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Design an anticancer copper(II) pro-drug based on the flexible IIA subdomain of human serum albumin

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ABSTRACT

Owing to the flexible IIA sub-domain of human serum albumin (HSA), we proposed to rationally design a metal agent with a leaving group, and then regulate a leaving group of metal agent to be displaced by His-242 for improving its delivery efficiency and selectivity. To confirm our hypothesis, we synthesized a copper(II) compound derived from 2-amino-5-chlorophenol 2-hydroxybenzaldehyde Schiff-base, containing a leaving group [pyridine, PRD], namely Cu(L)(PRD). The HSA complex structure revealed that Cu(L)(PRD) binds to the hydrophobic cavity in HSA IIA sub-domain, His242 of HSA replaces the pyridine ligand in Cu compound, coordinating with Cu^{2+}. HSA complex enhances cytotoxicity by about 1.4-fold in cancer cells but has no effect on normal cells *in vitro* through selectively accumulating into cancer cells. Interestingly, HSA complex has stronger anticancer capacity relative to unbound Cu(L)(PRD).

1. Introduction

On one hand, *cis*platin and its derivatives are highly effective in treating a variety of cancers, on the other hand, cures with *cis*platin and its derivatives are still limited because of dose-limiting side effects and inherited or acquired resistance phenomena [1–4]. Therefore, the alternative metal anti-cancer compounds have been extensively developed [5–9].

Excitingly, a large number of metal compounds have been evaluated *in vitro* and *in vivo* and some have reached clinical trials [10–12]. However, we still face the major challenge on how to improve the efficiency and selectivity of metal drugs [13]. Currently, the drug delivery systems and the pro-drug strategy have been the most promising for overcoming the above problems [14–17]. Among drug delivery systems, human serum albumin (HSA)-based drug delivery systems are promising and have been extensively studied owing to unique advantages relative to other drug carriers [18–21]. HSA is the most abundant protein in plasma, not only contains many special active residues, such as lysine and cystine, but also has several binding sites for a diverse group of endogenous and exogenous compounds [22–27]. Thus we can apply the nature of an HSA carrier to improve efficiency and the targeting of anticancer agents. For example, we can conjugate drugs to the special residues of albumin by a chemical linker [28]. Importantly, we can form the HSA complex by drugs directly binding to the HSA without introducing a chemical linker [29–35].

However, we may face two potential problems when the metal agent is delivered to cancer cells *via* complexation with an HSA carrier *in vivo*. Firstly, the metal agent is released from HSA during blood circulation because of weak binding. Secondly, the metal agent is not released at all from the HSA carrier in tumor cells due to tight binding. Therefore, to overcome the above-mentioned potential problems, Yang et al. proposed and confirmed that we can design a metal pro-drug based on the N-donor residues of the HSA IIA sub-domain [36–38]. Owing to the flexible IIA sub-domain of HSA, the binding mode of the drug in the HSA IIA sub-domain is directly tied to its molecular structure [39–44]. Therefore, we proposed to design a metal pro-drug with a leaving group, and then regulate the leaving group in the metal compound to be replaced by His242 in the HSA IIA sub-domain, which allows the metal compound to bind with HSA by a coordination bond, forming a stable HSA complex (Fig. 1). Subsequently, His242 of HSA is protonated in the cancer cell's lysosomal acidic environment, which decreases its coordination ability with metal ions, and allows the metal

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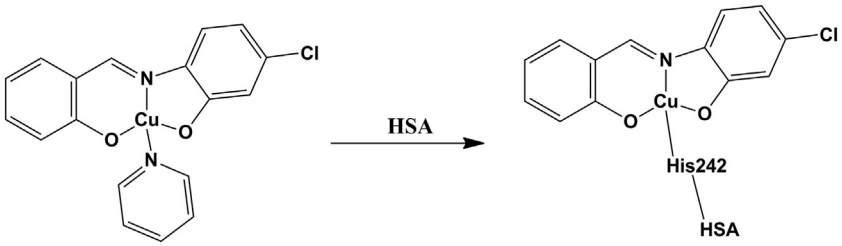

Fig. 1. The hypothesis of developing Cu pro-drug based on the flexible HSA IIA subdomain.

agent to be released from the HSA carrier [37,45].

Liver cancer has become very common worldwide and is responsible for causing a significant number of cancer deaths [46–49]. Furthermore, a Cu compound may be promising for next generation metal-based anti-cancer agents because Cu is an essential element for human physiological functions due to its bioactivity and oxidative nature [50]. Taking into consideration the above factors, we used a Cu(II) compound derived from Schiff-base and liver cancer cells (HepG2) to confirm our hypothesis. Our results may be helpful to develop the novel strategy of targeting cancer therapy with metal agents.

2. Materials and methods

Fatty acid free human serum albumin (catalogue number A3782) was purchased from Sigma Chemical Company. The other chemicals and solvents used had high purity levels and are available from commercial sources. The H_2O used in the reactions was distilled prior to use. Elemental analyses (C, H, and N) were carried out on a Perkin-Elmer 2400 analyser.

2.1. Development of Cu(L)(PRD)

2.1.1. Synthesis of Cu(L)(PRD)

2-amino-5-chlorophenol (0.5 mmol) and 2-hydroxybenzaldehyde (0.5 mmol) were dissolved in an aqueous methanol solution (20 mL) and stirred for 1 h to give an orange solution, which was added to a methanol solution (20 mL) of $Cu(NO_3)_2{\cdot}H_2O$ (0.5 mmol) and pyridine (0.5 mmol). The mixture was stirred for another 1 h at 60 °C to give a translucent solution and then filtered. The filtrate was kept in open air for one week, forming blue-black crystals. The crystals were isolated, washed three times with distilled water and dried in a vacuum dessicator containing anhydrous $CaCl_2$. Yield: 83%. Anal. Calcd for $C_{18}H_{13}ClCuN_2O_2$ (388.3): C, 55.68; H, 3.37 and N, 7.21. Found: C, 55.53; H, 3.35 and N, 7.42. IR(KBr, cm^{-1}): 1607ν(C = N), 689, 644, 462, 442 ν(Cu-N/Cu-O).

2.1.2. Characterization of Cu(L)(PRD)

X-ray crystallographic data were collected on a Bruker SMART Apex II CCD diffractometer using graphite-monochromated Mo-Kα (λ = 0.71073 Å) radiation. Empirical adsorption corrections were applied to all data using SADABS. The structures were solved by direct methods and refined against F^2 by full-matrix least-squares methods using the SHELXTL version 5.1 [51]. All of the non-hydrogen atoms were refined anisotropically and other hydrogen atoms were placed in geometrically ideal positions and constrained to ride on their parent atoms. The crystallographic data for Cu(L)(PRD) are summarized in Table S1. Selected bond lengths and angles are given in Table S2. Crystallographic data for the structural analyses have been deposited at the Cambridge Crystallographic Data Centre, reference numbers 1028868 for Cu(L)(PRD). The crystallographic data can be obtained free of charge from the Cambridge Crystallographic Data Centre *via* http://www.ccdc.cam.ac.uk/data_request/cif.

2.2. Determining binding affinity of Cu(L)(PRD) to HSA

The HSA solution (1 μM) was titrated by successive additions of the Cu(II) compound using micropipettes for all of the experiments. The fluorescence emission spectra were scanned from 300 to 420 nm after excitation at 280 nm. The binding constant of HSA for the compound can be analyzed according to the Scatchard eq. [52]:

$\log[(F_0 - F)/F] = \log K + n \times \log(Q)$where F and F_0 are the fluorescence intensities of protein in the presence and absence of the quencher, respectively; n is the number of binding sites; K is the binding constant and [Q] is the quencher concentration. From the plot of $\log[(F_0 - F)/F]$ *versus* log [Q], the number of binding sites (n) and the binding constant (K) were calculated.

2.3. Structural evidence of the Cu pro-drug design based on the flexible HSA IIA sub-domain

The complex of the Cu compound and HSA was prepared as published [36]. In brief, 100 μL HSA (100 mg/mL), 1.2 mL palmitic acid (PA) (2.5 mM) and 90 μL Cu compound (5 mM) were mixed overnight, and then the mixture was concentrated to 100 mg/mL with a Millipore spin filter (10,000 Da cutoff). Crystallization was carried out by sitting drop vapor diffusion at room temperature. An equal volume of the HSA complex was mixed with the reservoir solution, consisting of 28–32% (*w/v*) polyethylene glycol 3350, 50 mM potassium phosphate (pH 7.5), 5% glycerol, and 5% DMSO. Crystals were directly selected from the drop solution and then frozen in liquid nitrogen.

X-ray diffraction data were collected under Cryo-conditions (100 K) at BL17U beamline of the Shanghai Synchrotron Radiation Facility and then integrated and scaled with HKL2000 [53]. The HSA complex structure was solved by molecular replacement using PHASER in PHENIX suites with the initial model of a HSA-MYR structure (PDB:1BJ5) but stripped the ligand as initial searching model, all ligands were built into the model by Ligand Fit in PHENIX and manually modified and adjusted in COOT [54–56]. Figures depicting the structure were prepared by PyMOL [57]. Data collection details and unit cell parameters are given in Table 1.

2.4. Anti-cancer properties of the HSA complex in vitro

The Culture medium DMEM (with $_L$-glutamin), Antibiotic-Antimycotic and Fetal bovine serum (FBS) were from E.U. Gibco BRL. The human liver cancer cell lines HepG2 and human liver cell lines HL-7702 were purchased from the American Type Culture Collection and the German Collection of Microorganisms and Cell Cultures and were maintained in DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 mg/mL streptomycin at 37 °C and 5% CO_2.

Cytotoxicity assay (MTT). To gain deeper insight into the effect of HSA's interactions on the activity of the compound, two sets of experiments were performed: (1) the Cu(II) compound was dissolved in PBS with 1% DMSO and incubated with HSA for 24 h at room temperature, after which the HSA complex was incubated with the above cell lines for 48 h, and (2) the free Cu(II) compound was dissolved in PBS with 1% DMSO and then tested. The 180 μL of cell