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Apo-neocarzinostatin: A protein carrier for Cu(II) glycocomplexes and Cu(II) into U937 and HT29 cell lines

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ABSTRACT

In the field of pharmaceuticals there is an increasing need for new delivery systems to overcome the issues of solubility, penetration, toxicity and drug resistance. One of the possible strategies is to use biocarriers such as proteins to encourage the cell-penetration of drugs. In this paper, the use of the apo-protein neocarzinostatin (apo-NCS) as a carrier-protein for two Cu(II) glycocomplexes, previously characterized, and Cu(II) ions was investigated. Its interaction with the metallic compounds was analyzed using microcalorimetry. The dissociation constants were shown to be in the micromolar range. The Cu(II) glycocomplexes, in absence of apo-NCS, were found to be cytotoxic in the U937 and HT29 cell lines whereas the corresponding glycoligands showed no toxicity. The leukemic cell line (U937) seems to be more sensitive to glycocomplexes than the colon cancer cell line (HT29). Interestingly, apo-NCS was shown to increase systematically the antiproliferative activity by a factor of 2 and 3 for Cu(II) glycocomplexes and Cu(II) respectively. The antiproliferative activity detected was not related to proteasome inhibition. This result stresses the importance of new molecular tools for the delivery of Cu(II) to tumor cells using non-covalent association with carriers proteins.

1. Introduction

Nowadays, while a large variety of chemotherapeutic drugs are used to treat cancer, there is a crucial need for new active antitumoral derivatives to solve issues such as delivery, penetration in tumor cells and also selectivity. Metal-based therapeutics [1–4] is an alternative that has stimulated research in bioinorganic chemistry [5–8]. In particular, some copper complexes have been described for their antiproliferative properties [9–12]. Glycoligands are a family of metal-chelating compounds that we have been developing since 2005 and that uses sugar as a central platform with appended moieties that contain Lewis bases [13]. They have been shown to induce a control of the geometry and of the magnetic properties [14], to predetermine the stereochemistry at the metal center [15–17] and they have also been used in the design of fluorescent ligands [18,19] or glycosiderophores [20]. This is an original approach as, except in a few examples, sugars are used in coordination chemistry, not as central structures, but as moieties to be appended to design complexes for asymmetric catalysis [21,22], to develop chelators [20,23], ligands for biodetection [18,24–27] or for biomedical applications [28,29]. In the present paper, the antiproliferative activity of two Cu(II)-glycocomplexes that were previously fully characterized [17] was evaluated and the use of a protein for carriage inside cells, namely apo-neocarzinostatin or apo-NCS, was investigated. This study presents two main innovative aspects: (1) thermodynamic evaluation of the interaction of Cu(II) and Cu(II)-glycocomplexes with apo-NCS and (2) exploration of the possible use of apo-NCS as a carrier for Cu(II) and Cu(II)-glycocomplexes inside cancer cells.

Among other possibilities [28–30], carrier proteins [31–34] can be used for the delivery of drugs including metal complexes [35], to promote their cell penetration and also to specifically target tumor cells. Neocarzinostatin (NCS) is a DNA-nicking protein, isolated from *Streptomyces carzinostaticus*. The NCS holo-protein consists of a fluorescent chromophore (MW 659) tightly but non-covalently bound ($K_D = 10^{-10}$) [36] to an 11,100 Da protein (apo-NCS). The holo-NCS has a broad activity *in vivo* against human leukemia, bladder cancer, liver metastasis and sarcoma [37]. Apo-NCS shows no cytotoxic activity (see also

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Table 2, line control) and is believed to serve both as a carrier and to protect the chromophore from decomposition before reaching the cell [38]. The holo-NCS, labeled by fluorescence, was able to penetrate inside eukaryotic cells as demonstrated by fluorescence microscopy [39].

The glycoligands **1** and **2**, and their corresponding Cu(II) complexes (**3** and **4** for the monometallic unit, see below, Section 2.1), were previously published and fully characterized [17] (see Scheme 1). In the present study, their cytotoxic effects have been evaluated on the histiocytic lymphoma cell line U937 and on the colorectal adenocarcinoma cell line HT29 in the presence of apo-NCS or in its absence. The interaction between Cu(II) glycocomplexes or Cu(II) ion, and apo-NCS was characterized by microcalorimetry (isothermal titration calorimetry, ITC).

In the literature, metal complexes have been described to alter proliferation through inhibition of the proteasome which is an emerging target in anticancer drug development [40,41]. In this context, proteasome inhibition assay was performed on U937 cell line to test whether glycoligands and Cu(II) glycocomplexes inhibit the proteasome pathway as do many Cu(II) complexes [9,42–44], which was not the case for **3** and **4**.

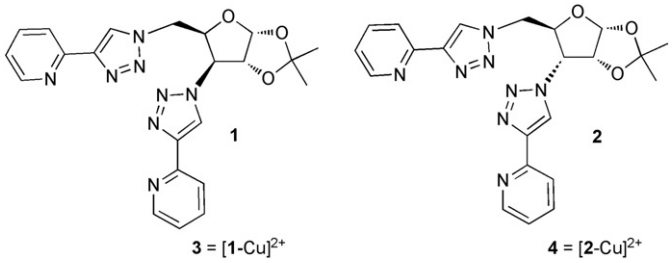
Interestingly, only the Cu(II)-glycocomplexes, and not the glycoligands, were shown to be cytotoxic, and apo-NCS was shown to improve the activity of the complexes. To the best of our knowledge, this is the first example in the literature of the delivery of metal complex by the carrier protein apo-NCS, although other proteins have already been described [35].

2. Material and methods

2.1. Chemicals and protein

Ligands **1** and **2** were obtained as previously described [17] and the corresponding copper(II) complexes were previously fully characterized [17]. They were obtained by mixing 1 equivalent of ligand **1** or ligand **2** in DMSO and 1 equivalent of $Cu(NO_3)_2 \cdot 3H_2O$ in water solution or $CuCl_2 \cdot 2H_2O$ in DMSO solution. The analytical and spectroscopic data indicated a 1:1 stoichiometry for both copper(II) complexes [17]. A dimeric structure (2L:2Cu(II)) was previously found in the solid state and in water solution down to 10^{-4} M. In the following, interaction with apo-NCS is evidenced to occur with the monomeric Cu(II) structures that will be labeled **3** and **4** (see Scheme 1). The precise structure of the mononuclear form of the complexes is not known and they were investigated by minimization (see Supplementary Information). The coordination of two pyridyl-triazolyl claws from the same ligand to one Cu(II) seemed too constraint. The most probable structures are those involving the coordination of either one of the two pyridyl-triazolyl claws coordinated to one Cu(II) with two additional water molecules (see Fig. SI7).

Wild type apo-neocarzinostatin was expressed in *Escherichia coli* containing a synthetic gene coding for neocarzinostatin inserted into the expression vector pET12A (NOVAGENE®). The apo-protein was purified as previously described [45].



Scheme 1. Schematic structure of the glycoligands **1** and **2**. The glycoligands and the corresponding Cu(II)-complexes **3** and **4** were previously characterized (see [17]).

2.2. Microcalorimetry

Calorimetric measurements were carried out with a VP-ITC MicroCal precision calorimeter. Titrations were performed at 293 K by successive addition of 10 μL of Cu(II)-complexes water solution ([**3**], [**4**] or $[Cu^{II}]$ = 300 μM, that will be called *titration solutions* and labeled T-solutions, containing a maximum of 3% DMSO) to an apo-NCS solution (v_{cell} = 1.4 mL, [apo-NCS] = 25 μM, pH = 7.2, [phosphate buffer] = 50 mM). We avoided the use of a 3% DMSO apo-NCS solution in order to minimize possible protein destabilization induced by DMSO. To take into account the heat of dilution due to the presence of a small amount of DMSO from the T-solutions, a background titration, consisting of the T-solution and only buffer in the measurement cell was subtracted from each experiment. The solution in the measurement cell was stirred at 370 rpm by the syringe to ensure rapid mixing. Typically, the T-solution was delivered over a 100 min period with a time-interval between the 10-μL injections that allows complete equilibration (240 s). Titrations have been performed up to 3 equiv. to ensure that no additional phenomena appeared. The data were collected automatically and subsequently analyzed with a one-class of binding site model by the Windows based Origin Software package supplied by MicroCal, after discarding the first injection as usual in this kind of measurements. To improve quality of the fit, in some titration curves an additional artifactual data point was removed for fitting: this led to a better fitting and did not change the overall calculated values. The Origin Software uses a nonlinear least-squares algorithm (minimization of χ^2) and the concentrations of the T-solution and the sample to fit the heat flow per injection to an equilibrium binding equation, providing best fit values of the stoichiometry (n), change in enthalpy (ΔH), and an apparent dissociation constant (K_d) expressed as the ratio [apo-NCS][Guest]/[NCS-adduct], in M and equivalent to a dissociation constant (see Table 1). Confidence intervals for each parameter correspond to the uncertainty of the fit of the data to the model binding used.

2.3. Experiments with human cell lines

The human colon adenocarcinoma HT29 and the histiocytic lymphoma (U937) cell lines were obtained from the American Tissue Culture Collection (Rockville, MD). Cell lines were maintained in the exponential phase at 37 °C in a 5% carbon dioxide atmosphere using a culture media containing 10% fetal calf serum (Lonza, Verviers, Belgium), antibiotics (50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin), and 2 mM L-glutamine: (i) RPMI-1640 medium (Lonza, Verviers, Belgium) with 25 mM HEPES buffer for U937; (ii) DMEM medium (Lonza, Verviers, Belgium) for HT29. Determination of cell numbers and viabilities were performed using the trypan blue exclusion test. HEPES has been chosen as it is a non-coordinating buffer.

Table 1
Thermodynamic parameters of the complexation of apo-NCS by the different Cu(II) complexes **3** and **4** and Cu(II), with different counter-ions, as determined by ITC at 298 K.

Host: apo-NCS			
Guest:	Type of counter-ion	Stoichiometry (host:guest)	Apparent K_d (M)[a]
3	NO_3^-	1:1	$(0.54 \pm 0.17) \times 10^{-6}$
4	NO_3^-	1:1	$(0.35 \pm 0.08) \times 10^{-6}$
Cu(II)	NO_3^-	1:1	$(0.18 \pm 0.04) \times 10^{-6}$
3	Cl^-	1:1	0.16×10^{-6}[b]
4	Cl^-	1:1	$(0.25 \pm 0.04) \times 10^{-6}$
Cu(II)	Cl^-	1:1	$(0.63 \pm 0.08) \times 10^{-6}$

[a] Equivalent to [apo-NCS] ∗ [Guest]/[complex] a quantity homogenous to M.
[b] Only an order of magnitude of the thermodynamic parameters could be estimated using a one-class binding site model.