DEVELOPMENT OF BIOCONJUGATES AND THEIR MODUL CONSTRUCTS FOR TARGETED THERAPY OF CANCERS WITH HIGH MORTALITY

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Tandem mass spectrometry of daunorubicin-containing peptide conjugates

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Introduction

Daunomycin (Dau) is an anthracycline anticancer drug, commonly used for the treatment of several types of leukemia.[1] It consists of an anthraquinone aglycon moiety and a daunosamine sugar linked to the tetracycline by an *O*-glycosidic bond. Daunorubicin is used in our laboratory for the development of anticancer peptide-drug conjugates.[2-6] These constructs showed efficient antitumor activity; therefore, these are promising candidates for targeted cancer therapy. Peptide-drug conjugates have complex structures, and for this reason mass spectrometry is generally used for verification of the composition, as well as for analytical characterization. However, mass spectrometric analysis of anthracycline-containing peptide-drug conjugates is still challenging.[7,8] Electrospray ionization (ESI) is the most widely used ionization method for peptides and proteins. This is a soft ionization technique that produces intact, singly or multiply protonated species from peptides. Besides the determination of molecular mass and verification of elemental composition, tandem mass spectrometry (MS/MS) can also be used to sequence peptides and verify modification sites. However, mass spectrometric analysis of daunomycin-containing peptide conjugates is hindered by the degradation of the compounds during electrospray ionization (ESI). Daunomycin-containing bioconjugates show significant in-source fragmentation.[7] This process results in the spontaneous dissociation of the glycosidic bond before the high vacuum region of the mass spectrometer and leads to the appearance of conjugate fragments with sugar loss. These fragment ions are usually detected in the full scan ESI-MS mass spectra as base peaks. Therefore, the aim of our work was to investigate the mass spectrometric behavior of daunomycin-containing peptide conjugates in details. MS/MS fragmentation properties

were studied by higher-energy collision-induced dissociation (higher-energy C-trap dissociation, HCD) in energy-resolved tandem mass spectrometric experiments.

Results

Daunomycin-containing peptide conjugates were studied by HCD fragmentation using a Thermo Scientific Q Exactive Focus (Bremen, Germany) mass spectrometer equipped with a heated electrospray ionization source (HESI). MS/MS breakdown graphs were recorded to explore the energy evolution of the fragmentation pathways which lead to the appearance of conjugate ions that have lost the sugar moiety.[8] For this study, linear peptide conjugates were selected in which a drug molecule is attached to an aminooxyacetic acid (Aoa) linker at the *N*-terminus of the peptides using oxime bond.[2] MS/MS spectra of various protonated peptide ions were recorded as a function of the HCD collision energy (CE) over the range from 10-100 eV.[8] Under our experimental conditions, all studied peptide conjugates showed an intensive in-source fragmentation, resulting in the loss of the sugar moiety. Intact singly or multiply protonated molecules were also detected, however, these fragment ions appear as the most intense signals in the mass spectra.

To demonstrate the extent of the in-source fragmentation for these compounds and the facile loss of the sugar moiety, Figure 1 shows the full scan ESI-MS spectra of two different peptide conjugates using direct sample infusion. Peptides were dissolved in a solvent mixture composed of acetonitrile and water (1:1, v/v) and 0.1% acetic acid. In the case of these linear constructs, singly and multiply protonated molecules can be identified in the full scan ESI-MS spectra (Figure 1). Ion ratios of the protonated molecules depend on the molecular weight of the constructs and the sequence of the peptide.[8] As shown in Figure 1, beside the intact protonated molecules, additional intense signals can be detected in the mass spectra. These peaks belong to fragment ions formed by the loss of the daunosamine sugar molecule (129 Da mass decrease). The glycosidic bond between the daunosamine sugar and the tetracycline aglycone moiety can be cleaved under acidic conditions during the synthesis of the conjugates as well. This side reaction results in significantly decreased biological effects. Therefore, it is crucial that the homogeneity and the structure of the synthesized compounds are verified using suitable mass spectrometric techniques.

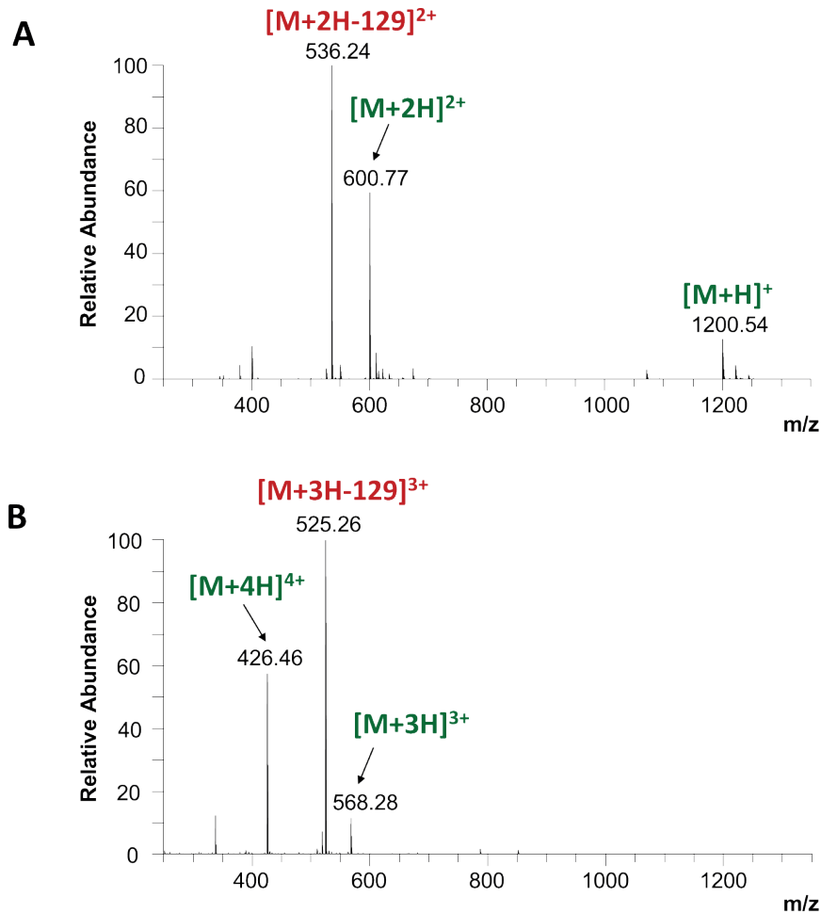

Figure 1. Full scan ESI-MS spectra of daunomycin-containing peptide conjugates. A) *Dau=Aoa*-SKAAKN-*OH*; B) *Dau=Aoa*-GFLGKSKAAKN-*OH*.

To investigate the formation of fragments with sugar loss in detail, energy-dependent fragmentation experiments were performed for various peptide conjugates. In these studies, MS/MS data of different precursor charge states (+1, +2, +3) were acquired in a wide collision energy range. Survival yield curves were calculated for the precursor ions, as well as ion intensity changes for the most important fragment ions.[8]

Energy-dependent experiments showed that the HCD collision energy required to induce the fragmentation of the conjugates greatly depended on the molecular weight of the peptides as well as the charge state of the selected precursor ions. Peptide conjugates with a lower molecular weight and of a higher charge state were much more prone to fragmentation at lower collision energy values. Loss of the sugar is the leading fragmentation pathway, after which the charge can be located on the remaining peptide ion or on the sugar. In the latter case, the protonated sugar molecule can be detected at *m/z* 130, together with the charge-reduced counterpart peptide ion which has lost the protonated sugar. An example of such an MS/MS spectrum is shown in Figure 2.

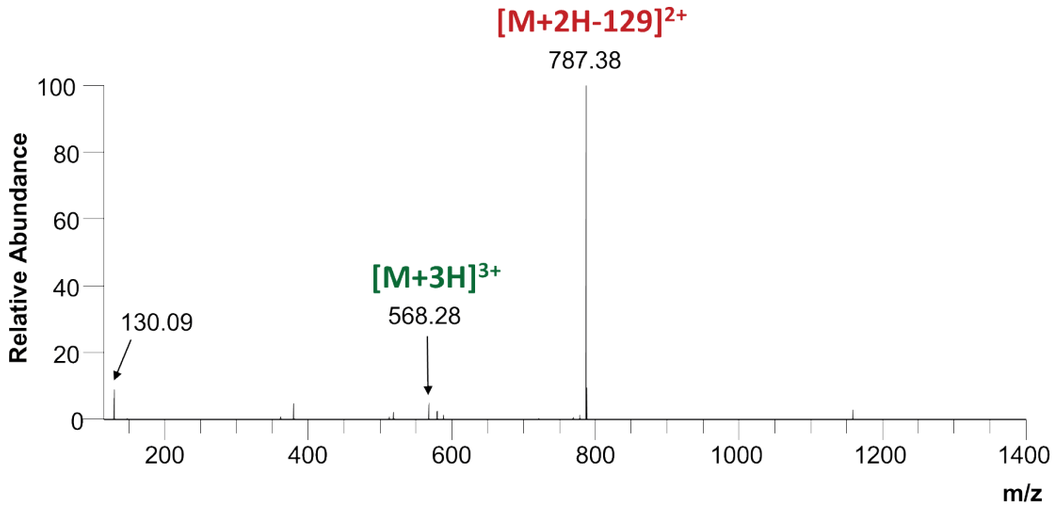

Figure 2. MS/MS spectrum of the triply protonated *Dau=Aoa*-GFLGKSKAAKN-*OH* (green) conjugate at 10 eV HCD collision energy. Charge-reduced fragment ion with sugar loss is detected as a base peak (red) together with the lower intensity protonated sugar.

Figure 2. demonstrates the HCD MS/MS spectrum of a triply charged peptide conjugate. This ion exhibits very low stability, and it is almost completely decomposed even under the lowest possible fragmentation energy of the instrument that was used for the experiments (10 eV). Interestingly, the fragment ion lacking the sugar moiety but bearing two charges only is dominant in this MS/MS spectrum. Our results point at an unusual instability of these multiply charged peptide conjugate ions in general. We observed that the charge separation process, in which a protonated sugar is eliminated from the ions, resulting in the appearance of the charge-reduced fragment ions is a key fragmentation pathway in the case of daunomycin-containing peptide conjugates. This process is demonstrated in Figure 3. Figure 3 shows the plots of normalized intensities of a fragment ion with sugar loss in the function of the collision energy in the case of different multiply charged precursor ions. The results demonstrate that the charge separation process, which leads to the formation of a fragment ion with a lower charge state (green line), is dominant for this compound (Figure 3.). MS/MS fragmentation of the precursor ion with charge state +3 leads to the dominant formation of the charge-reduced fragment (+2), even at very low collision energies (green line). MS/MS fragmentation of the doubly-charged precursor ion with +2 charges produces the same species at higher collision energies, and in this case other fragment ion types can also be observed (blue line).

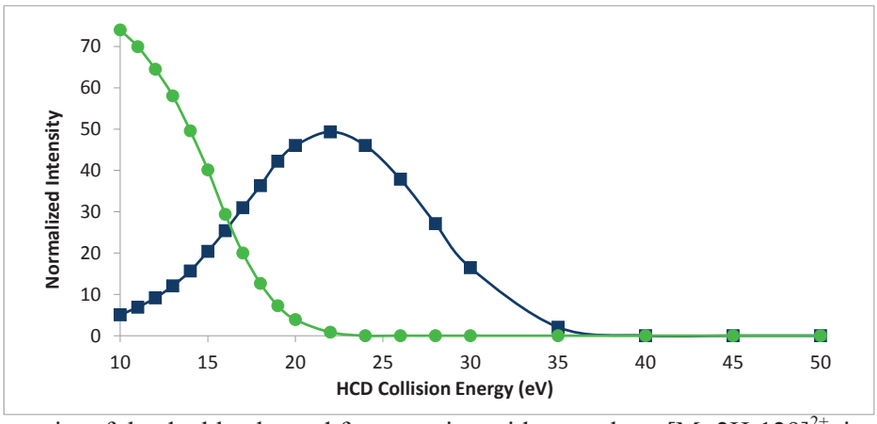

Figure 3. Intensity of the doubly charged fragment ion with sugar loss, $[M+2H-129]^{2+}$, in the function of the HCD collision energy for *Dau=Aoa*-GFLGKSKAAKN-*OH*. Precursor ions: doubly protonated molecule, $[M+2H]^{2+}$ (blue), and triply protonated molecule, $[M+3H]^{3+}$ (green). Intensity values are normalized to the sum of all peak intensities in the respective spectrum.

In conclusion, our research was focused on the MS/MS fragmentation of daunorubicin-containing peptide conjugates, which results predominantly in fragment ions with the loss of the daunosamine sugar. Charge separation is a key fragmentation pathway for these multiply protonated ions. This process can also occur during the ionization, and results in complex mass spectra. Tune parameters of the mass spectrometer can slightly influence the in-source fragmentation, but we suggest the use of appropriate neutral buffers to decrease the charge state of the ions and thereby to prevent the dissociation of the highly charged species. These results could be used to predict the mass spectrometric behavior of these PDCs and could help in the evaluation of the mass spectrometric results as well as in the optimization of their MS detection.

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