Dear Editor,

Peptide-drug conjugates (PDCs) are promising anticancer agents. Most of the conventional cytotoxic compounds used in chemotherapy lack the optimal pharmacokinetic properties, such as solubility in water, oral bioavailability and metabolic stability. In addition, conventional drugs cause side effects that decrease patients’ quality of life during treatment. PDCs are a type of prodrug, in which drug molecules are attached to peptides through covalent coupling via specific linkers. The peptide acts as a tumour-homing agent that can also be selected to improve the required physicochemical properties of the drug. PDCs are therefore promising candidates for personalized cancer treatments.

Daunorubicin (Dau) is an anthracycline anticancer drug used for the treatment of a variety of cancers including varying type of leukemia. Daunorubicin was used for the development of a large number of peptide-drug conjugates in our laboratory that showed efficient anti-tumor activity not only in vitro, but also in vivo.5 Daunorubicin consists of an anthraquinone aglycon part and a daunosamine sugar moiety linked to the tetracycline by a glycosidic bond. Due to the complex structure of PDCs, tandem mass spectrometry is an indispensable tool for verification of the structure and purity of synthetic products. High sensitivity of mass spectrometers enables detailed analysis of PDCs structure and the changes thereof even at very low analyte concentrations. Mass spectrometry is widely used to determine the release of drugs from PDCs, and to identify their metabolites.6-8

In the case of anthracycline containing PDCs, however, mass spectrometric analysis is hindered by the degradation of the compounds during electrospray ionization (ESI). ESI is a soft ionization technique, producing intact, singly or multiply protonated molecules from peptides. However, daunorubicin containing bioconjugates show significant in-source fragmentation under the commonly used experimental conditions, such as ESI ionization from slightly acidic solutions.8 Spontaneous dissociation of the glycosidic bond in the ion source results in the appearance of intensive fragments with sugar loss, which hinders the mass spectrometric analysis significantly.8 Therefore, our aim was to investigate in detail the fragmentation properties of daunorubicin containing peptide conjugates, using energy-resolved tandem mass spectrometric (MS/MS) experiments. Breakdown graphs were used to explore and compare the energy evolution of the fragmentation pathways which can lead to the appearance of ions which have lost the sugar. In this work, higher energy collision-induced dissociation (higher-energy C-trap dissociation, HCD) experiments were performed on an orbitrap mass spectrometer. In the case of collision-induced dissociation, the fractional ion yields as a function of collision energy can provide qualitative, important information to the energy evolution of the fragmentation pathways of protonated peptide ions.

Three bioactive linear peptide conjugates were selected for this study with one daunorubicin molecule attached at the N-terminus through an aminoxyacetic acid (Aoa) linker with oxime bond. The peptides were prepared by solid phase peptide synthesis using standard Fmoc/Bu strategy. Boc-Aoa-OH was attached to the N-terminus before cleavage of the peptides from the resin. Dau was conjugated to the purified peptide in solution (0.2 M ammonium acetate buffer, pH 5.1), as described before.10 Structures and relevant analytical data are summarized in Table 1. In these constructs, the tumour-homing peptide is based on a Frizzled-receptor-specific sequence (CKAAKN),11,12 in which the cysteine residue has been replaced by serine (SKAAKN).13 This peptide was elongated with a lysine residue as well (KSKAAKN), bearing an additional basic amino group close to the position of the daunorubicin. The third construct was built using a spacer peptide (GFLG), resulting in a larger sequential distance between the daunorubicin and the basic lysine amino groups (peptide sequence GFLGKSKAALK). These molecules were used to analyse the effect of the location and number of possible protonation sites has on the cleavage of the sugar from the daunorubicin moiety.

The experiments were performed on a Thermo Scientific Q Exactive Focus (Bremen, Germany) mass spectrometer, equipped with a heated electrospray ionization source (HESI) in positive ionization mode. Peptides were dissolved in an acetonitrile–water (1:1, v/v) solvent mixture, containing 0.1% acetic acid. Energy-resolved fragmentation was measured using a continuous flow of the peptide solution (10 μL/min) infused into the ion source using a syringe pump. The HCD mass spectra of the ions were recorded as a function of collision energy (CE) over the range from 10–100 eV with 5 eV steps in the case of the singly charged precursor ions, [M + H]⁺. For the precursor ions with higher charge states, [M + 2H]²⁺ and [M + 3H]³⁺, a 10–50 eV collision energy range was used with 1 eV steps between

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and 20 eV, 2 eV steps between 20 and 30 eV, and 5 eV steps from 30 to 50 eV. The instrumental parameters were as follows: ESI spray voltage (4 kV), capillary temperature (320 °C), sheath gas flow (5.0 a. u.), auxiliary gas (3.0 a.u.), probe heater temperature (50 °C). All solvents were UHPLC–MS grade and purchased from VWR International Kft. (Debrecen, Hungary).

All three PDCs showed intensive in-source fragmentation with a facile loss of the sugar moiety. Singly, doubly and triply protonated molecules were detected; however, the most intense signals were associated to protonated molecules suffering a neutral loss of sugar. Formation of these fragment ions can be explained by the fragmentation properties of the drug moiety.\textsuperscript{14} Bond breakage at the glycosidic bond in a daunorubicin molecule is initiated by the protonation of the oxygen. Two types of inductive cleavages are depicted on Figure S1 showing the formation of the \( m/z \) 130 fragment ion as well as the neutral loss of the sugar. Bond breakages at the oxygen atom can result in fragment ions with the oxygen atom remaining on the sugar moiety or, alternatively, on the aglycon part (Figure S1). Similar processes can be observed in the case of daunorubicin-containing PDCs, in which this drug molecule is conjugated to a peptide.

To uncover the general trends in the energy-dependent fragmentation of daunorubicin containing peptide conjugates, the MS/MS data of three precursor charge states (+1, +2, +3) of each synthetized

| Peptide | Monoisotopic mass (calc.) | Monoisotopic mass (meas.\textsuperscript{a}) | Mass error (ppm)\textsuperscript{a} | Retention time (min)\textsuperscript{b} |
|---------|---------------------------|------------------------------------------|---------------------------------|-------------------------------|
| Dau = Aoa-SKAACKN-OH | 1199.5346 | 1199.5346 | 0.0 | 11.7 |
| Dau = Aoa-KSKAAKN-OH | 1327.6296 | 1327.6303 | 0.5 | 11.4 |
| Dau = Aoa-GFLGKSKAACKN-OH | 1701.8250 | 1701.8253 | 0.2 | 12.9 |

Note: A flow rate of 1 ml/min was used at ambient temperature. Peaks were detected at \( \lambda = 220 \) nm. Dau stands for daunorubicin, Aoa stands for aminooxyacetic acid.

\textsuperscript{a}ESI-MS data measured on a Thermo Scientific Q Exactive focus mass spectrometer.

\textsuperscript{b}Analytical RP–HPLC was performed using a Waters Symmetry C18 column (150 × 4.6 mm, 5 μm, 100 Å). Linear gradient elution was used: 0 min 0% B; 2 min 0% B; 22 min 90% B with eluent a (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile-water (80:20, v/v)).
compound were acquired. Different charge states of one given compound were compared to each other. In addition, the same precursor charge states of the different compounds were likewise compared. Because of the m/z range limitation of the instrument, fragment ion peaks under m/z 129.0 were not considered. To facilitate the data analysis, only peaks with an intensity of at least 5% of the base peak were considered. The survival yield for the precursor ion and the yield for fragments were used to compare the fragmentation pathways. For precursors, the expression is $SY = \frac{I_p}{I_m + \sum I_f}$ while for fragments, the expression is $Y = \frac{I_f}{I_m + \sum I_f}$, where $I_p$ and $I_f$ are the intensity of the precursor and fragments, respectively, and the summation extended to all the fragment intensities.\textsuperscript{15,16}

It was found that, in general, the collision energy required to induce any kind of fragmentation of the investigated PDCs greatly depended on the molecular weight of the peptides as well as the charge state of the selected precursor ions. PDCs with a lower molecular weight and of a higher charge state were much more prone to fragmentation at lower CE values than their higher molecular weight, lower charge state counterparts.

The most prominent fragmentation pathway, which could be observed in the case of all conjugates in all charge states was the facile loss of the sugar moiety of the daunorubicin. An example is shown in Figure 1 for the peptide $\text{Dau} = \text{Aoa-SKAAKN-OH}$. The structure of the peptide is also depicted in Figure 1, showing the linkage of the daunorubicin to the N-terminus of the SKAAKN peptide through an oxime bond. It is important to note that aminooxyacetylated peptides were used for conjugation to the oxo group in the daunorubicin molecule, which resulted in the formation of the oxime bond (Figure 1). During MS/MS fragmentation, loss of the sugar is the leading fragmentation pathway; after which, the charge is located on the remaining peptide ion or on the sugar. In the latter case, the protonated sugar could be detected in the tandem mass spectrum as well, together with the protonated, but charge-reduced sugar-lost peptide (Figure 1).

The intensity of this fragmentation showed a dependency on the length of the peptide chain (Figure 2): the longer the peptide, the

**FIGURE 2** Intensity of the precursor and product ions: (A) in the case of the singly charged precursor and fragment ions; (B) in the case of precursor ions of +2 charge state and the singly charged, sugar eliminated product ions; (C) in the case of precursor ions of +2 charge state and the doubly charged, sugar eliminated product ions. Intensity values are normalized to the sum of all peak intensities in the respective spectrum.
larger the CE must be to eliminate the sugar moiety. Figure 2 shows the normalized intensities of the singly protonated sugar-loss fragment ions [M + H-129]$^+$ derived from the singly protonated precursor peptides. These fragment ions are the major products of the fragmentation process, reaching approximately 50% of the normalized intensity at the maximum abundance collision energies for all studied peptides. CE requirement of this specific pathway depends on the length of the peptide chain: the shift of the maximum ion intensity to higher CE can be observed for longer peptides (Table 2).

The length of the peptide chain affected the fragment ratios and the charge separation phenomena. Conjugates with longer peptide chains are more likely to retain multiple charges after fragmentation as shown in Figure 2. An increase of the CE of both fragmentation processes as the molecular weight increases can be observed. This phenomenon can be explained with the degree of freedom effect, which predicts a linear increasing of the characteristic collision energy as the molecular weight of the compound increases (proportional to the number of vibrational degrees of freedom of the compound).\(^{17-19}\)

In Figure 2, the increments in CE values are not completely linear due to the addition of heterogeneous amino acids to the peptide chain that add variations in the excitation voltage and entropy. Besides, the basicity of the peptide chain also plays an important role. In fact, if the peptide sequence was elongated by only a single basic amino acid (SKAAKN vs. KSKAAN), it can be observed that there is an increase of CE for both, the neutral elimination and the charge separation fragmentation processes. In other words, these trends indicate that the positive charges are better stabilized as the basic group numbers increase; thus, higher CE is needed to effectively fragment the molecule.

A doubly protonated ion of the shortest peptide, Dau = Aoa-SKAAN-OH, showed the lowest stability among the studied molecules. In this case, both the singly protonated, sugar eliminated ions—originating from the charge separation process—and the doubly protonated, sugar eliminated ions are formed rapidly at very low collision energies. The appearance of the singly charged products requires higher collision energies compared to the formation of the doubly protonated fragments (Table 2). These facts can be explained as a competition of the neutral sugar loss fragmentation process with the charge separation process.\(^{16}\) Generally, for all PDCs, the doubly charged state with a neutral sugar loss remains as the most probable fragmentation pathway at lower CE values. In contrast, at high CE values, the charge separation processes are the most likely to occur.

Formation of a charged sugar fragment at m/z 130 was not significant for singly protonated PDC species, but it is a prominent fragment ion for multiply charged species, for example for the doubly protonated molecules. The calculated abundance patterns indicate that the competitive regio-protonation between the basic amino acid residues and the amino moiety from the sugar part could incline the balance towards the [M + H-129]$^+$ fragment production.

In conclusion, the mass spectrometric analysis of daunorubicin-containing peptide-conjugates is difficult due to the facile loss of the sugar moiety during ionization. The in-source fragmentation of the molecules results in complex mass spectra, which could lack peaks of important protonated PDC molecules in their intact forms. Fragmentation of this type of PDCs depends on several parameters. In addition to being dependent on instrument settings and solvent composition, fragment ion intensities and fragment ion types are dependent on structure. Our investigation verified that the MS/MS peak intensities and ratios of the sugar eliminated +1 or +2 charged product ions were strongly influenced by the amino acid chain length and the basicity of the sequence. These results could be used to predict mass spectrometric behaviour 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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### TABLE 2  
HCD collision energy necessary to achieve the maximum fragmentation for the three main fragmentation pathways of the peptide conjugates

| Peptide               | [M + H]$^+$—[M + H-129]$^+$ (eV) | [M + 2H]$^{2+}$—[M + H-129]$^+$ (eV) | [M + 2H]$^{2+}$—[M + 2H-129]$^{2+}$ (eV) |
|-----------------------|----------------------------------|--------------------------------------|----------------------------------------|
| Dau = Aoa-SKAAN-OH    | 41                               | 17                                   | 10                                     |
| Dau = Aoa-KSKAAKN-OH  | 50                               | 20                                   | 13                                     |
| Dau = Aoa-GFLGSKAAKN-OH | 68                              | 30                                   | 22                                     |
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