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CISPLATIN CONJUGATE WITH CYANOCOBALAMIN: SOFT IONIZATION/HIGH RESOLUTION MASS SPECTROMETRY AS A NEW BLADE FOR ITS CHARACTERIZATION

Conjugate of cisplatin with cyanocobalamin (CNCbl-cisplatin) can be considered as a prodrug for Pt-based antineoplastic drug delivering to cancer cells. Using the CNCbl-cisplatin conjugate as an example, we demonstrate here that detailed molecular analysis can be obtained by either matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) or electrospray ionization Fourier-transform MS (ESI FTMS) using an orbital trap (Orbitrap).

Cisplatin, *cis*-diamminodichloro platinum(II) (Fig. 1A) is a platinum-based drug used for the treatment of various kinds of cancers (e.g. ovarian and testicular) and head and neck solid tumors [1, 2]. Despite its effectiveness, the toxic side effects and tumor resistance associated with cisplatin have led to the search for newer Pt-containing drugs with enhanced anticancer activities and lower side effects [3, 4]. Indeed, carboplatin and oxaliplatin are recognized as third generation platinum-based drugs [5, 6]. More recently, however, an innovative drug delivery system has been proposed to make the attack more effective against cancer cells, using lower dosages. The production of prodrugs within cancer cells is particularly interesting; vitamin B_{12} in the form of cyanocobalamin (*CNCbl*) functionalized with cisplatin could be regarded as a potential prodrug candidate

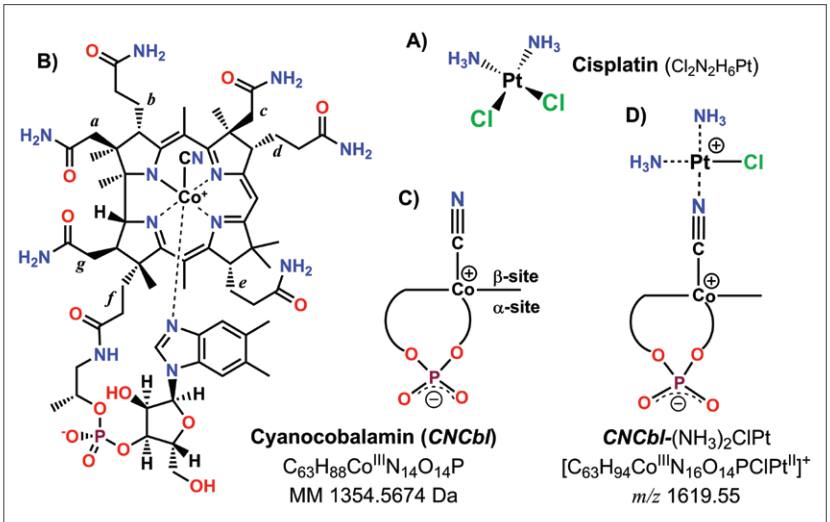

Fig. 1 - A) *cis*-diamminedichloroplatinum(II) commonly named cisplatin; B) chemical structure of cyanocobalamin (*CNCbl*), also known as vitamin B_{12}, along with its empirical formula and monoisotopic mass (MM); C) schematic representation of *CNCbl*; D) schematic chemical structure of the conjugate species between *CNCbl* and cisplatin in which the chlorido ligand has been replaced by a cyanide-bridged (Co-CN-Pt). The conjugate species was prepared in good yield in aqueous solution at 60 °C for 16 h. The monoactivated form of cisplatin (i.e., [*cis*-Pt$(NH_3)_2Cl]^+$) reacts with the cyano ligand of Co(III)-cyanocobalamin forming a positively charged species at *m/z* 1619.55

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because *CNCbl* uptake in mammalian cells is mediated by specific, high-affinity receptors that are overexpressed in numerous human tumors [7-10]. Moreover, the affinity of *CNCbl*-cisplatin conjugates for cell surface transcobalamin II receptors is high enough providing the rationale for targeted drug delivering to cancer cells [9].

Fig. 1B shows the molecular structure of *CNCbl* in which the central Co(III) ion is equatorially coordinated to four nitrogens of a corrin ring consisting of four reduced pyrroles and seven acetamide side chains (i.e., *a-f*). The lower axial coordination site (*α-side*) is occupied by a dimethylbenzimidazole (DMB) base tethered to the acetamide chain f via a nucleotide loop; the upper (*β-side*) axial ligand identifies different Cbls (e.g. -CN in the *CNCbl*) as illustrated in the simplified structure of Fig. 1C. Reaction in the aqueous phase of cisplatin and *CNCbl* generates the conjugate shown in Fig. 1D as demonstrated by Alberto and coworkers, using X-ray crystallography and nuclear magnetic resonance (NMR) [11].

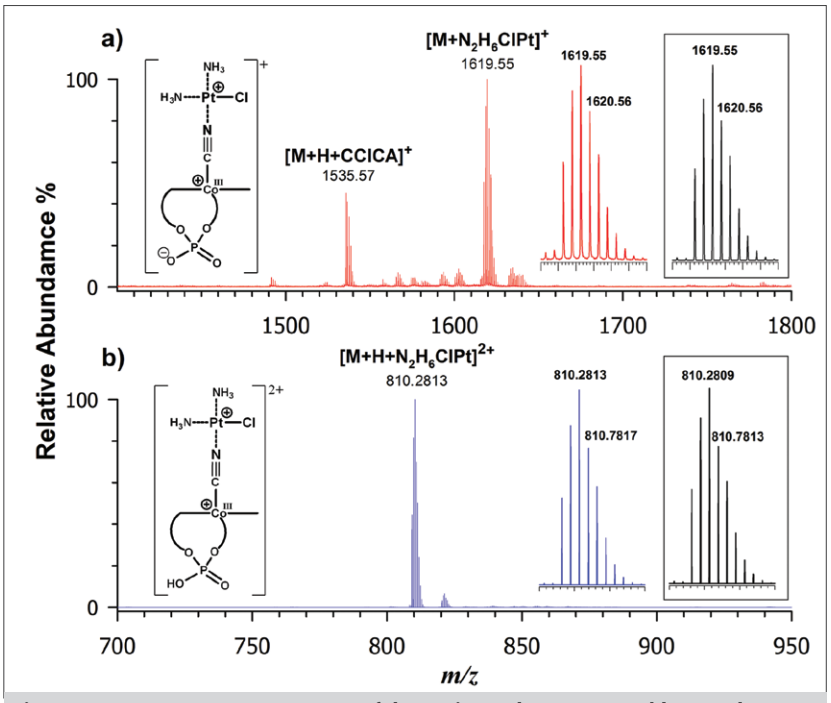

Fig. 2 - a) MALDI-ToF mass spectrum of the conjugate between *CNCbl* (M) and monochloro cisplatin ([M+*cis*-Pt(NH$_3$)$_2$Cl]$^+$) where 4-chloro-α-cyanocinnamic acid (CClCA) was used as a matrix; b) mass spectrum obtained by ESI-FTMS of the same conjugate that exhibits a doubly charged ion as the predominant peak. Insets show the enlarged isotopic cluster at (a) *m/z* 1619.55 and (b) *m/z* 810.2813 compared with simulated profiles using the following empirical formulae, [C$_{63}$H$_{94}$ClCoN$_{16}$O$_{14}$PPt]$^+$, and [C$_{63}$H$_{95}$ClCoN$_{16}$O$_{14}$PPt]$^{2+}$, respectively (see boxed frames). Simulated data were obtained by Xcalibur software 2.2 SP1.48 (Thermo Scientific). The peak signal at *m/z* 1535.57 is due to an adduct of *CNCbl* with the matrix

Inductively coupled plasma mass spectrometry (ICP-MS) [12, 13], is the golden standard analytical tool for the quantification of elemental Pt and has been used in several studies for monitoring cisplatin uptake and formation of Pt-DNA adducts [12]. To the best of our knowledge, soft ionization mass spectrometry (MS) methods and especially matrix assisted laser desorption ionization (MALDI) has not yet been fully evaluated for the characterization of CNCbl-Pt(II) drug conjugates, one reason being the failure to detect the intact conjugate at the expected *m/z* value of 1619.55 using conventional (first generation) matrices. Here it is clearly shown that rationally designed MALDI matrices can overcome this issue. Furthermore, MALDI MS is compared with electrospray ionization (ESI) Fourier transform (FT) high resolution MS using an orbital trap analyzer [14, 15].

Fig. 2 shows typical positive ion mode MALDI and ESI mass spectra of the conjugate formed between *CNCbl* and monochloro-cisplatin, [*cis*-Pt(NH$_3$)$_2$Cl]$^+$ prepared by reacting (at 60 °C for 16 h in aqueous solution) a cisplatin/CNCbl mixture (molar ratio >100:1). The MALDI spectrum (Fig. 2a) was obtained using 4-chloro-α-cyano-cinnamic acid (CClCA) as matrix [16]. Note that conventional proton transfer matrices such as α-cyano-4-hydroxycinnamic acid, does not permit detection of the intact *CNCbl*-cisplatin conjugate in the gas phase, most likely because the labile Co(III)-CN bond of *CNCbl* is homolytically dissociated during the desorption process [17]. Using CClCA, an ion cluster with the most intense signal at *m/z* 1619.55 is observed with the following empirical formula, [C$_{63}$H$_{94}$ClCoN$_{16}$O$_{14}$PPt]$^+$, being in excellent agreement with that expected for the *CNCbl*-monochloro cisplatin conjugate ([M + *cis*-Pt(NH$_3$)$_2$Cl]$^+$). This species is generated by the elimination of one easy-leaving chloride from the Pt complex and the formation of a Co-CN-Pt bridge, which produces the

single and positively charged ion (Fig. 1D). To our knowledge, the CClCA matrix is the only one available to preserve CN labile axial ligand loss during the MALDI MS analysis [17], which is an indirect confirmation of the linking site.

ESI or MALDI ionization sources greatly influence the resulting spectra of the *CNCbl*-monochloro cisplatin conjugate. Fig. 2 (plot b) shows the mass spectrum obtained by ESI-Orbitrap FTMS: the base peak is due to a doubly charged platinum containing ion at *m/z* 810.2813. Experimental isotopic pattern matches very well with the chemical composition $[C_{63}H_{95}ClCo^{III}N_{16}O_{14}PPt^{II}]^{2+}$ as illustrated in the boxed frame, with a mass accuracy better than 0.5 ppm. Definitively, both ionization sources allowed us to obtain reliable and informative pictures of cisplatin interplay with *CNCbl*, at the molecular level. As cisplatin produces its pharmacological effects by cross-linking the DNA nucleobases, forming intra- and inter-strand cisplatin-DNA adducts, the use of these high resolution and sensitive MS techniques can qualitatively characterize and elucidate their chemical structure. Though cellular uptake studies of cisplatin can be addressed by elemental speciation analysis at biological relevant concentration levels (i.e. ca. 5 μM), no detailed structure information can be obtained. Conversely, the accurate quantification of intact *CNCbl*-cisplatin is possible also in complex biological samples, especially when ESI-FTMS is coupled to liquid chromatography, thus preserving the molecular identity of Pt-based drugs (manuscript in preparation). Compared to LC-ICP-MS, the molecular identity of Pt-based drugs is conserved without losing molecular details, which might influence the cellular uptake and drug selectivity. As many authors have revealed, a higher number of DNA-adducts in the patients responding to Pt drugs, the coupling of LC with ESI-FTMS will afford the examination of such adducts including those formed with *CNCbl*-cisplatin. The key for successful identification can be retrieved by acquiring MS/MS spectra and comparing fragmentation pattern obtained by different MS instruments.

Acknowledgments

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Analisi MS della cianocobalamina cisplatinata

La cianocobalamina funzionalizzata con cisplatino è un esempio di pro-farmaco che potrebbe consentire il rilascio mirato del cisplatino, un noto agente antitumorale a base di Pt(II); in questo studio la spettrometria di massa ad alta risoluzione con sorgenti di ionizzazione soft MALDI o ESI è utilizzata per ottenere informazioni sulla composizione chimica e strutturale di specie a base di Pt.