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A comparative study on the interactions of human copper chaperone Cox17 with anticancer organoruthenium(II) complexes and cisplatin by mass spectrometry

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ABSTRACT

Herein we report investigation of the interactions between anticancer organoruthenium complexes, [(η^6-arene)Ru(en)(Cl)]PF_6 (en = ethylenediamine, arene = *p*-cymene (**1**) or biphenyl (**2**)), and the human copper chaperone protein Cox17 by mass spectrometry with cisplatin as a reference. The electrospray ionization mass spectrometry (ESI-MS) results indicate much weaker binding of the ruthenium complexes than that of cisplatin to apo-$Cox17_{2s\text{-}s}$, the functional state of Cox17. Up to tetra-platinated Cox17 adducts were identified while only mono-ruthenated and a little amount of di-ruthenated Cox17 adducts were detected even for the reactions with 10-fold excess of the Ru complexes. However, ESI-MS analysis coupled with liquid chromatography of tryptic digests of metalated proteins identified only three platination sites as Met4, Cys27 and His47 residues, possibly due to the lower abundance or facile dissociation of Pt bindings at other sites. Complexes **1** and **2** were found to bind to the same three residues with Met4 as the major site. Inductively coupled plasma mass spectrometry results revealed that ~7 mol Pt binding to 1 mol apo-$Cox17_{2s\text{-}s}$ molecules, compared to only 0.17 (**1**) and 0.10 (**2**) mol Ru to 1 mol apo-$Cox17_{2s\text{-}s}$. This is in line with the circular dichroism results that much larger unfolding extent of α-helix of apo-$Cox17_{2s\text{-}s}$ was observed upon cisplatin binding than that upon organoruthenium bindings. These results collectively indicate that Cox17 might not participate in the action of these anticancer organoruthenium complexes, and further verify the distinct anticancer mechanism of the organoruthenium(II) complexes from cisplatin.

1. Introduction

The human cytochrome c oxidase assembly protein 17 (Cox17; 7 kDa) is a key copper chaperone protein responsible for delivery of copper ions to mitochondrion [1,2]. It contains 63 residues with 6 conserved cysteines leading to three oxidation states based on the number of disulfide bonds. $Cox17_{2s\text{-}s}$, the intermediate state of Cox17, with two disulfide bonds and two free cysteine residues has been thought to be the functional state for transferring Cu(I) in the mitochondrial intermembrane space (IMS) [3]. The NMR solution structure showed a coiled coil-helix-coiled coil-helix domain in $Cox17_{2S\text{-}S}$ stabilized by two disulfide bonds involving Cys26–Cys55 and Cys36–Cys45 preceded by a flexible and completely unstructured N-terminal tail [1]. A number of researches have showed that human copper transport system is involved in the cytotoxic and resistance mechanism of cancer cells to the famous clinical anticancer drug cisplatin [4]. The resistance mechanism include reduced uptake by cancer cells, enhanced detoxification, elevated drug efflux, *etc.* [4,5]. For example, the P-type ATPases, ATP7A and ATP7B, the two copper efflux transporters, regulate the efflux of cisplatin [6]. Antioxidant Protein 1 (Atox1), receiving Cu from Ctr1 and delivering it to ATPase in the secretory compartment, can also interact with cisplatin and is involved in cisplatin resistance [7–9]. Copper transporter protein 1 (Ctr1), the major copper influx transporter, has been found to facilitate the uptake of cisplatin and its analogues as well [10,11]. More recent *in vitro* studies have also shown that Cox17 protein participates in cisplatin transfer to mitochondria and contributes to the overall cytotoxicity of cisplatin [12]. The interaction results showed

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that cisplatin bound to the $Cox17_{2s\text{-}s}$ protein at the thiols of the cysteinyl residues which are the copper coordination sites and such binding resulted in the copper release from the protein. Moreover, the platination of Cu^{I}-bound Cox17 by cisplatin is much higher than that of apo-Cox17 and such promotion effect was thought to be the Cu-Pt interactions in the platination adducts [12]. Interestingly, the presence of glutathione enhances the reactivity of cisplatin to Cox17, whereas reduces the reactivity of transplatin to Cox17 [13].

Cisplatin has been thought to trigger cancer cell apoptosis by attacking DNA [14,15], and is widely used to treat many solid human malignancies in clinic [16,17]. However, the acquired drug resistance and the toxicity to normal cells restricts its further wider applications [18]. Therefore many other transition metal complexes with potential anticancer activity have been developed [19–21]. Ruthenium complexes were among the most promising candidates since the spectrum of kinetics for their ligand substitution reaction is similar to that of platinum complexes and the versatile ligands make the structure and property of them more diverse. Two ruthenium(III) complexes, namely NAMI-A (New Antitumour Metastasis Inhibitor A, *trans*-$[RuCl_4(DMSO)(Im)]ImH$, Im = imidazole) [22] and KP1019 (*trans*-$[RuCl_4(Ind)_2]IndH$, Ind = indazole) [23], have entered the clinical trials. Distinct therapeutic effects were shown by these two ruthenium(III) complexes, despite their structure similarities. KP1019 is active against primary tumors, whereas NAMI-A is more active against metastases than against primary tumors [24,25]. It is believed that these ruthenium(III) complexes might be reduced to ruthenium(II) to exert their anticancer effect. With regard to this, a series of the half-sandwich ruthenium(II) based organometallic complexes ($[(\eta^6$-arene)Ru(en)(Cl)$]PF_6$, en = ethylenediamine, arene = benzene, *p*-cymene (**1**), biphenyl (**2**), tetrahydroanthrance, *etc.*) have been developed and show promising cytotoxicity both *in vitro* and *in vivo*, and even cytotoxic towards cisplatin-resistant cancer cell lines [26–28]. As for cisplatin, DNA is a potential target for the Ru(II) arene complexes, most of which bind selectively to N7 of guanine [29–32]. However, it has been proposed that proteins could play more important roles in the action of ruthenium drugs than in that of platinum drugs [33]. Previous results have also showed that these ruthenium arene complexes are also reactive towards peptides and proteins like glutathione (GSH) [34,35], human serum albumin (HSA) [36], human transferrin [37], protein tyrosine phosphatase 1B [38] and human glutathione-S-transferase π (GSTπ) [39] through binding to cysteine, methionine and/or histidine residues. Such interactions cannot be avoided as most of anticancer metallodrugs are administrated intravenously and the formation of drug-protein complexes will significantly influence the transport, distribution, bioavailability and toxicology of metallodrugs [40]. For example, cisplatin covalently bound to serum proteins like transferrin is hardly released inside cells and greatly reduces its amount reaching nuclei, severely decreasing its cytotoxicity, while the coordination to transferrin has little effect on the cytotoxicity of organometallic ruthenium(II) complexes [37]. These findings imply that protein bindings may play different roles in the action of metal-based anticancer complexes. Therefore, the involvement of Cox17 in the mechanism of action of cisplatin intrigues us to probe the roles of Cox17 in the action of ruthenium arene anticancer complexes, of which little has been reported so far.

In the present work, by using cisplatin as a reference, the interactions of organoruthenium(II) complexes **1** and **2** (Chart 1) and apo-$Cox17_{2s\text{-}s}$ were investigated by mass spectrometric method. The binding sites were identified by trypsin digestions combined with high performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) and the binding stoichiometry was also determined by inductively coupled plasma mass spectrometry (ICP-MS). Additionally, circular dichroism (CD) spectroscopy was applied to characterize the effect of ruthenium complexes binding on the conformation of $Cox17_{2s\text{-}s}$ protein.

2. Experimental section

2.1. Materials

Organoruthenium(II) complexes $[(\eta^6$-arene)Ru(en)(Cl)$]PF_6$ (arene = *p*-cymene (**1**) or biphenyl (**2**), en = ethylenediamine; Chart 1) were synthesized as described in the literature [27]. Cisplatin was purchased from Alfa Aesar. Trypsin (sequence grade) was bought from Promega, formic acid (FA) from Acros and acetonitrile (HPLC grade) from Merck. Microcon centrifugal ultrafilteration units with 3 kDa molecular weight cut-off were purchased from Millipore. Aqueous solutions were prepared using MilliQ water (Milli-Q Reagent Water System).

2.2. Expression and purification of Apo-$Cox17_{2s\text{-}s}$

The Cox17 protein was expressed in *Escherichia coli* and redox state was controlled as reported previously [12]. In brief, the coding sequence of human Cox17 was amplified by PCR and then inserted into a pST-SG vector using the ligation independent cloning (LIC) method. The recombinant plasmid was transformed into Origami B (DE3) competent cells for the expression of His_6-TEV site-$hCox17_{3S\text{-}S}$ fusion protein. The fused protein was purified using Ni^{2+}-NTA affinity chromatography. Then, the His_6-tag was removed by tobacco etch virus (TEV) protease. The protein was further purified through gel filtration, and the concentration was determined using Bradford method. The oxidized state protein $Cox17_{3S\text{-}S}$ was obtained through this process. The partially reduced state $Cox17_{2S\text{-}S}$ was gained by adding 1 mM DL-1,4-Dithiothreitol (DTT) into $Cox17_{3s\text{-}s}$. The excess DTT was removed by gel filtration or desalting.

2.3. Preparation of metalated $Cox17_{2s\text{-}s}$ adducts

In a typical reaction, apo-$Cox17_{2s\text{-}s}$ (20 μM) in 20 mM MES buffer (pH 6.0) was incubated with ruthenium complex **1**, **2**, or cisplatin at a molar ratio of 1:10 for 24 h at 310 K. Unbound ruthenium complexes or cisplatin was removed by ultrafilteration three times with a 3 kDa

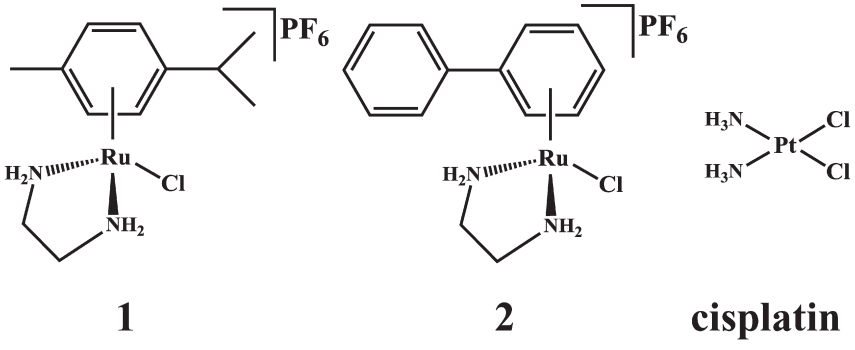

Chart 1. Chemical structure of organoruthenium(II) complexes and cisplatin used in this work.