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Characterizing activation mechanisms and binding preferences of ruthenium metallo-prodrugs by a competitive binding assay

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ABSTRACT

The activation mechanisms and reactivity of ruthenium metallo-prodrug lead structures were investigated in detail using capillary zone electrophoresis mass spectrometry (CZE–MS) in a time-dependent manner and by exposing to a protein/oligonucleotide mixture. The competitive assays were performed with sodium *trans*-[$RuCl_4(HInd)_2$] where Hind = indazole (NKP-1339), [(η^6-*p*-cymene)$RuCl_2$(pta)], where pta = 1,3,5-triaza-7-phosphaadamantane (RAPTA-C) and [(η^6-biphenyl)RuCl(1,2-ethylenediamine)]PF_6 (RM175). Molecular and quantitative information on binding preferences was obtained by coupling CZE to electrospray ionization MS (ESI-MS) and inductively coupled plasma MS (ICP-MS), respectively. A score system is presented that ranks the binding preferences of Ru complexes with nucleotides and demonstrated the following trend of decreasing selectivity after 24 h: RM175 (0.89) > RAPTA-C (0.78) > NKP-1339 (0.40). As expected, the organometallic drug candidates RM175 and RAPTA-C underwent a halido/aqua ligand exchange reaction at the metal center and showed distinct reactivity towards the biomolecules. In particular, the protein/DNA binding sites of RAPTA-C in a mixture of protein (ubiquitin) and oligonucleotide (5′-dATTGGCAC-3′) were located at single-amino acid and single-nucleotide resolution, respectively. Activated RAPTA-C bound selectively to Met1, adenine and cytosine in this setting, which contrasts with the selectivity of RM175 for guanine. Finally, activation products of NKP-1339 were detected corresponding to $Ru^{II}(Hind)_2$ fragments coordinated to the oligonucleotide, which represents one of the few examples of a directly observed Ru^{II} adduct.

1. Introduction

The cytotoxic mode of action of cisplatin, one of the leading metal-based anticancer drugs, is mediated by its DNA binding ability [1]. Similarly, early ruthenium-based coordination compounds were thought to bind to DNA as the main mechanism of action [2]. However, in recent years it has emerged that the therapeutic effect of next-generation non-platinum drug candidates – especially ruthenium-based – may likely be caused by interaction with proteins rather than DNA, although the exact mechanisms of action are often not yet elucidated and direct targets unknown [3,4]. Several ruthenium metallodrugs were shown to interact with DNA and proteins and examples include the advanced drug candidates sodium *trans*-[$RuCl_4(HInd)_2$], where HInd is indazole (NKP-1339) [5,6], imidazolium *trans*-[$RuCl_4(HIm)_2$] NAMI-A, where HIm is imidazole [7,8] and [(η^6-*p*-cymene)$RuCl_2$(pta)], where pta = 1,3,5-triaza-7-phosphaadamantane (RAPTA-C) [9,10].

A fundamental question to be addressed in the discovery process is whether a metallodrug shows binding preference to DNA or protein. So far, this cannot be determined by classical screening assays on a molecular level. In fact, crystal-soaking with the nucleosome core particle (NCP) is the only method to-date for obtaining such insight [11]. The NCP is an advanced model for competitive binding to biomolecules consisting of a double-stranded 145 bp DNA oligonucleotide and a histone protein octamer that can sample binding preferences of metallodrugs. It is intriguing to note that simple ligand modifications of organometallic drug candidates dictate target preferences in this setting and that metallodrugs engage in coordinative binding to biomolecules upon hydrolysis [12,13]. However, the X-ray diffraction experiments with the NCP are not suitable for screening purposes, which prompted us to develop a capillary zone electrophoresis-mass spectrometry

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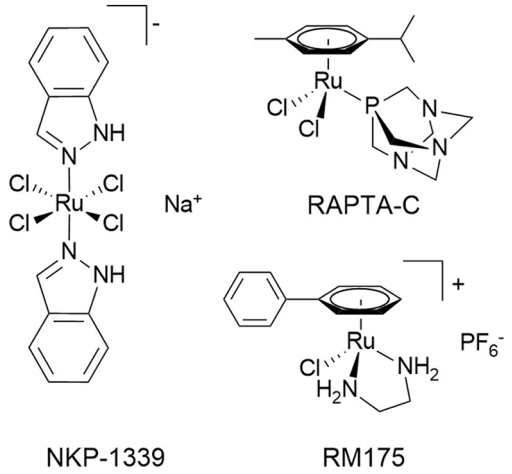

Fig. 1. Structures of the metal-based anticancer drug candidates investigated in this study: NKP-1339, RM175 and RAPTA-C.

(CZE–MS) approach based on competitive binding experiments of intact proteins and oligonucleotides with metallodrugs [14]. Mass spectrometric methods are increasingly used for studying the reactivity of metallodrugs to biomolecules [4,15–18] and also in competition experiments by stand-alone electrospray ionization mass spectrometry (ESI-MS) [19–21], but these are rare with simultaneous exposure to protein and DNA models [14,22]. In particular, the hyphenation of CZE separation to mass spectrometric detection holds promise as an ideal combination for analyzing metallodrugs. This is related to analytes being separated by CZE according to their charge and size [23–25] and activation mechanisms of metallodrugs often involve charge variations by ligand exchange reactions, which are in some cases accompanied by redox processes.

Here, we report on an extended investigation on metallodrug activation pathways and binding preferences to protein or DNA (oligonucleotide) in a competitive approach. Three representative ruthenium lead structures were included, the Ru^{III} anticancer agent NKP-1339 and the organometallic Ru^{II}(arene) derivatives RM175 and RAPTA-C (Fig. 1). The experiments were performed in a time-dependent manner over 72 h and distinct molecular reactivities were observed for each of the ruthenium metallo-prodrugs.

2. Materials and methods

2.1. Materials

NKP-1339, RAPTA-C and [(η^6-biphenyl)RuCl(1,2-ethylenediamine)] PF_6 (RM175) were synthesized according to literature procedures [9,26,27]. Ubiquitin (ub) (from bovine erythrocytes) was purchased from Sigma Aldrich and the oligonucleotide 5′-dATTGGCAC-3′ (DNA) (lyophilized, HPLC purified) was a product of Integrated DNA Technologies (Coralville, USA) or Microsynth (Balgach, Switzerland). [Tri(acetylacetonato)cobalt(III)] (99.99% trace metals basis) and 3-nitrobenzyl alcohol (for mass spectrometry, ≥ 99.5%) were received from Sigma-Aldrich, ammonium bicarbonate (BioUltra, ≥ 99.5%), formic acid (p.a. for mass spectrometry) and ammonium hydroxide solution (≥ 25% in H_2O, eluent additive for LC-MS) from Fluka and acetic acid (99.8%, for analysis) from Acros. Methanol and 2-propanol (both HPLC grade) were from Fisher. NaOH solution (1 M) was purchased from Agilent Technologies and inorganic standards (P, S, Pt, Ru, Re; 1000 ± 3 µg/mL) from CPI. Ultrapure water was taken from a Millipore Advantage A10 system (18.2 MΩ, ≤ 5 ppb TOC, 185 UV, Darmstadt, Germany).

2.2. Sample preparation

Aqueous stock solutions of ubiquitin (ub), DNA-oligomer (DNA, 200 µM each), and the metal complexes RAPTA-C, RM175 and NKP-1339 (1 mM each) were mixed to yield final concentrations in the reaction mixtures of 50 µM for each ub/DNA and 200 µM for the respective metallodrug. Reaction mixtures were incubated at 37 °C at constant shaking (400 rpm) in the dark. Aliquots were taken right after sample preparation, 1, 6, 24 and 72 h of incubation and stored at − 20 °C until measurement.

2.3. Instrumentation

All measurements were performed on a G7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (190–400 nm). The capillary electrophoresis system was hyphenated to a high-resolution time-of-flight (TOF) mass spectrometer (maXis 4G UHR-TOF, Bruker, Bremen, Germany) to acquire high resolution mass spectra or the Agilent ICP MS 7800 (for RM175 studies) and 8800 (for RAPTA-C and NKP-1339 experiments, Waldbronn, Germany).

2.4. CZE–MS

The capillary (fused silica, undeactivated, 75 µm, Agilent Technologies, USA) with a total length of 70 cm and an effective length of 23 cm (UV/Vis window) was cleaned with isopropanol before installation. New capillaries were flushed successively with HCl (1 M, 5 min), water (1 min), NaOH (1 M, 10 min), water (1 min) and background electrolyte (BGE, 20 min). The capillary cassette temperature was set to 25 °C. NH_4HCO_3 (25 mM, pH 7.9) was used as the background electrolyte (BGE). The separations were performed at 25 kV and capillaries were flushed successively with NaOH (1 M), water and BGE (1 min each, 950 mbar) after each run. Before injection, the capillary was rinsed with BGE for 1 min (950 mbar). Typical instrument parameters are listed in Table 1.

2.4.1. CZE–ESI-MS

For CZE hyphenation to the ESI mass spectrometer, a coaxial sheath-flow CZE–ESI-MS Sprayer Kit (Agilent Technologies) was used. The sheath liquid was isopropanol, methanol, water, formic acid and 3-nitrobenzyl alcohol (25/25/50/0.2/0.5, v/v/v/v/v). The latter was added to obtain higher charge states of the protein [28]. The sheath flow of 3 µL/min was provided by a syringe pump (KDS 1000, KD Scientific Inc., Holliston, USA). Samples were injected hydrodynamically for 5 s at 50 mbar. Extracted ion electropherograms in the figures were obtained by selecting the appropriate mass-to-charge signal including an offset of ± 0.05 *m/z*.

2.4.2. CZE–ESI-MS^2

For fragmentation experiments (ESI-MS^2) *in source* collision-induced dissociation (ISCID) energy was increased (90–170 eV). All other settings were similar to MS^1 experiments.

2.4.3. CZE–ICP-MS

A custom-made interface was used for CE hyphenation to the ICP-mass spectrometer. The sheath liquid for ICP-MS experiments was acetic acid (20 mM) containing 20 ppb Re as external standard and was provided by the nebulizer pump with an uptake of 0.03 or 0.1 rpm. [Tris(acetylaetonato)cobalt(III)] with a cobalt concentration of 50 mg/L was used as an internal standard that was diluted to a final concentration of 5 mg/L cobalt in the reaction mixtures. Samples were hydrodynamically injected for 10 s at 50 mbar. Phosphorous was followed as ^{31}P on the Agilent 7800 (for RM175) and as ^{47}PO on the Agilent 8800 ICP-MS (for RAPTA-C and NKP-1339). For measurements on the 8800 ICP-MS instrument an oxygen cell gas flow of 30% was