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Improving the Tumor-Specific Delivery of Doxorubicin in Primary Liver Cancer

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1. Introduction

Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality. More than 500 000 new cases are currently diagnosed yearly, with an age-adjusted worldwide incidence of 5.5–14.9 per 100 000 population (Jemal et al., 2011). Doxorubicin remains the first line of treatment for liver cancer ever since its discovery in 1971 (Yoshikawa & Kitaoka, 1971). Unfortunately, clinical effectiveness of this class of drugs is limited by cumulative cardiotoxicity which occurs in significant percentage of patients at cumulative dose in the range 450-600 mg/m² (Ganz et al., 1993). Therefore, various strategies have been developed to reduce cardiotoxicity of doxorubicin and its analogues (Haley & Frenkel, 2008). Commercialized doxorubicin has high cytotoxicity, good solubility and high affinity to nuclear (Viale & Yamamoto, 2008). There is no need to improve its ability to penetrate cell membrane and accumulation into cell nuclear. The aim of delivery techniques is to alter its in vivo distribution, enhance its deposition in the liver tumor sites and reduce its cardiotoxicity. Formulations of doxorubicin should have the ability to specifically release the drug in response to the tumor micro-environment.

Doxorubicin can insert in the double strand of DNA and preferentially bind to double-stranded 5′-GC-3′ or 5′-CG-3′ to form tightly coupled complex without chemical bond links (Vicent, 2007). Once the DNA was digested, the doxorubicin can be released. Cationic polymers are often used as carriers for the DNA drugs' delivery because they can combine DNA to form nanoscale particles by the interaction between their positive charge and the negative charge on the DNA chain (Bodley et al., 1989). Upon these two aspects, we developed a nanoscale formulation of doxorubicin. Doxorubicin was complexed into polyGC double-strand DNA fragments to form the DOX-polyGC intercalation. After that, the DOX-polyGC intercalation was combined by a bio-degradable cationic polymer, cationic gelatin, to form nanoscale particles. Cationic gelatin can be effectively digested by gelatinase (GA) that is a mixture of two kinds of matrix metalloproteinase (MMP) highly expressed by the tumor tissue (Eliyahu, 2005). This makes the complex composed by cationic gelatin, DNA and doxorubicin (CPX1) can be specifically digested and release the doxorubicin in tumor sites (Figure 1). To avoid the accumulation into the liver which also produces gelatinase in a relatively high level (Emonard & Grimaud, 1990), a pH-sensitive material, histamine-modified alginate (His-alginate) was used to cover CPX1 to form CPX2. His-alginate has a pKa of about 6.9 which shows a cationic state when pH < 6.9 and

an anionic state when pH > 6.9. His-alginate combined CPX1 at physiological pH (7.2) via its anion interact with the cation on the surface of CPX1. In tumor micro-environment, the pH varied from 6.2-6.7 according to different physical states. At such a pH, His-alginate turned it anionic state to a cationic one and dissociated from CPX2. To enhance the ability of CPX2 to escape from the reticuloendothelial system, PEG 2000 was conjugated to His-alginate to form PEG-His-alginate (PHA). The construct scheme was shown in Figure 1.

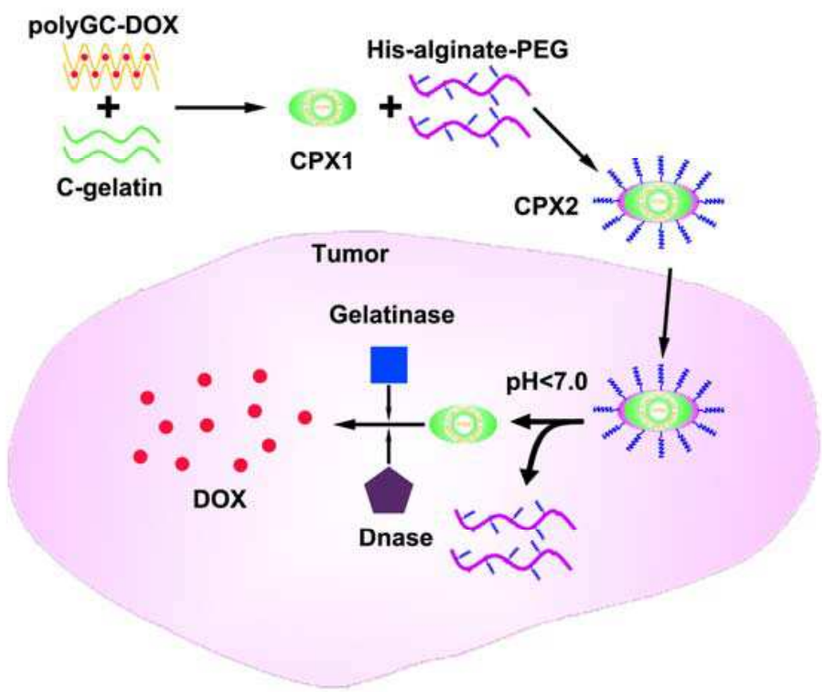

Fig. 1. Fabrication scheme of CXP1 and CPX2.

2. Materials and methods

2.1 Materials synthesis

Poly-GC and Poly-AT were synthesized by Invitrogen (Shanghai, China) with a length of 20 bases. Cationic gelatin was synthesized by using Ethylenediamine-modification according to the reported methods (Matsumoto et al., 2006). The conversion rate of carboxyl groups to amino groups was measured by the TNBS method to characterize the product (Eklund, 1976). PEG-His-modified alginate was synthesized according the following methods: A total of 500 mg of alginate (Sigma) was dissolved in 20 ml of 10 mM N,N,N′,N′-tetramethylethylenediamine (TEMED) / HCl buffer solution (pH 4.7). A total of 1.0 g of EDC was added to this solution and stirred at 40 ◦C for 24 h. Then, different1.5 g histidine were added and stirred for another 24 h at 40 ◦C. The resulting His-alginate were dialyzed for 4 days against Milli Q water and lyophilized. The resulted His-alginate was examined for its pKa value. The following Pegylation was achieved by conjugating a 2000 Da mPEG-NH2 to the His-alginate using a Pegylation kit (YARE Chem, Shanghai, China). Rhodamine-labled

PEG-His-alginate and FITC-labled cationic gelatine was synthesized by using fluorescence-label kits (DAZHI Biotech, Nanjing, China) injected into liver tumor bearing mice.

2.2 Cells and animals

Hepa 1-6 is a mouse hepatoma cell line derived from the BW7756 mouse hepatoma which arose in C57/L mouse (ATCC). Hepa 1-6 cells were cultured in DMEM supplemented with 10% FCS. Female ICR mice (18-20g) of the same background were purchased from the experimental animal center of Nanjing Medical University. All animals received human care according to Chinese legal requirements. To generate the allograft model of liver cancer, Hepa 1-6 cells (1×10^7 cells/ml, 10 μl) were injected into the hepatic lobe of anesthetized mice using a microinjector (Cheng et al., 2011).

2.3 Preparation of CPX1 and CPX2

PolyGC-DOX intercalation was obtained by mixing 2 mg/ml DOX with DNA solutions with different concentrations to find the DOX/DNA ratio at which free DOX can insert into DNA chain completely. The fluorescence (exciting: 480 nm; emission: 590nm) of DOX quenched when inserting in the DNA. By detecting the fluorescence of DOX, the formation of the intercalation could be determined. CPX1 was formed by mixing PolyGC-DOX intercalation with cationic gelatin solution at different DNA/Gelatin weight ratio. CPX2 was obtained by mixing CPX1 solution with the some volume of 2mg/ml PEG-His-alginate solution by gently agitation for 1 hour. The physical stability of each complex was studied by agarose gel electrophoresis (0.8% agarose in TAE buffer). The diameters and zeta potential of the complex were analyzed by photon correlation spectroscopy by using 90 Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY). The DOX fluorescence of CPX1 was also examined to investigate whether the combination between the intercalation and cationic gelatin release the DOX from DNA chain.

2.4 Dox release from the complexes

CPX1 and CPX2 were digested by gelatinase and Dnase I in PBS buffers with different PH values, and the resulted solution was examined by DOX fluorescence quantification.

The digestion abilities of mouse plasma, the supernatant of the tumor homogenate (THS) and liver homogenate (LHS) on CPX1 and CPX2 were also examined. CPX1 and CPX2 solutions were mixed with mouse plasma or the tissue homogenate supernatants for 2 hours. After that, the mixtures were examined by quantification of the DOX fluorescence.

To further confirm the tumor-specific drug-release ability of CPX2, in vivo experiments was performed. Rhodamine labeled PEG-His-alginate (PHA) and FITC-labeled cationic gelatin was used to form CPX2. Hoechst 33258 was used to substitute for DOX because Hoechst 33258 also possess high DNA-affinity like DOX and it show blue fluorescence which could be discriminated from the red fluorescence of PHA and green fluorescence of C-gelatin. PolyAT was used to substitute for polyGC because Hoechst 33258 specifically binds AT in DNA (Jong et al., 1991). This PHA-C-gelatin-polyAT-Hoechst complex was injected into the tumor and liver separately. One hour later, sections of the liver and tumor was examined under microscope.

2.5 Bio-distribution of doxorubicin

CPX1 and CPX2 solutions and free DOX solution were separately injected into liver tumor bearing mice via tail vain at a dose of 20 mg/kg body weight. Different organs were harvested from the experimental mice bearing implanted tumors. Doxorubicin in the organ was extracted according a reported method and quantified by the examination of its fluorescence intensity at 590 nm (Bigotte, 1985).

2.6 Toxicology investigation

Healthy mice were given CPX1, CPX2 or free DOX to examine their toxicity. CPX1, CPX2 and DOX were intravenously injected into animals at the doses of 20 mg DOX/kg body weight and 30 mg DOX/kg body weight. The changes of their body weights were examined every day for one week. The mortality of the animals was also calculated. For histological examination, different tissues were harvested at the 4th day after the drugs were given and sectioned and analyzed by hematoxylin and eosin staining. Animal plasma activity of alanine transaminase (ALT) and creatine kinase (CK) were determined for the evaluation of the functions of livers and hearts.

2.7 Anti-cancer activity

Animals bearing implanted liver tumors were intravenously given CPX1, CPX2 and free DOX at a dose of 10 mg DOX/kg body weight every two days from day 5 after the allograft model establishment. All tumors were separated, weighed and sectioned for pathological analyze on day 21. For the calculation of survives, testing animals bearing tumors were divided into 4 group, 10 animals each. CPX1 and CPX2 at a dose of 10 mg DOX/kg body weight were given intravenously every two days with free doxorubicin and saline as controls.

2.8 Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M). The differences between groups were analyzed by Mann-Whitney U test and, if appropriate, by Kruskal-Wallis ANOVA test. Survival curves were analyzed by the Kaplan-Meyer log-rank test. Changes in body weight were compared by use of the Wilcoxon matched-pair signed-rank test. A value of $P < 0.05$ was considered significant.

3. Results

3.1 Preparation of CPX1 and CPX2

The anthracycline class of drugs, including doxorubicin, has fluorescence properties that become quenched after intercalation into DNA. Fluorescence spectroscopy was used to examine the binding of doxorubicin to DNA and the drug encapsulation efficiency of CPX1 and CPX2. A series of weight ratio of DOX/polyGC was tested. DOX could be completely combined by polyGC at the weight ratio of 2:1. Cationic gelatin could combine with DOX-polyGC intercalation at a weight ratio of polyGC/C-gelatin = 1:2. In CPX1, weight ratio of DOX/polyGC/C-gelatin = 2:1:2. Its zeta potential was +18 mV. PEG-His-alginate was added to the solution of CPX1 at the C-gelatin/PEG-His-alginate ratio of 1:1

to form CPX2. The zeta potential of CPX2 was -1.2 mV. Figure 2A show the fluorescence intensity of doxorubicin, DOX-polyGC, CPX1 and CPX2. Figure 2B show the particle sizes of CPX2.

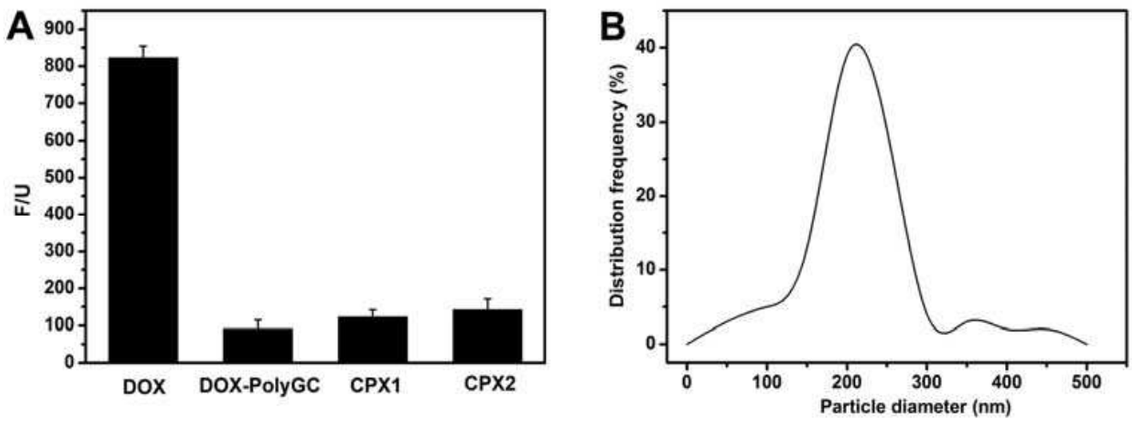

Fig. 2. Preparation of CPX1 and CPX2. A) Fluorescent intensity of free DOX,
DOX-polyGC intercalation (weight ratio : DOX:polyGC = 2:1),
CPX1 (weight ratio: C-gelatin : polyGC : DOX = 2:1:2) and
CPX2 (weight ratio: PEG-His-alginate : C-gelatin : polyGC : DOX = 2:2:1:2) at 590nm;
B) Size distribution of CPX2 in saline.

3.2 DOX release from the complexes

The most important property of the complex is to response to PH and gelatinase. PHA-C-gelatin-polyAT-Hoechst complex was used in vivo instead of CPX2 to test the drug release property of CPX2 in response to tumor acidic microenvironments. Results shown in Figure 3A demonstrated that the three kinds of fluorescence were completely overlapped in liver sections. In liver tumor sections, blue "drug" could be seen in a separated area from the green and red fluorescence.

The ability of gelatinase and Dnase I (DA1) to free DOX from CPX1 and CPX2 in PBS buffers with different pH values was tested. The results in Figure 3B demonstrated that DOX in CPX1 could be released under both PH values of 7.2 and 6.5 while CPX2 could only be digested when PH was 6.5. CPX1 and CPX2 were incubated with tumor tissue homogenate supernatant (THS) and then were examined for the released DOX. Serum and normal liver tissue homogenate supernatant (LHS) was used as the control. The results were shown in Figure 3C and Figure 3D. DOX in CPX1 or CPX2 was not released in serum while THS could efficiently release free DOX from CPX1 and CPX2. LHS could only release free DOX from CPX1. CPX2 remained stable in LHS. Figure 3E demonstrated that more than 60% DOX was released from both CPX1 and CPX2 within 2 hours when incubated with THS. When incubated with LHS, CPX1 was also destructed and released DOX very quickly while CPX2 remained stable and kept the DOX from release for more than 20 hours.

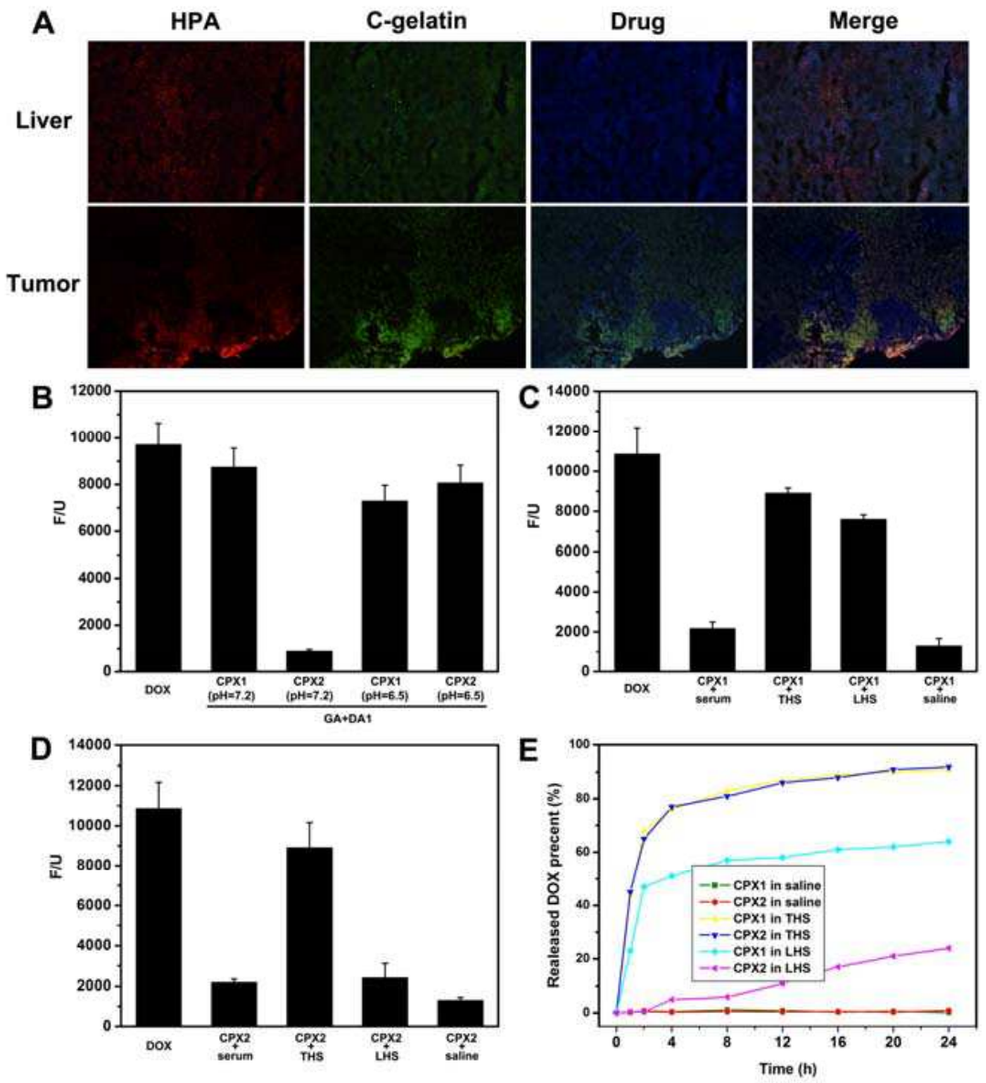

Fig. 3. Release of DOX from CPX1 and CPX2. A) Tumor-specific drug release of CPX2. 0.2 mg Hoechst 33258 contained in multi-fluorescence-labeled CPX2 (polyGC was substituted by polyAT) was injected into the tumor or animal liver after the animals were anesthetized 1 hour before the tissues were separated, sectioned and analyzed by microscope. B) Fluorescent intensity of CPX1 and CPX2 digested by 5 U/ml gelatinase (GA) and 0.5 U/ml Dnase I (DA1) in PBS buffer with PH value of 7.2 or 6.5 for 2 hours at 37 ˚C at 590nm; C) Fluorescent intensity of CPX1 digested by plasma, THS or LHS for 2 hours at 37 ˚C at 590nm; D) Fluorescent intensity of CPX2 by plasma, THS or LHS for 2 hours at 37 ˚C at 590nm; E) DOX release rates from the complexes in THS or LHS. In all experiments, the concentration of DOX contained in CPX1 and CPX2 in the solution is 0.2mg/ml.

3.3 Bio-distribution of doxorubicin

Free DOX, CPX1 and CPX2 were administrated into mice via tail vein at a dose of 20 mg DOX per kg body weight. Different tissues and organs were harvested 48 hours later. The

concentration of DOX in these tissues was measured. Data in Figure 4A indicated that CPX2 efficiently enhanced the accumulation of DOX in tumor tissue and reduced the deposition of the drug in heart and the other organs. As a control, CPX1 not only enhanced the drug concentration in tumor but also in liver. The concentration of DOX delivered by CPX1 was 2 times higher than free DOX. Figure 4B and Figure 4C described the variation of DOX concentrations in tumor and liver after CPX1, CPX2 or free DOX was administrated. CPX2 dramatically increased the deposition of DOX in tumor and decreased the liver concentration of the drug. CPX1 could increase the concentration of DOX in both tumor and liver.

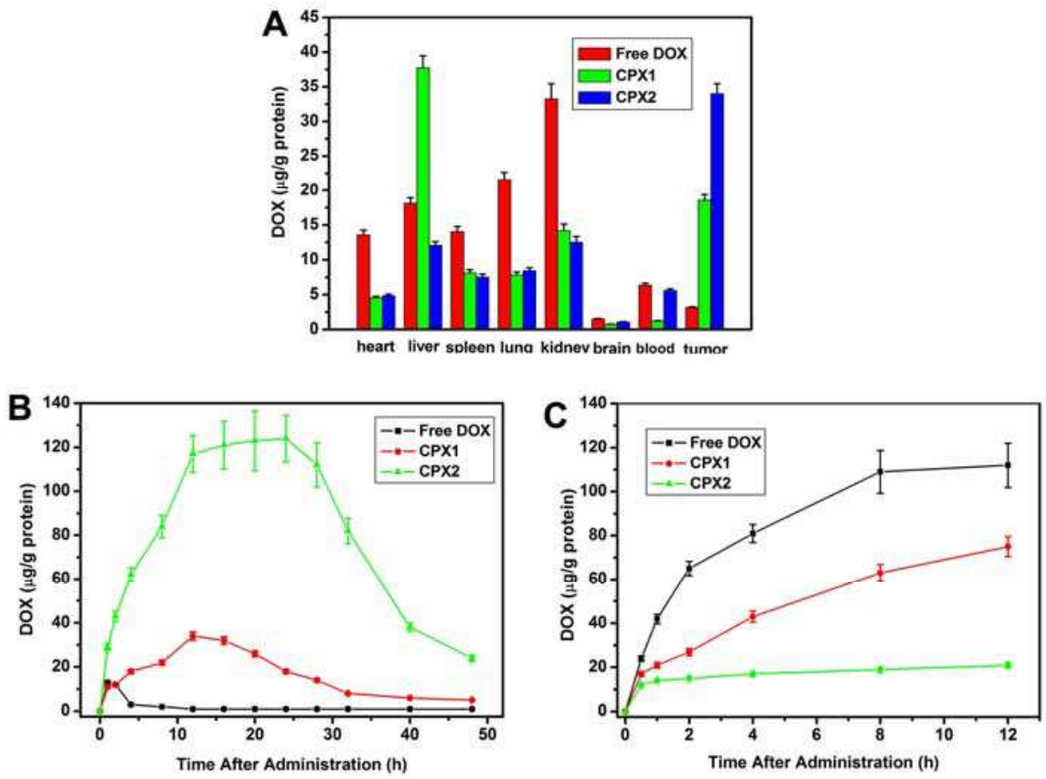

Fig. 4. A) Bio-distribution of free DOX or delivered by CPX1 and CPX2 given i.v. at a dose of 20 mg/kg body weight; B) DOX concentrations in tumor after free DOX, CPX1 or CPX2 were injected i.v. at the dose of 20 mg/kg body weight; C) DOX concentrations in liver after free DOX, CPX1 or CPX2 were injected i.v. at the dose of 20 mg/kg body weight.

3.4 Toxicology investigation

Daily injection of doxorubicin exhibited serious toxicity which resulted in loss of weight, cardiac toxicity and death. CPX1 and CPX2 remarkably reduced the toxicity of doxorubicin, which caused less weight loss than free DOX (Figure 5A). 30 and 20 mg/kg body weight of DOX caused 100% and 60% death in 5 days. The same dose of DOX in the form of CPX1 caused 50% and 20% death in up to 8 days while 20 mg/kg body weight DOX in the form of CPX2 cause no death and the extreme high dose of 30 mg/kg body weight only caused 10% death (Figure 5B).

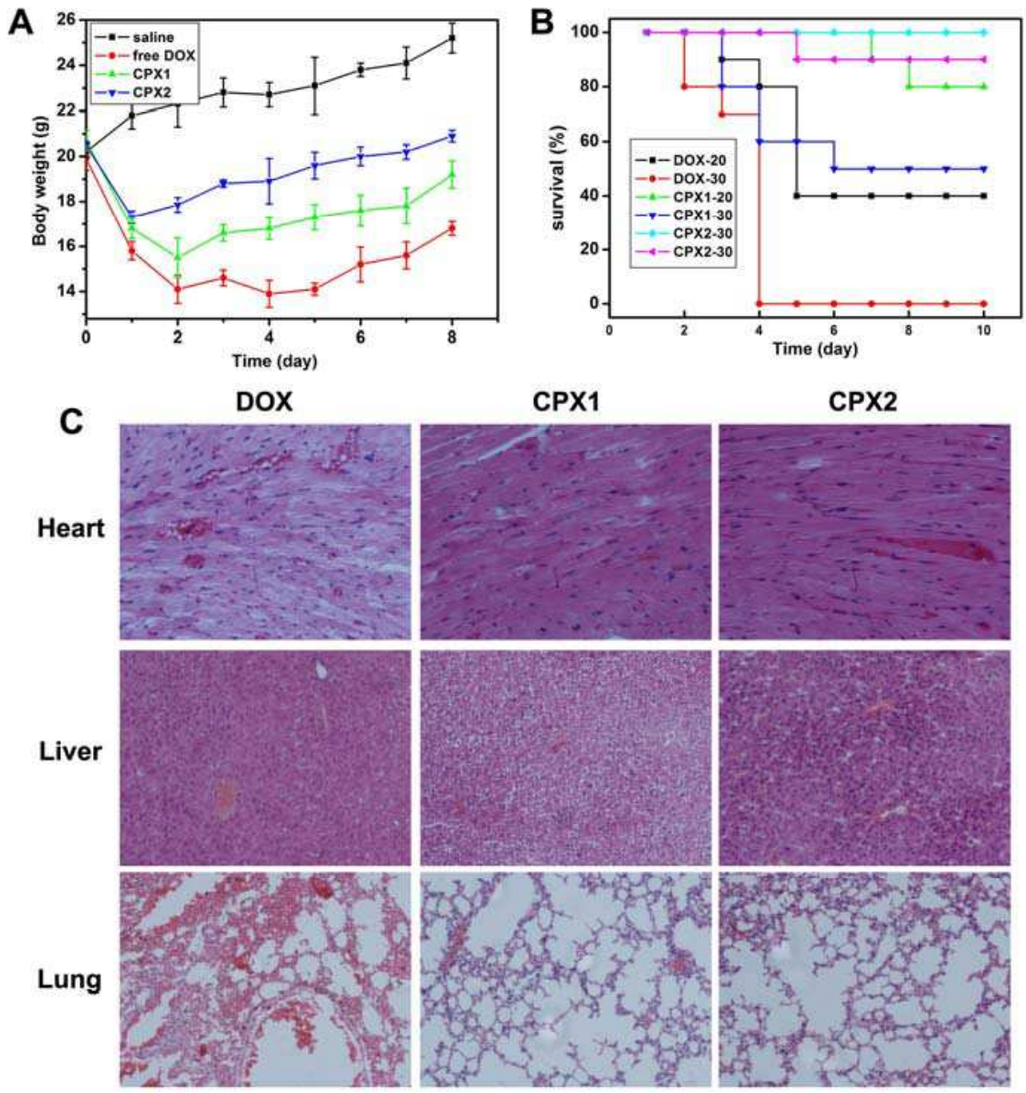

Fig. 5. Toxicology analysis. A) Body weight changes of animals received i.v. injections of saline, free DOX, CPX1 or CPX2 at a dose of 20 mg/kg body weight; B) Mortality caused by free DOX, CPX1 or CPX2 at different doses; C) H & E stained heart, liver and lung tissue sections of animal treated by 20 mg/kg body weight free DOX or delivered by CPX1 and CPX2.

Treatment with free doxorubicin caused massive myocardial degeneration (Figure 5C), which resulted in the increase of the plasma level of CK and LDH (Table 1) and heart failure which induced significant pulmonary congestion (Figure 5C). This toxicity was greatly reduced when DOX was delivered by CPX1 and CPX2 (Figure 5C and Table 1). CPX1 showed more hepatotoxicity than free doxorubicin at the same dose, which resulted in the necrosis of hepatocytes (Figure 5C) and increase in plasma levels of ALT (Table 1) while CPX2 did not cause this toxicity. CPX2 also reduced the nephrotoxicity of doxorubicin which was demonstrated by the decrease of BUN level (Table 1).

	ALT (IU)	AST (IU)	CK (U/ml)	LDH (U/l)	BUN (U/l)
Healthy mice	26.9	32.1	0.23	489.2	10.3
Mice given DOX	29.5	29.7	1.28	562.9	12.7
Mice given CPX1	42.3	34.1	0.26	461.3	10.9
Mice given CPX2	28.3	30.5	0.24	462.7	10.2

Table 1. Serological analysis of mice treated with free DOX, CPX1 and CPX2.

3.5 Anti-cancer activity of CPX1 and CPX2

The anti-tumor activity was evaluated in a mouse orthotopic allograft liver cancer model using a hepatoma cell line, Hepa 1-6. Figure 6A shows the photograph of tumors separated from animals 16 days after they received free DOX, CPX1, CPX2 and saline in vein. From the results of histological analyze of tumor sections (Figure 6B), massive cancer cell remissions could be observed from the sections of tumors treated with CPX1 and CPX2. CPX2 exhibited the most efficiency on prevention of cancer cell's expansion.

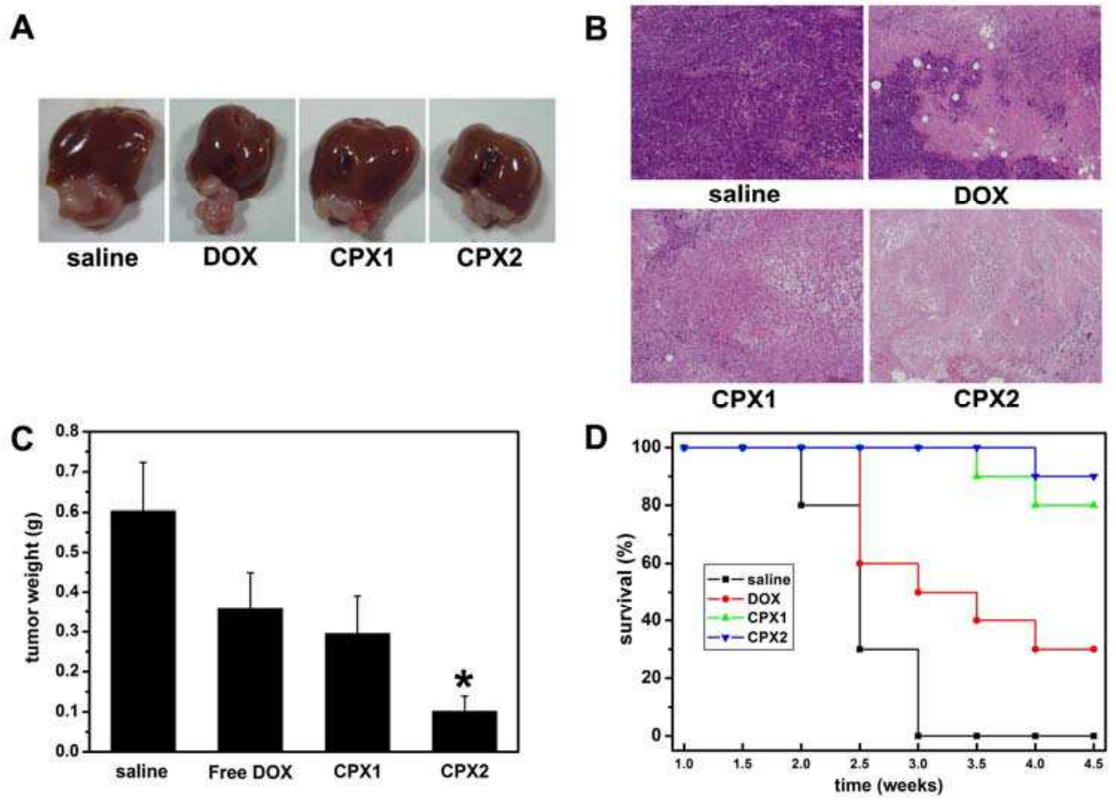

Fig. 6. Anti-tumor activity. A) Representative Tumors separated from animals received intravenous injections of saline, free DOX, CPX1 or CPX2. B) Sections of tumor tissues taken from animal received intravenous injections of saline, free DOX, CPX1 or CPX2. C) Mean weights of tumors separated from animals received of saline, free DOX, CPX1 or CPX2 intravenously. D) Survive rates of animals implanted tumors received treatments with saline, free DOX, CPX1 or CPX2 in more than 4 weeks. *$p < 0.05$ compared with saline treated group.

The mean weight of the tumors isolated from the liver was shown in figure 6C. CPX2 exhibited more than 1 times higher anti-tumor activity than free DOX. Results of the examination of animal survive shown in Figure 6D. Animals bearing tumors and received saline as control died within 4 weeks. Free doxorubicin kept 30% animals from death within 5 weeks while CPX2 rescued 90% of them in more than 4 weeks.

4. Discussion

Doxorubicin, as a chemotherapy drug, has been commonly used in the treatment of hepatocellular carcinoma, but is thwarted by a key obstacle-cumulative cardiotoxicity. To improve its anti-cancer activity and reduce its toxicity, a number of doxorubicin formations such as pegylated liposomal doxorubicin, doxorubicin-eluting-bead has been performed in liver cancer clinical trials recently. Among them, anthracycline-DNA complexes as therapeutic reagents have been used for the treatment of liver cancer for a long history, due its easy-fabrication, no change in the structure of the drug molecules and its anti-cancer activity (Trouet, 1979a). Clinical trials were also carried out to evaluate their enhancement in the performances in effectiveness and toxicology compared to free anthracycline (Trouet, 1979b). However, except a few of *in vitro* reports about successfully reducing the toxicity of the anthracycline by using the complexes, there is no convincing results demonstrating the advantages of using the complexes instead of free anthracycline (Dorr et al., 1991). One of the most important reasons underlies the ineffectiveness of the intercalation between DNA and anthracycline is that there is abundant Dnase in circulation and body fluids which digests un-protected DNA molecular with extremely high efficiency (Paik & Kim, 1970). The anthracycline-DNA complexes were destroyed soon after their injection into the body. The anthracycline in complexes was released very quickly and did not change their pharmaceutical properties any more.

Protection of DNA from destroying by Dnase is necessary for gene delivery technology (Tranchant et al., 2004). Non-viral gene delivery practices take the advantage of using cationic polymers to combine DNA, which prevents the exposure of DNA molecules to Dnases (Wagner, 2004). DNA complexed with cationic polymers could totally resist the degradation of Dnase I or serum Dnases in reported *in vivo* and *in vitro* tests (Paleos et al., 2009). These researches instruct us cationic polymers could be used to combine the intercalation of anthracycline-DNA to form an anthracycline-DNA-cationic polymer ternary complex. Such a complex can avoid the unexpected early release of anthracycline into serum by protecting DNA from the digestion of Dnases. In our experimets, cationic gelatin was used to combine DNA-DOX intercalation. This simple process generated regular nanocomplexes that could be observed. It is difficult to release DOX in short time using Dnase I alone to digest the complex, which suggested that DNA is well protected by cationic gelatin. Additionally, in the present study, synthesized GC oligonucleotide was used to substitute the natural DNA fragments, which increased the drug-loading capacity of DNA. Moreover, the combination between DOX and polyGC is more stable than DOX-natural DNA intercalation fragments which may release DOX when combined by some kind of cationic polymer. No DOX release was observed in the study when DOX-polyGC was combined by cationic gelatin.

A lot of cationic polymers are used as gene carriers, such as polyether imide (PEI), polylysine (PLL), cationic polysaccharides and other polycation materials (Bodley et al.,

1989). To ensure the sufficient release of anthracycline in tumor sites, the cationic polymer used to form the anthracycline-DNA-cationic polymer ternary complex must be biodegradable, especially in tumor. Cationic gelatin is a derivate of gelatin by conjugating ethylenediamine, spermine or other compounds with multiple amino groups to the gelatin molecules. Cationic gelatin is a proved effective DNA carrier for gene delivery *in vitro* and *in vivo*. It can protect DNA and even RNA from the degradation of Dnases (Kushibiki et al., 2003). More importantly, cationic gelatin can be easily digested by gelatinase (Ganea et al, 2007). Gelatinase belong to the matrix metallopeptidase (MMP) family. It is composed of MMP-2 and MMP-9 (Eliyahu, 2005). Recent investigations suggest that gelatinase is involved in the genesis, development and metastasis of most kinds of tumors, which is highly expressed in solid tumors (Deryugina & Quigley, 2006). That means cationic gelatin could be effectively digested in tumor. To address this, tumor homogenate supernatant was tested for its ability to digest cationic gelatin in CPX1 and release DOX. Compared to plasma, THS exhibited much higher efficiency of releasing the DOX from CPX1. This implies CPX1 can be destroyed and DOX can be released specifically in tumor sites.

However, only gelatinase-response is not sufficient to enable the tumor-specific drug release ability of the delivery system because gelatinase is relatively high-expressed in liver (Emonard & Grimaud, 1990). In our experiments, CPX1 notablely enhanced the liver accumulation of DOX and caused serious hepatotoxicity which weakened the efficacy of the drug. To avoid this, a pegylated PH-responsive alginate was introduced in the system. After the surface of CPX1 was covered with this polymer (Formed CPX2), it could resist the digestion of gelatinase under physiological conditions. The environment intra tumor is acid which could trigger a change of the charge on the polymer from negative to a positive one, which resulted in the dissociation of the polymer form CPX1 and the digestion of CPX1 by gelatinase in the tumor. Because of the pegylation, CPX2 acquired stronger enhanced permeability and retention (EPR) effect that increased the distribution of the drug in tumor than CPX1 and greatly reduced the liver accumulation.

Cardiotoxicity is the main drawback of anthracycline, which restraints more effective application of this drug to liver cancer patients (Ferreira et al., 2008). Myocardial degeneration is the most commonly encountered side-effects of anthracycline (Combs & Acosta, 1990). It can cause serious heart failure and even death (Ferrans, 1978). In our study, CPX2 efficiently reduced the accumulation of DOX in heart, which greatly alleviated the cardiotoxicity of DOX. It completely rescued animals from death caused by high doses of DOX. Additionally, controlled-release and tumor site specifically release of DOX also eased the acute toxicity-induced loss of body weight.

5. Conclusion

In our present study, a PH-/enzyme responsive complex composed by self-assemble of doxorubicin, CpG DNA fragments, cationic gelatin and a PH-sensitive alginate was developed. This complex increased the accumulation of doxorubicin in tumor, reduced its deposition in heart and could specifically release doxorubicin in tumor sites, which resulted in the enhanced anti-cancer activity and decreased cardiotoxicity of doxorubicin. When tested in animal model of implanted tumor, the complex exhibited high effective in preventing the growth of the tumors and dramatically alleviated toxicity compared to free

doxorubicin. All results suggest that this easy- manufactured, cost-effective nanoscale formulation of doxorubicin hold great promise to be used to clinical practices.

6. Acknowledgements

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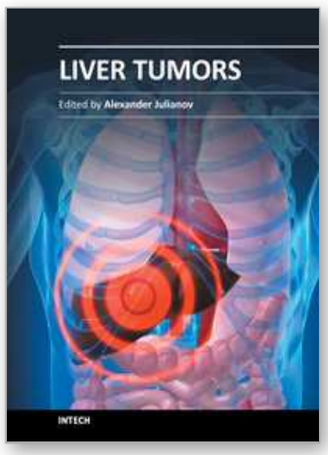

Liver Tumors

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This book is oriented towards clinicians and scientists in the field of the management of patients with liver tumors. As many unresolved problems regarding primary and metastatic liver cancer still await investigation, I hope this book can serve as a tiny step on a long way that we need to run on the battlefield of liver tumors.

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