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Synthesis of doxorubicin–peptide conjugate with multidrug resistant tumor cell killing activity

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Abstract—Cell penetrating peptide TAT was introduced into doxorubicin structure. Synthesized doxorubicin–TAT conjugate showed different intracellular distribution pattern and cell killing activity from those of free doxorubicin. Unlike free doxorubicin, doxorubicin–TAT conjugate was highly permeable to drug-resistant cells and was able to kill drug-resistant tumor cells efficiently.

Clinical resistance to anticancer drugs is the major reason for cancer treatment failure. Now nearly 50% of human cancers are either completely resistant to chemotherapy or respond only transiently, after which they are no longer affected by commonly used anticancer drugs. This phenomenon is generally referred to as multidrug resistance (MDR).[1] Various mechanisms are presumed to be responsible for the MDR phenotype of a cell including intracellular conversion of a toxic drug into less harmful metabolites and decreased expression of topoisomerase II in MDR tumor cells. By far the best characterized mechanism leading to decreased intracellular drug levels is the over-expression of energy-dependent drug efflux pump proteins such as P-glycoprotein (Pgp). This integral membrane protein is able to remove drugs from the cytoplasm and thus reduce intracellular anticancer drug concentrations.[2]

Recently, several peptides have been demonstrated to translocate across the plasma membrane of eukaryotic cells by a very efficient and energy-independent pathway.[3] These peptides, termed as cell penetrating peptides (CPPs), have been used successfully for the intracellular delivery of molecules with various molecular weights.[4] Recent research shows that hybrids consisting of a cell penetrating sequence and a brain impermeable compound can cross blood–brain barrier (BBB), a P-glycoprotein highly expressed endothelial cell layer, to get into the brain.[5] The objective of this study was to examine if cell penetrating peptide could also bring cell impermeable drug into P-glycoprotein highly expressed drug-resistant tumor cells to overcome drug resistance in cancer therapy.

Conjugate from a common anticancer drug, doxorubicin, and a well-known cell penetrating peptide, TAT peptide, was synthesized. The cell penetration as well as tumor killing activity of doxorubicin–TAT conjugate was examined on both drug-sensitive and drug-resistant tumor cells.

Doxorubicin was dissolved in DMSO and then dispersed in a phosphate buffer solution (3.0 ml, pH 8.0) at a concentration of 0.3 mg/ml. After addition of 20 μl of triethylamine (TEA), 200 μl of succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, 10 mg/ml) was added. The mixture was incubated at room temperature for 2 h. After adjusting the pH of the mixture to 5.5, 300 μl of synthesized TAT peptide (CGGGYGR KKRRQRRR) solution (15 mg/ml) was added. The mixture was kept at room temperature for another 2 h. SMCC is a widely used cross-linker for the conjugation of compounds with amino and thiol groups, respectively, especially in protein/peptide related conjugation.[6] A scheme of doxorubicin–TAT conjugate synthesis is demonstrated in Figure 1.

Unreacted doxorubicin, SMCC and TEA, were removed by passing the reaction mixture through a heparin column connected to FPLC and washed with PBS. Because TAT is a positively charged peptide, TAT peptide and

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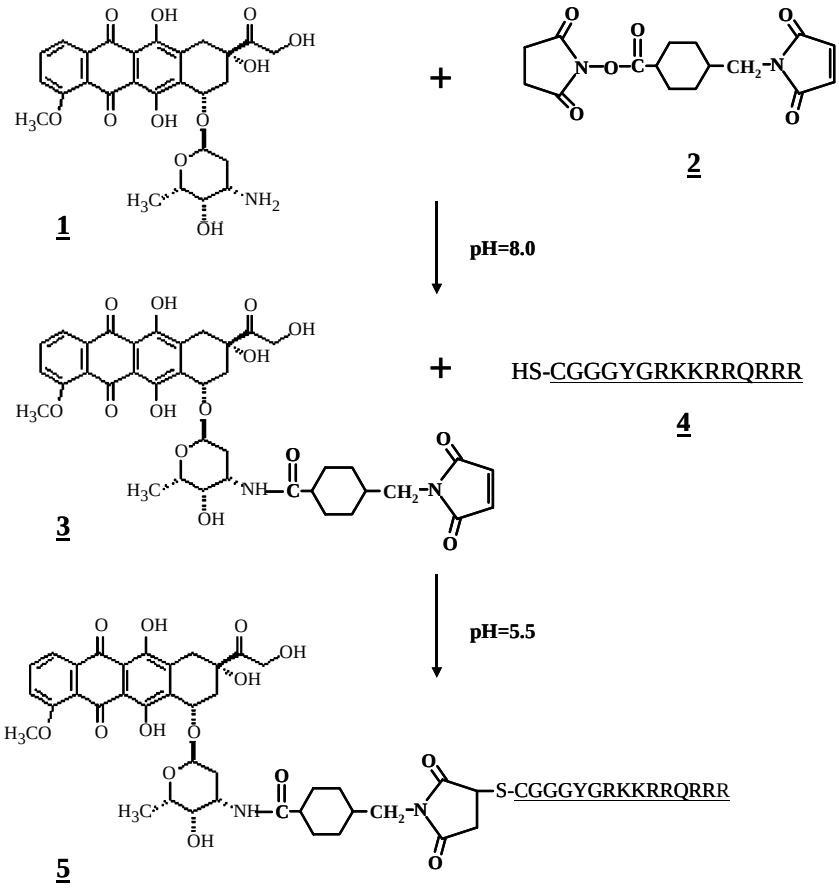

Figure 1. Scheme for doxorubicin–TAT conjugate synthesis. **1**, doxorubicin; **2**, SMCC; **3**, doxorubicin–SMCC; **4**, TAT peptide; **5**, doxorubicin–TAT conjugate.

its conjugation production would show strong affinity to heparin column. Unreacted TAT peptide and doxorubicin–TAT conjugate were eluted from heparin column through a linear elution using 2.0 M NaCl (Fig. 2A) according to established method.[7] Peak #2 in Figure 2A had the same retention time as pure TAT peptide and thus was unreacted TAT peptide. Collected peak #1 in Figure 2A was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) assay[7] and two peptide bands were found (Fig. 2B). The molecular weight of one band was about 1700 Da, which was slightly higher than the molecular weight of TAT peptide (1678 Da). However, the molecular weight of another band was about 3,500 Da, more than two times higher than that of TAT peptide. Since doxorubicin is a fluorescent compound, the ultraviolet image of the same SDS–PAGE gel was generated and is shown in Figure 2C. Unlike TAT peptide band, both peptide bands from peak #1 in Figure 2A were visible under ultraviolet light, implying that peptides in these two bands had been already linked with doxorubicin. This result also excluded the possibility that the high molecular weight peptide band was TAT peptide dimer formed by a disulfide bond (TAT–S–S–TAT). The conjugate mixture was subjected to a further separation on a C_8 reverse-phase HPLC using a shallow gradient of acetonitrile (10–40% over 40 min) as described previously.[7] Two doxorubicin–TAT conjugates with a molecular ratio of 1:1 (peak #1) and 1:2 (peak #2) were obtained (Fig. 2D) as confirmed by mass spectrometry assay. Yields of these two products were about 43% and 8% for peak #1 and peak #2, respectively. The additional TAT peptide in the conjugate with 1:2 doxorubicin/TAT ratio seemed to be from the direct reaction between the SH group of TAT peptide and the aromatic or sugar rings of doxorubicin. Since TAT peptide linked to aromatic ring or any other position of sugar ring might cause anticancer activity drop of doxorubicin,[8] only desired doxorubicin–TAT conjugate (doxorubicin/TAT = 1:1, Fig. 1) was characterized and used for further studies.

First, both the cell permeability and intracellular distribution pattern of doxorubicin–TAT conjugate were compared with those of free doxorubicin. MCF-7 breast cancer cells were incubated with 5.0 μM free doxorubicin or doxorubicin–TAT for 30 min. After washing with an acidic solution (28 mM NaAC, 120 mM NaCl, and 20 mM barbital, pH 2.5) to remove cell membrane-associated doxorubicin–TAT conjugate, cells were examined by fluorescence microscopy for doxorubicin uptake and intracellular distribution.[9] As shown in Figure 3, although both free doxorubicin and doxorubicin–TAT conjugate were permeable to MCF-7 cells, different intracellular distribution profiles were observed for these two compounds. Free doxorubicin accumulated at nuclei while most of doxorubicin–TAT conjugate located

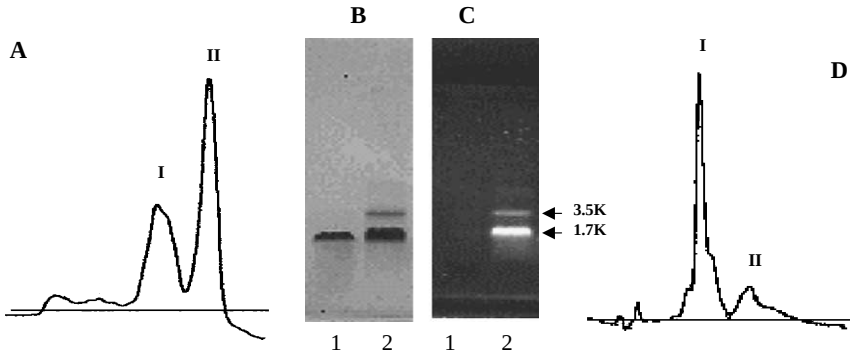

Figure 2. Purification and characterization of doxorubicin–TAT conjugates by heparin affinity FPLC (A), SDS–polyacrylamide gel electrophoresis (B and C), and C_8 reverse-phase HPLC (D). Lanes 1 and 2 in SDS–PAGE are for free TAT peptide and conjugation mixture (peak #1 in A). (B and C) Electrophoresis images obtained under white and ultraviolet light, respectively. Please refer to Ref. 7 for detailed experimental conditions.

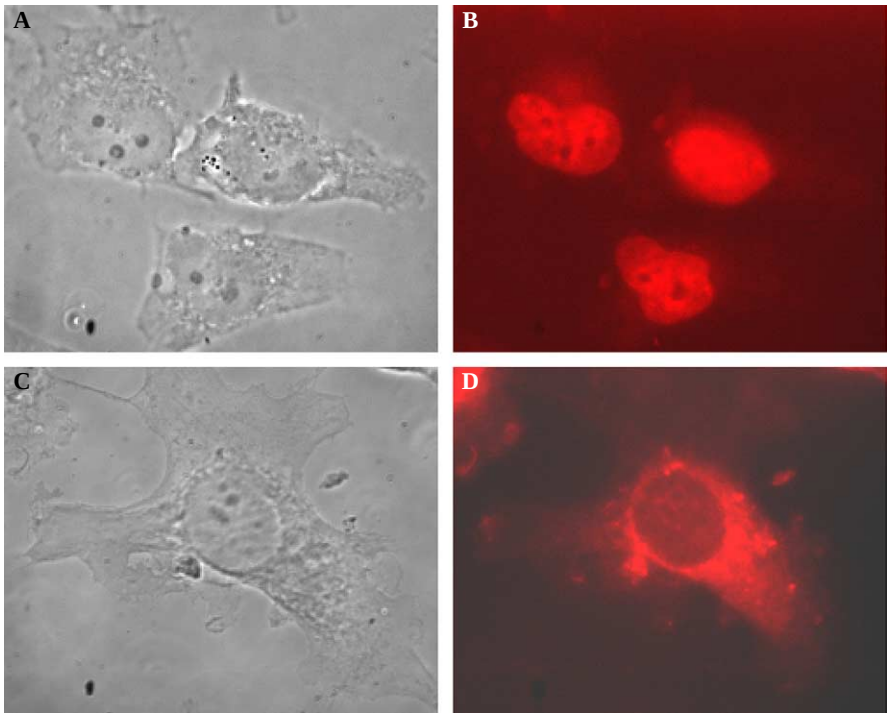

Figure 3. MCF-7 cells grown on chamber slides (Nalge Nunc, Naperville, USA). After incubation with drugs for 30 min, cells were washed with HBSS followed by cold acidic solution (28 mM CH_3COONa, 120 mM NaCl, and 20 mM barbital–HCl, pH 3.0) to remove all cell surface-bound drugs. Cells were fixed with PBS-buffered (pH 7) 4% formaldehyde/1.5% methanol solution. Cell observation was done with an inverted Zeiss laser scanning microscope (LSM410; Carl Zeiss, Jena, Germany). Maximum excitation was performed by a 488-nm line of internal argon laser, and fluorescence emission was observed at 590 nm. Images (A and B) are from doxorubicin treated cells, and images (C and D) are from doxorubicin–TAT conjugate treated cells. (A and C) Contrast and (B and D) fluorescent images, respectively.

in either perinuclear region or cytoplasm (Fig. 3D). Obviously, linked TAT peptide changed the intracellular distribution pattern of doxorubicin in doxorubicin–TAT conjugate.

Next, we compared tumor killing ability of doxorubicin–TAT conjugate with that of free doxorubicin. Cells were seeded in 96-well plates at a density of 10^3 cells per well. After incubation with various concentrations of doxorubicin–TAT or free doxorubicin for 48 h, cell viability was assessed by the MTT assay.[9] Fluorescence intensity measurement of doxorubicin (λ_{ex} = 470 nm; λ_{em} = 590 nm) in both doxorubicin–TAT conjugate and free doxorubicin solutions was used to determine and adjust drug concentrations. Cytotoxicity assay was first performed on drug-sensitive cells, MCF-7. As shown in Figure 4A, both doxorubicin–TAT conjugate and free doxorubicin showed dose-dependent growth inhibitory effects on MCF-7 cells. Interestingly, even though doxorubicin–TAT had different intracellular distribution pattern from that of free doxorubicin (Fig. 3D, Table 1), it showed the same MCF-7 killing ability as free doxorubicin (Fig. 4A, Table 1). Recent research shows that in addition to inhibiting DNA synthesis in nuclei, doxorubicin can also kill cells by interacting with cytoplasm components or apparatus and cause cell

Table 1. Cell permeability and killing activity of doxorubicin–TAT conjugate

	Intracellular concentration (%)		IC_{50} (μM)			
	Free Dox	Dox–TAT	Free Dox	Dox–TAT	Verapamil (10 μM)	
					Free Dox	Dox–TAT
MCF7	92.3	84.6	0.25	0.28	ND	ND
MCF7/ADR	4.5	58.4	45.2	5.8	6.7	5.2
AT3B-1	7.9	49.3	167.5	23.4	42.4	23.8

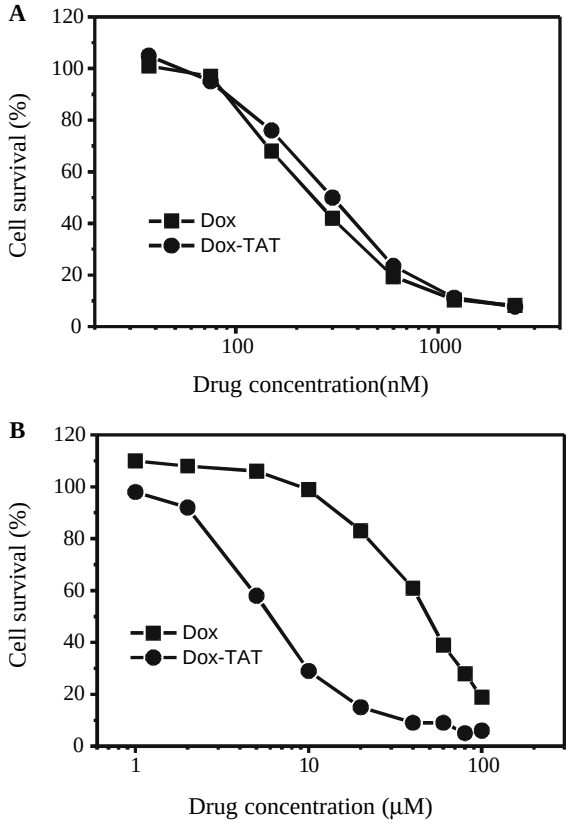

Figure 4. Cytotoxicity of free doxorubicin (Dox) and doxorubicin–TAT (Dox–TAT) to drug-sensitive (A) and -resistant (B) MCF-7 cells. Cells were seeded in 96-well plates at a density of 10^3 cells/well. Twenty-four hours later, cells were continuously exposed to free doxorubicin, doxorubicin–TAT for 48 h. Cell viability was then assessed by the MTT assay.

apoptosis.[10] Therefore, multiple intracellular acting sites of doxorubicin should be the reason why doxorubicin–TAT conjugate and free doxorubicin had the same cell killing activity (Table 1), even though they showed total different intracellular distribution profiles (Fig. 3).

Cell killing activities of doxorubicin–TAT conjugate and free doxorubicin were then tested on drug-resistant MCF-7 cells (MCF-7/ADR). Unlike on drug-sensitive cells, doxorubicin–TAT conjugate was much more potent than free doxorubicin in killing drug-resistant cells (Fig. 4B). The IC_{50} of doxorubicin–TAT conjugate was 8–10 times less than that of free doxorubicin (Table 1). The same result was also observed on another drug-resistant cell line, AT3B-1. Obviously, linked TAT peptide greatly enhanced the drug-resistant cell killing ability of doxorubicin.

To clarify drug-resistant cell killing mechanism of doxorubicin–TAT conjugate, intracellular doxorubicin concentrations in doxorubicin–TAT conjugate and free doxorubicin treated cells were compared. Intracellular doxorubicin was extracted by incubating cells with extraction solution (isopropyl alcohol solution containing 0.075 N HCl) at 4 °C overnight. Doxorubicin concentrations in extractives were determined using spectrofluorimeter by setting the excitation and emission wavelengths at 470 and 590 nm, respectively. As shown in Table 1, although nearly 90% of free doxorubicin could get into drug-sensitive cells, this number dropped to about 5% in drug-resistant cells, suggesting that doxorubicin lost its cell permeability because of the presence of the P-glycoprotein efflux pump in drug-resistant cell membrane. On the contrary, doxorubicin–TAT conjugate was insensitive to P-glycoprotein efflux pump, and doxorubicin–TAT conjugate was still highly permeable (58.6%) to drug-resistant cells. Since only doxorubicin–TAT but not a simple mixture of doxorubicin and TAT peptide killed drug-resistant cell efficiently (data not shown), TAT peptide inhibition of P-glycoprotein activity could not be the reason for the drug resistance overcoming ability of doxorubicin–TAT conjugate. In addition, P-glycoprotein inhibitor (verapamil) could partially resume free doxorubicin' toxicity to drug-resistant cells but it did not affect the overall cell killing ability of doxorubicin–TAT conjugate (Table 1). Therefore, it seemed that doxorubicin–TAT conjugate overcame drug resistance through a 'bypass' but not P-glycoprotein inhibition mechanism.

In conclusion, this study demonstrates that cell penetrating peptide-linked doxorubicin can bypass P-glycoprotein efflux pump and is able to kill drug-resistant cells. This finding shed the new light for more potent anticancer drug development.

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