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Comparison between capillary and nano liquid chromatography–electrospray mass spectrometry for the analysis of minor DNA–melphalan adducts

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Abstract

Nano liquid chromatography (nanoLC) coupled to electrospray mass spectrometry (ES-MS) was evaluated for the analysis of DNA adducts in melphalan-treated Jurkat cells. The detection limit of the nanoLC–ES-MS–MS system was assessed using a dAMP–melphalan adduct. Compared to capillary liquid chromatography (capLC) ES-MS the absolute detection limit could be improved by a factor 10, leading to the detection of 395 fg dAMP–melphalan adduct under single-ion monitoring conditions at a S/N of 14. Minor adducts such as cross-linked adducts could be detected in in vitro solutions of 2′-deoxynucleotides (dNMP) treated with melphalan using column-switching nanoLC–ES-MS. These adducts were not found using capLC–ES-MS. More detailed structural information of the alkylation sites was obtained by examining the nanoLC–ES-MS–MS data. Jurkat cells were treated with melphalan, the modified DNA was isolated and enzymatically hydrolyzed. Several modified dinucleotides were identified, the most abundant adducts were pdG$^{Mel(Cl)}$pdC (m/z=453, t_r=17.0 min) and pdG$^{Mel(OH)}$ pdC ring opened (m/z=453, t_r=39.5 min).

Keywords: DNA; Melphalan

1. Introduction

Alkylation of DNA is considered as the primary basis for the mutagenic and carcinogenic activities of alkylating agents [1]. Melphalan (L-phenylalanine mustard) is an antineoplastic alkylating agent that covalently binds to intracellular nucleophilic sites. Mono-adducts of mustards most frequently occur at the N^7 position of guanine [2]. Or as suggested by Charles et al. mono-adducts of melphalan also can occur at the N^3 of adenine [3]. Because melphalan is a bifunctional alkylating agent it can cause intra-strand and inter-strand cross-linked adducts. It is likely that intra-strand cross-linked adducts prevent DNA replication or DNA transcription to RNA because of the stereochemical effects introduced by the alkylation process. Inter-strand cross-linking is one of the important mechanisms for cell death or chromosome loss. If inter-strand cross-linked adducts are formed and not repaired, they will prevent the dissociation of DNA strands required for successful DNA replication. The specificity and kinetics of

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inter-strand and intra-strand bifunctional alkylation by nitrogen mustards at a G–G–C sequence was well described by Bauer and Povrik [4]. They concluded that secondary alkylation for melphalan was much slower compared to other mustards and they did not observe an intra-strand cross-link formed by melphalan. Tilby et al. [5] characterized several cross-linked products from the in vitro reaction of melphalan with dGMP or with GMP using an antibody specific for melphalan-alkylated DNA. They also detected the protonated molecules of the adducts using fast atom bombardment (FAB). Osborne et al. [6,7] claimed the presence of cross-linked adducts between two guanine residues and melphalan and between guanine and adenine. The structural data were however not elaborated. Recently Tilby et al. [8] synthesized a monofunctional derivative of melphalan, i.e., monohydroxy-melphalan, in which a hydroxyl group replaced one chlorine atom. In its reaction with dAMP only one adduct was found. But also here limited proof for the structure of the corresponding adducts was given. They claim, although this seems logical to us, that no cross-linked adducts were present. The used techniques did not give unambiguously structural information and several factors can influence the interaction between the antibody and the adduct. In particular, it has not been possible to determine if the antibodies, when applied to polymeric DNA, preferentially recognize bi- or monofunctional melphalan adducts. Furthermore it is interesting to note that many interesting studies are hampered by the limited amount of cellular material that can be obtained from patients treated with melphalan. In a previous paper [9] we have demonstrated the benefit and limitations of capillary liquid chromatography (capLC) combined with electrospray mass spectrometry (ES-MS) for the analysis of adducts formed by the interaction of melphalan with several 2′-deoxynucleoside-5′-monophosphates and with calf thymus DNA in vitro. In these experiments no cross-linked adducts were identified. Therefore we concluded that either the concentration of the cross-linked adducts formed in the analyzed mixtures was below the detection limit or that cross-linked adducts were not present at all. However, as shown by Vanhoutte et al. [10] the coupling of ES-MS with high-performance liquid chromatography (HPLC) columns with even smaller internal diameter (I.D. 75 μm) will result in a better detection limit since theoretically the mass sensitivity of the system is improved by a factor 18. Therefore the capLC–ES-MS system was replaced by a nanoLC system coupled to nano-electrospray tandem mass spectrometry (nanoES-MS–MS). This methodology allowed us, as we will show below, the detection and identification of some cross-linked adducts in dNMP samples, which are treated with melphalan in vitro. Furthermore both the qualitative and quantitative analysis of hydrolysates of DNA pellets isolated from melphalan-treated Jurkat cells were performed in combination with a column-switching system and the sensitivity of both the capLC–ES-MS system and the nanoLC–ES-MS system was compared.

2. Experimental

2.1. Materials

Melphalan, 2′-deoxyadenosine-5′-monophosphate (dAMP), thymidine-5′-monophosphate (TMP) and 2′-deoxyguanosine-5′-monophosphate (dGMP) were obtained from Sigma (Sigma–Aldrich, Belgium). Methanol, glacial acetic acid (analytical grade), ammonium acetate (analytical grade), $ZnCl_2$, NaOAc and 2′-deoxycytidine-5′-monophosphate (dCMP) were purchased from Acros (Geel, Belgium). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFiP) was purchased from Fluka (Sigma–Aldrich, Belgium). Nuclease P_1 (EC 3.1.30.1) from *Penicillium citrinum* was purchased from Sigma.

Jurkat cells were available in the hematology laboratory. The cultures were maintained between $3 \cdot 10^5$ and $9 \cdot 10^5$ cells/ml, 5% CO_2 at 37°C and the medium contains 10% fetal bovine serum (FBS) purchased from Biochrom (Berlin, Germany). Millipore Milli-Q purity water was used in all experiments.

Caution: the drug included in this study has toxic properties and is listed as a carcinogen to humans. Appropriate precautions were taken and melphalan was handled with great care.

2.2. Preparation of melphalan–2′-deoxynucleotide cross-linked adducts

Several reaction mixtures were prepared out of stock solutions. Each stock solution contained 2 mg

of a 2′-deoxynucleotide (dAMP, dCMP, dGMP or TMP) in 1.5 ml NH_4OAc (0.01 *M*, pH 6.9). To 350 μl of a stock solution of a 2′-deoxynucleotide, 350 μl of another stock solution of a 2′-deoxynucleotide was added. As such the following stock solutions, of the type dNMP–dN′MP were prepared: dAMP+dCMP, dAMP+TMP, dGMP+dCMP, dGMP+TMP and dCMP+TMP. To each of these solutions 250 μl of a melphalan solution [0.6% (w/v) melphalan in methanol] was added. The final concentration of the nucleotides in the samples injected on the pre-column is approximately 1.25 μg/μl. In order to be able to check for the presence of dNMP–melphalan–dNMP cross-linked adducts melphalan was also added to the pure dNMP stock solutions. In these samples no dN′MP–melphalan–dNMP cross-linked adducts can be formed. This facilitated the analysis of the mixed samples. The solutions were kept at 37°C and aliquots were taken after 3 h. Another set of solutions was kept at 37°C for 17 h.

2.3. Preparation of melphalan adducts in the Jurkat cells and the enzymatic hydrolysis to 2′-deoxynucleotides

Jurkat cells were treated with melphalan according to the following protocol. To a cell suspension of $1.6 \cdot 10^6$ cells/ml, a melphalan stock solution of 50 m*M* (in methanol) was added, the final concentration of melphalan is 0.5 m*M*. The solution was incubated at 37°C (5% CO_2). After 24 and 48 h the DNA was isolated out of the treated cells using a RapidPrep Macro Genomic DNA Isolation Kit for blood (Pharmacia Biotech). The resulting DNA pellet (approximately 250 μg) was dissolved in 90 μl NaOAc (0.1 *M*, pH 5.2) and 75 μl $ZnCl_2$ (2 m*M*) and hydrolyzed with nuclease P_1 (4 units) for 5 h. The complete hydrolysis procedure is described previously [9]. The enzymatically hydrolyzed cell DNA was stored at −20°C and used for further analyses.

2.4. Mass spectrometric conditions

Positive ion (+) and negative ion (−) electrospray mass spectra were recorded on a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). The drying gas (N_2) was used at a flow-rate of 250–300 l/h in the capillary set-up and 35–40 l/h in the nano set-up. The nebulizing gas (N_2), approximately 15 l/h, is only needed in the capillary set-up. The voltage on the capillary was between 2.3 and 3 kV. Low-energy collision activated decomposition (CAD) spectra were obtained using argon as collision gas. The collision cell pressure was ca. $3.0 \cdot 10^{-3}$ mbar and the collision energy was optimized for each component. The optimal collision energy varied between 5 and 60 eV. The source temperature was set to 80°C in the capillary set-up and 45°C in the nano set-up. The mass spectra were recorded in the continuum mode and in multiple channel acquisition (MCA) mode. The scan range of the first quadrupole was 400–1000 (scan time 2.5 s). For LC–MS–MS experiments the scan range of the second quadrupole was set from 20 to [M+10] u. (scan time 1.5 s). Single ion monitoring (SIM) was performed on $[MH]^+$ ions, the span was set to 0.1 u and the dwell time to 80 ms. Data were acquired and processed by the Masslynx data system.

2.5. Chromatographic conditions

2.5.1. Column switching

A Kontron HPLC system (Kontron Instruments, Milan, Italy) was used for the LC–ES-MS experiments. The system consists of a 325 HPLC pump, a 332 HPLC detector (λ=260 nm) and a capillary (or nano depending on the set-up) U-shaped flow cell (LC-Packings, Amsterdam, The Netherlands) with cell volumes of 40 nl and 3 nl, respectively. Aliquots of 6 μl (capillary set-up) and 10 μl (nano set-up) were injected with a FAMOS (fully automated micro sampling workstation, LC-Packings, Amsterdam, The Netherlands) equipped with a two-valve system that allows column-switching experiments. The column-switching system for the capLC–MS set-up was described previously [9]. The dimensions of the capillary column used were 15 cm×300 μm. The pre-column was 0.5 cm×500 μm. The packing for both columns was Hypersil BDS C_{18} (LC-Packings). In the nanoLC set-up 10-μl samples dissolved in HOAc (2%, v/v)–MeOH (99:1) were injected on a 5 mm×300 μm I.D. pre-column filled with Hypersil BDS C_{18} (LC-Packings). Adducts were captured by eluting the pre-column with an isocratic buffer consisting of HOAc (2%, v/v)–MeOH (99:1) at a flow-rate of 15 μl/min delivered by an additional pump (HPLC pump 422, Kontron Instruments).

Unmodified nucleotide material was sent to a waste (monitored by a second UV system equipped with a capillary U-shaped flow cell). After 90 s the adducts were backflushed to the analytical column (15 cm× 75 μm I.D.). Initially a mobile phase of HOAc (2%, v/v)–MeOH (80:20) is used, in 30 min the composition was changed to HOAc (2%, v/v)–MeOH (50:50). The latter conditions were maintained for 10 min after which initial conditions were restored in 15 min. The flow-rate was 150–350 nl/min. The column-switching system, used for the analysis of the hydrolysate of modified Jurkat DNA was identical to the system used for the analysis of the samples that only contain dNMPs but the adducts were backflushed to the analytical column after 5.0 min as explained by us [9]. They were separated using a gradient elution from HOAc (2%, v/v)–MeOH (80:20) to (50:50) in 30 min. The latter composition was maintained for another 10 min and changed to 100% MeOH in 15 min. These conditions were maintained for 5 min after which initial conditions were restored in 15 min.

2.5.2. Flow-injection analyses

When column-switching nanoLC–ES-MS experiments indicate that only one isomer of a cross-linked species is formed in the reaction mixture, the adducts can be infused into the mass spectrometer using a syringe pump (Harvard Apparatus, type 2111). At a flow-rate of 100–400 nl/min (depending on the volume of the sample) the samples were infused using the solvent in which the reaction took place as mobile phase and the low-energy CAD product ion spectra were recorded. These infusion experiments can be recorded in MCA mode and give a better signal-to-noise ratio (S/N) due to the fact that the average of the noise is recorded. The major advantage of this procedure is the gain in time, only 1/45 of the time needed for a column-switching nanoLC–ES-MS–MS experiment is used. The previous procedure can only be used for the analysis of the in vitro reaction mixtures of melphalan and 2′-deoxynucleotides. The modified DNA hydrolysate from the Jurkat cell cultures cannot be analyzed by this procedure because of the presence of salts. These salts are needed for the DNA isolation. In the case of column-switching nanoLC–ES-MS experiments the salts are removed by column switching.

3. Results and discussion

3.1. Sensitivity

Modification of one nucleotide per 10^7 nucleotides leads to a concentration of approximately 0.3 fmol adduct/μg DNA. If 100 μg DNA is isolated out of a biological sample, then the absolute detection limit of the used technique should be 30 fmol adduct. This corresponds to the isolation of pg-amounts of adducts, e.g., 18 pg for an adduct with molecular mass of 600. In order to compare the column-switching nanoLC–ES-MS system with the column-switching capLC–ES-MS system under ES(+) conditions an isolated dAMP–melphalan adduct was measured [9]. The $[MH]^+$ ion at $m/z=600$ was monitored under SIM conditions. A detection limit of 4 pg (6.58 fmol) on-column was found with a S/N of 8 and an RSD of 1.06% ($n=5$) using the column-switching capLC–ES-MS system. For the nano set-up a detection limit of 0.395 pg adduct (0.658 fmol) on-column was found with a S/N of 14 and an RSD of 0.93% (SIM) ($n=5$). In Fig. 1 the reconstructed ion chromatogram (RIC) of the isolated dAMP–melphalan adduct is shown. Against all expectations no lower concentrations could be measured reliably. The measured peak area of samples with a concentration lower than $6.6\cdot10^{-11}$ M hardly differed from a blank run. The detection limit of the capillary set-up using single reaction monitoring (SRM) was already described previously [9]. A detection limit of 29.8 pg (49.67 fmol) of the adduct injected on-column was achieved. SRM experiments were not performed in the nano set-up as it is less sensitive compared to SIM. From these results we can conclude that the mass sensitivity of the system was improved by a factor 10, leading to the detection 395 fg of an isolated dAMP–melphalan adduct under SIM conditions. Theoretically, downscaling from cap- to nanoLC should give an improvement of the mass sensitivity of 18 times [down-scaling factor $f=(\text{diameter}_{\text{conventional}}/\text{diameter}_{\text{miniaturized}})^2$].

3.2. Quantification

The linearity of the developed system was evaluated using 6 μl of different concentrations with a range from $1.1\cdot10^{-9}$ M to $3.3\cdot10^{-7}$ M of an isolated

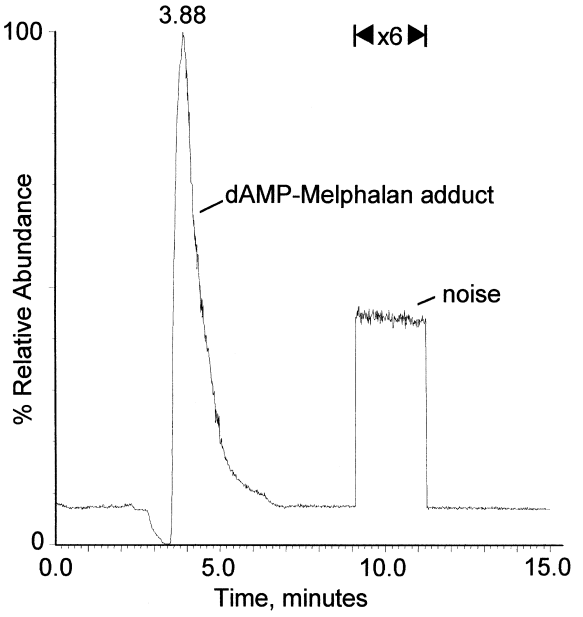

Fig. 1. Reconstructed ion chromatogram for $[MH]^+$ (m/z 600) of a SIM analysis. Injection of 10 μl of a $6.6 \cdot 10^{-11}$ *M* solution of an isolated dAMP–melphalan adduct (*MM*=599) using column-switching nanoLC–ES-MS [10 μl injected on the pre-column (0.5 cm×300 μm), back-flushed after 90 s].

dAMP–melphalan adduct. A r^2 value of 0.9997 was found for this concentration range using the column-switching capLC–ES-MS system ($y=6.8 \cdot 10^8 x+$ 262) [9]. The column-switching nanoLC–ES-MS system was evaluated by injecting 10 μl of different concentrations with a range from $6.6 \cdot 10^{-11}$ *M* to $6.6 \cdot 10^{-7}$ *M* of the aforementioned adduct. These experiments were performed under the chromatographic conditions described previously (Section 2.5.1) and an r^2 value of 0.8953 was measured ($y=3.0 \cdot 10^{10} x+$ 307). This lower r^2 value could be explained by scattering of the datapoints in the lower concentration region due to the delicacy of the nanospray and the nanoLC system. On the other hand the sensitivity of the column-switching nanoLC–ES-MS system was higher and the S/N was better. A sample with a concentration of $1.1 \cdot 10^{-9}$ *M* was measured in both systems. The S/N in the capLC–ES-MS system was 8 and the S/N in the nanoLC–ES-MS was 22, so a gain of approximately 2.5 times could be achieved. Quantification of this mono-alkylated dAMP–melphalan adduct in melphalan-treated Jurkat cells and in modified calf thymus DNA was also performed. The column-switching capLC–ES-MS system was the first choice for these experiments because of the higher reliability (and better linearity). Moreover we expected that the system should be sensitive enough to detect the dAMP adduct in melphalan-treated Jurkat cells and in modified calf thymus DNA. Usually these quantification experiments are performed using an internal standard. Due to the absence of a stable isotope labeled compound, external calibration was performed, keeping in mind that this is not the ideal way for quantification. In the Jurkat cells a 5.24 pg adduct was found (RSD=0.95%, $n=3$) and in the modified calf thymus DNA a 1166 pg adduct was found (RSD=10.6%, $n=3$).

3.3. The column-switching nanoLC–ES-MS and nanoLC–ES-MS–MS data

3.3.1. The cross-linked adducts dNMP–melphalan–dNMP resulting from the interaction of melphalan with dNMPs

In order to get an idea of which cross-linked adducts might be formed in Jurkat cell lines and in modified calf thymus DNA several reaction mixtures were prepared using stock solutions of regular dNMPs. These samples were analyzed by column-switching nanoLC–ES-MS. Product ion spectra were obtained either under ES(+) or (−). These product ion spectra were performed under flow-injection analysis (FIA) or in column-switching experiments.

3.3.1.1. The dAMP–melphalan–dAMP cross-linked adducts. In the following reaction mixtures dAMP–melphalan–dAMP cross-linked adducts were found: dAMP, dAMP+dGMP, dAMP+dCMP and dAMP+TMP. In Fig. 2 tentative structures are shown. The RICs for the $[MH]^+$ ions corresponding to dAMP–melphalan–dAMP cross-linked adducts (m/z 931, 913 and 760) are shown in Fig. 3. Also the $[MH]^+$ [m/z=600 (^{35}Cl)] of the mono-alkylated melphalan adducts of dAMP were reconstructed. The intensity of the signals of the $[MH]^+$ ions of the cross-linked adducts is very weak compared to the signals of the mono-alkylated adducts (e.g., the major cross-linked adduct with m/z 931 is 8.8-times lower in intensity

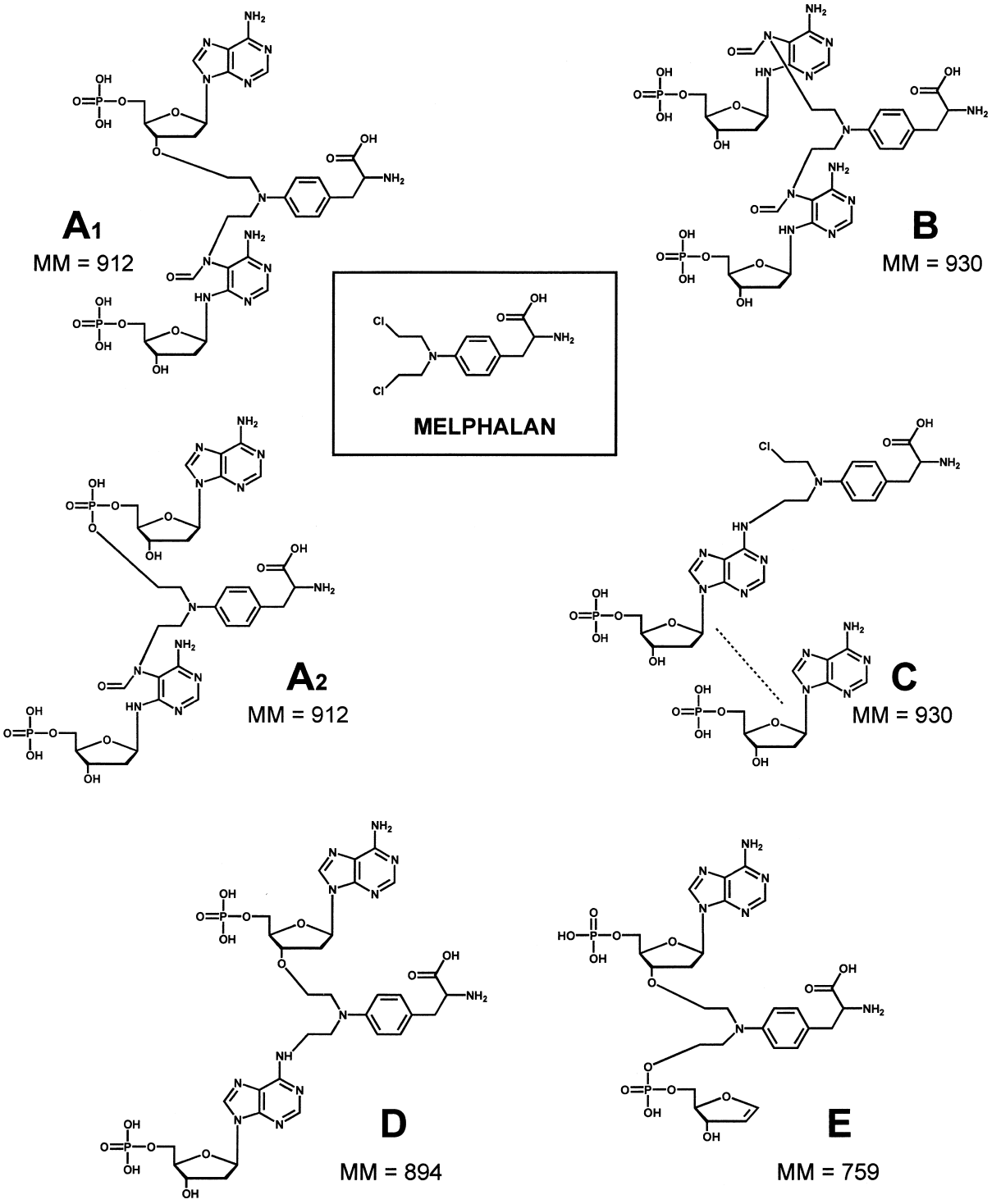

Fig. 2. The structure of melphalan and tentative structures for dAMP–melphalan–dAMP cross-linked adducts.

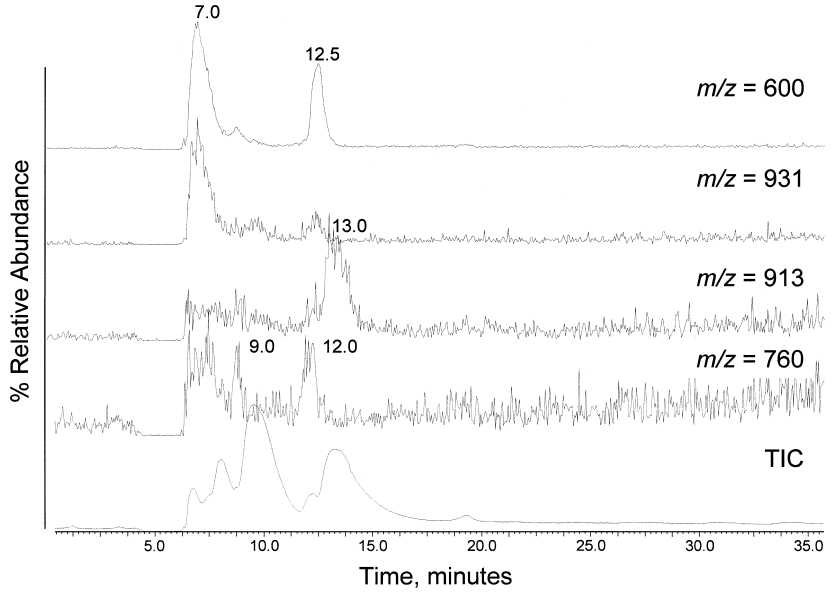

Fig. 3. Analysis of 10 μl of a dAMP–melphalan mixture by column-switching nanoLC–nanoES-MS (full scan). The reconstructed ion chromatograms (RICs) for the $[MH]^+$ ions corresponding to possible dAMP–melphalan–dAMP cross-linked adducts (m/z 931, t_r=7.0 and 12.5 min, 913, t_r=13.0 min and 760, t_r=9.0 and 12.0 min) are shown. Also the RIC for $[MH]^+$ [m/z 600 (^{35}Cl)] of the mono-alkylated melphalan adducts of dAMP is reconstructed for comparison.

than the major mono-alkylated adduct with m/z 600). The compound eluting at t_r=13 min probably corresponds to one of the structures under A (Fig. 2). The $[MH]^+$ ion appeared at a m/z value 18 u higher then expected for an intact dAMP–melphalan–dAMP cross-linked adduct. This observation suggested ring opening. Analogous observations were made by Leonard et al. [11] in a study investigating the reaction of diethyl pyrocarbonate with adenine. In order to get more insight in the structure of the isomeric dAMP–melphalan adducts, the low-energy CAD product ion spectra were recorded. The product ion spectrum of the adduct eluting at t_r=13 min ($[MH]^+$=913) showed the following ions: m/z 136, 332 and 582. These fragment ions can be explained by the protonated adenine moiety $[BH_2]^+$, protonated dAMP and $[MH]^+$–dAMP. This led us to the conclusion that the melphalan moiety linked the dAMP molecules from the N^7 position of base moiety to either the 5′-phosphate or 3′-OH of the sugar moiety. After N^7 alkylation the imidazole moiety underwent ring opening. The corresponding ES(−) product ion scans of $[M-H]^-$ (m/z 911) gave abundant ions at m/z 580 and 330. These ions corresponded with m/z 582 and 332 found in the ES(+) recordings and therefore pointed to the same structures under A (Fig. 2). The compound with $[MH]^+$ (m/z 931) (structure B, Fig. 2) produced a signal at t_r=7.0 and t_r=12.5 min and seemed to co-elute with the mono-alkylated dAMP adduct with m/z 600 (^{35}Cl). The most abundant ion in the product ion scan of m/z=931 eluting at t_r=7.0 min was found at m/z 600 which corresponded to a loss of 331 u, i.e., dAMP (Fig. 4). This result cannot be explained by structure B because of the absence of an intact adenosine moiety. Moreover, additional proof for the presence of dAMP in the structure came from two other ions: m/z 136 ($[BH_2]^+$ of adenine) and m/z 332 ($[dAMPH]^+$). The presence of the ions at m/z 663, 269 and 404 pointed to melphalan in which one chlorine atom was still present. The ion at m/z 404 corresponds with the mono-alkylated base. Furthermore the ion at m/z 269 is a diagnostic ion for the mono-alkylated nucleotide [9]. The isotope pattern of $[MH]^+$ was checked in the full scan spectra proving the presence of a chlorine atom (observed ratio ^{35}Cl:^{37}Cl=43.0%, calculated ratio ^{35}Cl:^{37}Cl=43.3%). Therefore the

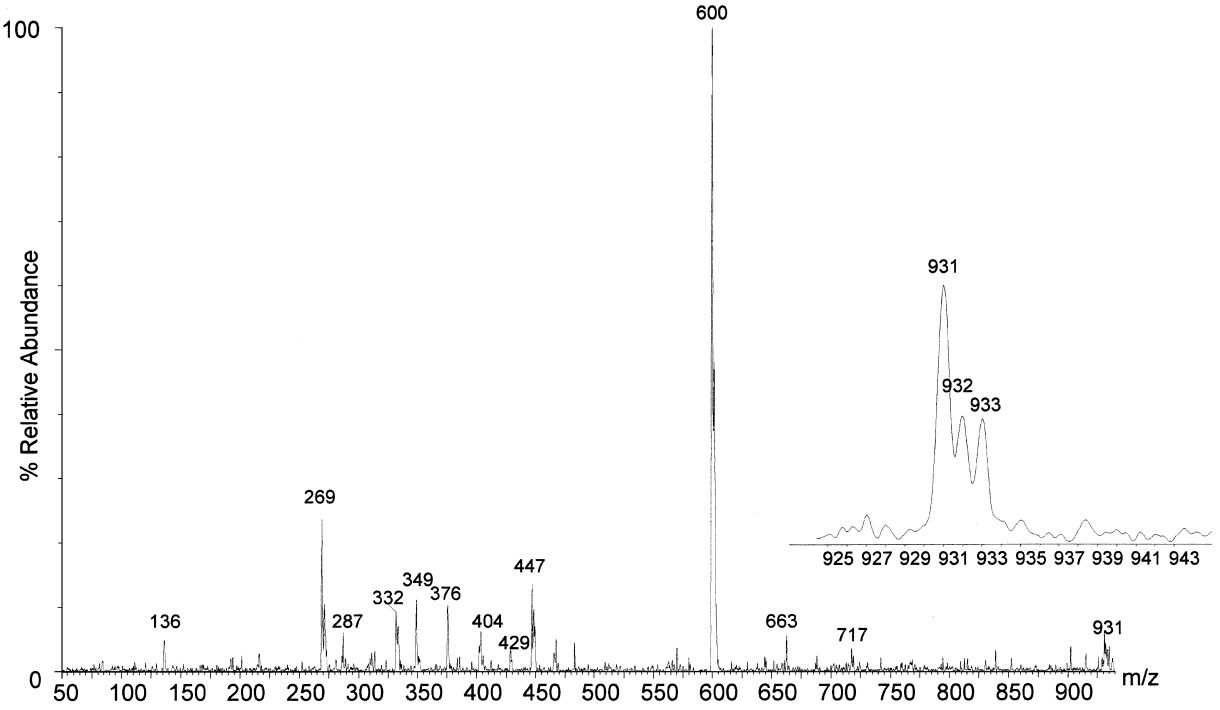

Fig. 4. Low-energy CAD product ion scans of m/z 931. In the insert the isotope pattern for m/z 931 is shown.

structure C was assigned to m/z 931: a non-covalent cluster of dAMP–melphalan and dAMP and not to the cross-linked adduct B. Remarkably the mixture showed no signal at m/z 895, the protonated molecule $[MH]^+$ expected for an intact cross-linked adduct such as structure D depicted in Fig. 2. Further examination of the total ion chromatogram indicated that a compound with $[MH]^+$ at m/z 760 eluted at t_r=9.0 min and t_r=12.0 min. A molecular mass of 759 probably corresponds to structure E (Fig. 2). No product ion scans could be recorded in because of the low abundance of these compounds. In source CAD at a cone voltage of 45 V gave product ions at m/z 662 and 625 for the compound eluting at t_r=12 min. This can refer to a loss of, respectively, a phosphate (loss of 98 u) or adenine (135 u) out of m/z 760. Additionally two other ions at m/z 429 and 644 were seen. The latter refers to a loss of 116 u, i.e., a 2′-deoxyribose moiety. This points to structure E: at least one free 5′-phosphate should be present. Cross-linking to the other phosphate group allows the elimination of the sugar moiety. The product ion at m/z 429 could be explained by the loss of an intact 2′-deoxyadenosine-5′-monophosphate unit. Also FIA experiments were performed. Precursor ion scans recorded in ES(−) for m/z 330, 195, 97 and 79 showed as precursor 758. This can be used as an additional evidence for the structure E. The compounds discussed here could also be found in all the other reaction mixtures containing dAMP.

3.3.1.2. The dGMP–melphalan–dGMP cross-linked adducts. A dGMP–melphalan mixture of which the main adducts have already been described in a previous article [9], was investigated for the occurrence of cross-linked adducts. In all reaction mixtures that contain dGMP cross-linked adducts dGMP–melphalan–dGMP were found. Both ES(−) and ES(+) low-energy product ion spectra were recorded. In the RIC ions were observed at t_r=5.5 min (m/z 945 [ES(+)]) and 6.5 min (m/z 927 [ES(+)]). The most useful structural information could be obtained from the ES(−) data. In panel I of Fig. 7 the product ion spectrum of $[M-H]^-$ at m/z 943 is shown. The molecular mass 944 could be explained as a cross-linked adduct in which one

imidazole ring-opening had occurred. The most abundant ion was found at m/z 596, which corresponded to a loss of dGMP out of $[M-H]^-$. Consecutive loss of a phosphate–sugar moiety (loss of 196 u), resulted in the ion at m/z 400 pointing to the presence of an intact 5′-phosphate 2′-deoxy-D-ribofuranosyl ring system. The ion at m/z 676 could be explained as the loss of 267 (loss of 2′-deoxyguanosine) out of m/z 943. Therefore the structure of the compound eluting at t_r=5.5 min was assigned to structure F (Fig. 5). The most abundant ion in the product ion spectrum of the adduct eluting at t_r=6.5 min ($[M-H]^-$=925, panel II) was found at m/z 346 showing the presence of an intact dGMP moiety. Several losses were found out of $[M-H]^-$: 267 (loss of 2′-deoxyguanosine) and 196 (loss of a 5′-phosphate sugar moiety). These processes gave rise to m/z 658 and 729, respectively. Further loss of *meta*-phosphoric acid (loss of 80 u) out of m/z 658 produced the ion at m/z 578. These data pointed to the presence of a structure G1 where two dGMP molecules were cross-linked from the 5′-phosphate group to the base moiety of the other dGMP unit. The observations ruled out a cross-linked structure

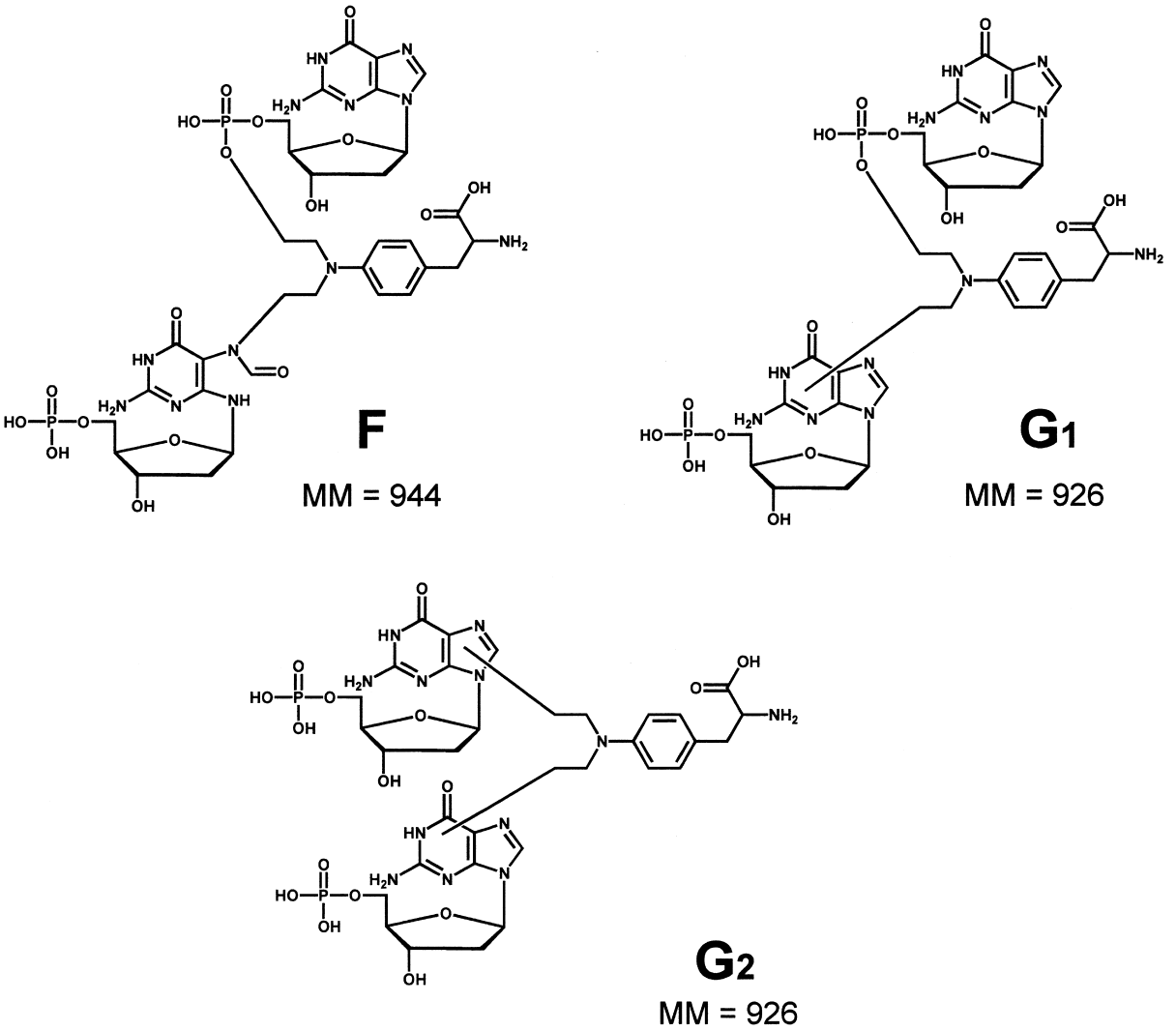

Fig. 5. The possible structures for dGMP–melphalan–dGMP cross-linked adducts.

between the two 5′-phosphate groups because in such a structure the loss of a dGMP molecule instead of a 2′-deoxyguanosine would have been expected. However further investigation of the product ion at *m/z* 729 (tentative structures are shown in Fig. 6) questioned this assignment. When *m/z* 729 was generated by in-source CAD and its product ion scan was recorded a loss of 151 and 196 u was observed. The former could be explained by a loss of a guanine moiety. This observation could be explained either by structure H1 or H2. The loss of 196 u pointed to the loss of again a 5′-phosphate 2′-deoxy-D-ribofuranosyl group and could only be expected in a structure such as H2. Therefore we had to conclude that the mass spectrometric data could not be rationalized by structure G1 alone. But they could be explained by the co-elution of the two isomers G1 and G2.

3.3.1.3. The dCMP–melphalan–dCMP cross-linked adducts and the TMP–melphalan–TMP cross-linked adducts. No dCMP–melphalan–dCMP cross-linked adducts could be detected. As TMP is the less reactive of the four nucleotides no cross-linked adducts were expected. Nevertheless the reaction mixtures that contain TMP were examined for the presence of TMP–melphalan–TMP cross-linked adducts. Both ES(−) and ES(+) full scan spectra were recorded but the results were all negative (Fig. 7).

3.3.2. The cross-linked adducts with dNMP–dN′MP

As expected not only cross-linked adducts of the type dNMP–melphalan–dNMP were formed in the reaction mixtures containing dNMP and dN′MP but also adducts of the type dNMP–melphalan–dN′MP. The study of this asymmetric substituted adducts can be interesting in order to investigate the preferential cross-linked sites in DNA (either from calf thymus DNA or Jurkat cells). Exactly the same column-switching procedure as described for the dNMP–melphalan–dNMP cross-linked species was used for the analysis of these samples. A longer reaction time was also evaluated as the formation of cross-linked adducts might be function of time. However no significant difference was observed between the samples reacted for 3 h at 37°C and those reacted for 17 h at 37°C.

3.3.2.1. The dAMP–melphalan–dGMP cross-linked adducts. In Fig. 8 the possible structures for dAMP–melphalan–dGMP adducts are depicted. For an intact dAMP–melphalan–dGMP adduct a protonated molecule at *m/z* 911 was expected. Only one signal was found for this ion in the reconstructed ion chromato-

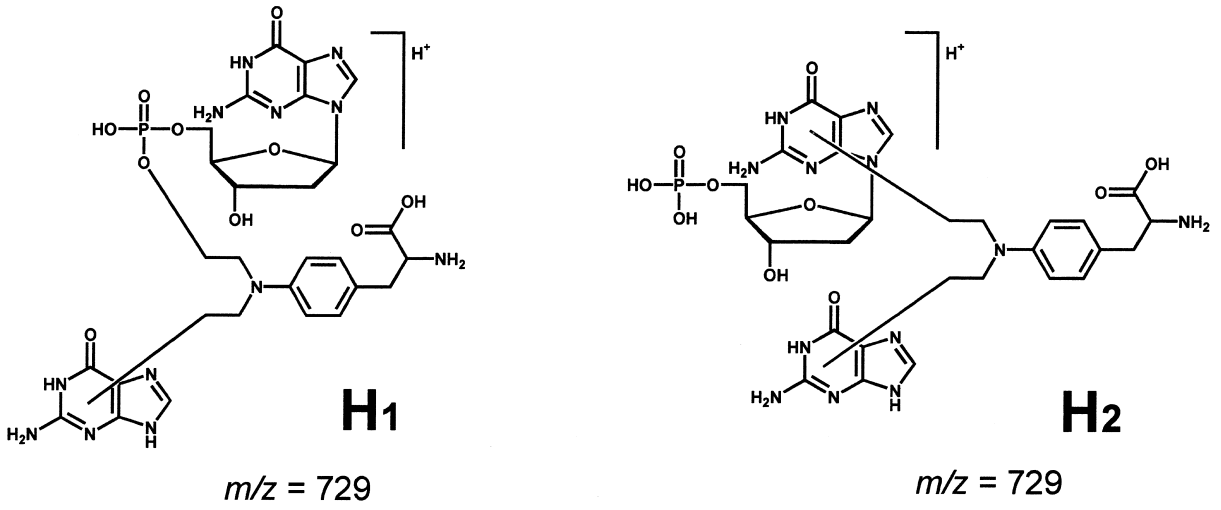

Fig. 6. Tentative structures for the product ion at *m/z* 729.

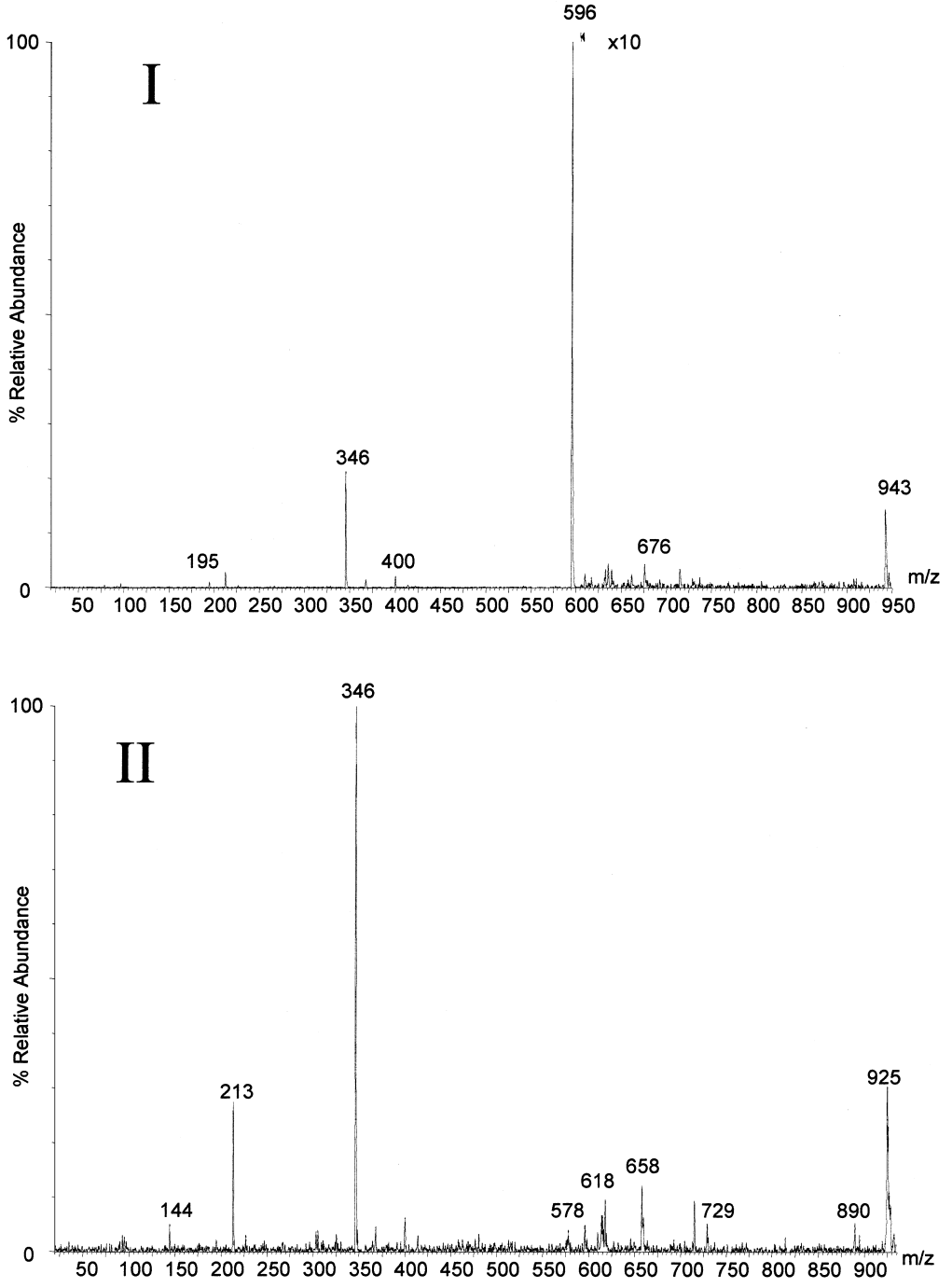

Fig. 7. Low-energy CAD product ion scan of m/z 943 (panel I, collision energy 25 eV) and m/z 925 (panel II, collision energy 15 eV) under ES(−) conditions.

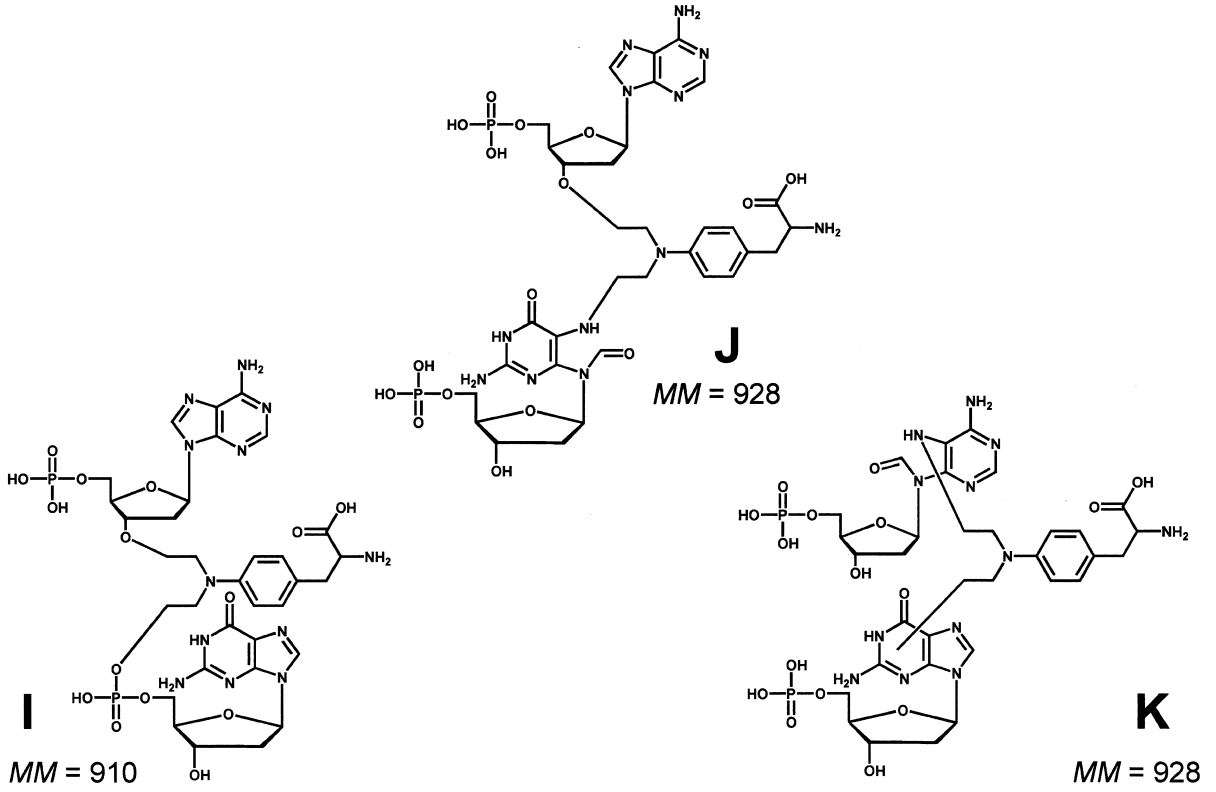

Fig. 8. Possible structures for dNMP–melphalan–dN′MP cross-linked adducts.

gram. The low-energy CAD products ion scans of both $[MH]^+$ (m/z 911) and $[M-H]^-$ (m/z 909) were recorded under FIA. For the ES(−) results the sample was diluted with HFiP (400 m*M* in 50% aqueous MeOH adjusted to pH 7.0 with triethylamine) prior to analysis [12]. The low-energy CAD spectra of the $[M-H]^-$ ion of the dAMP–melphalan–dGMP cross-linked adduct (m/z=909, collision energy 49 eV) showed ions at m/z 330 and 346 (see Fig. 9). Also the ions m/z 79, 97, 195 and 134 were present. The latter ions corresponded with the typical product ions of dAMP under ES(−). No fragment ions were found in which a melphalan residue was linked to the adenine or guanine base. In the corresponding ES(+) product ion spectra of m/z=911 (collision energy 20 eV) the ions 332, 348, 152 and 136 could be detected. Also an ion at m/z 644 was detected, this loss of 267 out of 911 pointed in the direction of the loss of 2′-deoxyguanosine. Based on these data the structure of this cross-linked adduct was assigned to structure I in Fig. 8. The compound with m/z 929 had a molecular mass 18 units higher than the aforementioned product. Therefore this molecule probably corresponded with a cross-linked adduct in which one imidazole ring was opened either in the adenine (structure K) – or guanine (structure J) moiety. Most likely ring opening at the guanine site is expected. The low-energy CAD product ion scans of the adduct were recorded under ES(−) and ES(+). In both modes a loss of 331 u was observed. This corresponded to the loss of an intact dAMP residue. The occurrence of 330 [ES(−)], the most abundant ion in the negative product ion scan and the presence of the ion at m/z 332 [ES(+)] favored the ring opening at the guanine residue as these ions stood for the presence of an intact dAMP molecule. In the positive product ion scan an ion at m/z 136 (intact adenine base) was present and no signal for m/z 152 (intact guanine base) was observed. This also pointed towards a ring

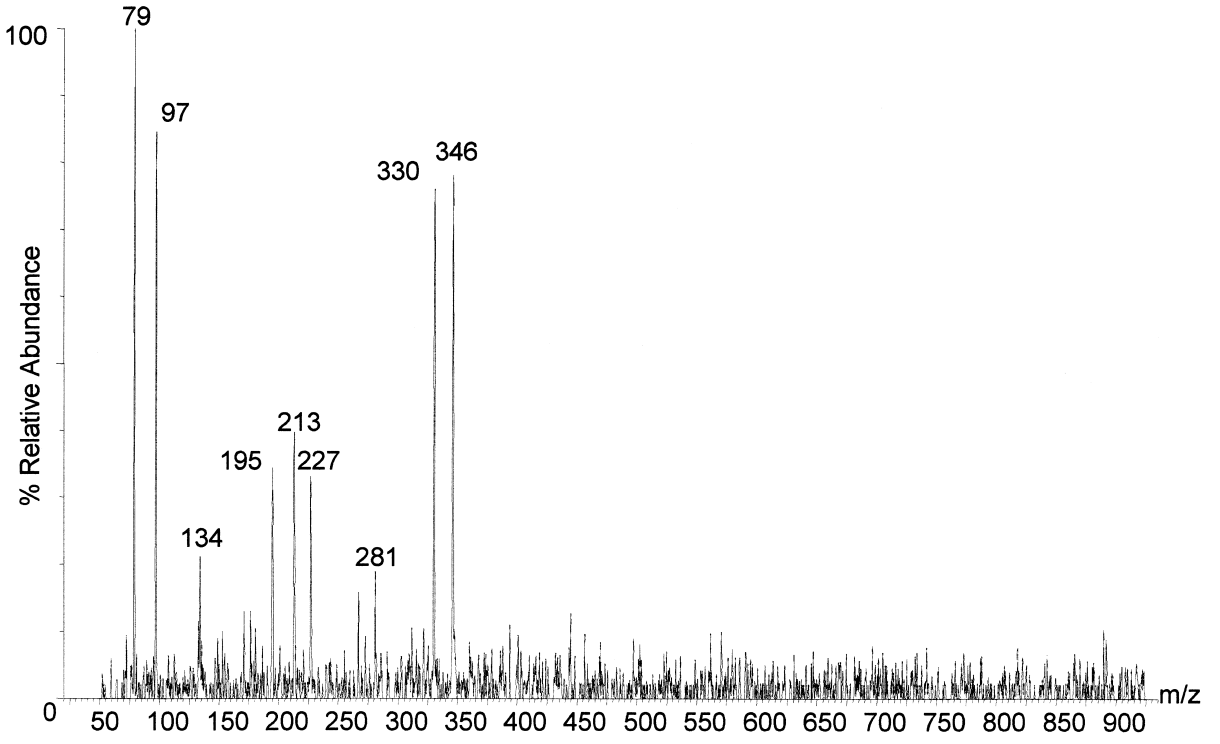

Fig. 9. The low-energy CAD spectrum of the $[M-H]^-$ ion of the dAMP–melphalan–dGMP cross-linked adduct (m/z 909, collision energy 49 eV). The sample was diluted with 1,1,1,3,3,3-hexafluoro-2-propanol (HFiP) (400 m*M* in 50% aqueous MeOH adjusted to pH 7.0 with triethylamine) prior to flow injection analysis.

opening at the guanine site. On the contrary, the presence of the ion at m/z 348 [ES(+)] referred to an intact dGMP moiety. These data could be explained by the co-elution of two isomers, one isomer in which melphalan was covalently bound to the N^7 position of the imidazole moiety and one in which the arm of the melphalan system was bound to the N^7 of the adenine moiety. However exact localization of the second alkylation site was hard to deduce from these spectra. Possible structures for these cross-linked adducts are depicted in Fig. 8 (structures J and K). No ion for a compound of molecular mass 759 (see Section 3.3.1.1) was detected.

3.3.2.2. The dAMP–melphalan–dCMP cross-linked adducts and the dGMP–melphalan–dCMP cross-linked adducts. The cross-linked adduct expected between dAMP and dCMP (*MM*=870) was of such low concentration that no MS–MS data could be recorded. Its adenine ring-opened analogue (*MM*=888) was not observed. In the reaction mixture containing dGMP, dCMP only mono-alkylated adducts were found.

3.3.3. Investigation of the cross-linked adducts in calf thymus DNA hydrolysates

The nanoLC–ES-MS–MS system was used for the evaluation of cross-linked adducts in calf thymus DNA hydrolysates. The preparation of these samples was already described previously [9]. Full scan experiments were performed using 10 μl injection on the pre-column. After 5 min the adducts were back-flushed to the analytical column. In these runs the corresponding masses of the cross-linked adducts were reconstructed. To our surprise no signals were observed for the different cross-linked adducts described above. However, the masses for known modified mono-alkylated dinucleotides and modified mono-nucleotides [9] gave nice chromatographic peaks. In order to enhance the sensitivity SIR

measurements were performed but also here the search for cross-linked adducts was negative.

3.3.4. Melphalan-treated Jurkat cells

As a start the enzymatically hydrolyzed DNA pellet from the melphalan-treated Jurkat cells was analyzed by the capLC–ES-MS system (for description of the system see Ref. [9]). As such, aliquots of 6 μl were injected on the pre-column, which was now eluted with an isocratic buffer consisting of HOAc (2%, v/v)–MeOH (99:1) at a flow-rate of 20 μl/min. Under these conditions not only the nucleotides but also the salts and impurities of the DNA isolation were sent to the waste prior to LC–MS analysis (column switching after 5 min). This approach revealed the presence of several adducts. In view of the results described earlier the chromatograms were reconstructed for the different mono-alkylated adducts which could be expected. Strangely enough the most important adduct in the hydrolysate of calf thymus DNA, namely mono-alkylated dGMP, characterized by the presence of a protonated molecule $[MH]^+$ at m/z 616 (^{35}Cl) with a characteristic chlorine isotope pattern was not found. Also no other mono-alkylated adducts (hydrolyzed or depurinated) were found. Only for m/z 420 (modified guanine) a very weak signal with a S/N less than 2 could be found. Unmodified dinucleotides namely pdGpT ($[MH]^+=652$) and pdCpdA ($[MH]^+=621$) could be found in the chromatogram but were not of interest to us. Next to these, mono-alkylated dinucleotides: $pdG^{Mel(Cl)}pdC$ ($[MH]^+=905$); $(pdGpdA)^{Mel(Cl)}$ ($[MH]^+=929$); $pdCpdC^{Mel(Cl)}$ ($[MH]^+=865$); $(pdCpdA)^{Mel(Cl)}$ ($[MH]^+=889$) and $pdApdA^{Mel(Cl)}$ ($[MH]^+=913$) were found. The most prominent dinucleotide adduct amongst them was the $pdG^{Mel(Cl)}pdC$ adduct eluting at 16 min. The structures of two important mono-alkylated dinucleotides are shown in Fig. 10. The low-energy CAD product ion spectra for m/z 453 (^{35}Cl) ($[M+2H]^{2+}$ ion) of these products are described previously [9]. The data of the column-switching capLC–ES-MS and capLC–ES-MS–MS experiments are not shown. Other modified dinucleotides gave such a weak diprotonated molecule that no product ion spectra could be recorded. Therefore further experiments were performed in which nanoLC is coupled to nanoES (Fig. 11). Again no signal was observed for m/z 616 nor for m/z 420 at the expected retention time. In search of the mono-alkylated dGMP adduct all fractions and supernatants of the DNA isolation were analyzed but m/z 616 was not detected. However when unmodified Jurkat DNA was isolated from untreated cells and then subsequently treated with melphalan

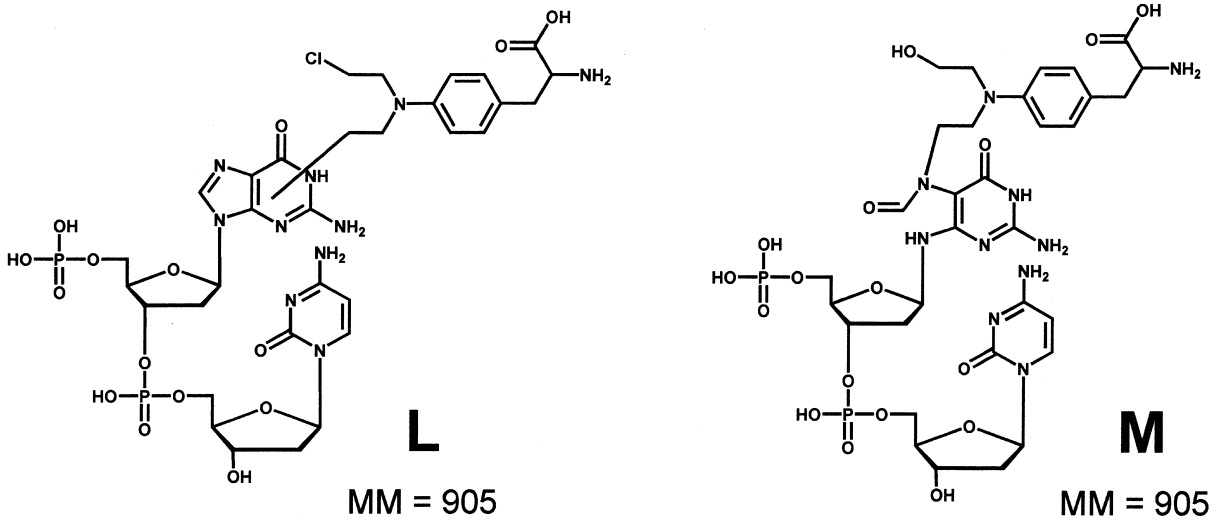

Fig. 10. Tentative structures for $pdG^{Mel(Cl)}pdC$ ($[M+H]^{2+}$ 453, $t_r=17.0$ min) and $pdG^{Mel(OH)}$ pdC ring opened ($[M+H]^{2+}=453$, $t_r=39.5$ min).

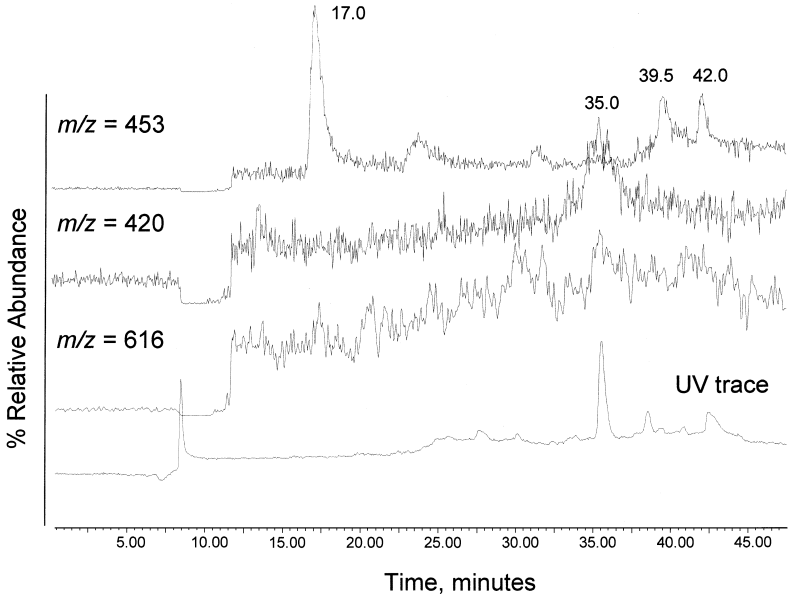

Fig. 11. Analysis of 10 μl of a DNA hydrolysate of melphalan-treated Jurkat cells by nanoLC–ES-MS using column switching. Reconstructed ion chromatograms for the $[MH]^+$ ions corresponding to $gua^{Mel(Cl)}$ (m/z 420), $dGMP^{Mel(Cl)}$ (m/z 616), $pdG^{Mel(Cl)}pdC$ ($[M+H]^{2+}=453$, $t_r=17.0$ min) and $pdG^{Mel(OH)}$ pdC ring opened (m/z 453, $t_r=39.5$ min).

for 3 h m/z 616 appeared. From this we can deduce that unmodified strands of DNA (calf thymus or Jurkat DNA) behave the same in an in vitro experiment. The modified mono-alkylated dinucleotides could also be detected using the column-switching nanoLC–ES-MS set-up. The peak eluting at $t_r=17$ min corresponded with the dinucleotide adduct pdGpdC modified at the G-site ($pdG^{Mel(Cl)}pdC$, $[MH]^+=905$, $[MH]^{2+}=453$). The compound eluting at $t_r=39$ min was the hydrolyzed imidazole ring-opened analogue ($pdGpdC^{Mel(OH)}$ (ring open), $[MH]^+=905$, $[MH]^{2+}=453$).

4. Conclusions

In order to be able to identify DNA adducts in small biological samples an approach based on the hyphenation of nanoLC to ES-MS using column switching has been developed. This technique was compared to the previously developed capillary column-switching system. The sensitivity of the system allowed the detection and structural identification of these adducts at the nucleotide level in the femtogram range. Cross-linked adducts were only detected in in vitro dNMP reaction mixtures. Some of these minor cross-linked adducts could be identified from a structural point of view. However we should point out that the interpretation of the MS–MS data not always defined the structure of the cross-linked adducts unambiguously. Therefore we feel that in depth study of the mass spectral behavior of these adducts is warranted. No cross-linked adducts were found neither in the melphalan-treated calf thymus hydrolysates nor in the melphalan-treated Jurkat cells even under SIR conditions. These observations are in contrast with the generally accepted idea that the antineoplastic activity of melphalan is due to the formation of cross-linked adducts. In the melphalan-treated Jurkat cells several mono-alkylated dinucleotides were present. Future experiments, both qualitative and quantitative are needed and will be conducted towards the analysis of DNA hydrolysates of DNA pellets isolated from the blood of patients treated with melphalan. This will enable us to analyze even smaller amounts of samples. This is an important requirement from

medical point of view especially for blood samples originating from melphalan-treated patients.

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