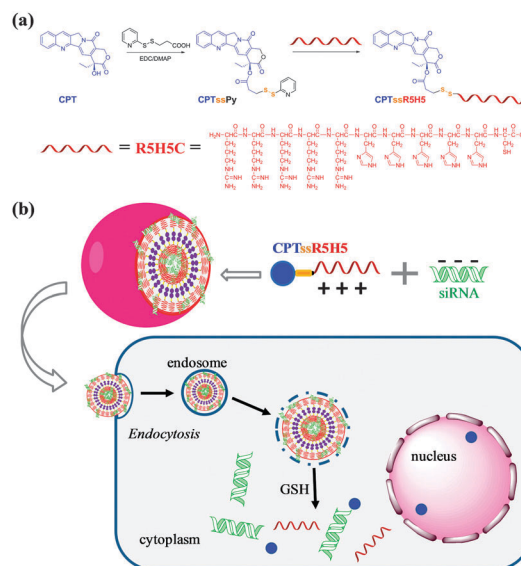
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We have reported the first effort towards directly using an anticancer prodrug (CPTssR5H5) as a carrier to co-deliver camptothecin (CPT) and small interfering RNA for multidrug resistant cancer chemotherapy. The results indicated that CPTssR5H5 is a promising co-delivery system for MDR cancer therapy.

The emergence of multidrug resistance (MDR) in cancer cells has been a significant impediment to the successful cancer chemotherapy,¹ since multidrug resistant tumor cells exhibit resistance to a number of structurally and functionally unrelated anticancer drugs.² Therapeutic strategies to overcome drug resistance as well as adverse side effects may greatly improve the efficacy of chemotherapy. Two approaches, the increase of drug doses and the suppression of cellular resistance to anticancer drugs, have been proposed and evaluated to treat MDR cancers.^{1a} Administration of high doses of drugs is expected to overcome MDR effects. However, the therapeutic drugs constitute only a minor portion in most of the nano-sized drug carriers to minimize the initial drug release in blood before reaching the target. The drug contents generally cannot exceed 10% in nanoparticles or liposomes.³ Therefore, large amounts of carriers have to be used to administer a high dose of the anticancer drug to overcome MDR effects. Repeated administration of high doses of low drug-loading carriers may cause severe toxicity⁴ and create a burden for the patients to absorb or excrete drug carrier materials. To circumvent this problem, an alternative strategy is to suppress the activities of proteins responsible for cellular defence induced by chemotherapy agents.⁵ Small interfering RNA (siRNA)-mediated RNA interference has recently emerged as a potent approach to induce specific silencing of a broad range of genes.⁶ Recently, there has been a surge of interest in developing platforms for co-delivery of anticancer drugs as apoptosis inducers and siRNAs as suppressors of cellular defence to enhance

chemotherapeutic effects. However, the lack of co-delivery systems limited the potential of the combinatorial therapy of siRNAs and anticancer drugs for MDR cancer.⁷ Moreover, a large amount of undesired toxic and non-degradable materials has to be used as carriers to deliver both siRNAs and anticancer drugs.^{5d-f}

To address these challenges in MDR cancer therapy, we synthesized an integrated and high drug-loading drug-peptide conjugate, CPTssR5H5, which can co-deliver the anticancer drug and siRNA (Scheme 1). The hydrophobic anticancer drug, CPT, was directly used as a component of the carrier, and functions as the hydrophobic block of the amphiphilic drug carrier. An oligo-peptide, RRRRRHHHHHC (R5H5), was used as a hydrophilic block of the carrier. Our previous study found that amphiphilic R5H5-cholesterol conjugates can condense nucleic acids and facilitate the endosomal escape of DNA-vector complexes to achieve high transfection efficiency.⁸ The two blocks were linked through a disulfide



Scheme 1 (a) Synthetic route of CPTssR5H5. (b) Illustration of a co-delivery system CPTssR5H5 to deliver CPT and MAP3K7-targeted siRNA simultaneously to MDR cancer cells for enhanced chemotherapy.

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bond that can be cleaved inside cells while providing high stability to the delivery system outside cells.⁹ CPTssR5H5 is expected to self-assemble into liposome-like vesicles with a high load of therapeutic agents (CPT and siRNA targeting mitogen-activated protein kinase 7 (MAP3K7) gene) and to deliver and release the drugs in cells to enhance the therapeutic outcome of chemotherapy.

CPTssR5H5 was synthesized by a three-step reaction (Scheme 1a) and purified by reverse phase high-performance liquid chromatography (HPLC) (Fig. S1, ESI†). The structure of CPTssR5H5 is confirmed by nuclear magnetic resonance (NMR) (Fig. S2, ESI†). The drug loading of CPTssR5H5 vesicle is 17%. CPTssR5H5 is expected to self-assemble into liposome-like vesicles. The critical micelle concentration (CMC) of CPTssR5H5 is 80 $\mu\text{g mL}^{-1}$ in H_2O (Fig. S3, ESI†). When observed under a transmission electron microscope (TEM), CPTssR5H5 vesicles showed a uniform spherical shape with a size of about 20 nm (Fig. 1a). The size of CPTssR5H5 vesicles measured by dynamic light scattering (DLS) was about 40 nm by number distribution with a zeta-potential of 14 mV (Fig. S4, ESI†). The discrepancy between TEM and DLS measurements is attributed to factors associated with the high vacuum conditions of TEM and the hydrodynamic and electro-kinetic effects operative in DLS measurements.¹⁰ Circular dichroism (CD) measurements showed that CPTssR5H5 vesicles did not form any secondary structures under neutral and acidic conditions (Fig. S5, ESI†).

The size and surface charge of drug carriers are two important factors that influence the delivery efficiency of anticancer drugs and siRNA *in vitro* and *in vivo*. The size and zeta-potential of CPTssR5H5–siRNA complexes were characterized. As shown in Fig. 1b, as the N/P ratio increases from 10 to 50, the size of complexes follows a decreasing trend, while the zeta potential follows an increasing trend. At the N/P ratio of 10, the size of complexes was 56 nm with a zeta-potential of 6 mV, which indicated that the cationic CPTssR5H5 at this concentration was able to condense siRNA into complexes. Above the N/P ratio of 30, the size of complexes decreased slightly and reached 37 nm at the N/P ratio of 50 with a zeta-potential of 14 mV. The morphology of CPTssR5H5–siRNA complexes was similar to that of CPTssR5H5 vesicles and the size of complexes at the N/P ratio of 45 was about 30 nm as observed by TEM (Fig. S6, ESI†). It was reported that liposomes with a size of less than 100 nm in diameter were optimal for drug retention and tumor extravasation.¹¹ The net positive charge of carrier–nucleic acid complexes facilitates their cellular uptake because of the interaction of the negatively charged cell membrane and the positively charged complexes. However, if the charge density of the complexes is too high (>30 mV), it might induce cellular toxicity¹² and reduce the specificity for target cells due to the elevated nonspecific binding to serum proteins and cells.

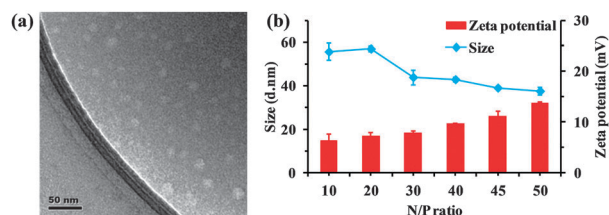


Fig. 1 (a) CPTssR5H5 vesicles observed by TEM (scale bar of 50 nm). (b) The size and zeta potential of CPTssR5H5–siRNA complexes.

Results show that CPTssR5H5 vesicles can efficiently condense siRNA to the desired size and surface potential *in vitro*.

To evaluate the binding affinity of CPTssR5H5 to siRNA, CPTssR5H5–siRNA complexes formed at various N/P ratios were analysed by agarose gel electrophoresis. As shown in Fig. S7a (ESI†), part of siRNA used for condensation run through the agarose gel at low N/P ratios (10 and 20), indicating that the complexes formed at these two ratios were not compact enough to prevent siRNAs from running through the gel. As the N/P ratio increased, the complete retardation of siRNA was achieved at the N/P ratio of 30. We hypothesized that the low intracellular redox potential of cells will induce the cleavage of the disulfide bond between CPT and the R5H5 peptide, which will lead to the release of CPT and siRNA. The decomplexation of CPTssR5H5–siRNA complexes in the reducing environment was determined by agarose gel electrophoresis (Fig. S7, ESI†). When complexes at N/P ratios of 30 and 45 were incubated with 25 mM dithiothreitol (DTT) for 1 hour at 37 °C, the majority of siRNAs were released from the complexes treated with DTT. Under the same conditions, no siRNA was released from the complexes without DTT treatment. Results demonstrate the disulfide linkage enables CPTssR5H5–siRNA complexes to unpack and release siRNA upon entering the reducing environment of the cytosol, while complexes are stable outside cells.

The ability of CPTssR5H5 as a vector to deliver MAP3K7 siRNA to cancer cells was observed by confocal laser scanning microscopy (CLSM) (Fig. 2a). After incubation with CPTssR5H5–MAP3K7 siRNA complexes for 6 hours, nearly all cells displayed red fluorescence in the cytoplasmic space, especially in perinuclear regions of the cytoplasm, indicating efficient delivery of siRNA by CPTssR5H5 into cells. As seen in Fig. 2a, no significant signals of red fluorescence were detected in the nuclei of cells, which is an advantage for RNA interference since this process takes place in the cytoplasm.¹³ A similar phenomenon was observed in cells incubated with lipofectamine–siRNA as a control, but with higher fluorescence intensity (Fig. S8, ESI†).

The efficiency of CPTssR5H5–MAP3K7 siRNA complexes to silence the MAP3K7 mRNA expression in MDA-MB-231 cells was studied by the quantitative reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 2b, MAP3K7 mRNA expression levels in

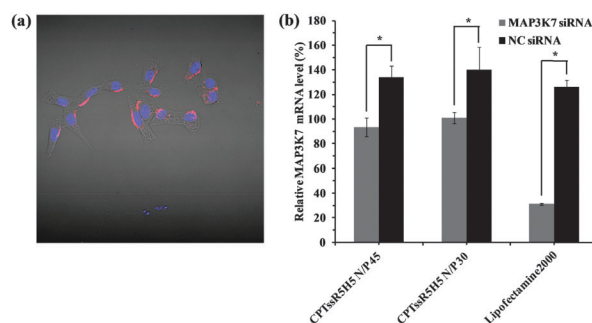


Fig. 2 (a) The cellular internalization of CPTssR5H5–MAP3K7 siRNA complexes at the N/P ratio of 45 after 6 hour incubation. The siRNA was stained by TM-rhodamine (red) and cell nuclei were stained by Hoechst 33342 (blue). (b) Relative MAP3K7 mRNA levels after 48 hour incubation. mRNAs of GAPDH and 18S rRNA genes were used as internal control, and mRNA expression levels were normalized to control (no treatment). The data are the mean \pm standard error for $n = 3$. * $p < 0.05$.

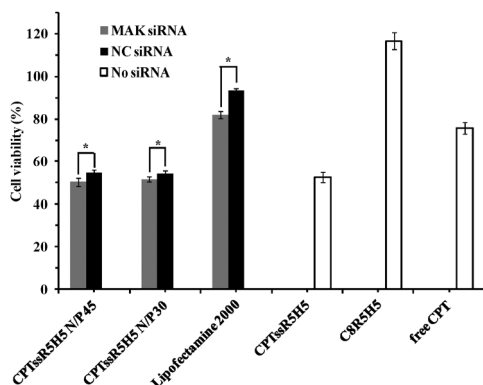


Fig. 3 Cell viability in the presence of different drug formulations at a CPT-equivalent dose of 36 μ M to MDA-MB-231 cells for 48 hours of incubation. * $p < 0.01$.

cells treated with CPTssR5H5–MAP3K7 siRNA complexes decreased by 30% and 27% at siRNA concentrations of 100 nM and 150 nM, respectively, compared to the cells treated with CPTssR5H5–negative control (NC) siRNA. For cells treated with lipofectamine 2000–siRNA complexes as a control, the mRNA reduction induced by MAP3K7 siRNA was 75% compared to that obtained for cells treated with NC siRNA. The results were consistent with those of the previous study.¹⁴ We observed that cells treated with NC siRNA complexed with either CPTssR5H5 or lipofectamine2000 showed higher expression of MAP3K7 mRNA than cells with no treatment. The overexpression of the MAP3K7 gene induced by CPTssR5H5 was in agreement with previous findings that anticancer drugs induce the activation of cellular resistance.^{5d} However, for the lipofectamine-induced overexpression of MAP3K7 mRNA, the mechanism was unclear. It is possible that lipofectamine 2000 is toxic to cells as a foreign reagent, which activates cellular defence and increases the MAP3K7 mRNA level.

The cytotoxicity of different CPTssR5H5–MAP3K7 siRNA formulations with the same CPT-equivalent dose of 36 μ M toward MDA-MB-231 cells was evaluated by MTT assay (Fig. 3). The non-CPT control, C8R5H5, which can also form liposome-like vesicles showed no cytotoxicity to cells. The results confirmed that the cationic surfactant structure of CPTssR5H5 is not toxic to cells. Compared to free CPT, CPTssR5H5 alone leads to much lower cell viability, indicating that this prodrug improves the solubility of CPT and thus facilitates CPT to travel into cells. As shown in Fig. 3, cells incubated with CPTssR5H5 complexed with either NC siRNA or MAP3K7 siRNA showed much lower cell viability, 55% and 51%, respectively, compared to cells incubated with lipofectamine 2000–siRNA (~88%). The results indicate that CPTssR5H5, as a prodrug, releases active CPT once delivered into cancer cells to suppress cell viability. CPTssR5H5 with MAP3K7 siRNA induced a higher cytotoxicity than CPTssR5H5–NC siRNA complexes.

Cell apoptosis was analysed to confirm the enhanced cytotoxicity resulting from increased apoptosis instead of necrosis by CLSM using Click-IT[®] TUNEL Alexa Fluor[®] 488 imaging assay. No green fluorescence was observed in cells without treatment (Fig. S9a, ESI[†]) and in cells treated with lipofectamine–MAP3K7 siRNA (Fig. S9c, ESI[†]), which indicated that no apoptosis took place in cells under both conditions. Weak green fluorescence was observed in the cells treated with free CPT, indicating that apoptosis took place only in few cells while most cells underwent necrosis. In contrast, when incubated with

CPTssR5H5–siRNA complexes containing an equivalent dose of CPT, strong green signals were detected in 50% of the cell population, and fragmented nuclei could be observed due to cell apoptosis (Fig. S9d and S9e, ESI[†]). The results indicated that CPTssR5H5 as a prodrug releases active CPT to induce cell apoptosis. Further investigation is underway to understand the mechanism of apoptosis induced by the simultaneous delivery of CPT and MAP3K7 siRNA using CPTssR5H5 and thus improve the chemotherapy efficacy of CPT.

In summary, we have demonstrated the first effort towards directly using the anticancer prodrug, CPTssR5H5, as a carrier to co-deliver CPT and siRNA. CPTssR5H5 can self-assemble into uniform liposome-like vesicles (20 nm), condense siRNA into compact vesicles above the N/P ratio of 10 and efficiently release siRNA under reducing conditions. CPTssR5H5–siRNA complexes were rapidly taken up by MDA-MB-231 cells, and the loaded MAP3K7 siRNA reduced the level of MAP3K7 mRNA. The results indicated that CPTssR5H5 is a promising co-delivery system for MDR cancer therapy, but it needs to be further optimized to achieve desired therapeutic results. This multifunctional prodrug-based drug delivery system is designed as a versatile platform, which can be readily adapted to co-deliver other MDR gene or oncogene silencing siRNAs and anticancer drugs to improve therapeutic effects of MDR cancer therapy.

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