International Journal of Pharmaceutics 434 (2012) 209–214

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Rapid communication

Self-assembled peptide (CADY-1) improved the clinical application of doxorubicin

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ARTICLE INFO

Article history:
Received 10 April 2012
Received in revised form 13 May 2012
Accepted 1 June 2012
Available online 9 June 2012

Keywords:
Self-assembled peptide
Doxorubicin
Cell penetrating peptide
Blood residence time

ABSTRACT

CADY-1 is an amphipathic peptide that possesses cell-penetrating activity. As an amphipathic peptide, CADY-1 is capable of forming complexes by self-assembly, and they are these complexes that possess cell-penetrating activity. This distinct characteristic of CADY-1 makes it a potent cell-penetrating drug delivery system. Doxorubicin is a widely used cytotoxic anti-cancer drug but is limited by its toxicity. Although the liposomal formulation of doxorubicin ameliorates its toxicity, its complicated synthesis remains an obstacle to its wide clinical use. In this study, our findings revealed that CADY-1 and doxorubicin form a stable complex at optimised molar ratios in a self-assembling manner. Formation of the complex extended the blood residence time of doxorubicin in a similar fashion to that of liposomal doxorubicin. In addition, the complex was capable of carrying doxorubicin across the cell membrane, which increased the therapeutic index of doxorubicin. Experimental animals treated with a CADY-1/doxorubicin complex exhibited better tolerance and anti-tumour activity than animals treated with either liposomal doxorubicin or the free form of doxorubicin. Collectively, the findings in this study support the advantages of using complexes formed by the self-assembled peptide CADY-1 and suggest that CADY-1 is a potent drug delivery system.

1. Introduction

Doxorubicin is a cancer chemotherapy drug used in wide range of cancers such as breast cancer, bladder cancer, and leukaemia (Carter, 1975). It is an anthracycline antibiotic, and like all anthracyclines, it interacts with DNA by intercalation and inhibition of macromolecular biosynthesis (Hanada et al., 1998). However, the cardiotoxicity of doxorubicin limits its clinical utility even though it exhibits remarkable clinical efficacy (Yi et al., 2006). Most experimental studies of the cardiotoxicity of doxorubicin support the hypothesis that the doxorubicin causes oxidative stress in the heart and that protection from cell death is afforded by administration of antioxidants (Oliveira et al., 2004). It is believed that oxidative stress results from the generation of oxygen-derived free radicals in the presence of doxorubicin (Oliveira et al., 2004; Spallarossa et al., 2004). Studies revealed that encapsulation of doxorubicin inside liposomes decreases the cardiotoxicity associated with the free form of the drug whilst maintaining anticancer activity (Gabizon et al., 1983; Herman et al., 1983). A variety of liposomal doxorubicin formulations have been investigated to assess their therapeutic value in animal models. Unfortunately, the traditional 'passive entrapment' strategies of liposomal formulation failed to prepare liposomal doxorubicin due to poor retention (Gabizon et al., 2006). A pH gradient system was established in 1989, and this procedure has been extremely useful in the formation of liposomal doxorubicin, which results in the reduction of cardiotoxicity (Mayer et al., 1990). Liposomal doxorubicin was approved by the FDA (US Food and Drug Administration) in 1999 under the trademark name Doxil. Doxil exhibits less toxicity than free doxorubicin, with a two-fold increase in the LD_{50} (50% lethal dose) (17 mg/kg for free doxorubicin and 32 mg/kg for Doxil) (Kanter et al., 1993). In 2008, the FDA provided an alert on the shortage of Doxil, due to the complicated steps required for its synthesis (Cui et al., 2007; Sachdeva, 1998).

Self-assembled peptides have been recognised as a drug carrier system during the last decade (Prongidi-Fix et al., 2007; Reches and Gazit, 2006). Self-assembled peptides contain two domains: a hydrophilic domain which interacts with the negatively charged domain of drug and a hydrophobic domain (Wang et al., 2011; Zheng et al., 2011a,b). Self-assembled peptides are capable of forming a stable complex with the drug inside (Fernandez-Carneado et al., 2004). The hydrophilic domain is

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http://dx.doi.org/10.1016/j.ijpharm.2012.06.003

located on surface of this supermolecular complex, which results in its tendency to interact with the cell membrane (Hudecz et al., 2005; Zheng et al., 2011a,b). As a self-assembled peptide, CADY-1 was also reported to be a cell-penetrating peptide (CPP), but the mechanism of its cell-penetrating activity remains unclear (Crombez et al., 2008, 2009; Konate et al., 2010). In order to improve anticancer activity and reduce systemic toxicity of doxorubicin, several drug carrier were employed, such as PEG-b-PLA polymeric micelles (Batrakova, 2011), monomethoxy poly(ethylene glycol)–poly(epsilon-caprolactone) (MPEG–PCL) (Gou et al., 2011), poly(epsilon-caprolactone)–poly(ethylene glycol)–poly(epsilon-caprolactone) (PCL–PEG–PCL, PCEC) (Gou et al., 2009). Self-assembled peptides are attracting increasing attention as drug delivery agents, as the technologies for peptide development and manufacture continue to mature. Concurrently, with booming research in nanotechnology for biomedical applications, peptides have been studied as an important class of components in nanomedicine (Galdiero et al., 2012), and they have been used either alone or in combination with nanomaterials of every reported composition. Peptides possess many advantages, such as smallness, ease of synthesis and modification, and good biocompatibility.

The aim of this project was to investigate the potential utility of CADY-1 as a drug carrier, which could contribute to the improvement of selected drugs in clinical use. In this study, CADY-1 was used to create a stable complex containing doxorubicin. The physiological properties of the doxorubicin/CADY-1 complex were investigated, including effect on cell viability, blood residence time and toxicity in a cell model and in rats.

2. Materials and methods

2.1. Peptide synthesis

The self-assembled peptide CADY-1 (GLWWKAWWKAWWK-SLWWRKRKRKA) was purchased from Sangon Biotech Co. (Shanghai; HPLC-purified; purity > 95%, identified by MS). The freeze-dried peptide was weighed and dissolved in saline to make 10 mg/ml stock solutions for further analysis. Doxorubicin and liposomal doxorubicin (Doxil) were kind gifts from Dr. Wei Liu in Tianjin Medical University General Hospital.

2.2. Analysis of the mixture of doxorubicin and the self-assembled peptide-CADY-1 by HPLC

Doxorubicin and CADY-1 were mixed at various molar ratios and incubated at 4 °C overnight. Varying amounts of CADY-1 were used (100 μg/ml, 500 μg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml), whilst the concentration of doxorubicin remained at 100 μg/ml. The final molar ratios of doxorubicin to CADY-1 were 1:1, 1:5, 1:10 and 1:20, respectively. Mixtures (10 μl) were analysed by a Surveyor HPLC system through a C18 analytical column (Thermal Scientific, USA). The column was eluted at a flow rate of 0.5 ml/min in a gradient mode with a mixture of mobile phase A (H_2O + 20% acetonitrile) and mobile phase B (100% acetonitrile). Mobile phase A was eluted for 10 min, and thereafter mobile phase B was increased from 20% to 100% over a 60 min period. HPLC analyses were performed at ambient temperature and the UV detection wavelength was set at 210 nm. Ten microlitre aliquots of doxorubicin stock solution (100 μg/ml) and CADY-1 stock solution (10 mg/ml) were injected into the C18 column of the HPLC as the controls.

2.3. MTT assay

The cytotoxicity of free doxorubicin, liposomal doxorubicin and the complex of CADY-1 and doxorubicin were determined by the MTT assay. The Hela or MCF7 cells (1×10^5 cells/100 μl/well) were cultured in 96-well plates at 37 °C and 5% CO_2. Aqueous solutions of free doxorubicin or the complex of doxorubicin and CADY-1 (molar ratio at 1:10) were dissolved in culture medium at final concentrations of 0.1, 0.5, 1, 5, 10 and 20 μM. After an incubation time of 4 h, the MTT solution (2 mg/ml PBS) was added, the plates were incubated for 4 h, and the cells were lysed with 50% N,N-dimethylformamide containing 20% SDS, pH 4.5. The absorbance at 570 nm was measured for each well by the SpectraMax M5 instrument (Molecular Devices, CA). The absorbance of control cells was taken as 100% viability, and the values of the treated cells were calculated as a percentage of control.

2.4. HPLC–MS measurement

Three ThermoFinnigan quadrupole mass spectrometers (TSQ7000) with XCALIBUR and LCQUAN software were equipped with an electrospray ion source and a divert valve. The chromatographic system consisted of an HPLC pump (model 616) and Controller 616 S from Waters (Milford, USA) and an autosampler Series 200 from Perkin-Elmer (Norwalk, USA). The Oasis HLB chromatography column (30 mm particle size, 1 mm × 50 mm) from Waters was used. Electrospray-ionisation was performed in the positive ion mode. The heated capillary was set at 245 °C and the spray voltage at 4.5 kV. Nitrogen was used as the sheath and auxiliary gas and set at 90 lb/square inch and 25 arbitrary units, respectively. The argon collision gas pressure was set to 2.5 mTorr. The plasma samples or cell lysis solutions (50 μl) were chromatographed at ambient temperature on the Oasis HLB column. Solvent A was acetonitrile and solvent B was 5 mM sodium trifluoroacetate in 0.1% aqueous formic acid. The analyses were chromatographed with a 0.8 ml/min flow rate with the following linear gradient: 0 min, 5% A; 0.5 min, 5% A; 5.5 min, 82% A. The flow was diverted to the mass spectrometer for quantitative analysis.

2.5. Blood residence time measurement

Female ICR mice (6–8 weeks old) were obtained from Shanghai Laboratory Animal Co., China Academy of Sciences (Shanghai, China). Groups of four mice per experimental point were given the specified treatment as a single intravenous dose via the lateral tail vein. Free doxorubicin, liposomal doxorubicin or the mixture of doxorubicin and CADY-1 (molar ratio at 1:10) were administrated at a dose of 10 mg/kg body weight. Blood samples collected from the carotid artery (100 μl) were placed in an EDTA-treated centrifuge tube and centrifuged (200 × *g*) for 10 min. The blood retention profiles of free doxorubicin, liposomal doxorubicin and the complex of CADY-1 and doxorubicin were determined by HPLC–MS. During this study, triton (1%) was added to the plasma sample to disassociate the doxorubicin from the liposome and complex.

2.6. Cell penetrating activity determination using HPLC–MS assay

The HeLa cells were lysed with a cell lysis buffer from Cell Signal Technology, Inc. (Danvers, USA) following incubation with doxorubicin, liposomal doxorubicin or the complex of CADY-1 and doxorubicin (10 μM/100 μl/well) for 1 h. Cell lysis solutions (50 μl) were separated by centrifugation (1500 × *g*) at 4 °C and analysed by HPLC–MS. RPMI 1640 medium containing 10 μM doxorubicin was analysed by HPLC–MS as control. The intracellular doxorubicin concentration from cells treated with either liposomal doxorubicin or

the complex of CADY-1 and doxorubicin were normalised against the control sample.

2.7. Median lethal dose (LD_{50}) of the CADY-1/doxorubicin complex

To investigate the acute toxicity of the CADY-1/doxorubicin complex, we determined the median lethal dose (LD_{50}) by the Litchfield–Wilcoxon method. Male ICR mice (weighing 22–25 g) were divided into six groups ($n = 5$/per group). Experimental animals were injected intravenously with free doxorubicin, liposomal doxorubicin or the doxorubicin/CADY-1 complex at various doses from 2 to 200 mg/kg. The lethality of the mice was observed 4 days after administration.

2.8. Treatment of human tumour xenograft mice

Female DBA/2 mice (weighing 16–20 g) were housed in barrier facilities on a 12 h light/dark cycle. Food and water were supplied ad libitum. On day zero, five groups of mice were inoculated via i.p. injection with L1210 tumour cells (2.5×10^6) in 0.5 ml of RPMI1640. Treatment was initiated 1 day after injection of tumour cells and was administrated as a single i.v. dose via the lateral tail vein. The animals were treated with liposomal doxorubicin or the CADY-1/doxorubicin complex at the specified dose (20 mg/kg body

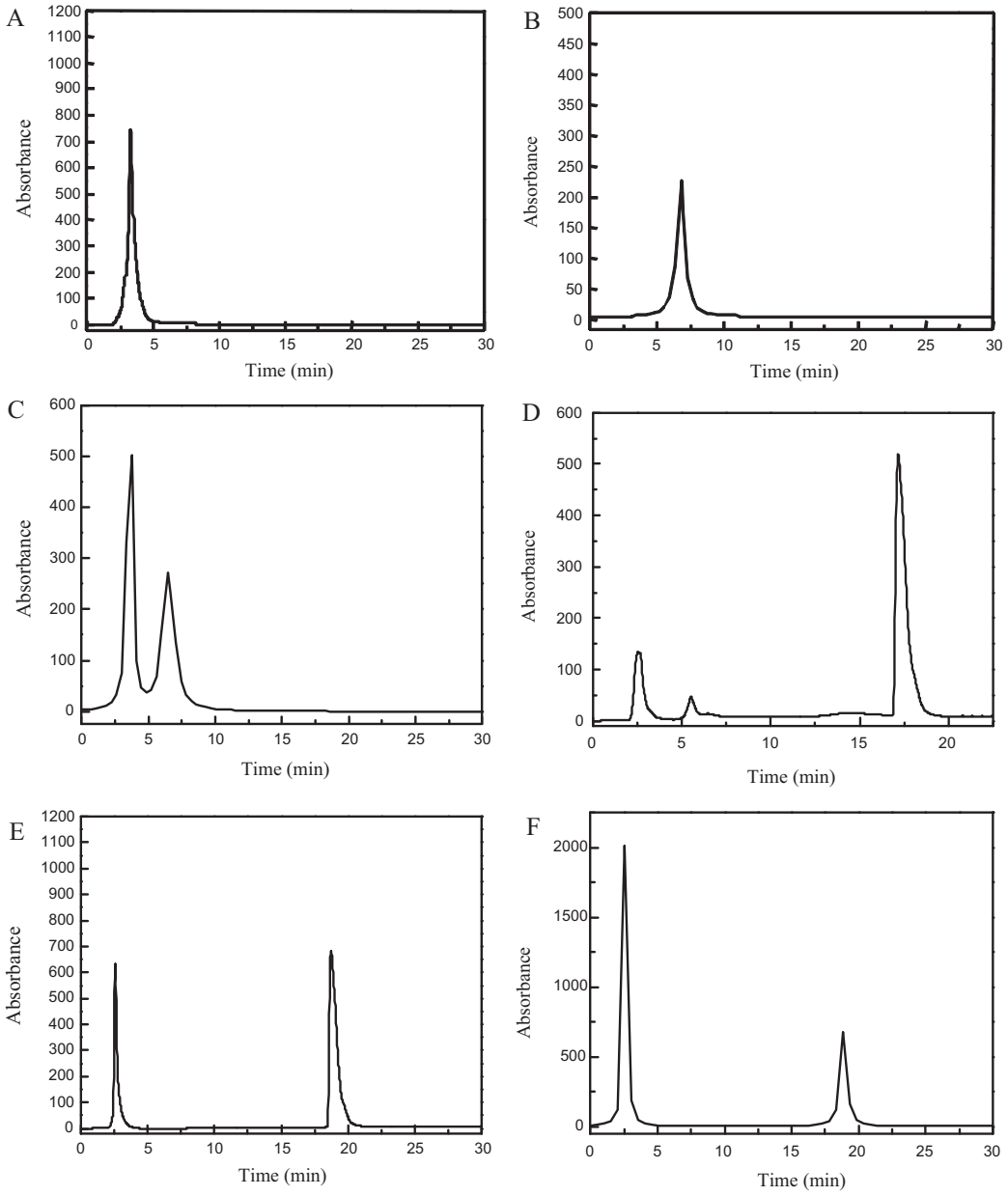

Fig. 1. HPLC comparison of CADY-1, doxorubicin and the CADY-1/doxorubicin mixture at different ratios. Panel A: HPLC spectrum of CADY-1. Panel B: HPLC spectrum of doxorubicin. Panel C: HPLC spectrum of the mixture of CADY-1 and doxorubicin at a ratio of 1:1. Panel D: HPLC spectrum of the mixture of CADY-1 and doxorubicin at a ratio of 1:5. Panel E: HPLC spectrum of the mixture of CADY-1 and doxorubicin at a ratio of 1:10. Panel F: HPLC spectrum of the mixture of CADY-1 and doxorubicin at a ratio of 1:20. *Conditions*: samples were injected into the C18 column of the HPLC at 0.5 ml/min in a solution containing 40% acetonitrile and 0.1% trifluoroacetic acid. The UV detector was set at 222 nm. The retention times of purified CADY-1 and doxorubicin were 3.7 min (A) and 6.4 min (B), respectively. A peak, which had a retention time of 18.1 min, was visible with an increasing ratio of CADY-1 to doxorubicin (D–F). At 1:10 (GLP-1:CADY-1) and 1:20 (GLP-1:CADY-1), the complete disappearance of the doxorubicin peak and appearance of a peak at 18.1 min indicated doxorubicin might interact with CADY-1 and form a stable complex (E and F).

weight). Control groups were treated with CADY-1. Survival time was recorded in days after tumour injection. The mean and median survival time and the statistical significance of the results were determined employing a two-tailed Wilcoxon's ranking test. All data obtained for repeated experiments were pooled and utilised for statistical analysis.

2.9. Statistics

Statistical significance was evaluated by using the two-tailed Student's *t*-test.

3. Results

3.1. Self-assembled supermolecular formulation of CADY-1 and doxorubicin

In this study, the formation of CADY-1/doxorubicin complex was analyzed by RP-HPLC. The complex of CADY-1/doxorubicin exhibited a distinct characterization, compared with either doxorubicin or CADY-1. Fig. 1A shows that purified CADY-1 has a retention time of 3.7 min, and doxorubicin possesses a retention time of 6.4 min (Fig. 1B). When CADY-1 and doxorubicin were mixed at a ratio of 1:1, two peaks remained at 3.2 min and 6.7 min; no extra peak was detected in the spectrum (Fig. 1C). At a ratio of 1:5 (doxorubicin:CADY-1), the peaks of both CADY-1 and doxorubicin almost disappeared, and a new peak with a retention time of 18.1 min was visible (Fig. 1D). The peak of doxorubicin completely disappeared at the ratios of 1:10 and 1:20, suggesting that all the doxorubicin had interacted with CADY-1, as shown in Fig. 1E and F. The HPLC results suggested that the peak with a retention time of 18.1 min might be the CADY-1/doxorubicin complex.

3.2. The cell-penetrating ability of the CADY-1/doxorubicin complex improved the anti-tumour effect

In this study, the anti-tumour activity of CADY-1/doxorubicin was determined by the MTT assay. Compared to free doxorubicin, the CADY-1/doxorubicin complex exhibited remarkable cell death activity. The MTT assay revealed that the cells treated with 0.5 mM of the CADY-1/doxorubicin complex showed 48% cell viability at 4 h, as shown in Fig. 2. However, free doxorubicin, only at a concentration of at least 10 mM, produced the same effect. HPLC–MS was employed to investigate the intracellular concentration of doxorubicin disassociated from the complex. It was presumed that the increased cell killing activity resulted from an enhanced efficiency of cell membrane penetration. The HPLC–MS results indicated that the intracellular concentration of doxorubicin was increased when in association with the complex. Fig. 3 indicates that the intracellular concentration of doxorubicin was remarkably increased when compared to free doxorubicin and liposomal doxorubicin. Compared to control, approximately 63% of the doxorubicin was detected inside the cells treated with the complex for 1 h; however, only 25% and 18% of the doxorubicin was detected in the cells treated with free doxorubicin or liposomal doxorubicin, respectively.

3.3. Blood residence time of the CADY-1/doxorubicin complex

For drug carriers like liposomes, the enhancement of drug stability in the blood is an important evaluation criterion. The blood residence time was also investigated to determine whether the formation of a complex with CADY-1 contributes to an extended blood residence time for doxorubicin. The results from animals treated with the complex showed a similar blood residence time (72 h) compared to liposomal doxorubicin (72 h), as seen in Fig. 4.

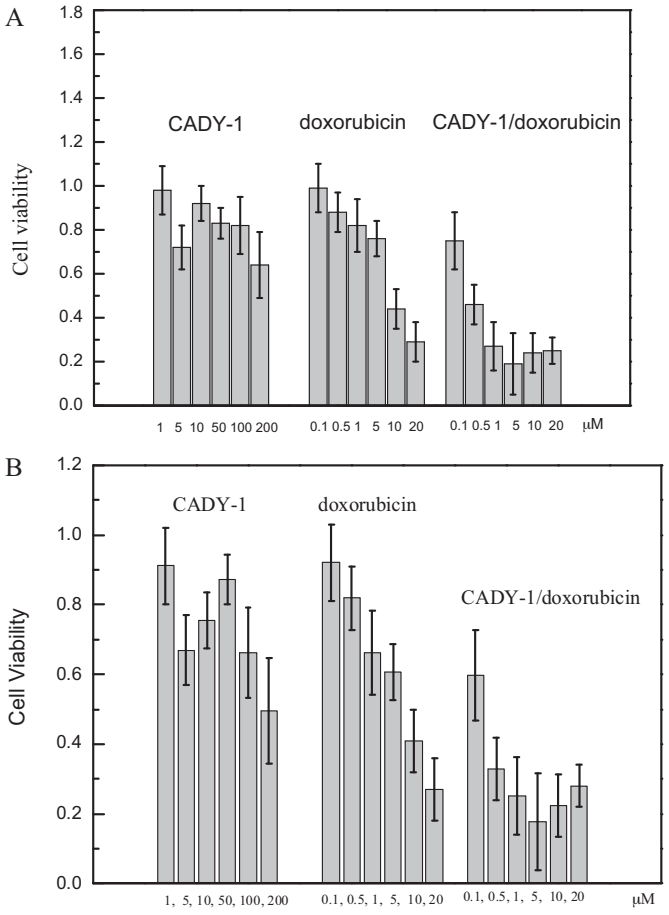

Fig. 2. Effects of various concentrations of the CADY-1/doxorubicin complex on cell viability. Panel A: effects of CADY-1, doxorubicin and the CADY-1/doxorubicin complex on HeLa cell viability. Panel B: effects of CADY-1, doxorubicin and the CADY-1/doxorubicin complex on MCF7 cell viability. *Condition*: the cells (1×10^5 cells/100 μl/well) were cultured in a 96-well plate at 37 °C and 5% CO_2. Subsequently, the cells were incubated with free doxorubicin or the doxorubicin/CADY-1 complex (at a molar ratio of 1:10) at final concentrations of 0.1, 0.5, 1, 5, 10 and 20 μM. After 4 h, the MTT assay was performed, and data were collected by the SpectraMax M5 (Molecular Devices, CA). The absorbance of control cells was taken as 100% viability, and the values of the treated cells were calculated as a percentage of control. Compared to the free doxorubicin, the CADY-1/doxorubicin complex exhibited remarkable cell death activity. The MTT assay revealed that cells treated with 0.5 mM of the CADY-1/doxorubicin complex showed a 48% cell viability within 4 h. However, the cells were treated with free doxorubicin at a concentration of at least 10 mM before reaching this level of viability.

Compared to the blood residence time of free doxorubicin (12 h), forming a complex with CADY-1 resulted in an extended blood residence time for doxorubicin, similar to that of liposomal doxorubicin. Considering its cell membrane penetrating activity and its improvement of drug stability, CADY-1 can be considered an outstanding drug carrier.

The toxicities of free doxorubicin, liposomal doxorubicin and the complex containing doxorubicin and CADY-1 were determined by 14-day dose–response survival studies in male ICR mice. As shown in Table 1, an LD_{50} of 17 mg/kg was observed in animals treated with free doxorubicin, which is comparable to that obtained in previous studies (Kanter et al., 1993). This value was increased to 30 mg/kg when administered with the CADY-1/doxorubicin complex, which was similar to the LD_{50} of liposomal doxorubicin at 32 mg/kg.

Mice, bearing Hela tumour cells, were administered free doxorubicin, liposomal doxorubicin or the doxorubicin/CADY-1 complex, using an intermittent schedule (days 4, 6, 8, 10). The results

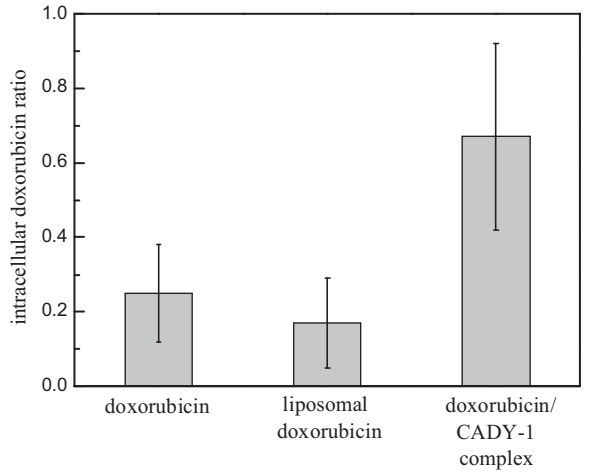

Fig. 3. Cell penetrating activity of the CADY-1/doxorubicin complex. *Condition*: HeLa cells were incubated with free doxorubicin, liposomal doxorubicin or CADY-1/doxorubicin (10 μM) for 1 h. The cells were lysed and analysed by HPLC–MS after centrifugation (1500 × *g*). RPMI 1640 medium containing free doxorubicin (10 μM) was analysed as a control. The detected amounts of doxorubicin were normalised to control. The level of doxorubicin recovered in the plasma of mice treated with free doxorubicin (open circles), CADY-1/doxorubicin complex (solid circles) or liposomal doxorubicin (solid squares). Doxorubicin was determined by HPLC–MS. The results indicated that the intracellular concentration of doxorubicin released from the complex was significantly increased compared to free doxorubicin or liposomal doxorubicin, $p < 0.05$.

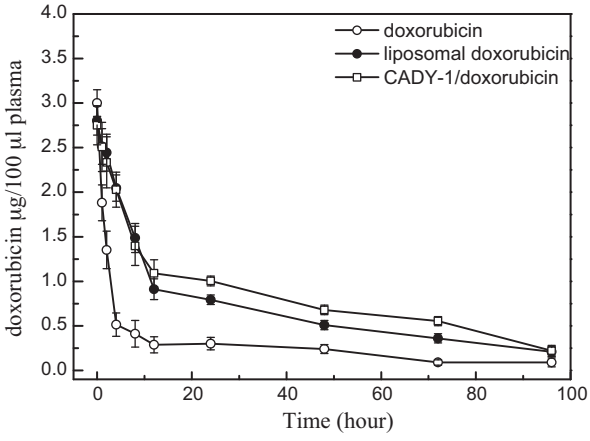

Fig. 4. Extended blood residence time of the CADY-1/doxorubicin complex. *Condition*: female ICR mice were administrated free doxorubicin, liposomal doxorubicin or the CADY-1/doxorubicin complex (10 mg/kg body weight). The plasma samples were collected and analysed by HPLC–MS as indicated in Section 2. Doxorubicin levels obtained from the plasma of mice treated with free doxorubicin (○), liposomal doxorubicin (●) or the CADY-1/doxorubicin complex (□) (10 mg/kg body weight) were determined by HPLC–MS. The results indicated that the formation of the CADY-1/doxorubicin complex protected doxorubicin against degradation and blood clearance. The retention time of the CADY-1/doxorubicin complex remained at a similar level to liposomal doxorubicin. Each point represents the average of four animals, and the error bars indicate the standard deviation, $p < 0.05$ ($n = 4$).

Table 1
Effect of the CADY-1/doxorubicin complex on the toxicity of doxorubicin. *Condition*: male ICR mice (weighing 22–25 g) were divided into six groups ($n = 5$/per group). Experimental animals were injected intravenously with free doxorubicin, liposomal doxorubicin or the doxorubicin/CADY-1 complex at various doses from 2 to 200 mg/kg. The lethal responses were observed for 4 days after administration. An LD_{50} of 17 mg/kg was observed for the animals treated with free doxorubicin. This value was increased to 30 mg/kg when administered with the CADY-1/doxorubicin complex and was similar to the LD_{50} of liposomal doxorubicin at 32 mg/kg.

Preparations	LD_{50} (mg/kg)
Free doxorubicin	17.2 ± 3.6
Liposomal doxorubicin	32.8 ± 5.7
Complex of CADY-1 and doxorubicin	30.3 ± 7.4

revealed that the administration of CADY-1/doxorubicin increased the survival rate of experimental mice.

4. Discussion

Liposomal doxorubicin was developed to avoid the toxicity associated with free doxorubicin and was a successful improvement in the clinical utility of doxorubicin. However, the multiple steps in the synthesis of liposomal doxorubicin are difficult to scale-up, which results in the limitation of liposomal doxorubicin in clinical usage. The FDA has provided an alert on the shortage of liposomal doxorubicin since 2008. In 2005, a PEG-Doxorubicin conjugate was developed as a novel formulation, but it displayed limited efficacy in extending survival time and was not retained long-term in an animal model. The discovery of self-assembled peptides provides a platform for a drug carrier system. Self-assembled peptides possess a self-assembly property which depends on amphipathic domains. In this study, the self-assembled peptide, CADY-1, was employed as a drug carrier system to extend the blood residence time of doxorubicin and increase the intracellular concentration of doxorubicin. An increased ratio between the intracellular concentration of doxorubicin and the total administrated doxorubicin is presumed to enhance the therapeutic index. In this study, HPLC was performed to identify the presence and formulation of complexes containing doxorubicin and CADY-1. The results indicated that CADY-1 is capable of forming a complex with doxorubicin at an optimised ratio of 1:10 (molar ratio of doxorubicin to CADY-1) (Fig. 1). Furthermore, blood residence time measurements were performed in mice and revealed an extended blood residence time of the complex compared to free doxorubicin (Fig. 3). In addition, this assembled complex showed a similar blood residence time to liposomal doxorubicin at approximately 72 h, suggesting that CADY-1 is a drug carrier similar to liposomes. Subsequently, cell viability was measured for the doxorubicin/CADY-1 complex using the MTT assay. The CADY-1/doxorubicin complex showed a remarkable cell death-inducing activity at a very low concentration of 0.5 mM. In HPLC–MS assays, the results showed that the increased intracellular concentration of doxorubicin was associated with increased

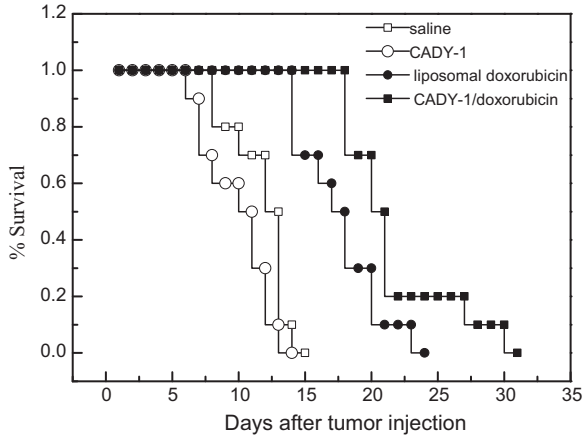

Fig. 5. Anti-tumour effect of the CADY-1/doxorubicin complex in tumour-bearing mice. *Condition*: on day zero, mice were inoculated via i.p. injection of L1210 tumour cells (2.5×10^6) in 0.5 ml of RPMI1640. Treatment was initiated 1 day after injection of tumour cells and was administrated as a single i.v. dose via the lateral tail vein. The animals were treated with liposomal doxorubicin or the CADY-1/doxorubicin complex at the specified dose (20 mg/kg body weight). Control groups were treated with saline or CADY-1. Survival time was recorded in days after tumour injection. All data obtained for repeated experiments were pooled and utilised for statistical analysis. The group treated with the CADY-1/doxorubicin complex (■) exhibited efficient anti-tumour activity as shown. Experimental animals showed an improved survival time compared to liposomal doxorubicin (●). In addition, CADY-1 alone (○) or saline alone (□) did not show any anti-tumour activity, $p < 0.05$ ($n = 5$).

cell viability, as shown in Figs. 2 and 3. In agreement with the MTT and HPLC–MS results, the CADY-1/doxorubicin complex showed an increased LD_{50} of 30 mg/kg in mice compared to 17 mg/kg in mice treated with free doxorubicin, as shown in Table 1. In addition, the CADY-1/doxorubicin complex exhibited efficient anti-tumour properties in tumour-bearing mice (Fig. 5).

In conclusion, the assembled peptide, CADY-1, is capable of forming a complex with doxorubicin. The formation of this complex delayed the degradation of doxorubicin and extended its blood residence time. In addition, the characteristic surface of the assembled complex possesses cell-penetrating activity, which augments the intracellular concentration of doxorubicin. Collectively, these findings suggest that the assembled CADY-1/doxorubicin complex possesses a more efficient anti-tumour activity than free doxorubicin. Compared to liposomal doxorubicin, the synthesis of the assembled peptide is less complicated and easy to scale-up with current technology. Collectively, these results suggest that the assembled peptide CADY-1 has potential as a doxorubicin carrier for further development.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was supported by the sub-project (Grant No. 2010CB735602) of the Major State Basic Research Development Program of China (973 Program) (Grant No. 2010CB35600) and the Funds for Creative Research Groups of China (Grant No. 2011ZX09401-009).

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