Analysis of the DNA adducts of phenyl glycidyl ether in a calf thymus DNA hydrolysate by capillary zone electrophoresis–electrospray mass spectrometry: evidence for phosphate alkylation

Dieter L.D. Deforce1, Filip Lemièrè2, Ilse Hoes3, Rebecca E.M. Millemamps1, Eddy L. Esmans3, Andreas De Leenheer1 and Elfriede G. Van den Eeckhout1,3

1Laboratory for Pharmaceutical Biotechnology, University of Ghent, Harelbekestraat 72, B-9000 Ghent, and 3Department of Chemistry, Nucleoside Research and Mass Spectrometry Unit, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium
2To whom correspondence should be addressed

Email: Elfriede.VandenEeckhout@rug.ac.be

Calf thymus DNA was reacted in vitro with phenyl glycidyl ether (PGE) and was hydrolysed enzymatically, to the 5’-monophosphate nucleotides using deoxyribonuclease I (DNA-ase I) and nuclease P1. The adducts were concentrated using solid phase extraction (SPE), on a polystyrene divinylbenzene copolymer in order to remove the unmodified nucleotides. The adducts could be identified using capillary zone electrophoresis–electrospray tandem mass spectrometry (CZE ES–MS/MS), using sample stacking. In addition to the base alkylated 2’-deoxynucleotides present in the DNA-hydrolysate, also phosphate alkylated 2’-deoxynucleotide adducts were identified for TMP and dAMP. An additional adduct, dUMP alkylated on the uridine base moiety was found originating from the hydrolytic deamination of dCMP alkylated on N3 of the cytosine moiety. Enzymatic hydrolysis using nuclease P1 was incomplete as shown by the presence of dinucleotides alkylated on the base moiety. They were successfully hydrolysed to the corresponding 2’-deoxynucleotides by snake venom phosphodiesterase (SVP). Data are shown indicating that alkylations on the pyrimidine bases were more resistant to enzymatic hydrolysis with nuclease P1 than the purine alkylated products.

Introduction

The interaction of xenobiotics with DNA and the resulting adducts have been studied extensively, in order to understand how they exhibit their cytotoxic and carcinogenic properties (1,2). It is within this philosophy that our group has focused on simple unsaturated epoxides on which until now only little research has been performed (3–6). As lead compound phenyl glycidyl ether (PGE5) was chosen, which is an important industrial epoxide mainly used in the paint and the resin industry. We have investigated the application of CZE ES–MS (Capillary Zone Electrophoresis–Electrospray Mass Spectrometry) (7) for the detection of nucleotides modified with PGE as an alternative to the popular 32P post-labeling technique. Although the 32P post-labeling (8,9) technique is very sensitive, one of its major drawbacks is that it gives no structural information. The combination of CZE as a high efficiency separation technique to electrospray tandem mass spectrometry (ES–MS/MS) proved to be very promising in the field of DNA-adduct research, enabling identification and structure elucidation of unknown adducts (7,10). In order to be able to load enough sample on the capillary to obtain ES–MS/MS spectra, sample stacking was performed as described by Wolf, Vousos and ourselves (7,10,11). The identification of unknown adducts and their structure can be important to explain or better understand the working mechanism of cytotoxic agents.

In the field of DNA adduct research, most research is focused on the adducts formed by the interaction of the alkylating agent with the purine or pyrimidine moiety (12). In a previous report (13) we were able to identify alkylation on the phosphate backbone of the DNA structure. Subsequent spontaneous hydrolysis of this alkylated backbone was shown to be responsible for the breakdown of the DNA structure into shorter oligonucleotides. Phosphate alkylation was also found in the reaction of mustards with DNA (14–16) and nitrosoarene with RNA. In the latter case it was held responsible for the degradation of the RNA string (17).

In our former experiments no phosphate alkylated adducts were detected in the enzymatically hydrolysed DNA pellet (7). In this report, a sample pretreatment step, using solid phase extraction (SPE), was elaborated in order to concentrate the modified nucleotides and to remove the unmodified 2’-deoxynucleotides, resulting in better stacking efficiency and loadability. The enzymatic hydrolysis of the DNA pellet was investigated especially for phosphate alkylated adducts because the presence of phosphate alkylated products in the DNA hydrolysate would be indicative for splicing of the DNA molecule at this site (13). This would result in an alkylated oligonucleotide which was then hydrolysed by the enzymatic procedure.

Materials and methods

DNA adduct formation and enzymatic hydrolysis

Calf thymus DNA (highly polymerized DNA Type I from calf thymus, Sigma, St. Louis, MO) was reacted, during 48 h, with PGE (Aldrich, Steinheim, Germany) and hydrolysed enzymatically to the nucleotide level as described in our previous work (7). This DNA-hydrolysate was subjected to a sample clean up procedure in order to remove the salts and the excessive amount of unreacted nucleotides. This was accomplished by using Chromabond HR-P SPE columns ( Macherey-Nagel, Düren, Germany) of which the amount of solid phase was reduced to 125 mg. 200 µl of the DNA-hydrolysate was applied to the column and rinsed with 6 ml of HPLC-grade water. The nucleotide adducts were then eluted using 2 ml of methanol/water 50/50, followed by 5 ml of methanol. The eluate was evaporated to dryness using a stream of nitrogen gas, and redissolved in 500 µl HPLC-grade water.

Since the presence of adducted dinucleotides revealed incomplete (7) hydrolysis using nuclease P1, 200 µl of the DNA-hydrolysate was incubated at 37°C for 4 h with 5 units SVP (Snake Venom Phosphodiesterase, from Crotalus amandateus, Pharmacia Biotech, Upsalla, Sweden), in order to investigate hydrolysis of the remaining dinucleotides.

Capillary zone electrophoresis

CZE separations were done on a Lauerlab Prince system equipped for on-column detection with a Kontron UV detector (type HPLC 332). Data

*Abbreviations: PGE, phenyl glycidyl ether; CZE ES–MS, capillary zone electrophoresis–electrospray mass spectrometry; SPE, solid phase extraction.
collection was performed with the PC Integration Pack version 3.90 (Kontron Instruments). The CZE capillary was a 75 µm i.d. fused silica capillary. The distance of the inlet of the CZE capillary to the UV detector was 61.5 cm. Detection was performed at a wavelength of 270 nm. The total length was 91.5 cm. The buffer system used was a 100 mM ammonium carbonate buffer system (pH 9.68). Electrophoresis was performed using a constant voltage of 17 kV. In order to load sufficient sample on the capillary, sample stacking was performed as described in our earlier work (7). The samples were injected applying a pressure of 120 mbar during 0.22 min prior to sample stacking.

Coupling CZE to electrospray mass spectrometry.

In order to obtain more structural information, the CZE system was coupled on-line to ES–MS (electrospray mass spectrometry) and ES–MS/MS (VG Quattro II triple-quadrupole system, Micromass) as previously published (7). The sample obtained as a result of the sample clean up procedure described above was concentrated ten times by evaporating the volume to 50 µl using a stream of nitrogen gas. The buffer system used to perform electrophoresis was a 100 mM ammonium carbonate buffer system (pH 9.68). Electrophoresis was performed using a constant voltage of 13 kV. In order to load sufficient sample on the capillary, sample stacking was performed as described in our earlier work (7). The samples were injected applying a pressure of 120 mbar during 0.22 min prior to sample stacking.

Results

CZE results using UV detection

Using SPE-sample clean up the salts present in the DNA-hydrolysate were efficiently removed. As a result not only the adducts were efficiently concentrated by selective removal of the unmodified nucleotides but also a low conductive sample plug was obtained which led to a better stacking efficiency of the analytes (Figure 1). Furthermore, by removing the former compounds prior to CZE analysis a larger amount of adducts could be injected by the sample stacking technique (see Materials and methods) without overloading the CZE column. The recovery of the modified 2’-deoxynucleotides was ~99% (as determined with a reference reaction mixture of TMP with PGE).

The adducted dinucleotides were efficiently hydrolysed using an additional digestion with SVP (Figure 2). However, since no new adducts were found after this additional hydrolytic procedure, we decided to perform the analysis of the DNA-hydrolysate after nuclease P1 treatment.

CZE ES–MS and CZE ES–MS/MS results

Compared to previously reported data (7) this approach revealed the presence of additional adducts hitherto not detected (Figure 3).

A compound was found at \( t_R = 8.55 \) min characterized by the presence of a \([M-H]^–\) at m/z 457. This corresponds to a molecular mass of 458 which could correspond to the mono-alkylated PGE adduct of dUMP. Although dUMP does not occur in Calf thymus DNA a study published by Lemiére et al. (4) proved that the N⁰ mono-alkylated adduct of PGE with dCyd underwent hydrolytic deamination and produced the corresponding PGE adduct of dUrd. A product with \([M-H]^–\) at m/z 456, corresponding to the mono-alkylated PGE-dCMP was not found in the DNA-hydrolysate. This would signify that all initially formed \(N^3\) PGE/dCMP was deaminated.

In order to prove the structure of this PGE/dCMP adduct, the low energy collisional activated decomposition (LE CAD) product ion spectrum of m/z 457 was recorded (Figure 4). The ion observed at m/z 261 represents the \(N^3\) alkylated fragment of PGE with uracil. For the mono-alkylated PGE-adduct \([M-H]^–\) at m/z 480) three signals were observed at respectively \( t_R = 5.25, 7.20 \) and \( 7.75 \) min. One of them, i.e.
the adduct at \( t_R = 5.25 \) min has a lower electrophoretic mobility which suggested 5’-phosphate alkylation. This assumption was proven by interpreting the LE CAD product ion spectrum of [M-H]⁺ ion at m/z 480 with \( t_R = 5.25 \) min (collision energy 25 eV). This spectrum showed diagnostic ions at m/z 134 and 345 (Figure 5). These ions can be assigned to the unmodified purine moiety B⁻ and the phosphate alkylated 2’-deoxyribofuranosyl moiety respectively as a result of the cleavage of the anomeric bond C’1-N1.

The isomers found at retention times 7.20 and 7.75 min were both identified as being the purine alkylated PGE adducts of dAMP. They most likely represent the N¹ and the N⁶ alkylated dAMP adducts (18). However, as we stated before (4,5) differentiation between these two isomers is only possible either at the nucleoside stage or by comparison of the (+)ES data of the nucleotide with the (+)ES data of the corresponding nucleoside adducts. As can be seen in Figure 3 the reconstructed ion chromatogram for m/z 471 ([M-H]⁺ PGE/TMP) shows two signals at \( t_R = 6.21 \) and 8.25 min, respectively. This is one adduct more than reported previously (7). Despite the low concentration of the compound eluting at 6.21 min the product ion spectrum of m/z 471 (collision energy 30eV) showed an ion at m/z 125 (Figure 6) which represents the unmodified B⁺ ion. Other ions at m/z 247 and 153 point to phosphate alkylation of 5’-TMP.

As can be seen in panel B of Figure 6 the other isomer showed a prominent ion at m/z 195 ([S]⁻, 2’-deoxyribofuranosyl 5’-monophosphate ion) and a less intense ion m/z 275 (PGE alkylated thymine moiety). This base alkylated PGE/TMP adduct occurred in a much higher concentration and was therefore detected by our group in earlier experiments without SPE clean-up (7).
Fig. 4. Low energy CAD product ion spectra of the [M-H]- ion at m/z 457 of the PGE-dUMP adduct (collision energy was set at 25 eV; for conditions see Materials and methods) present in the DNA-hydrolysate after sample clean up.

Fig. 5. Low energy CAD product ion spectra of the [M-H]- ions at m/z 480 of the PGE-dAMP adducts (collision energy for both spectra was 25 eV; for conditions see Materials and methods) present in the DNA-hydrolysate after sample clean up. (A) Product ion spectrum of the product with t_R = 5.25 min, identified as the phosphate alkylated dAMP. (B) Product ion spectrum of the products with t_R = 7.20 and 7.75 min, both identified as dAMP alkylated on the base moiety. These adducts most likely represent the N1 and the N6 alkylations, however, as noted in the text, we could not differentiate between the two isomers in these experiments.
DNA adducts of PGE

Fig. 6. Low energy CAD product ion spectra of the [M-H]– ions at m/z 471 of the PGE-TMP adducts (collision energy for both spectra was 30 eV; for conditions see Materials and methods) present in the DNA-hydrolysate after sample clean up. (A) Product ion spectrum of the product with \( t_R = 6.21 \) min, identified as the phosphate alkylated TMP. (B) Product ion spectrum of the product with \( t_R = 8.25 \) min, identified as TMP alkylated on the base moiety.

Table I. AUC (Area Under the Curve) ratios of the different adducts, to unmodified dAMP present in the DNA-hydrolysate, before and after addition of SVP\(^a\) (conditions see text)

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Before SVP hydrolysis AUC ratio</th>
<th>After SVP hydrolysis AUC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP phosphate alkylation ( t_R = 5.25 ) min</td>
<td>0.0019</td>
<td>0.0444(^b)</td>
</tr>
<tr>
<td>dAMP base alkylation ( t_R = 7.20);7.75 min</td>
<td>0.0433</td>
<td>0.2134 (× 4.9)(^a)</td>
</tr>
<tr>
<td>TMP base alkylation ( t_R = 8.25 ) min</td>
<td>0.0467</td>
<td>0.3852 (× 8.3)(^a)</td>
</tr>
<tr>
<td>dUMP base alkylation ( t_R = 8.55 ) min</td>
<td>0.0131</td>
<td>0.1999 (× 15.2)(^a)</td>
</tr>
<tr>
<td>dGMP-N(^2) alkylation ( t_R = 7.50 ) min</td>
<td>0.0083</td>
<td>0.1949(^f)</td>
</tr>
<tr>
<td>dGMP-N(^7) alkylation ( t_R = 8.65 ) min</td>
<td>0.0428</td>
<td>0.0271 (× 0.6)(^a)</td>
</tr>
<tr>
<td>Imidazole ring opened dGMP adduct</td>
<td>0.0048</td>
<td>0.0119 (× 2.5)(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Relative increase of the adduct detected after SVP hydrolysis.
\(^b\)Ratio of phosphate alkylation to base alkylation.
\(^f\)Ratio of dGMP-N\(^2\) alkylation to dGMP-N\(^7\) alkylation.

Finally, as can be seen from Figure 3, two products with [M-H]– at m/z of 496 were found (\( t_R = 7.50 \) min and \( t_R = 8.65 \) min). In Table I we can see that the ratio of their abundance increases by a factor of 8.43 after additional hydrolysis of the dinucleotide adducts with SVP. This is due to the increase of the abundance of the product with \( t_R = 7.50 \) min and the simultaneous decrease of the product with \( t_R = 8.65 \) min. This decrease can be explained by the observation of Vanhoutte et al. (6) that the N\(^7\) alkylated adduct of dGuo with bisphenol A, undergoes hydrolytic imidazole ring opening. This hypothesis is confirmed by our experimental data where we can observe an increase in the imidazole ring opened product of the N\(^7\) alkylation of dGMP with PGE ([M-H]– at m/z 514, \( t_R = 8.65 \) min). Both products have identical electrophoretic behavior. These data indicate that the product with \( t_R = 8.65 \) min is the N\(^7\) alkylated adduct of dGMP with PGE. The product with \( t_R = 7.50 \) min, which is according to these data, not susceptible to imidazole ring opening, could be either the N\(^2\) or the O\(^6\)-alkylated adduct of dGMP with PGE, in reference to the data obtained on the nucleoside...
adducts, published by Van den Eeckhout (18). The CZE ES–MS/MS data obtained of the compounds with [M-H]– at m/z 496 (collision energy 23 eV) indeed revealed two distinct adducts (Figure 7). The product ion spectrum of the adduct with t_R = 8.65 min is characterized by the presence of the ions at m/z 195 and 300, originating from the cleavage of the glycosidic bond. In the N7 alkylated dGMP this glycosidic bond is prone to cleavage because of the vicinity of the quaternary N7 (Figure 8). Bearing in mind the data on the imidazole ring opening we could identify this product as being the N7 alkylated dGMP. In the product ion spectrum of the adduct with t_R = 7.50 min, only the ions at m/z 79 and 97 were observed, both originating from the phosphate group. This indicates that alkylation could be present on N2. In this case the glycosidic bond is stronger due to the absence of a quaternary N7, which explains the absence in this spectrum of the ion at m/z 300, and the very low abundance of the ion at m/z 195.

In order to investigate whether phosphate- or base alkylation was responsible for the incomplete digestion of dinucleotide adducts, the abundance of the phosphate- and base alkylated adducts was investigated before and after additional hydrolysis with SVP. Both samples were analysed by CZE ES–MS without sample clean up so that the unmodified nucleotides were present in the sample and could serve as an internal standard. The ratio of the alkylated adducts versus dAMP was calculated, before and after SVP hydrolysis (Table I). Because only the ratio of the base alkylated adducts increased it was obvious that the base alkylation and not phosphate alkylation was responsible for the incomplete digestion by the nuclease P1 enzyme. Hence, these data suggest that the dinucleotide adducts are all modified on their base moiety which is consistent with the reactivity of SVP (19).
DNA adducts of PGE

Fig. 9. Reconstructed ion electropherogram of the DNA hydrolysate using nuclease P1 hydrolysis, without sample clean up procedure, using full-scan conditions (75–900 Da, at a scan speed of 550 Da/s; CZE ES–MS conditions see Materials and methods). [M-H]⁻ values at m/z represent: m/z 784, dAMP-TMP dinucleotide alkylated on the base moiety; m/z 775, TMP-TMP dinucleotide alkylated on the base moiety; m/z 760, dCMP-TMP dinucleotide alkylated on the base moiety. (The identity of all the products was determined by CZE ES–MS/MS.)

Fig. 10. Low energy CAD product ion spectrum of the [M-H]⁻ ion at m/z 784. Identified as a mixture of two co-eluting adducts of pApT(PGE), structure (A) the specific fragments for molecule A are denoted in the spectrum with A and pTpA(PGE), structure (B), with specific fragments (collision energy was 33 eV; for conditions see Materials and methods) present in the DNA-hydrolysate after sample clean up.
Fig. 11. General fragmentation scheme for the [M-H]– ions of the adducted dpNdpN dinucleotides under LE-CAD.

In order to get additional evidence the CZE ES–MS/MS data were obtained for the most abundant dinucleotide adducts. LE CAD spectra (collision energy 33 eV), were obtained for the products with deprotonated molecules [M-H]– at m/z 760, 775 and 784. The reconstructed ion chromatogram (RIC) obtained for these ions in a CZE ES–MS run is shown in Figure 9. The dinucleotide with molecular mass 785 ([M-H]– at m/z 784) corresponds to a mono-alkylated dinucleotide pApT or pTpA. Analysis of the LE CAD product ion spectrum of [M-H]– 784 (Figures 10 and 11) revealed the presence of two co-eluting isomers. The product ions at m/z 284 and 275 were assigned to the alkylated [B]– ions of the Ade- and Thy-moiety respectively. Other ions were found at m/z 321 (b-type ion) and m/z 401 (d-type ion) proving the presence of a pTpA(PGE) dinucleotide while m/z 330 (b-type ion) and 410 (d-type ion) could be explained out of the pApT(PGE) isomer, which is the most abundant.

The product with [M-H]– at m/z 775, was identified as the mono-alkylated adduct of pTpT. In this case two possible isomers were possible, i.e. pT(PGE)pT and pTpT(PGE). Because of the presence of product ions at m/z 321 (b-type ion) and 401 (d-type ion) and because of the absence of ions at 471 (b-type ion) and m/z 551 (d-type ion) the product ion spectrum was assigned to the pTpT(PGE) isomer. Base alkylation was proven by the occurrence of the B– ion at m/z 275.
Discsecond

By introducing a SPE sample clean up we were able to identify phosphate alkylated adducts of TMP and dAMP in DNA hydrolysates of calf thymus DNA reacted in vitro with PGE by using CZE ES–MS/MS. Since in in vivo samples generally 1 modification occurs per 10^7 nucleotides (21) a sample clean up procedure to remove unmodified nucleotides is a prerequisite. The reaction of PGE on the phosphate backbone, producing unstable phosphotriesters (17) is probably of major biological significance, since we were able to prove that these alkylations are responsible for the hydrolysis of DNA resulting in strand breaks (13).

Additional hydrolysis with SVP removed the dinucleotides which could not be completely hydrolysed by the nuclease P1 enzyme. This indicates that the SVP enzyme is less sensitive to inhibition by modified nucleotides. In the field of DNA adduct research it may therefore be interesting to include this additional enzyme in order to achieve complete digestion of modified DNA to the 5'-mononucleotides. The ratios of change of the signal intensity before and after hydrolysis with the SVP enzyme, as depicted in Table I, indicate that alkylations on the pyrimidine bases (TMP and dCMP converted to dUMP by hydrolytic deamination) are more resistant to hydrolysis with the nuclease P1 enzyme than the purine alkylated adducts (dGMP and dAMP). This was confirmed by the CZE ES–MS/MS data obtained for the most abundant mono-alkylated dinucleotides, where most alkylations were located on the pyrimidine bases. These data also confirmed that the base alkylations and not the phosphate alkylated adducts were responsible for the incomplete hydrolysis by nuclease P1.

Alkylation on the base moiety of dCMP resulted in hydrolytic deamination to PGE modified dUMP, in the DNA hydrolysate no dCMP adducts were observed, this indicates that all dCMP adducts were hydrolysed to the dUMP adduct. Since this reaction occurs only to a small extent in the reaction mixture of the dCMP nucleotide with PGE, it is possible that this hydrolytic deamination is favored by the conditions used to enzymatically hydrolyse the modified DNA.

For dGMP two alkylation products were found, a N7 alkylated adduct and a N2 or O6 alkylated adduct. The N7 alkylated adduct is susceptible to imidazole ring opening, and to subsequent deglycosylation (6). By this mechanism the N7 modified guanine would be removed from the DNA chain and could thus be repaired by repair enzymes (22). The N2 or O6 alkylated adduct is not susceptible to imidazole ring opening and deglycosylation and may result in more cytotoxic lesions than the N7 alkylated product. This N2 or O6 alkylated product was not formed in the reaction between dGMP and PGE and is thus specific for interaction of PGE with DNA, in which it is the most abundant species (see Table I).

In summary, CZE in combination with ES–MS and ES–MS/MS proves to be very promising in the field of DNA-adduct research, enabling structure elucidation of unknown adducts and contributing to the elucidation of cytotoxic reaction mechanisms.

Acknowledgements

We wish to thank the FWO-Vlaanderen for grant number 32013394. One of us (E.E.) is indebted to the Flemish Government for financial support (GOA-action).

References


Received on December 8, 1997; revised on February 5, 1998; accepted on February 5, 1998.