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# Tumor homing cell penetrating peptide decorated nanoparticles used for enhancing tumor targeting delivery and therapy

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ABSTRACT

Specific targeting ability and good tissue penetration are two critical requirements for tumor targeted delivery systems. Systematical selected peptides from a library may meet these two requirements. RLW was such a cell penetrating peptide that could specifically target to non-small cell lung cancer cells (A549). In this study, RLW was linked onto nanoparticles (RNPs) and then the RNPs were used for lung cancer targeting delivery. A traditional cell penetrating peptide, R8 (RRRRRRRR), was used as control. *In vitro* cellular uptake study demonstrated that modification with RLW specifically enhanced the uptake by A549 cells rather than human umbilical vein endothelial cells, while modification with R8 increased the uptake by both cells. Furthermore, the modification with RLW specifically elevated the penetration into A549 tumor spheroids rather than glioma cell (U87, used as *in vivo* control) spheroids. And the *in vivo* imaging further demonstrated RNPs could target to A549 xenografts rather than U87 xenografts. Importantly, the distribution of RNPs in normal organs was approximately the same as that of unmodified nanoparticles. However, R8 modified nanoparticles elevated the distribution in almost all the tissues. These results demonstrated that RLW was superior in A549 tumor targeted delivery. After loaded with docetaxel, an anti-microtube agent, different formulations could effectively induce the A549 cell apoptosis, and inhibit the growth of A549 spheroids *in vitro*. While *in vivo*, RNPs displayed the best antitumor effect. The tumor volume was significantly lower than other groups, which was only 33.3% as that of saline group. In conclusion, *in vitro* RLW could specifically target to A549 cells and enhance the cytotoxicity of docetaxel. *In vivo*, RLW could significantly enhance the A549 xenografts targeting delivery and led to improved antitumor effect.



## 1. Introduction

Nanoparticles (NPs) are widely used as drug delivery systems to improve specific tissue/organ/cell targeting (Bae and Park, 2011; Davis et al., 2008; Peer et al., 2007). To further enhance the targeting effect, ligands were modified onto the surface of NPs. Recently, several ligand-modified nanomedicines were under evaluation in clinic (Allen, 2002; Florence, 2012; van der Meel et al., 2013; Yu et al., 2013). Generally, ligands can be classified into two types. One kind of ligands are characterized by specific targeting effect with poor penetration effect, which could be recognized by specific receptors, carriers, *etc.* (Bae and Park, 2011; Guo et al., 2012; Liu et al., 2013; Yu et al., 2013). The other kind of ligands could interact with cell membranes without selectivity, for example, cell penetrating peptides (CPPs) (Foged and Nielsen, 2008; Wender et al., 2000; Wu et al., 2012). CPPs were first discovered in 1988 from the HIV and gained much attention because of their ability of facilitating cargoes across cell membranes (Bechara and Sagan, 2013; Fonseca et al., 2009). However, the poor selectivity of CPPs hampered their application in systemic delivering drugs into specific tissues and led to increased risk of drug originating side effects (Koren and Torchilin, 2012).

To overcome this problem, several strategies were developed. Anchoring CPPs with cell-selective residue and enabling CPPs with environment-responsive ability were two successful methods (Alberici et al., 2013; Gu et al., 2013; Jiang et al., 2004; Zhang et al., 2013). Additionally, systematic selection from a library is a promising direction for finding CPPs with specific cell targeting effect. Nowadays, researchers developed a sequence of peptides that possessed both cell penetrating property and specific cell

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selectivity using mRNA display technology (Kondo et al., 2012). RLW (with a sequence of RLWMRWYSPRTRAYG), which could specifically target to A549 cells, is one of those peptides (Kondo et al., 2012). However, the application of this kind of peptides has not been evaluated, and the potential superiority over traditional CPPs needed to be further explored.

With an incidence of 1.5 million new cases every year, lung carcinoma is one of the most common cancers in the world (Le Chevalier, 2011; Lopez-Gonzalez et al., 2012). Non-small cell lung carcinoma (NSCLC) accounts for approximately 85% of all the lung carcinoma. Although, surgery remains to be the most successful treatment strategy for early detected patients, 90% would recur in the following 5 years after surgery (Biswas et al., 2013; Lopez-Gonzalez et al., 2012). Chemotherapy is also a common strategy to manage the NSCLC. However, the limited drug concentration in tumor is still an unsolved problem in chemotherapy owing to the whole body distribution of drugs (Brannon-Peppas and Blanchette, 2004). Targeted delivery systems may circumvent the above mentioned problems and improve the antitumor effect (Bae and Park, 2011).

In this study, RLW was anchored onto poly(ethyleneglycol)-poly (ε-caprolactone) (PEG-PCL) nanoparticles for lung cancer targeting delivery and therapy. R8 (RRRRRRRR), one of the most famous and widely used traditional CPPs (Futaki et al., 2001; Nakamura et al., 2013), was used for comparison. Docetaxel (DTX), a taxane derivative, was an inhibitor of microtubule depolymerization that widely used for treatment of many kinds of tumors (Gao et al., 2012a). Coumarin-6 was a dye with high fluorescent efficiency that widely used in *in vitro* and *in vivo* tracking of particles. DiR is a near infrared dye with excitation/emission wavelengths of 748/780 nm, thus it was commonly used for *in vivo* imaging. Therefore, in this study, DTX was used as a model drug and coumarin-6 and DiR were used for tracking the behavior of particles.

## 2. Materials and methods

### 2.1. Materials

DTX was purchased from Knowshine (Shanghai, China). Methoxyl PEG–PCL (MPEG–PCL) (Mw: 3–15 k) and carboxyl PEG–PCL (HOOC–PEG–PCL) (Mw: 3.4–15 k) were synthesized as previously described (Gao et al., 2012b). RLW and R8 were synthesized by Sangon Biotech (Shanghai, China). *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxy-succinimide (NHS), 2-(*N*-morpholino) ethanesulfonic acid (MES), and coumarin-6 were purchased from Sigma (Saint Louis, MO, USA). The near infrared dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine Iodide (DiR), was purchased from Biotium (Hayward, CA). DAPI, Hoechst 33, 342, and Annexin V-FITC apoptosis detection kit were purchased from Beyotime (Haimen, China). Hitrap desalting column was purchased from GE Healthcare Biosciences (Uppsala, Sweden). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Co., Ltd., (Wuxi, China). The HUVEC, A549 and U87 cell line were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle Medium (high glucose) cell culture medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY, USA). All other chemicals were of analytical reagent grade and were purchased from Sinopharm Chemical Reagent (Shanghai, China).

BALB/c nude mice (male, 4–5 weeks, 18–22 g) were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China) and maintained under standard housing conditions. All animal experiments were carried out in accordance with protocols evaluated and approved by the ethics committee of Sichuan University.

### 2.2. Preparation and characterization of NPs

The PEG–PCL NPs were prepared by an emulsion/solvent evaporation method described previously (Gao et al., 2011). Briefly, 1 mL of dichloromethane containing 28 mg of MPEG–PCL and 2 mg of HOOC–PEG–PCL was added into 5 mL 0.6% sodium cholate hydrate solution. After pulse sonication for 75 s at 200 W on ice using a probe sonicator (Scientz Biotechnology Co., Ltd., China), the emulsion was applied to dichloromethane removal using a rotary evaporator and the NPs were condensed to a fixed concentration by ultrafiltration at 4000 g.

For the RLW and R8 conjugation, the carboxyl units of NPs were activated by EDC and NHS in a pH 6.0 MES buffer for 0.5 h. The MES buffer was then replaced by phosphate-buffered saline (PBS) (pH 7.4) using a Hitrap™ desalting column, and 50 μg of RLW or 25 μg of R8 in 1 mL of PBS (pH 7.4) was added into the NP suspension and stirred for 4 h in the dark. The product was then subjected to ultrafiltration to remove the unconjugated peptide, and the RLW modified NPs (RNPs), and R8 modified NPs (TNPs) were collected. DTX, coumarin-6- and DiR-loaded NPs were prepared using the same procedure except that the materials were dissolved in 1 mL of dichloromethane, which contained 1 mg of DTX, 30 μg of coumarin-6, or 600 μg of DiR.

The particle sizes and zeta potentials were determined by dynamic light scattering using a Malvern zeta sizer (Malvern, NanoZS, UK). The morphology of particles was examined by transmission electron microscope (TEM, Hitachi, H-600, Japan) after staining with 2% (w/v) phosphotungstic acid solution.

To characterize the release profile, 1 mL of DTX–RNPs or DTX–TNPs in PBS (pH 7.4) or 10% plasma was added into a dialysis bag and then the bag was immersed in 9 mL of corresponding release medium. At pre-set time points, 50 μL of medium was sampled and an equal volume of fresh medium was appended. The concentration of DTX was determined by high performance liquid chromatography (Agilent, 1200, USA) which consisted of a quantum pump, an on line degasser, and a variable wavelength detector. The conditions were as follows: diamond C18 column (150 mm × 4.6 mm, pore size 5 μm), a mobile phase of $CH_3CN$–$H_2O$ (1:1, v/v), a flow rate of 1.0 mL/min, and a measured wavelength of 230 nm. The methodology has been well evaluated by us previously (Gao et al., 2012a).

To evaluate the stability in FBS, RNPs, and TNPs were suspended in PBS (pH 7.4) supplemented with FBS (0% and 50%). Then, 0.2 mg/mL RNPs and TNPs were incubated in a 37 °C shaker. The absorbance of the RNPs and TNPs at 570 nm was measured at pre-set time points.

### 2.3. Cellular uptake

HUVEC and A549 cells in the logarithmic growth phase were seeded in 24-well plates at a density of $1 \times 10^4$ cells/mL. Twenty four hours later, different concentrations of coumarin-6-loaded NPs, RNPs, or TNPs were added into the wells and incubated for different periods of time. The adsorptive and free particles were removed by washing with ice-cold PBS. Fluorescent intensity of cells was directly observed by fluorescent microscope (Leica, Germany). To quantitatively determine the fluorescence intensity, the cells were digested, and detected by a FACS Aria cell sorter (BD, USA).

### 2.4. Tumor spheroid penetration

A549 and U87 (a human glioma cell line) three-dimensional spheroids were established as previously described (Gao et al.,