Research Article

Rapid Communications in Mass Spectrometry

Received: 22 November 2012 Revised: 9 January 2013 Accepted: 11 January 2013 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2013**, *27*, 835–841
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6515

Analysis of novel melphalan hydrolysis products formed under isolated lung perfusion conditions using liquid chromatography/tandem mass spectrometry

Jasper Boschmans[1], Ernst de Bruijn[2], Paul Van Schil[3] and Filip Lemière[1]*

[1]University of Antwerp, Chemistry, Center for Proteomics, Groenenborgerlaan 171 V.417b, Antwerp, BE 2020 Belgium
[2]University of Leuven, Lab Experimental Oncology, UZ Gasthuisberg, CDG Building, Herestraat 49, Leuven, BE 3000 Belgium
[3]Antwerp University Hospital, Thoracic and Vascular Surgery, Wilrijkstraat 10, Antwerp, BE 2650 Belgium

RATIONALE: Melphalan is a widely used cytotoxic agent in cancer treatments. This phenylalanine analog has been shown an effective drug in the treatment of breast cancer, multiple myeloma and melanoma of the extremities. A good knowledge of the drug's degradation and metabolism are crucial for understanding its activity during cancer treatments.
METHODS: The formation of hydrolysis products of melphalan is studied using ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS). Aqueous melphalan solutions were incubated at elevated temperatures and analyzed by UPLC/MS/MS. Two previously described hydrolysis products, mono- and dihydroxymelphalan (MOH and DOH), were formed *in vitro* and could be characterized during MS/MS and high-resolution experiments.
RESULTS: Novel compounds with *m/z* values >500 Da were discovered. Comparison of the fragmentation patterns of these new molecules with those of MOH and DOH show great similarities. The higher masses are explained by the presence of two or more melphalan units. In total, more than 15 new hydrolysis products were found. Experiments were set up to study the formation and the chemical structures of these molecules.
CONCLUSIONS: The hydrolysis of melphalan is studied in the scope of a phase II clinical trial (isolated lung perfusion, ILuP). Patient samples were screened for the presence of all documented and novel melphalan hydrolysis products. This study reports the formation of a new class of oligomeric compounds in both *in vivo* and *in vitro* samples.

Melphalan (Mel; Fig. 1) is a widely used chemotherapeutic agent in cancer treatments. This phenylalanine analog has been shown an effective drug in the treatment of breast cancer, multiple myeloma, bladder cancer and melanoma of the extremities.[1] Nitrogen mustards like Mel, cyclophosphamide and chlorambucil are alkylating agents because they can form covalent bonds with the nucleobases of DNA, thereby adding an alkyl group on the DNA strand and forming a DNA adduct (Fig. 1).[2–4] The dose of orally or intravenously administrated Mel is limited by bone marrow suppression. A solution to overcome this toxicity is to apply Mel locoregionally. The Department of Thoracic and Vascular Surgery of the University Hospital Antwerp (UZA) started investigating the use of Mel during an isolated lung perfusion (ILuP).[5–8] To treat pulmonary (micro)metastases, the lungs are isolated from the rest of the blood circulation and perfused with a high dose of Mel.

It is known that Mel hydrolyzes spontaneously in aqueous solutions (e.g. blood, perfusion fluid).[9–12] Earlier studies report two hydrolysis products: monohydroxymelphalan (MOH), which further hydrolyzes to dihydroxymelphalan (DOH; Fig. 1). Using liquid chromatography/tandem mass spectrometry (LC/MS/MS) we here identified multiple earlier unknown Mel hydrolysis products.

* *Correspondence to:* F. Lemière, University of Antwerp, Chemistry, Center for Proteomics, Groenenborgerlaan 171 V.417b, Antwerp, BE 2020 Belgium.
E-mail: filip.lemiere@ua.ac.be

EXPERIMENTAL

Chemicals and reagents

Melphalan (Mel; min. 95%) was purchased from Sigma-Aldrich (Bornem, Belgium). Acetonitrile (ACN; HPLC grade for gradient analysis) and formic acid (FA; 99+ %) were obtained from Acros (Geel, Belgium). Reversed osmosis (RO) water was prepared using a Silex water filtering system from Eurowater (Nazareth-Eke, Belgium). Ethanol (EtOH) was obtained from Carlo Erba Reagenti (Rodano, Italy).

Samples

Reactivity and oligomerization samples
A 5 mM stock solution of Mel was prepared using RO water. Because of the low solubility of Mel in water the solution was vortexed and sonicated to improve solubility. This dilution was incubated at 37 °C for 3 h using a gas chromatography (GC) oven (HP, type 5890) or Thermomixer (Eppendorf, type 5436)

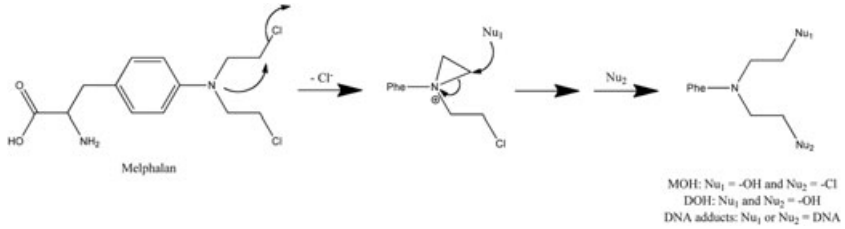

Figure 1. Nucleophilic substitution on melphalan (*m/z* 305) and formation of hydrolysis products MOH (*m/z* 287), DOH (*m/z* 269) or DNA adducts.

depending on the sample volume. The solution was filtered prior to MS analysis using a 0.2 µm Nylon filter (4 mm HPLC syringe filter; Alltech, Lokeren, Belgium). Samples used for product ion analysis were concentrated 10 times.

ILuP samples

During the isolated lung perfusion (ILuP) the blood circulation of the treated lung is isolated from the general circulation and flushed with a perfusion fluid containing melphalan. This perfusion fluid contains melphalan, Plasma-Lyte A (Baxter; Lessines, Belgium), Voluven (6 %, Fresenius Kabi; Schelle, Belgium), heparin and blood. Before and during the ILuP samples are taken from the perfusion fluid. All samples are immediately snap-frozen and stored at −80 °C. Preceding our analyses, samples had already been thawed once for LC-UV quantitation of Mel.

Sample preparation

ILuP samples

A volume of 50 µL of the perfusion fluid sample was mixed with 900 µL ice-cold ethanol (EtOH) and cooled at −20 °C for 60 min. Afterwards the sample was centrifuged for 15 min at 13 500 rpm and 10 °C (Eppendorf centrifuge, type 5415 R). Samples were evaporated to remove all EtOH and reconstituted in 50 µL H_2O. Samples were centrifuged for 4 min at 14 000 rpm (Eppendorf miniSpin plus) in order to remove small particles, since usage of a filter was not possible due to small sample volumes.

LC-MS conditions

Acquity and nanoAcquity UPLC

A binary gradient was developed on a Waters Acquity LC system (Milford, MA, USA). Guard column: 2.1 µm × 5 mm 1.8 µm Vanguard HSS T3. Analytical column: 2.1 µm × 100 mm 1.8 µm Acquity HSS T3 (both Waters, Milford, MA, USA). Column temperature: 30 °C. Gradient method used both acidified (0.1% formic acid; FA) H_2O (A) and ACN (B) for analysis as well as wash purposes. The gradient had a run time of 4 min, starting with 0.89 min of on-column focusing. Going from 99% A to 60% B in 3.20 min, the gradient is followed by 0.20 min of washing (99% B) and 0.30 min of equilibration at initial conditions. Injection: 5 µL full loop (overfill factor 1).

A similar method was developed on a nanoAcquity UPLC system, for coupling with the Q-Tof II mass spectrometer. Trapping column: 180 µm × 20 mm 5 µm Symmetry C_{18}. Analytical column: 100 µm × 100 mm 1.7 µm Acquity BEH130 C_{18} column (both Waters, Milford, MA, USA).

Xevo TQ MS

Full scan MS spectra, product ion spectra and MRM analyses were performed on a Xevo TQ mass spectrometer (*in vivo* samples).

Electrospray experiments in positive ion mode (ES+) were performed on a Xevo TQ mass spectrometer (Waters, Milford, MA, USA). Capillary voltage: 1 kV. Cone voltage: 27 V. Source temperature: 150 °C, desolvation temperature: 500 °C. Resolution settings in MS/MS mode were 2.9 for both LM 1 and LM 2. For HM 1 and HM 2 resolution was set to 15.

Cone voltage and collision energy were optimized for each of the 12 compounds (Table 1). QuanLynx software (Waters, Milford, MA, USA) was used to integrate the areas of all peaks recorded in the multiple reaction monitoring (MRM) method.

Q-Tof II

Accurate mass data were acquired on a Q-Tof II mass spectrometer (Micromass, Manchester, UK), equipped with a chip-based NanoMate source (Advion, Ithaca, NY, USA). The NanoMate was connected with the nanoAcquity pump using an LC-coupler. A spray voltage of 1.7 kV was applied. Cone voltage: 20 V. Source temperature: 80 °C. Desolvation temperature: 60 °C.

TOF MS scans were performed with the collision energy at 5 eV. A mass range for studying dimers and trimers: *m/z* 100 to 900. Mass ranges and MS profile settings were adjusted when studying tetramers. Scan time: 0.9 s, inter-scan delay: 0.050 s.

Collision energies for product ion scans (MS/MS): 25 to 50 eV. The mass range for dimers and trimers: *m/z* 50–600 or *m/z* 50–900, for tetramers: *m/z* 100–1200. Scan time: 0.9 s, inter-scan delay: 0.050 s.

RESULTS AND DISCUSSION

Formation of hydrolysis products and oligomers

Separation of a 5 mM incubated solution of Mel results in a complex chromatogram (Fig. 2). Based on existing literature only three compounds are expected: Mel, MOH and DOH. These are detected at retention time (t_R) 1.72 min, 2.99 min and 3.77 min and their identity is confirmed by relative elution order in RP chromatography and their MS spectra. All other compounds present were initially unknown.

Both MOH and DOH are formed by reaction of Mel with H_2O: one or two Cl atoms are substituted for a hydroxyl group. In this reaction H_2O is the nucleophile; however, other molecules in solution can act as a nucleophiles. Potential

Rapid Communications in Mass Spectrometry

Table 1. Optimized cone voltages and collision energies for a total of 12 compounds as used in the developed MRM method

Exact mass	Chemical formula	Retention time	Name	Transitions	Cone voltage	Collision energy
269.1501	$C_{13}H_{21}O_4N_2Cl_0^+$	1.72	DOH	269 > 176; 287 > 150	25	30; 30
287.1162	$C_{13}H_{20}O_3N_2Cl_1^+$	2.99	MOH	287 > 228; 287 > 150	25	25; 30
305.0824	$C_{13}H_{19}O_2N_2Cl_2^+$	3.77	Mel	305 > 246; 305 > 194	20	35; 35
501.2712	$C_{26}H_{37}O_6N_4Cl_0^+$	2.69	MOH-eDOH	501 > 251; 501 > 234	35	25; 30
519.2819	$C_{26}H_{39}O_7N_4Cl_0^+$	2.54	MOH-DOH	519 > 251; 519 > 234	35	25; 30
519.2374	$C_{26}H_{38}O_5N_4Cl_1^+$	3.05	Mel-eDOH	519 > 269; 519 > 252	30	30; 35
537.2036	$C_{26}H_{35}O_4N_4Cl_2^+$	3.38	Mel-eMOH	537 > 252; 537 > 168	35	35; 35
537.2480	$C_{26}H_{38}O_6N_4Cl_1^+$	3.00	MOH-MOH	537 > 251; 537 > 234	30	25; 30
537.2480	$C_{26}H_{38}O_6N_4Cl_1^+$	2.92	Mel-DOH	537 > 269; 537 > 252	30	25; 30
555.2141	$C_{26}H_{37}O_5N_4Cl_2^+$	3.49	MOH-Mel	555 > 251; 555 > 234	25	25; 30
555.2141	$C_{26}H_{37}O_5N_4Cl_2^+$	3.39	Mel-MOH	555 > 269; 555 > 252	30	25; 30
573.1802	$C_{26}H_{36}O_4N_4Cl_3^+$	3.80	Mel-Mel	573 > 269; 573 > 252	35	25; 30

nucleophiles are organic solvents used during reaction (e.g. alcohols), carboxylate or other anions or Mel itself. Reaction between two Mel molecules results in dimeric structures with masses >500 Da and MS signals and isotope patterns characteristic for their chlorine content.

Dimeric structures can be formed between an electrophile (Mel or MOH) and a nucleophile (Mel, MOH or DOH). Potential nucleophilic sites present in Mel, MOH and DOH are the carboxyl and amine groups of the phenylalanine moiety and the hydroxyl group on the mustard side of a (partially) hydroxylated Mel molecule (MOH or DOH). All of these compounds have a characteristic isotope pattern and exact mass. Potential dimeric structures, together with their chemical formulas, exact masses and isotope patterns are summarized in Fig. 3 and Table 2. This list compromises a total of 15 dimers including several isomers, resulting in only four unique masses.

Apart from signals for MOH and DOH, additional peaks at other retention times were observed in the RICs. Analysis of the MS/MS data reveals that these peaks originate from Mel dimers. As far as we know, dimerization or oligomerization has never been described in the literature before.

Identification of melphalan dimers, isomers and isobars

RICs for *m/z* 573 and 537 contain multiple peaks. Peaks arising from $[2M+H]^+$ clusters of Mel, MOH and DOH are also present in these RICs but these are easily identified as such. Multiple chromatographic peaks are also observed in RICs for the predicted dimers with mass-to-charge ratios *m/z* 555 and 519. The presence of multiple chromatographic peaks, identical accurate masses and matching isotope patterns indicate that for some dimers multiple isomers are formed.

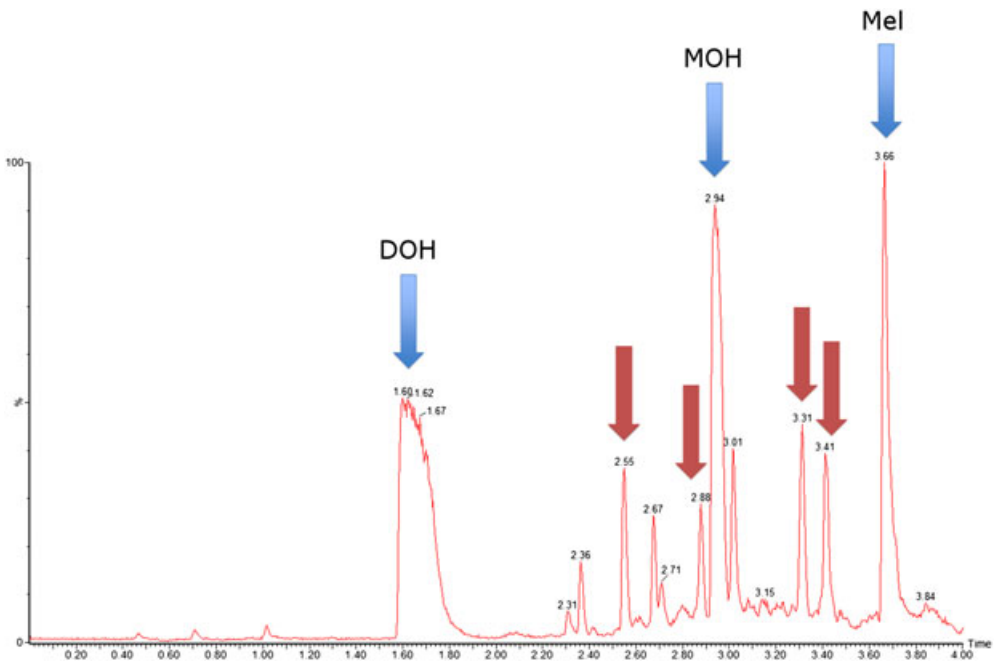

Figure 2. UPLC/MS analysis (TIC) of a melphalan incubation sample (3 h, 37 °C). Apart from melphalan and its hydrolysis products, MOH and DOH, numerous other compounds are present. The polar DOH elutes early in the gradient resulting in a poor peak shape.

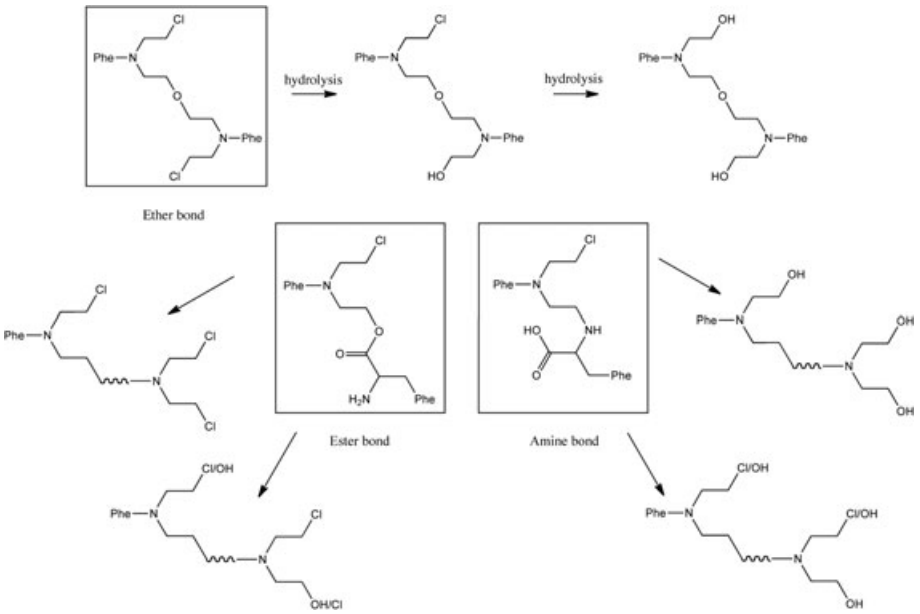

Figure 3. In aqueous solution melphalan oligomerizes and forms dimers. Possible structures are classified by their bond type: ethers, esters and amines. Further hydrolysis of these compounds originates in more products.

When assigning a chemical structure to the detected dimers, we need to identify the bond between two Mel molecules. Four possible bond types are suggested: an ether, pyrazine, amine or ester bond (see Fig. 3). A dimer with an ether linkage (a) has a structure with two free phenylalanine groups and (one or two) unreacted chlorine atoms. The hydroxyl group of MOH or DOH acts as the nucleophile that binds to the electrophilic Mel or MOH. A pyrazine bond (b) is formed when the NH_2 group of the amino acid of a first molecule (Mel, MOH or DOH) reacts with both chloro ethyl groups of a Mel molecule. An ester (or amine; c or d) bond is formed when the carboxyl (or amine) group acts as the nucleophile.

Melphalan-related compounds are characterized by their typical fragmentation in which the bond between Mel and its reaction partner is broken adjacent to Mel's ethyl group.[13] Depending whether the second ethyl group of the mustard is unmodified (−Cl) or hydrolyzed (−OH) this fragment is found at *m/z* 269 or 251. Further loss of ammonia yields a product ion at *m/z* 252 and 234, respectively. This results in two fragmentation patterns: 269/252 for the unmodified mustard (−Cl) and 251/234 for the hydrolyzed mustard (−OH). The accurate mass data of the product ions is in agreement with the elemental composition of these proposed product ions.

Knowing the structural possibilities (i.e. bond types) of a Mel dimer, tentative chemical structures can be assigned to all observed compounds and their isomers.

m/z 573

A single chromatographic peak is found for the product ions of *m/z* 573. The isotope pattern and accurate mass of *m/z* 573.1793 (t_R = 3.80 min; $C_{26}H_{36}O_4N_4Cl_3^+$, calc. 573.1802 Da) reveal three chlorine atoms in its structure. In a Mel dimer with an ether or pyrazine linkage, a maximum of only two chlorine atoms can be present excluding these bond types for *m/z* 573. Only the formation of an amine or ester bond between two Mel molecules yields a dimer with three chlorine atoms (*m/z* 573) as found at t_R = 3.80 min. The product ion spectrum shows a signal at *m/z* 269 and one at *m/z* 252

Table 2. Dimeric structures: chemical formulas, exact masses and isotope patterns

Exact mass	Chemical formula	Name(s)		Bond type	Retention time (s)	
519.2819	$C_{26}H_{39}O_7N_4Cl_0^+$	MOH-MOH		Ether	–	
		MOH-DOH		Ester	2.54	
		MOH-DOH		Amine	2.54	
537.2480	$C_{26}H_{38}O_6N_4Cl_1^+$	Mel-MOH		Ether	–	
		Mel-DOH	MOH-MOH	Ester	2.92	3.00
		Mel-DOH	MOH-MOH	Amine	2.92	3.00
555.2141	$C_{26}H_{37}O_5N_4Cl_2^+$	Mel-Mel		Ether	–	
		Mel-MOH	MOH-Mel	Ester	3.39	3.49
		Mel-MOH	MOH-Mel	Amine	3.39	3.49
573.1802	$C_{26}H_{36}O_4N_4Cl_3^+$	Mel-Mel		Ester	3.80	
		Mel-Mel		Amine	3.80	

Rapid Communications in Mass Spectrometry

typical for a structure with a Mel residue with a remaining Cl atom. Based on our current data, ester or amine linkage cannot be distinguished.

m/z 555

Two peaks are observed in the RIC for *m/z* 555 (t_R = 3.39 and 3.49 min). The mass difference with the previous compound (18 Da) and the difference in their isotope pattern (one Cl atom) can be explained by the hydrolysis of the chlorine atom of *m/z* 573. Hydrolysis can take place for any of the chlorines present (see Fig. 4): substitution for -OH of -Cl_A or -Cl_B leads to identical dimers due to free rotation of the mustard part (C–N bond). Substitution of Cl_C leads to an isomeric molecule with identical exact mass but with different retention time.

MS spectra at both retention times show an ion with a double chlorine isotope pattern at *m/z* 555.2120 ($C_{26}H_{37}O_5N_4Cl_2^+$, calc. 555.2141 Da), indicating that these compounds are isomers. The product ion spectrum for each chromatographic peak shows the presence of the 269/252 (t_R = 3.49 min; $–OH_C$ position) or the 251/234 (t_R = 3.39 min; $–OH_A$ or $–OH_B$ position) fragmentation pattern, identifying the position of the hydrolysis.

m/z 537

In the RIC for *m/z* 537, three chromatographic peaks are found. For each compound the product ion spectrum is recorded. The compounds eluting at t_R = 2.92 and 3.00 min (*m/z* 537.2469; $C_{26}H_{38}O_6N_4Cl_1^+$, calc. 537.2480 Da) show a single chlorine isotope pattern whereas the peak at t_R = 3.38 min (*m/z* 537.1995; $C_{26}H_{35}O_4N_4Cl_2^+$, calc. 537.2036 Da) contains two chlorine atoms. The presence of the chlorine atoms is confirmed by the accurate mass data.

The product ion spectrum of the peak at t_R = 2.92 min shows the presence of the *m/z* 269/252 fragment ions (Fig. 4). The 251/234 fragments are observed for the compound with identical accurate mass eluting at t_R = 3.00 min.

The compound at t_R = 3.38 min also contains the 269/252 fragments. When comparing all three data sets, additional fragment ions (with low intensities) are observed at t_R = 3.38 min. These additional fragments are formed after the loss of e.g. 28 Da, 45 Da or 101 Da from the $[M+H]^+$ ion. We were not yet able to assign all of these signals to specific fragment ions.

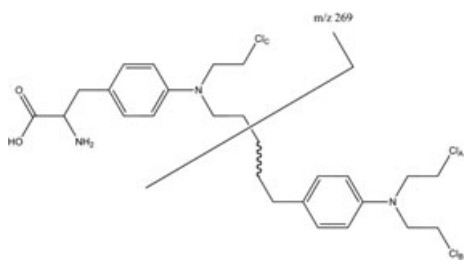

Figure 4. Independent of whether the melphalan dimers are linked through an ester or amine bond, substitution can take place on the three unreacted Cl atoms (Cl_A, Cl_B or Cl_C). Fragmentation of the ion results in an *m/z* 269 ion which, after additional loss of NH_3, leads to an *m/z* 252 ion. Substitution on Cl_C by –OH gives a 251/234 fragmentation pattern.

The elemental composition as derived from its accurate mass, including the presence of two Cl atoms for the *m/z* 537.2036 compound (t_R = 3.38 min), implies that this structure is the result of an elimination of water from the *m/z* 555.2141 dimer (t_R = 3.49 min; $C_{26}H_{37}O_5N_4Cl_2^+$). Further hydrolysis of this compound results in a dimer at *m/z* 519.2411 ($C_{26}H_{36}O_5N_4Cl_1^+$, calc. 519.2374 Da) and *m/z* 501.2724 ($C_{26}H_{37}O_6N_4Cl_0^+$, calc. 501.2713 Da). These compounds are indeed detected at t_R = 3.05 and 2.69 min, respectively.

m/z 519

Plotting the RIC for *m/z* 519 shows the presence of two chromatographic peaks at t_R = 2.54 and 3.05 min. MS spectra for these peaks reveal that both compounds have isobaric masses. The isotope patterns reveal that the compound at t_R = 2.54 min contains no chlorine atoms, whereas the other structure (t_R = 3.05 min) contains a single chlorine atom.

The product ion spectrum for the dimer at t_R = 2.54 min (*m/z* 519.2827, $C_{26}H_{39}O_7N_4Cl_0^+$, calc. 519.2819 Da) shows the 251/234 fragmentation pattern, confirming the absence of a chlorine atom in the part of the ion that is split off and analyzed.

The presence of the Cl atom in the product ion pattern (269/252) for the ion present at t_R = 3.05 min reveals that both chlorines of the second Mel moiety were hydrolyzed followed by elimination of H_2O resulting in the elemental composition as detected for this product (*m/z* 519.2411, $C_{26}H_{36}O_5N_4Cl_1^+$, calc. 519.2374 Da).

Higher-order oligomers

Observation and identification of the Mel dimers in our samples led us to the assumption that higher-order oligomers of Mel can also be formed. Theoretical structures, their masses and isotope patterns were calculated and spectra and RICs were searched for the presence of these compounds.

Spectra show clear but low-abundance signals in RICs for predicted masses at *m/z* 751.4000, 769.3691, 769.4136, 1001.5348 and 1019.5009 (data not shown). Cluster ions do not appear to contribute to these signals.

Chromatographic data shows that our method is not optimized for the separation of these low-abundance trimers and tetramers resulting in peak broadening and overlapping chromatographic peaks.

Multiply charged ions

In the MS spectra of the dimers, intense signals were observed at half the *m/z* value of the analyzed compounds. Closer investigation showed that for dimeric compounds, $[M+2H]^{2+}$ ions are observed while in the spectra of trimeric compounds additional $[M+3H]^{3+}$ ions are observed as well. The number of Mel subunits determines the maximum charge state.

MRM analysis of ILuP samples

An MRM method was developed on the Xevo TQ MS detector and included Mel, MOH, DOH and all dimers (see Table 1).

Four perfusate samples acquired at different time points for each of two treated patients were analyzed: before the start of the ILuP (ILuP blank) and 5 min, 15 min and 30 min after the start of ILuP. For patient 2, an extra sample, i.e. arterial blood,

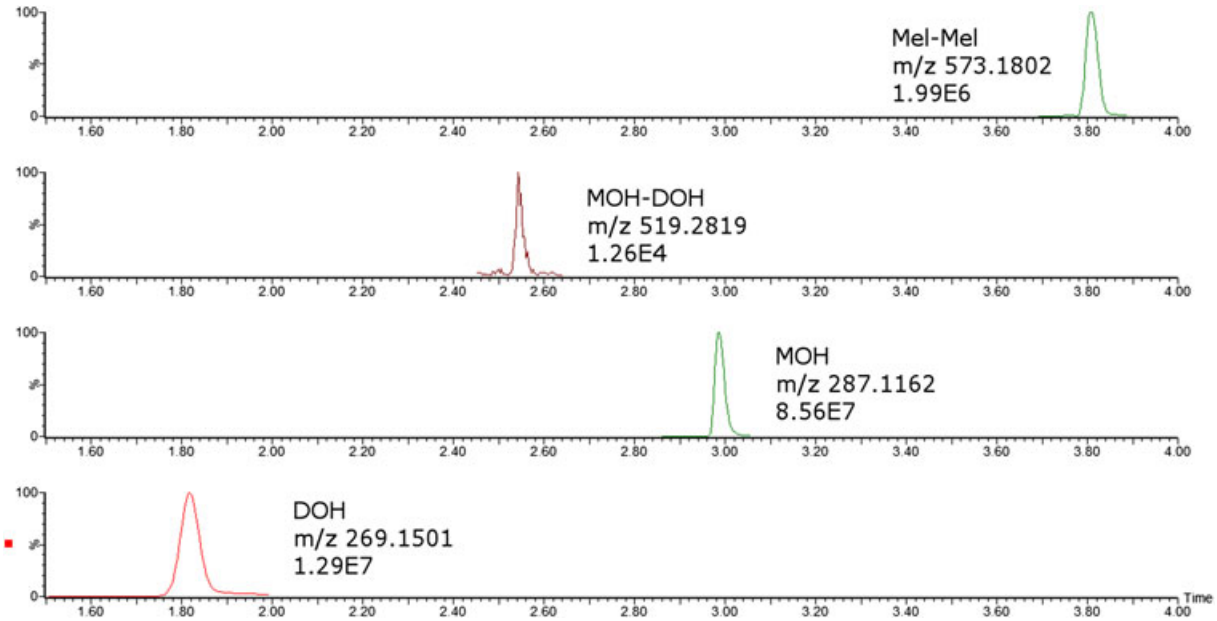

Figure 5. MRM analysis of an ILuP sample showing the presence in an *in vivo* sample of MOH, DOH and two of the newly discovered dimeric compounds. Other dimeric compounds are present (data not shown).

was available that was acquired 60 min after injection of Mel (or 30 min after end of ILuP). The samples were analyzed in triplicate, separated by blanks (H_2O) to prevent and check for carryover.

Analysis of the perfusate samples acquired before the start of the perfusion (ILuP blanks) shows no signal for Mel, MOH or DOH, nor for the analyzed dimers as expected, because no Mel has been introduced in the circuit at this point. Samples acquired after the start of ILuP show signals for the monomeric hydrolysis compounds and at lower signal intensities the newly characterized dimeric compounds (see Fig. 5).

The arterial blood sample for patient 2 allowed to check for a leakage of Mel into the system after rinsing of the lung and decannulation (restoring of the normal blood circuit of the lung). Mel, MOH and DOH are detected in the blood samples (S/N >5). The dimeric compounds are below the detection limit.

The presence of Mel and its hydrolysis products in the arterial blood can be explained by a minor leakage of the perfusion fluid into the system. Even though after ILuP the lung is rinsed with fresh perfusion fluid, some remaining melphalan can enter the blood stream after decannulation of the pulmonary artery.

CONCLUSIONS

It is known that aqueous solutions of the nitrogen mustard and chemotherapeutic agent melphalan contain the (monomeric) hydrolysis products MOH and DOH. Based on LC/MS/MS data, we report the existence of a new class of hydrolysis products that were identified as oligomers of melphalan. Dimers were characterized based on accurate mass, isotope pattern and product ion spectra. Low-abundance signals that are attributed to trimeric structures were detected as well. The dimers differ in the number of remaining Cl atoms after elimination of H_2O or the hydrolysis of the chlorine atom.

Samples originating from two patients treated with an isolated lung perfusion were analyzed. In accordance with *in vitro* experiments (data not shown), ILuP samples show higher signals for all monomeric hydrolysis products compared to the signals for all dimeric products.

Different patients show different profiles of Mel hydrolysis during the ILuP treatment.

The availability of an arterial blood sample acquired post-perfusion allowed us to investigate the leakage of melphalan into the blood stream. Data shows that there are low but clear signals visible for melphalan, MOH and DOH (S/N >5).

Using the described LC/MS/MS method we were able to confirm the presence of mono- and dimeric hydrolysis products of melphalan in *in vivo* perfusate samples of individual patients. The effect of these hydrolysis products on the treatment of lung metastases during an isolated lung perfusion with melphalan is not yet known.

Interpretation of the differences and potential application of this information requires further development of the analytical method and patient follow-up in a larger study.

Acknowledgements

We would like to thank the Hercules Foundation for financial support; Professor P. Van Schil, M.D., for providing us information about and giving us the opportunity to attend the ILuPs; the surgeons from the Department of Thoracic and Vascular Surgery (UZA) for providing us with the patient samples; and Gunther Guetens and Gert De Boeck for helping us with the perfusion fluid sample preparation.

Rapid Communications in Mass Spectrometry

REFERENCES

[1] H. M. Kroon, M. Moncrieff, P. C. A. Kam, J. F. Thompson. Outcomes following isolated limb infusion for melanoma: a 14-year experience. *Ann. Surg. Oncol.* **2008**, *15*, 3003.

[2] M. Tattersall, M. Jarman, E. Newlands. Pharmaco-kinetics of melphalan following oral or intravenous administration in patients with malignant disease. *Eur. J. Cancer* **1978**, *14*, 507.

[3] B. Golding, M. Kebbell. Chemistry of nitrogen mustard [2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine] studied by nuclear magnetic resonance spectroscopy. *J. Chem. Soc.* **1987**, *6*, 705.

[4] T. Hamill, M. Colvin. NMR studies of the conjugation of mechlorethamine with glutathione. *J. Med. Chem.* **1990**, *33*, 1009.

[5] P. E. Van Schil, J. M. Hendriks, B. P. van Putte, B. A. Stockman, P. R. Lauwers, P. W. ten Broecke, M. J. Grootenboers, F. M. Schramel. Isolated lung perfusion and related techniques for the treatment of pulmonary metastases. *Eur. J. Cardiothorac. Surg.* **2008**, *33*, 487.

[6] J. M. Hendriks, P. E. Van Schil, G. De Boeck, P. R. Lauwers, A. A. Van Oosterom, E. A. Van Marck, E. J. Eyskens. Isolated lung perfusion with melphalan and tumor necrosis factor for metastatic pulmonary adenocarcinoma. *Ann. Thorac. Surg.* **1998**, *66*, 1719.

[7] J. M. H. Hendriks, M. J. J. H. Grootenboers, F. M. N. H. Schramel, W. J. van Boven, B. Stockman, C. A. Seldenrijk, P. ten Broecke, C. A. J. Knibbe, P. Slee, E. De Bruijn, R. Vlaeminck, J. Heeren, J. B. Vermorken, B. van Putte, S. Romijn, E. Van Marck, P. E. Y. Van Schil. Isolated lung perfusion with melphalan for resectable lung metastases: a phase I clinical trial. *Ann. Thorac. Surg.* **2004**, *78*, 1919.

[8] M. J. H. Grootenboers, J. M. H. Hendriks, W. J. van Boven, C. A. J. Knibbe, B. van Putte, B. Stockman, E. De Bruijn, J. B. Vermorken, P. E. Y. Van Schil, F. M. Schramel. Pharmacokinetics of isolated lung perfusion with melphalan for resectable pulmonary metastases, a phase I and extension trial. *J. Surg. Oncol.* **2007**, *96*, 583.

[9] S. A. Stout, C. M. Riley. The hydrolysis of L-phenylalanine mustard (melphalan). *Int. J. Pharm.* **1985**, *24*, 193.

[10] S.A. Stout, C.M. Riley. Hydrolysis of l-phenylalanine mustard (melphalan) II. Further observations on the effects of pH, chloride ions and buffers on the rate of reaction. *Int. J. Pharm.* **1987**, *37*, 257.

[11] G. Fortier, N. Bernier, D. Bates. Kinetic studies of melphalan thermodegradation by HPLC. *Anal. Lett.* **1991**, *24*, 961.

[12] G. De Boeck, K. Van Cauwenberghe, A. Eggermont, A. Van Oostertom, E. de Bruijn. Determination of melphalan and hydrolysis products in body fluids by GC-MS. *J. High. Resolut. Chromatogr.* **1997**, *20*, 697.

[13] B. Van den Driessche, F. Lemière, W. Van Dongen, E. L. Esmans. Alkylation of DNA by melphalan: investigation of capillary liquid chromatography–electrospray ionization tandem mass spectrometry in the study of the adducts at the nucleoside level. *J. Chromatogr. B* **2003**, *785*, 21.

