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Characterization and cellular uptake of platinum anticancer drugs encapsulated in apoferritin

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ABSTRACT

Clinical application of platinum-based anticancer drugs is largely limited by severe general toxicity and drug resistance. Drug delivery systems with tumor-targeting potential are highly desired for improving the efficacy and applicability of these drugs. This study describes an alternative strategy for the delivery of platinum drugs (cisplatin, carboplatin and oxaliplatin) by encapsulating each of them in the cavity of apoferritin (AFt). The encapsulation was achieved through manipulating the pH-dependent unfolding–refolding process of AFt at pH 2.0 and 7.4, respectively, in saturated drug solution. UV–vis spectrometry, circular dichroism spectrometry, dynamic light scattering, and inductively coupled plasma mass spectrometry were used to characterize the AFt–drug complexes. The loading capacity of AFt varies with respective drugs and the structural integrity of the protein shell remains intact after encapsulation. In vitro assays on the rat pheochromocytoma cell line (PC12) show that AFt–cisplatin inhibits the cells in a slow but sustaining mode and the cellular uptake of platinum is enhanced by AFt. AFt–carboplatin and AFt–oxaliplatin complexes only exhibit a marginal cytotoxicity towards this cell line under similar concentrations.

1. Introduction

Platinum-based anticancer drugs such as cisplatin (CDDP), carboplatin (CBDCA) and oxaliplatin (LOHP) (Fig. 1) have been widely used to treat various solid tumors [1]. However, the therapeutic window of these drugs is drastically narrowed by their severe systemic toxicity and drug resistance from tumors [2]. For example, CDDP produces nephrotoxicity, neurotoxicity, ototoxicity and induces inherent and acquired drug resistance; CBDCA shows cross-resistance to CDDP and causes myelosuppression; and LOHP engenders cumulative sensory peripheral nerve damage [3,4]. The toxicities are believed to be derived from the interactions of platinum drugs with healthy tissues during the transmission and distribution of the drugs in the body [5]; and the drug resistance may be associated with insufficient or ineffective cellular uptake of the drugs [6]. Therefore, how to reduce the general toxicity and to improve the efficacy become the major goals in the development of platinum-based drugs.

Targeted drug delivery to tumor tissues could abate the side effects. The realization of this intention, however, is heavily dependent on suitable targeting carriers. Copolymer- [7] or liposomal-based [8,9] carriers have been used to improve the delivery of platinum drugs for the enhanced permeability and retention (EPR) effect of macromolecules on tumors, but altered tissue distribution would lead to new toxicity profiles in clinical trials [10]. Recently, molecular containers with large cavities such as cucurbituril series were examined as drug delivery vehicles for platinum complexes [11], but poor water solubility and inefficient transport limit their potential application [12]. As an alternative, ligand–receptor-mediated delivery systems have attracted much attention because of their non-immunogenic and site-specific targeting potential to the ligand-specific bio-sites [13]. For instance, transferrin has been exploited for the delivery of CDDP into proliferating malignant cells that over-express transferrin receptors [14]. On the same score, biomolecules such as folic acid [15,16], estrogen [17], herceptin [18], and galactose residues [19,20] have also been incorporated into hybrid complexes to enhance the targeting property of the drugs towards tumor tissues with respective receptors. In our recent work, the demineralized ferritin, i.e. apoferritin (AFt), has been shown to be a promising vehicle for targeted delivery of platinum-based drugs [21]. AFt is a hollow cage with internal and external diameters of 8 and 12 nm, respectively [22]. Since ferritin-binding sites [23] and endocytosis of ferritin [24] have been identified in neoplastic cells, and receptors of ferritin have shown some potential in the delivery of anticancer drugs into the brain [25], AFt may enhance the drug selectivity for cell surfaces that express ferritin receptors.

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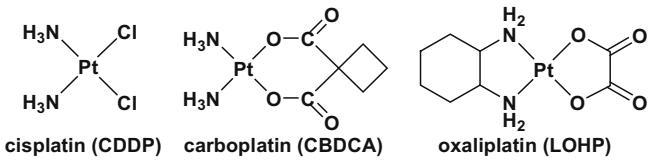

Fig. 1. Platinum-based anticancer drugs used in this work.

In this paper, we have extended our previous work to a wider range where LOHP is also involved in the encapsulation by AFt, and enriched the means of characterization for the AFt–drug complexes. More importantly, the dose-responsive and time-dependent cytotoxic profiles and the enhanced cellular uptake of AFt–CDDP complexes are observed against rat pheochromocytoma cell line (PC12). The three AFt–drug complexes are denoted hereafter as AFt–CDDP, AFt–CBDCA, and AFt–LOHP, respectively.

2. Experimental

2.1. Materials

Ferritin was purchased from Sigma. CDDP, CBDCA and LOHP were obtained from Shandong Boyuan Chemical Co., Ltd. Other chemical reagents were used as received without further purification. Doubly deionized water (18.2 MΩ cm at 25 °C) prepared on a Milli-Q (MQ) water system was used throughout all experiments.

2.2. Encapsulation of platinum drugs

AFt was obtained from the horse spleen ferritin by demineralization according to the established procedures [26]. The encapsulation of platinum-based drugs in the AFt cavity was carried out as described previously [21]. Briefly, the platinum drugs were dissolved, respectively, to get their saturated solutions (CDDP, 1 mg mL^{-1}; CBDCA, 15 mg mL^{-1}; LOHP, 5 mg mL^{-1}). AFt was added to each solution to reach a final protein concentration of 1 mg mL^{-1}. The dissociation of AFt into its subunits was induced by adjusting the pH of the mixed solution to 2.0 with HCl (1 M) and then maintaining this value for about 15 min. Afterwards, the pH was slowly adjusted back to 7.4 using NaOH (1 M). The resulting solution was stirred at 100 rpm under room temperature for 2 h and dialyzed against saline water (NaCl, 0.15 M, renewed three times) for 24 h to completely remove drug molecules outside of the protein shell. After that, the solution was centrifuged at 10,000 rpm for 10 min to remove the precipitates. The solutions of AFt–drug complexes were concentrated using Amicon Ultra-15 centrifugal filters (MWCO 50, Millipore, Bedford, MA). The concentration of AFt was determined in triplicate by the BCA protein assay kit (Pierce, Socachim, Switzerland) using bovine serum albumin as the standard.

2.3. Characterization of AFt and AFt–drug complexes

AFt and AFt–drug complexes were characterized by the following measurements. UV–vis absorption spectra were performed on a Shimazu UV-3100 spectrometer supplied with Perkin Elmer UV WinLab (version 1.1) computer software. Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter in the far-ultraviolet wavelength range of 190–250 nm in a quartz cell (0.1 cm) using following parameters: bandwidth, 1 nm; step resolution, 0.1 nm; scan speed, 10 nm min^{-1}; and response time, 1 s. The wavelength and optical rotation of the instrument have been calibrated by benzene vapor and d-10-camphorsulphonic acid, respectively. The data of each spectrum were the average of three scans. All samples were at the same protein concentration (0.5 mg mL^{-1}, 0.1 M NaCl). Hydrodynamic diameters were determined using a BI-200SM dynamic light scattering (DLS, Brookhaven Instruments Co., Holtsville, NY). The protein concentration of the samples is *ca.* 0.1 mg mL^{-1} in 0.1 M NaCl. All samples were filtered through a 0.45 μm filter before analysis and the average of triplicate values was adopted. The data were analyzed by the 9kdlsw_v3.50 software. Zeta potential (ζ) of AFt and AFt–drug complexes was measured in NaCl (0.1 M) with the protein concentration of *ca.* 0.1 mg mL^{-1} on a Malven Nano-Z instrument. The mean of triplicate measurements is taken as the final result.

2.4. Pt analysis

Pt analysis was performed on inductively coupled plasma mass spectrometer (ICP-MS) using a standard Plasma-Quad II instrument (VG Elemental, Thermo Optek Corp.). A three-point calibration curve was made for all measurements against Pt-containing solutions prepared by serial dilution of a certified reference standard. The most abundant isotope of Pt was monitored at m/z 195 [27]. The calibration was linear (typical $r > 0.999$) over the analytical working range (0.1–100 μg L^{-1}). The samples were diluted by 100-fold to make the final Pt concentration within the working range. The nitrolysis of the samples was carried out sequentially with concentrated HNO_3 at 95 °C for 2 h, H_2O_2 at 95 °C for 1.5 h and concentrated HCl at 37 °C for 0.5 h. Finally, the solution was diluted to 2 mL with MQ water and the Pt content was measured. The reported result of the sample is the average of three replicates. A Pt-free solution prepared likewise was used as a control in each test to detect any possible contamination; and in most cases the Pt content was below 0.5% of that in the samples. The Pt content in the final dialytic solutions was also determined to ensure the exhaustive dialysis.

2.5. Cytotoxicity assay

Growth inhibitory effect of the AFt–drug complexes on PC12 cells was tested by the MTT assay [28]. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (10%, v/v), streptomycin (0.1 mg mL^{-1}) and penicillin (100 U mL^{-1}) in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were seeded in 96-well plates at 5×10^3 cells per well in DMEM medium and incubated overnight, which were then treated in triplicate with fresh medium containing grade concentrations (on Pt) of AFt–drug complex with free drug and AFt as the references. The cells in the plates were incubated at 37 °C under 5% CO_2 for 72 h and were then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 10 μL) solution (5 mg mL^{-1}) in PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter) for 4 h. DMSO (150 μL) was added to each well after the medium was removed. The absorbance of the purple formazan was recorded at 490 nm using an ELISA plate reader. The cytotoxicity results were calculated based on the data of three replicate tests. The time-dependent growth inhibitory effect of AFt–CDDP on PC12 cells were carried out similarly at the IC_{50} concentration of AFt–CDDP (20 μM).

2.6. Cellular uptake

PC12 cells were seeded into a 12-well plate at 10^5 cells per well and incubated overnight. The medium was refreshed (1 mL per well) and the cells were treated with AFt–CDDP and CDDP, respectively, at the IC_{50} concentration of AFt–CDDP (20 μM) and incubated at 37 °C for 24 h. The medium was removed and the cells were incubated with trypsin solution for 1 min. After digestion,