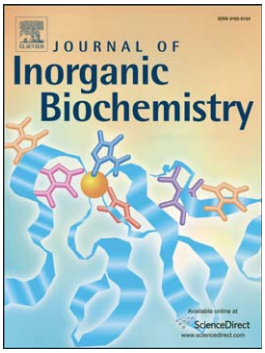

Enhanced anti-cancer efficacy to cancer cells by doxorubicin loaded water-soluble amino acids-modified β-cyclodextrin platinum complexes

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PII: S0162-0134(14)00089-0
DOI: doi: 10.1016/j.jinorgbio.2014.03.012
Reference: JIB 9494

To appear in: *Journal of Inorganic Biochemistry*

Received date: 16 December 2013
Revised date: 20 March 2014
Accepted date: 21 March 2014

Please cite this article as: Mei-Xia Zhao, Meng Zhao, Er-Zao Zeng, Yang Li, Jin-Ming Li, Qian Cao, Cai-Ping Tan, Liang-Nian Ji, Zong-Wan Mao, Enhanced anti-cancer efficacy to cancer cells by doxorubicin loaded water-soluble amino acids-modified β-cyclodextrin platinum complexes, *Journal of Inorganic Biochemistry* (2014), doi: 10.1016/j.jinorgbio.2014.03.012

Author contributions

M. X. Zhao, Y. Li and E.-Z. Zeng performed the experiments. M. X. Zhao, M. Zhao, E.-Z. Zeng, Y. Li, J.-M. Li, Q. Cao, C.-P. Tan, Z. W. Mao and L. N. Ji reviewed, analyzed and interpreted the data. M. X. Zhao and Z. W. Mao wrote the paper. All authors discussed the results and commented on the manuscript.

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Abstract: The effectively targeted delivery of insoluble anticancer drugs to increase the intracellular drug concentration has become a focus in cancer therapy. In this system, two water-soluble amino acids-modified β-cyclodextrin (β-CD) platinum complexes were reported. They showed preferable binding ability to DNA and effective inhibition to cancer cells. And they could bind and unwind pBR322 DNA in a manner, which was similar to cisplatin. Besides, our platinum complexes could effectively deliver the anticancer drug doxorubicin (Dox) into cells and had higher cell inhibition ratio, but less toxicity on the normal cells, compared with cancer cells. In this combination system, Dox was encapsulated into the hydrophobic cavities of β-CD at the optimum molar ratio of 1:1, which were validated by UV-visible (UV-vis) absorption spectroscopy, fluorescence spectroscopy and MTT experiments. Moreover, the combination system had higher cell inhibition ratio than free Dox and amino acids-modified β-CD platinum complexes. And the results of high content screening (HCS) showed that Dox-loaded amino acids-modified β-CD platinum complexes could permeate the cell membrane and enter cells, suggesting the efficient transport of Dox across the membranes with the aid of the β-CD. We expect that the amino acids-modified β-CD platinum complexes will deliver antitumor drug Dox to enhance intracellular drug accumulation and such combination system showed great potential as antitumor drug.

Keywords: Cyclodextrin, Platinum complexes, Doxorubicin, Drug delivery, Combination system

1. Introduction

The development of platinum complexes as antitumor drug is an area of considerable current interest, and platinum-based anticancer drugs have been constantly advanced and modified [1-6]. To achieve more effective antitumor activity, platinum complexes should be transported across the cell membrane and undergo hydrolysis [7-9]. Thus, solubility should be taken into consideration in the design of new active platinum complexes [10, 11]. At present, the popular approach to improve the bioavailability of insoluble anticancer drugs is carriers-based delivery and modified by biomolecules, but the application of some delivery systems have been limited by their complicated construction and poor performance in vivo due to uptake in non-specific tissues [12, 13]. The platinum complexes with better activity in tumour cells and less toxicity in normal cells could be obtained through linking bioactive groups to platinum compounds, e.g., amino acids, peptides, nucleosides, and nucleobases, etc. [14-17]. The platinum complexes with bioactive groups are revealed to be good DNA intercalators [18-20]. For example, Farrell et al. studied the mechanism of cytotoxic action of naphthoquinone-platinum(II) complexes [21]. The results showed that coordination and stabilization of the quinone structure could substantially affect the chemical and biophysical properties of the pro-ligands, thus

improving their DNA binding properties and generating compounds capable of cleaving DNA and catalytically inhibiting Topo I [21].

β-Cyclodextrin (β-CD) is a type of cyclic oligosaccharides consisting of seven glucose units with lipophilic central cavities and a hydrophilic outer surface. It can be used as a pharmaceutical excipient to improve the solubility, stability, and bioavailability of hydrophobic drug molecules [22-27]. There have been many reports about cyclodextrins as new drug carriers. For instance, Yao et al. synthesized glycol chitosan-carboxymethyl β-CD and studied their inclusion capability of delivering different hydrophobic anticancer drugs (5-fluorouracil, doxorubicin (Dox), and vinblastine), which could be successfully loaded into the cavities of the β-CD [28]. Gourevich and co-workers also reported the potential of a cyclodextrin-based nanocarrier as a drug delivery vehicle, and investigated the cell uptake routes of Dox encapsulated in the drug delivery vehicle [29]. The results showed that the uptake of encapsulated Dox into cancer cells was increased in amount by a factor of up to 5.5 when ultrasound was applied. Thus, β-CD derivatives are attractive carriers since they possess the hydrophobic cavities and they are highly desirable on the way to improve the bioavailability of insoluble anticancer drugs via anticancer drug carriers.

In the present work, we designed and synthesized two Dox loaded water-soluble cyclodextrin platinum complexes. In this system, amino acid ligands are attached to β-CD in order to evaluate whether the presence of these bioactive ligands can modulate the DNA binding ability and potential cytotoxic activity of the

platinumcomplexes (Scheme 1). The amino acids-modified β-cyclodextrin could improve the solubility and stability of the platinum complexes, and the application of L-Arg with positive charge and L-Phe with neutral charge could be preferably bound to platinum. The water-soluble β-CD platinum complexes with amino acid showed preferable ability to bind DNA and they bound and unwound pBR322 DNA in a manner similar to that of cisplatin. Besides, the platinum complexes could effectively deliver the anticancer drug Dox into cells and had less toxicity in the normal cells than in cancer cells (Scheme 1). The optimum molar ratio of Dox encapsulated into the hydrophobic cavities of β-CD was validated by UV-visible (UV-vis) absorption spectroscopy, fluorescence spectroscopy and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiments. In addition, the positive charge of L-Arg led to a considerable increase in the intracellular delivery of Dox. Moreover, we further investigated intracellular uptake of the combination system of amino acids-modified β-CD platinum complexes loaded with Dox by a high content screening (HCS) technique.

2. Experimental Section

2.1. Chemicals and reagents

β-CD, p-toluenesulfonyl chloride, triethanolamine, L-Arg, L-Phe, and K_2PtCl_4 were obtained from Shanghai Chemical Factory, China. β-CD was recrystallized twice from twice-distilled water and dried under vacuum for 12 h at 95 °C before use.

Other organic solvents were purchased from EM Sciences. Fetal bovine serum (FBS) and Dulbecco minimum essential medium (DMEM) were from Invitrogen Corporation. MTT and Dox were from Sigma.

2.2. Preparation of the ligands L-amino acid-modified β-cyclodextrin

L-Amino acid-modified β-CD was synthesized based on reported method with slight modification [30, 31]. Briefly, the mono[6-O-(p-toluene-sulfonyl)]-β-cyclodextrin and amino acid (L-Arg and L-Phe) at molar ratio of 1:3 were dissolved in water solution containing triethanolamine at 85 °C for 24 h under N_2. Most of the solvent was evaporated under reduced pressure, and the resulting solution was poured into vigorously stirred 500 mL ethanol, then the resultant mixture was stored in a 4 °C refrigerator for 18 h to produce a pale yellow precipitate. The precipitate then was purified by chromatography with deionized water as eluent, followed by repeated recrystallization from water and ethanol, to gain pale yellow product in 65.2% yield for L-Arg-β-CD and in 47.3% yield for L-Phe-β-CD.

Mass Spectrometry (MS) (Electrospray ionization (ESI), H_2O, m/z) L-Arg-β-CD: 1291.7 (M+H); ^{1}H NMR (DMSO, Tetramethylsilane (TMS)): 2.98-3.64 (m, 45H), 4.53-4.63 (m, 7H), 4.64-4.82 (m, 7H), 5.58-5.86 (m, 14H), 7.34-8.67 (m, 5H), 12.34 (s, 1H); Anal. Calcd for L-Arg-β-CD complex ($C_{48}H_{82}N_4O_{36}\cdot 10H_2O$): C 39.18; H 6.99, N 3.81; found: C 39.32, H 6.65, N 3.81.

MS (ESI, H_2O, m/z) L-Phe-β-CD: 1282.3 (M+H); ^{1}H NMR (DMSO, TMS): 2.78-3.34 (m, 50H), 4.47-4.82 (m, 7H), 5.60-5.71 (m, 14H), 7.20-7.21 (m, 5H); Anal.

Calcd for L-Phe-β-CD complex ($C_{51}H_{79}NO_{36}\cdot 8H_2O$): C 42.95, H 6.71, N 0.98; found: C 42.92, H 6.81, N 0.77.

2.3. Synthesis of L-amino acid-modified β-cyclodextrin platinum complexes

A total of 0.5 mmol of the L-amino acid-modified β-CD and 0.5 mmol of K_2PtCl_4 was suspended in 50 mL of H_2O, and the mixture was stirred at 60 °C for 48 h under argon in dark. After that, the reaction mixture was cooled to room temperature. The solids were then filtered out. And the raw products were separated by acetone precipitation followed by centrifugation. The solid products were collected by centrifugation and then purified by chromatography on a Sephadex G-25 column with deionized water as eluent, to give pale yellow product in suitable yield.

[Pt(L-Arg-β-CD)Cl_2]·10H_2O. Yield as light yellow powder (53.5%). ^{1}H NMR ([D6]DMSO, TMS): δ = 1.58-1.72 (m, 4H), 2.08 (m, 1H), 3.12-3.15 (m, 2H), 3.28-3.64 (m, 45H), 4.18-4.63 (m, 7H), 4.82-4.91 (m, 7H), 5.67-6.15 (m, 14H), 7.34-8.67 (m, 2H); elemental analysis calcd (%) for Anal. Calcd for $C_{48}H_{81}N_4O_{36}PtCl_2\cdot 10H_2O$: C 34.27, H 5.69, N 3.33; Found: C 34.15, H 5.89, N 3.16.

[Pt(L-Phe-β-CD)Cl_2]·8H_2O. Yield as light yellow powder (63.6%). ^{1}H NMR ([D6] DMSO, TMS): δ = 2.72-2.88 (m, 2H), 3.12-3.15 (m, 2H), 3.26-3.62 (m, 45H), 4.35-4.54 (m, 7H), 4.82-4.96 (m, 7H), 5.52-5.95 (m, 14H), 7.204-7.315 (m, 4H), 8.29 (m, 1H); elemental analysis calcd (%) for $C_{51}H_{78}Cl_2NO_{36}Pt\cdot 8H_2O$: C 36.23, H 5.61, N 0.83; found: C 36.29, H 5.58, N 0.85.

2.4. Spectral measurements

UV-vis absorption spectra were recorded in a quartz cuvette with a 1 cm path length using a Perkin-Elmer Lambda-850 UV-vis spectrometer (Perkin-Elmer, USA). 3 mL of Tris buffer solution was added to the reference pool with the sample cell as the baseline correction. 3 mL of 100 μM calf thymus DNA (CT-DNA) solution was added to sample cell, then same volume (6 μL) of 10 mM sample was added to the sample pool, so that the concentration ratio of the complexes and the DNA increased. Mixed for 24 h everytime. The ultraviolet-visible spectral changes upon the addition of the complexwere detected in the range of 180-500 nm.

2.5. Preparation of Dox-loaded β-CD platinum complexes

A general protocol for the Dox-loaded amino acids-modified β-CD platinum complexes formation was as follows. Dox and amino acids-modified β-CD platinum complexes were dissolved in phosphate-buffered saline (PBS) at various molar ratios (n_{Dox} : $n_{platinum}$ = 1:0.5, 1:1, 1:1.5, 1:2, 1:4), then sonicated for 4 h. Afterwards, the optimum molar ratio of Dox and Dox-loaded amino acids-modified β-CD platinum complexes were validated by UV-vis absorption and fluorescence emission spectroscopy, respectively.

The UV-vis absorption spectra of free Dox and Dox-loaded amino acids-modified β-CD platinum complexes were recorded using a Perkin-Elmer Lambda-850 UV-vis spectrometer (Perkin-Elmer, USA). The fluorescence emission spectra from 500 nm to 800 nm were obtained using a Perkin-Elmer Ls55 luminescence spectrometer (Amer-ican, Perkin-Elmer Co.).

2.6. Circular dichroism (CD) measurements

CD measurements were performed on a Jasco J-810 CD spectropo-larimeter at 37 ◦C in a rang of 200-400 nm and the cell length was 1 cm. Titrations were performed by incrementally adding aliquots of each complex to 25 mM CT-DNA in 10 mM Tris-HCl buffer with pH valueadjusted to 7.4. The concentration of each complex ranged from 0 to 20 mM. CD spectra were baseline-corrected for signal contributions due to the buffer. The CD spectrum of CT-DNA alone (25 mM) was recorded as control. After each addition of complex, the reaction was stirred and allowed to equilibrate for at least 24 h and a CD spectrum was collected with at least five scans. Final analysis of the data was carried out using Origin 7.5.

2.7. DNA binding study

Linear DNA fragments were isolated from pBR322 DNA cleavage products by platinum complexes and purified using a DNA gel extraction kit [32, 33]. Specifically, pBR322 DNA was incubated with Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2 in 20 mM HEPES buffer, pH 7.20, for a certain period of time (24 h), then 4 μL of loading buffer (0.05% bromophenol blue, 50% glycerol, and 2 mM Na_2H_2EDTA) was added to the mixture. Control reactions without platinum complexes were carried out under the same conditions. Then the samples were loaded directly onto a 0.9% agarose gel and electrophoresed in TBE buffer (89 mM tris-borate, 2 mM Na_2H_2EDTA) at a constant voltage of 80 mV for 100 min. Then the gels were placed in the ethidium bromide (EB) dilute solution and tained five minutes. The gels were visualized in the

electrophoresis documentation and analysis system 120. Densitometric calculations were performed using Image Tools 3.00. The intensities of supercoiled pBR322 DNA were corrected by a factor of 1.22 as a result of its lower staining capacity by EB [34].

2.8. Cell culture

K562, HepG2 and 7701 (normal liver cells) cells were cultivated in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin. These cells were allowed to grow in monolayer in a tissue culture flask incubated at 37 °C in a 95% humidified incubator with 5% CO_2. When the cells were maintained in an exponential growth phase by periodic subcultivation, the cells were counted by hemocytometer and seeded in 96-well microplate at a density of 5×10^4 cells/well.

2.9. Cell viability assay

The optimal molar ratio of Dox and amino acids-modified β-CD platinum complexes was performed using the MTT assay. Dox (5 μM) and amino acids-modified β-CD platinum complexes were dissolved in DMEM medium at various molar ratios (n_{Dox} : $n_{platinum}$ = 1:0.5, 1:1, 1:1.5, 1:2, 1:4). After being sonicated for 4 h, Dox and Dox-loaded amino acids-modified β-CD platinum complexes incubated with cells for 48 h to determine the optimal molar ratio at maximal cell inhibition rate.

In vitro cytotoxicity of the samples was determined by a modified MTT assay[35]. Briefly, the cells (5×10^4 cells/well) were seeded in 96-well microplate for

24 h, then the different concentrations of samples in DMEM medium (1-200 μM) were added to the wells in triplicates and incubated for 48 h. After the incubation period, 20 μL of MTT solution (2.5 mg mL^{-1} in PBS) was added to each well and the plates were incubated in the dark for another 4 h. Then the media were removed and the formazan crystals were dissolved in 100 μL of DMSO. Then the absorbance of each well at 570 nm was read on the Tecan Infinite F200/M200 multimode Plate readers. The percentage of cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100%. Cells treated with vehicle (1% DMSO) were used as controls. Data were averages of three independent experiments ± standard deviations.

2.10. Cellular uptake of Dox-loaded amino acids-modified β-CD platinum complexes in live cells

HepG2 cells in DMEM medium containing FBS (10 %) were seeded in 96-well microplate for 24 h. The Dox-loaded amino acids-modified β-CD platinum complexes containing medium were added to the cells at a concentration of 5 μM. After incubation for 6 h, the cells were thoroughly washed with PBS buffer to remove samples that were not permeated. After washing, the cell nuclei were stained with Hoechst 33342 solution. Then the cells were analysed by a high content screening (HCS) technique immediately under excitation at 488 nm.

3. Results and discussion

3.1. Synthesis and characterization of the platinum complexes

The complexes Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2 were prepared via the reaction of K_2PtCl_4 with ligands L-Arg-β-CD and L-Phe-β-CD in suitable yields. ESI-MS spectrometry is an important method for the investigation of the formation of platinum complexes. Fig. S1 and S2 in the Supporting Information showed the MS spectra of the Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2. The ESI-MS spectrum in Fig. S1 in the Supporting Information showed the molecular ion fragment peaks at *m/z* 1579.1 and *m/z* 798.0 which was assigned to the complexes $[Pt(L\text{-}Arg\text{-}\beta\text{-}CD)Cl_2+Na]^+$ and $[Pt(L\text{-}Arg\text{-}\beta\text{-}CD)Cl_2+H_2O]^{2+}$, respectively. Moreover, the ESI-MS spectrum in Fig. S2 in the Supporting Information showed the species of *m/z* 1546.3 which was assigned to the complex $[Pt(L\text{-}Phe\text{-}\beta\text{-}CD)Cl_2]^-$. These results provided strong evidence for the presence of the corresponding platinum complexes. Fig. S3 and S4 in the Supporting Information showed the ^1H NMR spectra of Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2 complexes. The disappearance of carboxylic acid signal and the shift of amido proton are both indicative of the coordination of platinum as shown in Scheme 1.

3.2. DNA binding properties of the platinum complexes

The DNA binding properties of the complexes to CT-DNA were investigated by various spectral techniques. CD is a useful technique to assess whether nucleic acids undergo conformational changes as a result of complex formation or environmental

changes. Fig. 1 showed the CD spectral change upon the titration of Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2 in Tris-HCl buffer at pH 7.4, respectively. In Fig. 1a, the presence of Pt(L-Arg-β-CD)Cl_2 induced increase in intensity at ~220 and ~245 nm but decrease at ~277 nm, indicating that there was perhaps non-covalent interaction between Pt(L-Arg-β-CD)Cl_2 and DNA [36]. In Fig. 1b, the presence of Pt(L-Phe-β-CD)Cl_2 displayed negaligible effect on the CD spectrum, indicating that perhaps the complexes could not bind to DNA [37]. This may be due to the disruption of base stacking, whichis required to optimize the binding interactions [38]. More significantly, in Fig. 1a a strong negative band at 295 nm was observed, suggesting the presence of Pt(L-Arg-β-CD)Cl_2 induced conformational change of the CT-DNA from B to Z-configuration [37]. These may reflect local untwisting of the CT-DNA helical backbone and changes in relative orientation of the bases to accommodate the probes within a particular base pair [39, 40]. The results indicated the different interaction modes or strength between the binding of the two complexes to CT-DNA.

UV-vis spectroscopy were also employed to investigate DNA binding ability of Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2. As shown in Fig. 2, the UV-vis absorption spectra both showed hyperchromic effect with the addition of Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2, and the hyperchromic effect became stronger with the increased complexes concentration. It was also noted that the UV-vis absorption at 256 nm split into two bands (231 nm and 272 nm) in the case of Pt(L-Arg-β-CD)Cl_2 (Fig. 2a). The hyperchromic effect might be ascribed to

electrostatic binding or partial uncoiling of the helix structure of DNA, exposing more bases of the DNA [41]. These results confirmed that the complexes could bind to DNA, in accordance with the results of CD, and further implied that the intercalation between the base pairs was not prevalent in these cases.

The binding abilities of cisplatin, ligands and complexes to pBR322 DNA were evaluated by gel electrophoresis. As shown in Fig. 3, the mobility of DNA decreased, after incubated with complex Pt(L-Arg-β-CD)Cl_2 and cisplatin for 24 h, whereas the ligands L-Arg-β-CD, L-Phe-β-CD and complex Pt(L-Phe-β-CD)Cl_2 had little effect on the mobility of DNA. It is obvious that the complex Pt(L-Arg-β-CD)Cl_2 could bind to pBR322 DNA in a similar way as cisplatin. The increaseed mobility indicates that the binding of the platinum complexes changes the sign of the writhe of DNA to a more positive value [42, 43]. It also indicates that complex Pt(L-Arg-β-CD)Cl_2 is able to bind to plasmid DNA more efficiently than ligands L-Arg-β-CD, L-Phe-β-CD and complex Pt(L-Phe-β-CD)Cl_2, which suggests a plausible synergistic effect between the Pt(II) ion and the positively- charged arginine residue.

The concentration effect was also evaluated for Pt(L-Arg-β-CD)Cl_2, Pt(L-Phe-β-CD)Cl_2 and cisplatin. As shown in Fig. 4, the mobility of DNA decreased with the increase of concentrations of Pt(L-Arg-β-CD)Cl_2 and cisplatin. However, such mobility disappeared when the concentration of Pt(L-Phe-β-CD)Cl_2 reaches 180 μM (Fig. 4b). Such difference suggested that Pt(L-Arg-β-CD)Cl_2 exhibit more effective binding activity with DNA compared to Pt(L-Phe-β-CD)Cl_2, and the binding

mode to DNA might be in a non-intrusive way similar to cisplatin [44]. A possible explanation is that the complex Pt(L-Arg-β-CD)Cl_2 has more positive charges and less steric than complex Pt(L-Phe-β-CD)Cl_2, which benefits the covalent interaction of Pt(L-Arg-β-CD)Cl_2 with DNA. On the other hand, the hydrophobic phenyl group in complex Pt(L-Phe-β-CD)Cl_2 may be embedded in the hydrophobic cavity of the β-CD, which increases the steric hindrance and hampers its interaction with DNA [45].

3.3 Drug delivery ability of platinum complexes

To evaluate the drug delivery ability of amino acids-modified β-CD platinum complexes as hydrophobic anticancer drugs, a strategy via host-guest interactions was proposed in Scheme 1. The two Dox-loaded amino acids-modified β-CD platinum complexes were prepared with the ultrasonic method at room temperature and protected from light. Dox was loaded into lipophilic central cavities of β-CD by hydrophobic interaction. UV-vis spectroscopy was used to prove the synthetic product and the optimum molar ratio of Dox-loaded amino acids-modified β-CD platinum complexes. The UV-vis spectra of Dox-loaded platinum complexes at different molar ratios of Dox to amino acids-modified β-CD platinum complexes are shown in Fig. 5. Dox had a maximum absorption peak at 498 nm, and this absorption increased upon the addition of two amino acids-modified β-CD platinum complexes, indicating that the β-CD formed inclusion complexes with drug Dox [46, 47]. Moreover, when the

molar ratio between Dox and amino acids-modified β-CD platinum complexes was 1:1, the absorption reached the maximum, suggesting that the inclusion complex was most stable at the ratio of 1:1 for Dox-loaded amino acids-modified β-CD platinum complexes, thus 1:1 was the matching molar ratio.

In order to further clarify the optimum molar ratio for producing the most stable complexes and forming inclusion complexes, the Dox-loaded amino acids-modified β-CD platinum complexes were characterized by fluorescence spectra. The fluorescence spectra of Dox-loaded amino acids-modified β-CD platinum complexes formed at different molar ratios of Dox to amino acids-modified β-CD platinum complexes were shown in Fig. 6. After adding amino acids-modified β-CD platinum complexes, the fluorescence intensity of the Dox-loaded amino acids-modified β-CD platinum complexes decreased sharply compared with free Dox, suggesting that both Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2 complexes had the ability to encapsulate Dox in PBS. Besides, when the molar ratio between Dox and both the two amino acids-modified β-CD platinum complexes was 1:1, the emission spectra reached the minimum, producing the most stable complexes.

In addition, MTT experiments were also carried out to validate the optimum molar ratio of the Dox to amino acids-modified β-CD platinum complexes. As shown in Fig. 7, the Dox-loaded amino acids-modified β-CD platinum complexes presented higher cell inhibition ratio compared to free Dox, confirming that Dox was encapsulated within lipophilic central cavities of β-CD. Cell inhibition ratio reached

maximum at the molar ratio of 1:1, which was consistent with the results of UV-vis and fluorescence spectroscopy. In other words, the intracellular drug concentration of the more stable Dox-loaded amino acids-modified β-CD platinum complexes was higher, leading to higher cell inhibition ratio of the drug combination system-treated cells compared to free Dox-treated (or platinum complex-treated) cells at the same concentration (5 μM).

The above results proved that amino acids-modified β-CD platinum complexes could solubilize hydrophobic anticancer drugs and form stable combination system of drug and amino acids-modified β-CD platinum complexes. Therefore, based on the combined results of UV-vis and fluorescence experiments, it can be summarized that amino acids-modified β-CD platinum complexes can encapsulate hydrophobic anticancer drugs into the cavities of β-CD to obtain drug combination system, thereby possessing very good loading ability for anticancer drugs.

3.4. Cell inhibition ratio of platinum complexes and Dox-loaded platinum complexes

In order to investigate the cell inhibition ratio of the Dox-loaded amino acids-modified β-CD platinum complexes and amino acids-modified β-CD platinum complexes, cytotoxic activity was examined by incubating the three types of cells (K562, HepG2 and 7701) with different concentrations of complexes, cisplatin and Dox, respectively, for 48 h. The IC_{50} values of the complexes, cisplatin and Dox by the MTT assay at 48 h were shown in Table 1. And the cell inhibition ratios of

Dox-loaded platinum complexes, platinum complexes, cisplatin and Dox were shown in Fig. 8. The cell inhibition ratio of L-Arg-β-CD and L-Phe-β-CD in K562, HepG2, and 7701 cells were all under 35%, which were relatively low even with higher concentrations, indicating that the ligands amino acids-modified β-CD had no significant growing inhibition to cells. For the same cell, Pt(L-Arg-β-CD)Cl_2 showed better activity than complex Pt(L-Phe-β-CD)Cl_2, L-Arg-β-CD and L-Phe-β-CD but it was much less active than cisplatin. Perhaps it was the positive charge of the L-Arg resulting in a considerable increase in the intracellular delivery of Dox. As expected, the cell growth inhibition efficiency of amino acids-modified β-CD platinum complexes-encapsulated Dox was higher than that of either amino acids-modified β-CD platinum complexes or free Dox. That is to say, encapsulated Dox via amino acids-modified β-CD platinum complexes is able to increase the cell inhibition ratio versus free Dox by increased accumulation of intracellular drug. The cell inhibition ratio of the combination system of Dox and amino acids-modified β-CD platinum complexes increased approximately 3 folds compared to free Dox. And the cell inhibition ratio under our experimental conditions (final concentration ≤ 1% DMSO) were in the order: Pt(L-Arg-β-CD)Cl_2/Dox > Pt(L-Phe-β-CD)Cl_2/Dox > Dox > cisplatin > Pt(L-Arg-β-CD)Cl_2 > Pt(L-Phe-β-CD)Cl_2 > free ligands (L-Arg-β-CD and L-Phe-β-CD). It was also observed that the two types of Dox-loaded platinum complexes had less toxicity in the normal 7701 cells than other two types of cancer cells. The synergistic effects of platinum complexes and Dox increased the

intracellular drug concentration and the percentage of cell death. Thus, the Dox-loaded platinum complexes had high cell inhibition ratio and certain selectivity for cancer cells.

3.5. Cellular uptake of Dox-loaded platinum complexes

We further studied the cellular uptake of Dox-loaded platinum complexes by high content screening (HCS) (Fig. 9). Fig. 9 showed that after 2 h incubation with the Pt(L-Arg-β-CD)Cl_2/Dox, the red fluorescence from Dox was observed in the cytoplasm of cells and the intensity was weak, indicating that the Dox was still loaded by β-CD. After 6 h of incubation, the red fluorescence started to aggregate and the intensity increased, indicating that Dox was released from the combination system Pt(L-Arg-β-CD)Cl_2/Dox.

Moreover, we compared the fluorescence intensity of free Dox and two Dox-loaded platinum complexes in cells. Fig. 10 showed that the red fluorescence from Dox was all observed in the cytoplasm of HepG2 cells after 24 h incubation with the free Dox and Dox-loaded platinum complexes. We can see that the intensity of the red fluorescence from free Dox was weak in the cytoplasm in HepG2 cells. However, the intensity of the red fluorescence displayed elevated increase in HepG2 cells based on the delivery of Dox by Pt(L-Arg-β-CD)Cl_2 and Pt(L-Phe-β-CD)Cl_2, suggesting the efficient transport of Dox across the membranes with the aid of the β-CD. Comparing the two combination system of Pt(L-Arg-β-CD)Cl_2/Dox and Pt(L-Phe-β-CD)Cl_2/Dox,

the red fluorescence signal of Dox delivered by Pt(L-Arg-β-CD)Cl_2 was stronger than that delivered by the Pt(L-Phe-β-CD)Cl_2, suggesting Pt(L-Arg-β-CD)Cl_2 possessed the higher Dox transport efficiency. This is probably because the Dox was loaded into the Pt(L-Arg-β-CD)Cl_2 more easily than the Pt(L-Phe-β-CD)Cl_2 due to the stronger positive charge of the Pt(L-Arg-β-CD)Cl_2. Thus, the combination system of Pt(L-Arg-β-CD)Cl_2/Dox and Pt(L-Phe-β-CD)Cl_2/Dox could affect Dox accumulation and showed great potential as antitumor drugs.

4. Conclusion

In conclusion, we reported two water-soluble amino acids-modified β-CD platinum complexes, which showed preferable binding ability to DNA and effective inhibition to cancer cells. They bind to and unwind pBR322 DNA in a manner similar to cisplatin. Besides, the combination system of Dox and amino acids-modified β-CD platinum complexes was synthesized and it could effectively deliver the anticancer drug Dox into cells and had higher cell inhibition ratio, with less toxicity in the normal cells than in cancer cells. The combination system of Dox and amino acids-modified β-CD platinum complexes in cells helped to promote the synergistic effects of the drug Dox and platinum, enhancing intracellular drug accumulation and potency to kill cancer cells. Hence, the amino acids-modified β-CD platinum complexes is capable of deliver antitumor drug to form the combination system and show great potential as antitumor drug.

5. Abbreviations

β-CD	β-cyclodextrin
Dox	doxorubicin
HCS	high content screening
FBS	Fetal bovine serum
DMEM	Dulbecco Minimum Essential Medium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CT-DNA	calf thymus DNA
CD	Circular dichroism
EB	ethidium bromide
PBS	phosphate-buffered saline
IC_{50}	50% inhibitory concentration
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid

Acknowledgements

This work was supported by the Project supported by the National Natural Science Foundation of China (No., 20901005, U1204201), Postdoctoral Science Foundation Funded Project of China (No. 2012M511570) and Department of Education Science and Technology Research Key Projects of Henan (13A150063).

References

[1] W. Liu, R. Gust, Chem. Soc. Rev. 42 (2013) 755-773.

[2] J.S. Butler, P.J. Sadler, Curr. Opin. Chem. Biol. 17 (2013) 175-188.

[3] K. Mitra, U. Basu, I. Khan, B. Maity, P. Kondaiah, A.R. Chakravarty, Dalton Trans. 43 (2014) 751-763.

[4] F. Bouyer, J. Moretto, D. Pertuit, A. Szollosi, M.A. Lacaille-Dubois, Y. Blache, B. Chauffert, N. Desbois, J. Inorg. Biochem. 110 (2012) 51-57.

[5] L. Cubo, A.G. Quiroga, J. Zhang, D.S. Thomas, A. Carnero, C. Navarro-Ranninger, S.J. Berners-Price, Dalton Trans. 18 (2009) 3457-3466.

[6] S.H. van Rijt, P.J. Sadler, Drug Discov. Today 14 (2009) 1089-1097.

[7] N.L. McGinely, J.A. Plumb, N.J. Wheate, J. Inorg. Biochem. 128 (2013) 124-130.

[8] B. Maity, S. Gadadhar, T.K. Goswami, A.A. Karande, A.R. Chakravarty, Eur. J. Med. Chem. 57 (2012) 250-258.

[9] E. Gabano, M. Ravera, D. Osella, Curr. Med. Chem.16 (2009) 4544-4580.

[10] V. Del Solar, A. Quiñones-Lombraña, S. Cabrera, J.M. Padrón, C. Ríos-Luci, A. Alvarez-Valdés, C. Navarro-Ranninger, J. Alemán, J. Inorg. Biochem. 127 (2013) 128-140.

[11] C. Martín Santos, S. Cabrera, C. Ríos-Luci, J.M. Padrón, I. López Solera, A.G. Quiroga, M.A. Medrano, C. Navarro-Ranninger, J. Alemán, Dalton Trans. 42 (2013) 13343-13348.

[12] S. El Kazzouli, N. El Brahmi, S. Mignani, M. Bousmina, M. Zablocka, J.-P. Majoral, Curr. Med. Chem. 19 (2012) 4995-5010.

[13] N.J. Farrer, J.A. Woods, L. Salassa, Y. Zhao, K.S. Robinson, G. Clarkson, F.S. Mackay, P.J. Sadler, Angew. Chem. Int. Ed. 122 (2010) 9089-9092; Angew. Chem. Int. Ed. Engl. 15 (2010) 8905-8908.

[14] D. Xu, Y. Min, Q. Cheng, H. Shi, K. Wei, F. Arnesano, G. Natile, Y. Liu, J. Inorg. Biochem. 129 (2013) 15-22.

[15] X. Wang, Z. Guo, Chem. Soc. Rev. 42 (2013) 202-224.

[16] L. Cubo, A.M. Pizarro, A.G. Quiroga, L. Salassa, C. Navarro-Ranninger, P.J. Sadler, J. Inorg. Biochem. 104 (2010) 909-918.

[17] E.R. Jamieson, S.J. Lippard, Chem. Rev. 99 (1999) 2467-2498.

[18] O. Pinato, C. Musetti, N.P. Farrell, C. Sissi, J. Inorg. Biochem. 122 (2013) 27-37.

[19] B. Liskova, L. Zerzankova, O. Novakova, H. Kostrhunova, Z. Travnicek, V. Brabec, Chem. Res. Toxicol. 25 (2012) 500-509.

[20] M. Vieites, P. Smircich, M. Pagano, L. Otero, F.L. Fischer, H. Terenzi, M.J. Prieto, V. Moreno, B. Garat, D. Gambino, J. Inorg. Biochem. 105 (2011) 1704-1711.

[21] A.P. Neves, M.X.G. Pereira, E.J. Peterson, R. Kipping, M.D. Vargas, F.P. Silva-Jr, J.W.M. Carneiro, N.P. Farrell, J. Inorg. Biochem. 119 (2013) 54-64.

[22] J. Zhang, P.X. Ma, Adv. Drug Delivery Rev. 65 (2013) 1215-1233.

[23] A. Magrì, F. D'Alessandro, D.A. Distefano, T. Campagna, G. Pappalardo, G. Impellizzeri, D. La Mendola, J. Inorg. Biochem. 113 (2012) 15-24.

[24] Á. Martínez, C.O. Mellet, J.M.G. Fernández, Chem. Soc. Rev. 42 (2013) 4746-4773.

[25] R. Kanasty, JR. Dorkin, A. Vegas, D. Anderson, Nat. Mater. 12 (2013) 967-977.

[26] D. Dorokhin1, S.-H. Hsu, N. Tomczak, C. Blum, V. Subramaniam, J. Huskens, D.N. Reinhoudt, A.H. Velders, G. J. Vancso, small 6 (2010) 2870-2876.

[27] D. Dorokhin, S.H. Hsu, N. Tomczak, D.N. Reinhoudt, J. Huskens, A.H. Velders, G. J. Vancso, ACS Nano 4 (2010) 137-142.

[28] H. Tan, F. Qin, D. Chen, S. Han, W. Lu, X. Yao, Carbohydr. Polym. 93 (2013) 679-685.

[29] D. Gourevich, O. Dogadkin, A. Volovick, L.J. Wang, J. Gnaim, S. Cochran, A. Melzer, J. Controlled Release 170 (2013) 316-324.

[30] M.-X. Zhao, J.-M. Li, L. Du, C.-P. Tan, Q. Xia, Z.-W. Mao, L.-N. Ji, Chem. Eur. J. 17 (2011) 5171-5179.

[31] M.-X. Zhao, Q. Xia, X.-D. Feng, X.-H. Zhu, Z.-W. Mao, L.-N. Ji, K. Wang, Biomaterials 31 (2010) 4401-4408.

[32] H. Mansouri-Torshizia, M. I-Moghaddama, A. Divsalarb, A.-A. Saboury, Bioorg. Med. Chem. 16 (2008) 9616-9625.

[33] P. de Hoog, M. Pitié, G. Amadei, P. Gamez, B. Meunier, R. Kiss, J. Reedijk, J. Biol. Inorg. Chem. 13 (2008) 575-586.

[34] R.P. Hertzberg, P.B. Dervan, Biochem. 23 (1984) 3934-3945.

[35] H.-Z. Wang, C.-H. Chang, C.-P. Lin, M.-C. Tsai, J. Ocul. Pharmacol. Ther. 12 (1996) 35-43.

[36] J.-T. Wang, Y. Li, J.-H. Tan, L.-N. Ji, Z.-W. Mao, Dalton Trans. 40 (2011) 564-566.

[37] M. Uemura, Y. Yoshikawa, M. Chikuma, S. Komeda, Metallomics 4 (2012) 641-644.

[38] T. Bugarcic, O. Novakova, A. Halamikova, L. Zerzankova, O. Vrana, J. Kasparkova, A. Habtemariam, S. Parsons, P.J. Sadler, V. Brabec, J. Med. Chem. 51 (2008) 5310-5319.

[39] Y. Nakamura, Y. Taruno, M. Sugimoto, Y. Kitamura, H.L. Seng, S.M. Kong, C.H. Nge, M. Chikira, Dalton Trans. 42 (2013) 3337-3345.

[40] Y.F. Long, Q.G. Liao, C.Z. Huang, J. Ling, Y.F. Li, J. Phys. Chem. B 112 (2008) 1783-1788.

[41] C. Pratviel, J. Bernadou, B. Meunier, Adv. Inorg. Chem. 45 (1998) 251-312.

[42] S.M. Valiahdi, A.E. Egger, W. Miklos, U. Jungwirth, K. Meelich, P. Nock, W. Berger, C.G. Hartinger, M. Galanski, M.A. Jakupec, B.K. Keppler, J. Biol. Inorg. Chem. 18 (2013) 249-260.

[43] Y. Shi, J.C. Dabrowiak, Inorg. Chim. Acta 393 (2012) 337-339.

[44] C. Bartel, A.K. Bytzek, Y.Y. Scaffidi-Domianello, G. Grabmann, M.A. Jakupec, C.G. Hartinger, M. Galanski, B.K. Keppler, J. Biol. Inorg. Chem. 17 (2012) 465-474.

[45] D. Prashar, Y. Shi, D. Bandyopadhyay, J.C. Dabrowiak, Y.-Y. Luk, Bioorg. Med. Chem. Lett. 21 (2011) 7421-7425.

[46] A. Okamatsu, K. Motoyama, R. Onodera, T. Higashi, T. Koshigoe, Y. Shimada, K. Hattori, T. Takeuchi, H. Arima, Bioconjugate Chem. 24 (2013) 724-733.

[47] M.S. Aw, M. Kurian, D. Losic, Chem. Eur. J. 19 (2013) 12586-12601.

Table legends:

Table 1. Summary of the cytotoxicity activity of complexes.

Samples	IC_{50} (μM)		
	K562	HepG2	7701
L-Arg-β-CD	> 200	> 200	> 200
Pt(L-Arg-β-CD)Cl_2	84.2±2.6	77.5±3.2	117.5±3.6
L-Phe-β-CD	> 200	> 200	> 200
Pt(L-Phe-β-CD)Cl_2	115.6±3.5	118.6±2.4	135.5±3.3
Cisplatin	22.6±3.4	22.9±2.2	25.6±2.7
Dox	9.4±2.8	9.3±3.1	9.6±2.6
Pt(L-Arg-β-CD)Cl_2/Dox	3.4±2.6	2.9±2.1	12.7±2.2
Pt(L-Phe-β-CD)Cl_2/Dox	3.7±3.3	3.1±2.7	10.2±3.2

Figure and Scheme Legends:

Scheme 1. Synthetic strategy of L-amino acid-modified β-CD platinum complexes and Dox loading into L-amino acid-modified β-CD platinum complexes and the cellular uptake of Dox-loaded amino acids-modified β-CD platinum complexes.

Fig. 1. CD spectra of CT-DNA modified by complexes at different complexes-to-DNA ratios. (a) DNA was titrated with Pt(L-Arg-β-CD)Cl_2 at r of 0, 0.5, 1.0, respectively; (b) DNA was titrated with Pt(L-Phe-β-CD)Cl_2 at r of 0, 0.5, 1.0, respectively.

Fig. 2. Absorption spectra of complexes (a) Pt(L-Arg-β-CD)Cl_2 and (b) Pt(L-Phe-β-CD)Cl_2 in Tris-HCl buffer in the presence of CT-DNA with increasing amounts of complexes. [CT-DNA] = 100 μM, [complexes] = 0-180 μM from bottom to top. Arrows indicate the change in absorbance upon increasing the complexes concentration.

Fig. 3. Agarose gel electrophoresis images of pBR322 DNA after incubated with cisplatin, ligands and complexes in 20 mM HEPES, pH 7.20 at 37 ^{o}C for 24 h. **Lane 0** (control), contained only 50 μM bp DNA ; **lane 1**, 50 μM DNA+50 μM of cisplatin, **lane 2**, 50 μM DNA+200 μM of ligand L-Arg-β-CD, **lane 3**, 50 μM DNA+200 μM of complex Pt(L-Arg-β-CD)Cl_2, **lane 4**, 50 μM DNA+200 μM of ligand L-Phe-β-CD, **lane 5**, 50 μM DNA+200 μM of complex Pt(L-Phe-β-CD)Cl_2.

Fig. 4. The concentration effect of complexes and cisplatin on their DNA (50 μM bp) unwinding activity, (a) Pt(L-Arg-β-CD)Cl_2, (b) Pt(L-Phe-β-CD)Cl_2, and (c) cisplatin, pH 7.20 at 37 ^{o}C for 24 h. Lane 1 for a, b, c: DNA control; lanes 2-10 for a, b: 20 μM, 40 μM, 60 μM, 80 μM, 100 μM, 120 μM, 140 μM, 160 μM, 180 μM; lanes 2-10 for c: 5 μM, 10 μM, 15 μM, 20 μM, 25 μM, 30 μM, 40 μM, 50 μM, 60 μM.

Fig. 5. UV-vis absorption spectra of (a) Pt(L-Arg-β-CD)Cl_2 and (b) Pt(L-Phe-β-CD)Cl_2 at different molar ratios of Dox to amino acids-modified β-CD platinum complexes with a fixed concentration of Dox (5 μM) in PBS solution.

Fig. 6. Fluorescence emission spectra of (a) Pt(L-Arg-β-CD)Cl_2 and (b) Pt(L-Phe-β-CD)Cl_2 at different molar ratios of Dox to amino acids-modified β-CD

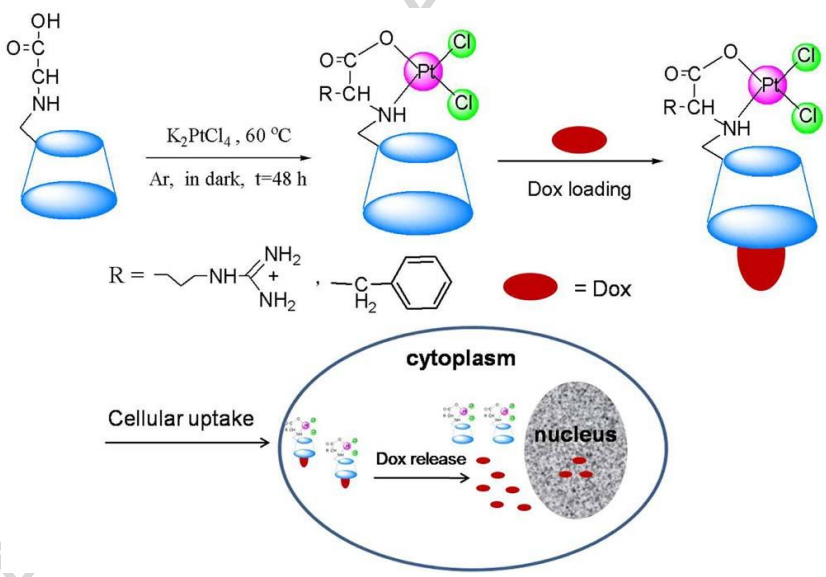
platinum complexes with a fixed concentration of Dox (5 μM) in PBS solution (Ex: 485 nm).

Fig. 7. The cytotoxicity of free Dox, Pt(L-Arg-β-CD)Cl_2/Dox and Pt(L-Phe-β-CD)Cl_2/Dox was evaluated by MTT assay, after incubation with Dox and Dox-loaded amino acids-modified β-CD platinum complexes at different molar ratios (the molar ratios between Dox and amino acids-modified β-CD platinum complexes are from 1:0.5 to 1:4) for 48 h in HepG2 cells. Cell viability was calculated by the ratio of absorbance of treated sample to that of untreated control. Dox concentrations in all samples were 5 μM.

Fig. 8. The cell inhibition ratio of Pt(L-Arg-β-CD)Cl_2/Dox, Pt(L-Phe-β-CD)Cl_2/Dox, free Dox, cisplatin, Pt(L-Arg-β-CD)Cl_2, Pt(L-Phe-β-CD)Cl_2, L-Arg-β-CD and L-Phe-β-CD in K562 (a), HepG2 (b), and 7701 (c) cells was evaluated by MTT assay.

Fig. 9. Time-dependent fluorescence imaging of Pt(L-Arg-β-CD)Cl_2/Dox (5 μM) in HepG2 cells. After the complexes adding to cells, fluorescence micrographs were obtained at 2 h, 6 h, and 12 h. Pt(L-Arg-β-CD)Cl_2/Dox entered cells after they were added into the cell culture for less than 2 h. The red fluorescence from Dox started to increase at incubation time of 6 h, indicating Dox separation from β-CD. Scale bars are 20 μm.

Fig. 10. Comparison of intracellular Dox accumulation among HepG2 cells treated with free Dox, Pt(L-Arg-β-CD)Cl_2/Dox, and Pt(L-Phe-β-CD)Cl_2/Dox for 24 h, with a fixed concentration of Dox and amino acids-modified β-CD platinum complexes at 5 μM. The nucleus is stained with Hoechst 33342, and Dox is indicated by red fluorescence.



Scheme 1

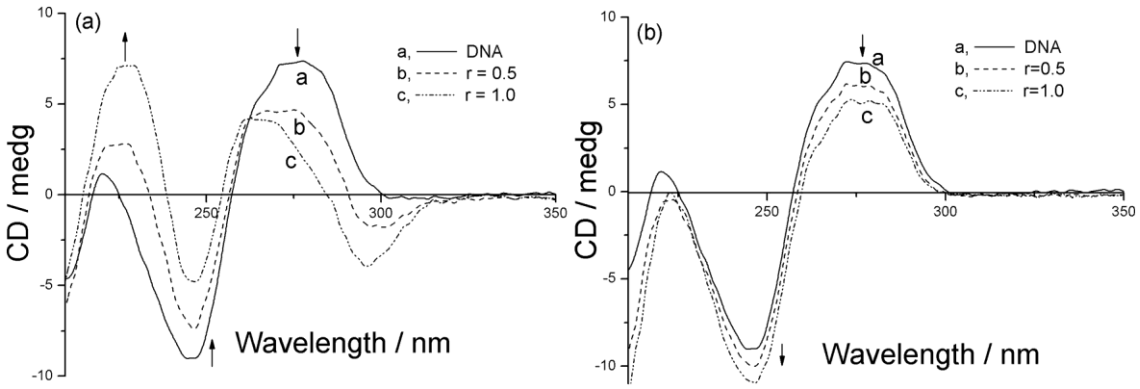


Fig. 1

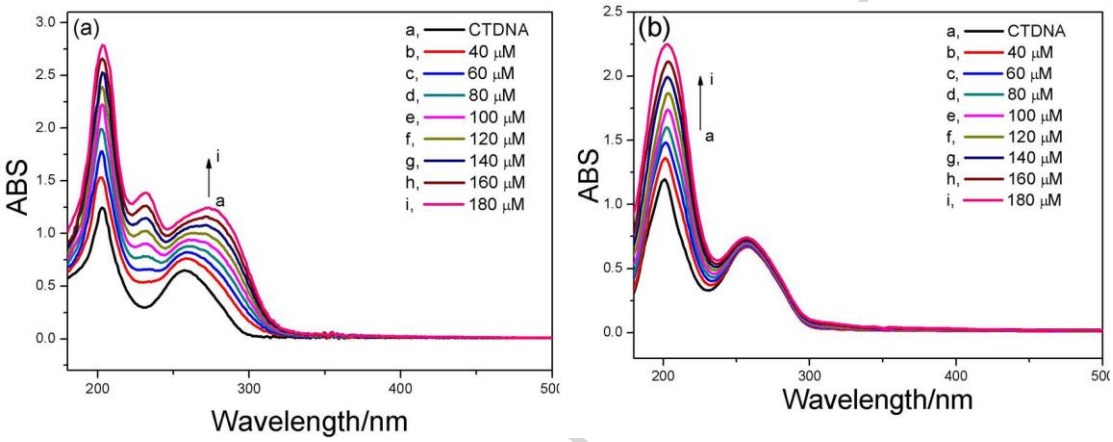


Fig. 2

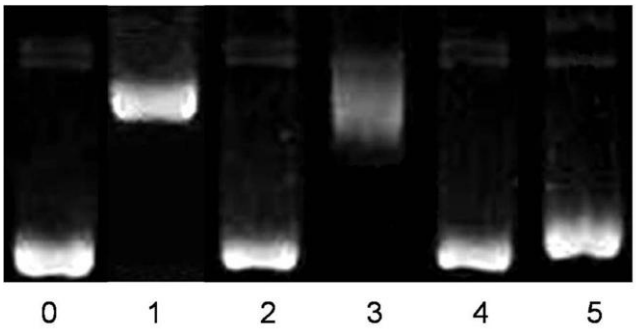


Fig. 3

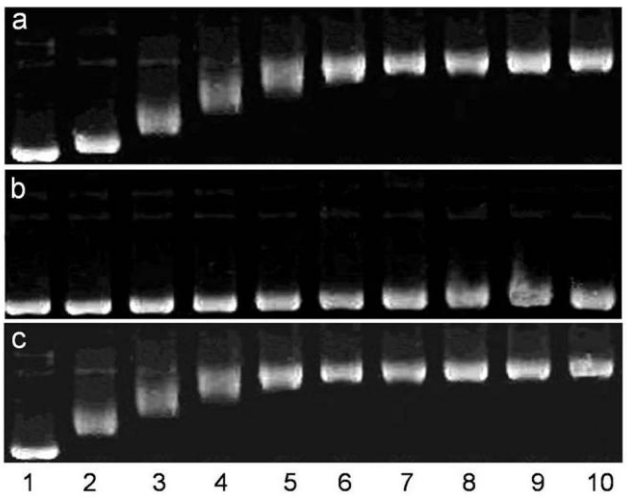


Fig. 4

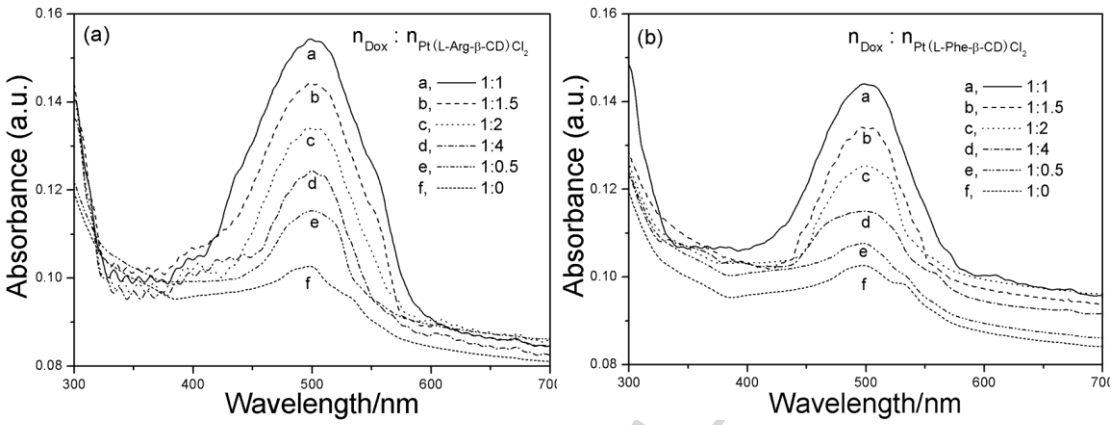


Fig. 5

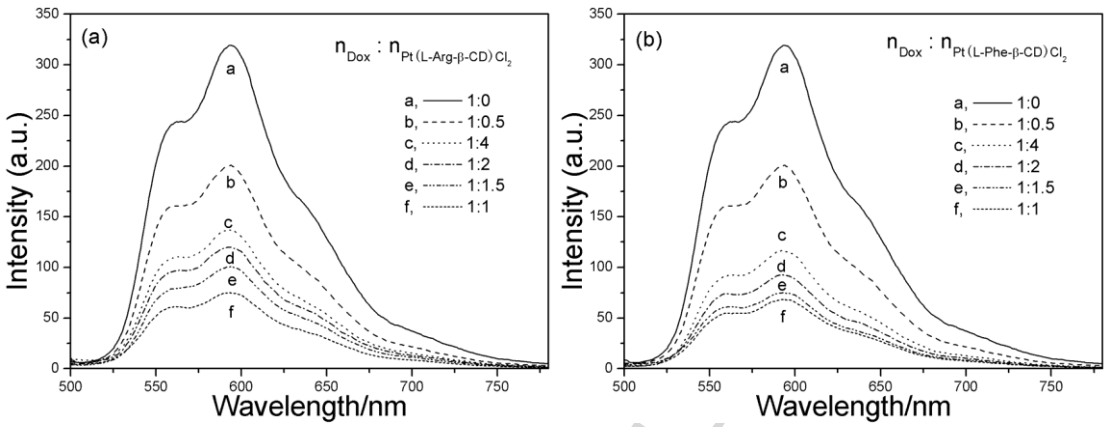


Fig. 6

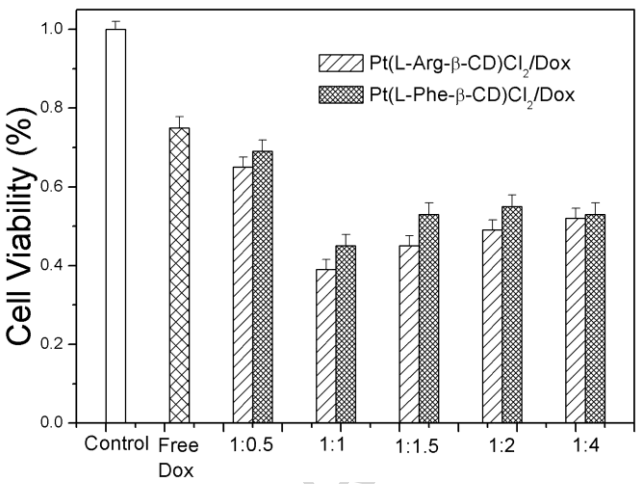


Fig. 7

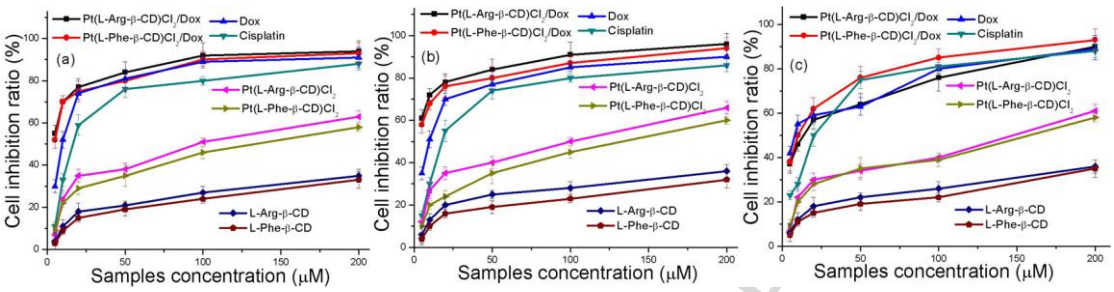


Fig. 8

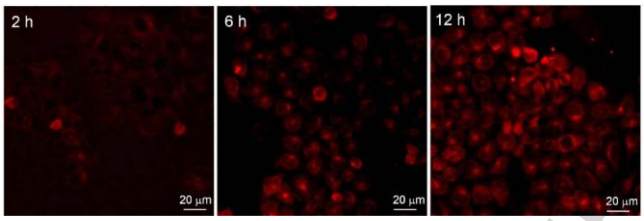


Fig. 9

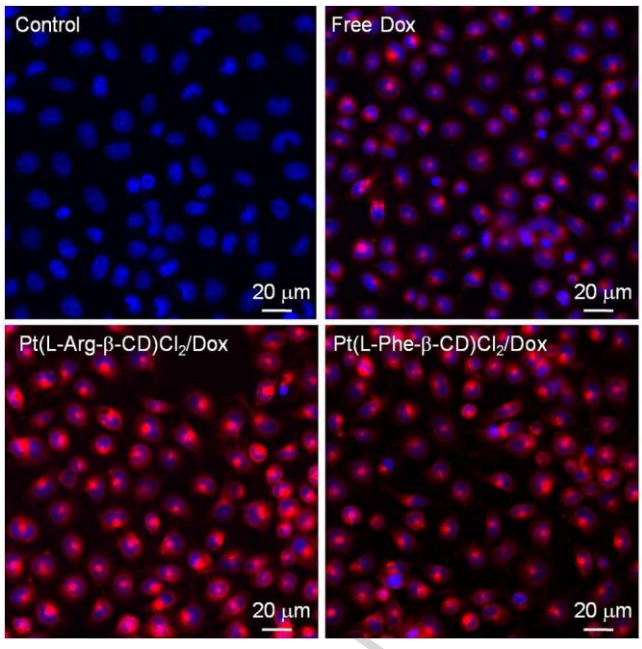
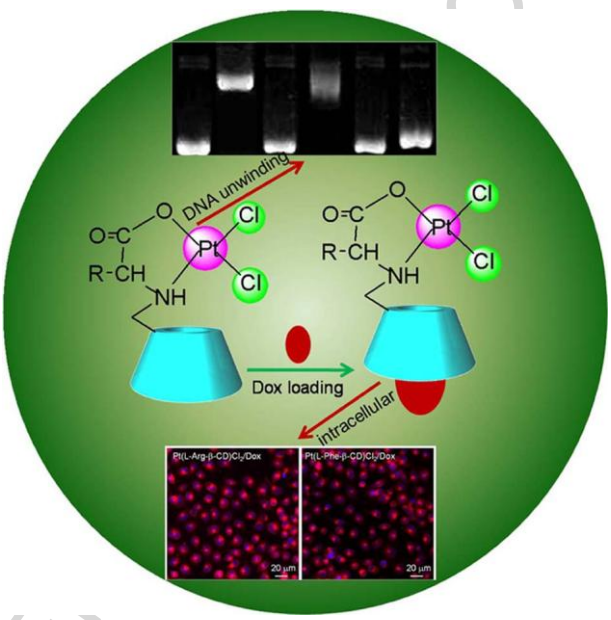


Fig. 10

Graphical abstract

Two water-soluble amino acids-modified β-CD platinum complexes can effective deliver the anticancer drug Dox, and showed preferable binding ability to DNA and effective inhibition on cancer cells. The amino acids-modified β-CD platinum complexes not only deliver antitumor drug Dox, but also show great potential as antitumor drug by enhancing intracellular drug accumulation.



Highlights

- Two water-soluble amino acids-modified β-CD platinum complexes were reported.
- The platinum complexes binds to and unwinds pBR322 DNA in a manner that is similar to that of cisplatin.
- The platinum complexes could effectively deliver Dox into cells and had higher cell inhibition ratio.
- The combination system helped to promote the synergistic effects of the Dox and platinum.
- The combination system enhance intracellular drug accumulation and increase potency of drug to kill cancer cells.