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Screening for DNA Alkylation Mono and Crosslinked Adducts with a Comprehensive LC-MS³ Adductomic Approach

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Abstract

A high-resolution/accurate-mass DNA adductomic approach was developed to investigate anticipated and unknown DNA adducts induced by DNA alkylating agents in biological samples. Two new features were added to a previously developed approach to significantly broaden its scope, versatility, and selectivity. First, the neutral loss of a base (guanine, adenine, thymine, or cytosine) was added to the original methodology's neutral loss of the 2'-deoxyribose moiety to allow for the detection of all DNA base adducts. Second, targeted detection of anticipated DNA adducts based on the reactivity of the DNA alkylating agent was demonstrated by inclusion of an ion mass list for data dependent triggering of MS² fragmentation events and subsequent MS³ fragmentation. Additionally, untargeted screening of the samples, based on triggering of an MS² fragmentation event for the most intense ions of the full scan, was included for detecting unknown DNA adducts. The approach was tested by screening for DNA mono and crosslinked adducts in purified DNA and in DNA extracted from cells treated with PR104A, an experimental DNA alkylating nitrogen mustard prodrug currently under investigation for the treatment of leukemia. The results revealed the ability of this new DNA adductomic approach to detect anticipated and unknown PR104A-induced mono and crosslinked DNA adducts in biological samples. This methodology is expected to be a powerful tool for screening for DNA adducts induced by endogenous or exogenous exposures.

Graphical Abstract

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SUPPORTING INFORMATION

Full details regarding cell culture conditions, *in vitro* reactions with PR104A, sample preparation, and results of the targeted and untargeted analyses.

SUPPORTING INFORMATION

Full details regarding cell culture, reactions of ctDNA and HT-29 cells with PR104A, DNA isolation, hydrolysis, and quantitation, and sample enrichment for this study. Results of the targeted analysis in ctDNA (Table S1), and results of the untargeted analysis in HT-29 cells (Table S2).

INTRODUCTION

Chemical modifications of DNA arise from exogenous chemical exposures and endogenous biological processes, and the resulting DNA adducts occur due to alkylation, oxidation, and deamination reactions.¹ If alkylation adducts, including monoadducts and crosslinks (intra- and interstrand), persist, they can interfere with DNA synthesis and transcription, leading to mutations or toxicity.² The particular potency of DNA alkylation interstrand crosslinking to induce cell death in cancer cells makes it one of the most important strategies for the design of cancer drugs.³

DNA adductomics is the identification and quantitation of chemical modifications of DNA and the study of the factors from which they originate.⁴ The DNA adductome resulting from chemical exposures or oxidative stress has been studied by untargeted mass spectrometric screening for DNA adducts involving the detection of modified nucleosides in hydrolyzed DNA samples by neutral loss monitoring for the loss of 2'-deoxyribose (dR),^{4–15} a diagnostic structural feature of DNA adducts (Figure 1A). LC-MSⁿ DNA adductomic studies are typically performed using triple quadrupole instrumentation operated in constant neutral loss (CNL) or “pseudo” CNL mode, and while this relatively simple approach provides for simple data analysis and low instrumentation costs, it is very limited in its selectivity and identification capabilities due to low resolution data acquisition and lack of fragmentation spectra.^{4,5} These limitations are not present when using instrumentation capable of high-resolution/accurate-mass (HRAM) MSⁿ data acquisition, such as an Orbitrap mass analyzer, as demonstrated recently with a DNA adductomic approach using accurate mass monitoring of the neutral loss of the 2'-deoxyribose moiety (116.0474 amu) and subsequent MS³ data acquisition.⁵ This approach provides a high level of selectivity allowing for minimal false positive detections as well as accurate mass measurements for the determination of likely elemental compositions and mass spectral data useful for the elucidation of adduct structure.⁵ This new approach represents a dramatic improvement in terms of selectivity and identification capability and is an important advancement in the ability to investigate DNA damage in complex biological samples.⁵ However, a limitation of this methodology is that it does not allow for the detection of those nucleosides modified at certain base positions (e.g. deoxyguanosine modified at the *N*7 position), which readily lose the 2'-deoxyribose moiety upon DNA hydrolysis resulting in the formation of base, rather than nucleoside, adducts (Figure 1B).

These base adducts comprise one of the most abundant forms of DNA damage and are frequently used as biomarkers of internal exposure due to their higher frequency compared

to DNA adducts alkylated at other positions in DNA.¹⁶ A chemical basis for their prevalence can be seen in the case of guanine where *N*7 is the most reactive position toward electrophilic alkyl groups,¹⁷ and alkylation at this position introduces a positive charge on the guanine heterocycle, thereby promoting depurination of the DNA adduct by cleavage of the glycosidic bond.^{16,18} Similarly, DNA alkylation at the *N*7/*N*3 position of adenine, *O*² position of cytosine, and *O*² position of thymine also accelerates the rate of deglycosylation.¹⁸ To our knowledge, a DNA adductomic approach that enables the detection of this important group of adducts (both mono and crosslinked), which represents a significant portion of the DNA adductome, has not been reported previously.

Therefore, in this study we present a new DNA adductomic approach with accurate mass neutral loss monitoring of the four DNA base moieties (G, 151.0494 amu; A, 135.0545 amu; T, 126.0429 amu; and C, 111.0433 amu) and the 2'-deoxyribose moiety (116.0474 amu) and subsequent triggering of MS³ fragmentation for identification and characterization of DNA adducts both with (nucleoside adducts) and without (base adducts) the 2'-deoxyribose moiety present (Figure 1). In addition, we tested the feasibility of using a targeted approach with a large mass list of anticipated DNA adducts to trigger MS² fragmentation, hypothesizing that this feature would enhance the sensitivity and selectivity of the analysis because the parent ion criteria would avoid fragmentation of extraneous background ion signal.

The DNA adductomic approach was tested and optimized by screening for DNA adducts induced by the experimental DNA alkylating nitrogen mustard prodrug PR104A¹⁹ in DNA purified from calf thymus (ctDNA) and in a human cancer cell line exposed to increasing concentrations of PR104A. *In vivo*, PR104A undergoes metabolic nitro-reduction to the hydroxylamine (PR104H) and amine (PR104M) metabolites (Scheme 1),^{19–21} and the characterization of DNA damage by comet assay and by γ H2AX formation in SiHa cells identified DNA interstrand crosslink (ICL) formation as a major mechanism of PR104A cytotoxicity.¹⁹ The targeted analysis presented here allowed us to detect anticipated mono and crosslinked PR104A-induced DNA adducts in biological samples, and was complemented by an untargeted analysis for detection of unknown PR104A-induced DNA adducts (Scheme 1).

EXPERIMENTAL SECTION

Caution

PR104A is a DNA alkylating agent. It should be handled with extreme caution in a well-ventilated hood and with personal protective equipment.

Chemicals

PR104A was purchased from Albany Molecular Research, Inc. (Albany, NY). The reference standards [pyridine-D₄]*O*²-[4-(3-pyridyl)-4-oxobutyl-1-yl]thymidine (D₄-*O*²-POB-dT),²² [pyridine-D₄]*O*⁶-[4-(3-pyridyl)-4-oxobutyl-1-yl]-2'-deoxyguanosine (D₄-*O*⁶-POB-dG),²³ [pyridine-D₄]*N*7-[4-(3-pyridyl)-4-oxobutyl-1-yl]guanine (D₄-*N*7-POB-G),²⁴ and [pyridine-D₄]*N*7-[1-hydroxyl-1-(3-pyridyl)but-4-yl]guanine (D₄-*N*7-PHB-G)^{24,25} were provided by

Dr. Pramod Upadhyaya of the Masonic Cancer Center, University of Minnesota. Calf thymus DNA was purchased from Worthington Biochemical Corporation (Lakewood, NJ). AKR1C3 was purchased from United States Biological Life Sciences (Salem, MA). Alkaline phosphatase was purchased from Roche Diagnostics (Indianapolis, IN). All the other chemicals and enzymes were purchased from Sigma-Aldrich. Cell culture medium and supplements were purchased from Invitrogen (Life Technologies, Switzerland). All solvents used for HPLC and MS analysis were of the purest grade commercially available.

DNA Samples

Full details regarding cell culture, reactions of ctDNA and HT-29 cells with PR104A, DNA isolation, hydrolysis, and quantitation, and sample enrichment for this study can be found in the Supporting Information.

LC-MS Parameters

2.5 μg (ctDNA samples) or 1.5–4.6 μg (HT-29 samples) of DNA was injected onto a NanoLC-Ultra 2D HPLC (Eksigent, Dublin, CA) system equipped with a 5 μL injection loop. Separation was performed with a capillary column (75 μm ID, 10 cm length, 15 μm orifice) created by hand packing a commercially available fused-silica emitter (New Objective, Woburn MA) with 5 μm Luna C18 bonded separation media (Phenomenex, Torrance, CA). The flow rate was 1000 nL/min for 5 min, then decreased to 300 nL/min with a 50 min linear gradient from 2 to 98% CH_3CN in 5 mM NH_4OAc aqueous buffer (pH 6.8) with a 5 min hold and a 5 min re-equilibration at 1000 nL/min 98:2 buffer/ CH_3CN . The injection valve was switched at 6 min to remove the sample loop from the flow path during the gradient. Samples were analyzed by nanoelectrospray using an LTQ Orbitrap Velos instrument (Thermo Scientific, Waltham, MA). The nanoelectrospray source voltage was 2.0 kV, and the capillary temperature was 350 $^\circ\text{C}$. The ion focusing and transfer elements of the instrument were adjusted for maximum signal intensity by using the automated instrument tuning feature while monitoring the background ion signal of m/z 371.1 (decamethylcyclotrisiloxane) to create the tune file used for data analysis. This resulted in an S-Lens RF level setting of 49%.

CNL-MSⁿ Data-dependent Scanning

Analysis was performed in real time by the instrument software with repeated full scan detection followed by MS^2 acquisition and constant neutral loss triggering of MS^3 fragmentation (data-dependent scanning). Full scan (200–2000 Da) detection was performed using the Orbitrap detector at a resolution of 60,000 (at m/z 400) with 1 microscan (one mass analysis followed by ion detection), automatic gain control (AGC) target settings of 1×10^6 , and a maximum ion injection time setting of 100 ms. MS^2 fragmentation was performed in the ion trap on the three most intense full scan ions listed in a parent mass list (targeted approach) or on the three most intense full scan ions from the full scan spectra (untargeted approach) with Orbitrap detection at a resolution of 7500, automatic gain control (AGC) of 2×10^5 , 1 microscan, maximum ion injection time of 100 ms, and full scan injection waveforms enabled. The parent mass list comprised 298 masses of the $[\text{M}+\text{H}]^+$ ions of anticipated mono and crosslinked DNA adducts induced in the four DNA bases by PR104A, PR104H, and PR104M, as well as DNA adducts induced by reaction of the

hydroxylamine group of PR104H with DNA. MS² fragmentation parameters were as follows: 3 amu isolation width, normalized collision energy of 35, activation Q of 0.25, and activation time of 10 ms. Data dependent parameters were as follows: triggering threshold of 10,000, repeat count of 1, exclusion list size of 500, exclusion duration of 60s, and exclusion mass width of ± 5 ppm. For the untargeted analysis, a reject mass list (56 ions) consisting of protonated 2'-deoxyribonucleosides and protonated 2'-deoxyribonucleoside artifacts was used with a mass tolerance of ± 5 ppm.⁵ MS³ HCD fragmentation (2 amu isolation width, normalized collision energy of 35, activation time of 0.1 ms) with Orbitrap detection at a resolution of 7500 was triggered upon observation of neutral losses (± 5 ppm) of 116.0474, 151.0494, 135.0545, 126.0429, and 111.0433 amu between the parent ion and one of the 50 most intense product ions from the MS² spectrum, provided a minimum signal of 1000 was observed. The following MS³ parameters were used: 1 microscan, repeat count of 1, AGC target setting 2×10^5 , maximum ion injection time of 100 ms. All spectra were acquired using the background ion signal of *m/z* 371.10124 Da (decamethylcyclopentasiloxane) as a lock mass. Instrument sensitivity was checked before each analysis by injection of 10 fmol of labeled standard (D₄-O⁶-POB-dG).

RESULTS AND DISCUSSION

The DNA adductomic approach was developed by reacting purified DNA with the DNA alkylating prodrug PR104A and the feasibility of increasing selectivity and sensitivity through the use of a list of expected PR104A-induced DNA adduct masses was tested as was the detection of base adducts using the neutral losses of the bases for triggering the MS³ fragmentation. For this purpose, ctDNA was allowed to react with PR104A and the enzyme aldo-keto reductase 1C3 (AKR1C3) at 37 °C for 24 h. AKR1C3 is thought to be responsible for bioactivation of PR104A to PR104H and PR104M under aerobic conditions (Scheme 1).²⁶ Reaction mixtures were enzymatically hydrolyzed, the enzymes removed by filtration, and hydrolysates were enriched by solid phase extraction, and fractionated in three portions (10%, 50%, and 100% CH₃OH in H₂O). Each fraction was analyzed separately on a nano flow HPLC system coupled by electrospray ionization to an ion trap-orbital trap mass spectrometer (LTQ-Orbitrap Velos). Two isotopically labeled DNA adducts (D₄-O⁶-POB-dG and D₄-O²-POB-dT)^{22,23} available in our laboratory were used as reference compounds to assure consistency of instrument sensitivity, chromatography, and DNA adduct recovery during sample preparation.

Analysis parameters were optimized, and resulted in a liquid chromatography (LC) linear gradient of 50 min from 2% CH₃CN to 98% CH₃CN and a mass spectrometry (MS) method programmed to perform three scan events: a full scan from *m/z* 200 to 2000 at a resolution of 60,000, and data-dependent MS² and MS³ analyses at a resolution of 7500 with the minimum signal threshold (counts) for fragmentation set at 10,000 and 1000 for MS² and MS³, respectively. In order to allow increased sensitivity and selectivity for the detection of PR104A-induced DNA adducts, a targeted analysis was performed by incorporation into the method of a parent mass list of anticipated masses of DNA adducts induced by PR104A, PR104H and PR104M to allow MS² fragmentation of the three most intense full scan ions listed in the parent mass list.

This method combined the accurate mass neutral loss of the four DNA bases (G, 151.0494 amu; A, 135.0545 amu; T, 126.0429 amu; and C, 111.0433 amu) with the neutral loss of 2'-deoxyribose, for a comprehensive MS³ triggering criteria allowing detection of both base and nucleoside adducts. This process was implemented after observing in preliminary studies that the neutral loss of guanine (G) or adenine (A) occurs upon MS² fragmentation of G and A base adducts. These adducts, e.g. those resulting from DNA alkylated at the N7 position of G and N7/N3 position of A, undergo hydrolysis of the glycosidic bond¹⁸ upon hydrolysis of the DNA and therefore cannot be observed by monitoring for neutral loss of 2'-deoxyribose. The addition of the base neutral loss to the MS³ criteria for base adduct detection was tested by analyzing a standard solution containing 25 fmol each of two deuterated reference N7-guanine DNA adduct standards available in our laboratory (D₄-N7-POB-G and D₄-N7-PHB-G).^{24,25} Both adducts triggered MS³ fragmentation upon observation of the neutral loss of guanine (151.0494 ± 0.0008 amu) from the full scan and MS² spectra using both the targeted and untargeted methods. For example, for the base adduct D₄-N7-POB-G, a dominant peak corresponding to [M + H]⁺ was detected in the full-scan-extracted ion chromatogram (*m/z* 303.1502 ± 0.0016, RT=15.63, Panel B in Figure 2). The detection of this *m/z* 303.1502 ion triggered an MS² fragmentation event (RT=15.65), and the observation of an ion corresponding to the neutral loss of 151.0494 amu (G) from the triggering parent mass in turn triggered an MS³ fragmentation event (RT=15.66) indicating the presence of a DNA adduct (Figure 2, Panel B) and providing additional mass information indicative of its chemical structure (Figure 2, Panel C).

The DNA adductomic method described above for the analysis of a standard mix of adducts was applied for the novel characterization of DNA damage profiles associated with the reaction of ctDNA with PR104A. This experiment allowed the detection of anticipated mono and crosslinked DNA adducts induced by PR104A, or its metabolites PR104H and PR104M (Table S1 and Figure 3). All DNA adduct masses that triggered an MS³ event were present in the 50% and 100% CH₃OH fractions of the solid phase extraction step. In the 50% CH₃OH fraction, four DNA adduct masses from the parent mass list were detected by accurate mass monitoring of the neutral loss of guanine or adenine. No DNA adducts containing the 2'-deoxyribose moiety were detected in the 50% CH₃OH fraction, probably due to the lower polarity of 2'-deoxyribose-containing DNA adducts. In the same fraction, one crosslinked DNA adduct was detected corresponding to a crosslink formed either by PR104H and a guanine and an adenine alkylated at the N7/N3 position, or by PR104M and two guanine bases alkylated at the N7 position (both have calculated mass *m/z* 595.2233, Table S1 and Figure 3B). In the 100% CH₃OH fraction, nine DNA adduct masses from the parent mass list were detected by accurate mass monitoring of the neutral loss of 2'-deoxyribose, guanine, or adenine (Table S1 and Figure 3). One crosslinked DNA adduct induced by PR104A alkylation of two guanine bases at one of several likely positions (N7, O⁶, N1 or N2) was detected in this fraction (*m/z* 741.2453, Table S1 and Figure 3B). The HRAM measurement by the Orbitrap mass analyzer allows for the creation of exact mass chromatograms characterized by defined analyte peaks free of background signal and interference from any co-eluting compounds (Figure 3A).

The LC-MS³ DNA adductomic approach described above was also used to analyze samples of DNA extracted from cells treated with increasing concentrations of the drug PR104A.

This experiment allowed us to test the sensitivity of the DNA adductomic approach in the context of a complex matrix of a cell and to examine how the reactivity of PR104A and its metabolites PR104H and PR104M with purified DNA (ctDNA) compares with its reactivity with cellular DNA. The cell line selected for this experiment was HT-29, a colorectal adenocarcinoma cell line with established AKR1C3 expression.²⁷ HT-29 cells were treated with increasing concentrations of PR104A and genomic DNA was isolated, enzymatically hydrolyzed, and fractionated. Similarly to the ctDNA reactions, all DNA adducts detected were found to be in fractions with 50% or 100% CH₃OH. Samples were analyzed with the targeted method with data dependent MS² fragmentation performed on ions from the parent mass list consisting of the masses of DNA adducts anticipated to be induced by PR104A, PR104H and PR104M.

A total of 14 mono and crosslinked DNA adducts induced by PR104A and PR104M were detected by accurate mass monitoring of the neutral loss of 2'-deoxyribose, guanine, adenine, or cytosine (Table 1). Ten of these were detected also in ctDNA (Tables 1 and S1). On the basis of mass values, structures were proposed for the anticipated DNA adducts detected in HT-29 cells by the targeted analysis (Figure 3B). Using the DNA adductomic approach, consistent observations were that more MS³ fragmentation events were triggered with increasing PR104A concentration (Table 1), and PR104A and its metabolite PR104M preferentially alkylated purine bases at their most nucleophilic sites (N7 position of guanine, and N7/N3 position of adenine), similar to other nitrogen mustards.²⁸

Experimental and technical reproducibilities of the approach were evaluated on the basis of percentage of adducts detected in all three replicates. The ctDNA experiment performed in triplicate served to evaluate the experimental reproducibility (Table S1), whereas LC-MS analysis of the HT-29 cell samples performed in triplicate served to evaluate the technical reproducibility (Table 1). For each replicate, the reproducibility was evaluated by dividing the number of DNA adducts detected in all three replicates by the total number of DNA adducts detected. The average resulting from these percent values was used to evaluate the reproducibility. For high abundant adducts (peak area > 10⁶), the experimental reproducibility was 100% and the technical reproducibility was 99%. For medium (10⁵ < peak area < 10⁶) and low (peak area < 10⁵) abundant adducts, both reproducibilities were found to be lower (the experimental reproducibility was 65% whereas the technical reproducibility was 60%). The high abundant adducts are more likely to be target candidates to be used as biomarkers of drug susceptibility, and their detection through this approach was demonstrated to be highly reproducible.

It has been observed previously in our laboratory that for a given nucleoside adduct a portion of the ions generated upon protonation during electrospray ionization undergoes in-source fragmentation with loss of 2'-deoxyribose to form the corresponding protonated base adduct ions. This process results in a full scan spectrum containing both the nucleoside and its corresponding base adduct ions. This phenomenon could result in ion signal from in-source fragmentation of a nucleoside adduct erroneously being attributed to its corresponding base adduct. To exclude this possibility, the accurate masses attributed to the base adducts (Tables 1 and S1) and the corresponding nucleoside adducts were extracted from the full scan data to check for co-elution. Using this criterion, none of the base adducts reported in Tables 1 and

S1 corresponded to nucleoside adducts undergoing in-source fragmentation (data not shown), confirming their identity as base adducts.

The dose-dependency of adducts formed in HT-29 cells treated with three different concentrations of PR104A was investigated. For this purpose, peak areas of adducts detected in all three PR104A concentrations tested and found in the 100% CH₃OH fraction (Table 1) were normalized on the basis of total DNA calculated by quantifying dGuo by HPLC-UV. Signal intensity (peak area/mg DNA) increased with increasing PR104A treatment concentration in similar proportions for the four most abundant adducts (Figure 4). While these results are not an absolute quantitation, they suggest that the approach could be amenable to future quantitative application, ideally performed with stable isotope labeled analogues of the analytes of interest to account for losses occurring during sample preparation as well as ion suppression effects.²⁹ In addition, attention should be paid when quantifying DNA adducts alkylated at those base positions that destabilize the glycosidic bond (*N*7-dG, *N*7/*N*3-dA, *O*²-dC, and *O*²-dT), since depending on the size of the alkyl group, depurination can take place during DNA extraction leading to an underestimation of the levels of the base adducts.¹⁶

To test the feasibility of using this DNA adductomic approach for detecting unknown DNA adducts, the same DNA samples obtained from PR104A-treated HT-29 cells that had been analyzed with the targeted method were re-analyzed using an untargeted approach. MS² fragmentation was performed for the three most intense full scan ions of each full scan. A total of 48 potential PR104A-induced DNA adduct masses were detected (Table S2). These DNA adducts were detected by accurate mass monitoring of the neutral loss of 2'-deoxyribose, guanine, adenine, cytosine, or thymine. Among them, three DNA adduct masses (*m/z* 476.1637, *m/z* 492.1586, and *m/z* 608.2059) detected by the targeted analysis were confirmed by the untargeted analysis (Tables 1 and S2). This experiment demonstrated the advantage of performing an untargeted analysis for obtaining a more comprehensive screening of DNA adducts induced by any DNA alkylating agent of interest. The combination of a targeted and untargeted analysis is of particular interest for those DNA alkylating agents whose reactivity with DNA is not completely understood and therefore a detailed list of anticipated DNA adducts cannot be predicted.

DNA damage by PR104A in cell lines has been investigated previously with a comet assay, by measuring γ H2AX formation, and on the basis of cytotoxicity in repair-defective cell lines. Data from these studies suggest that DNA ICLs are the basis of PR104A cytotoxicity.^{19,30,31} It has been hypothesized that cytotoxicity may also result from the formation of monoadducts, redox cycling due to biotransformation of the nitro group and generation of reactive oxygen species (ROS), and formation of reactive nitroso or hydroxylamine compounds.³⁰ The formation of mono and crosslinked DNA adducts by PR104A has never been observed directly; however, in this study their formation was observed by targeted and untargeted screening of PR104A-treated ctDNA and genomic DNA isolated from PR104A-treated HT-29 cells. Thus, a number of mono and crosslinked DNA adducts induced by PR104A, PR104H and PR104M were observed (Tables 1 and S1). On the basis of peak areas from DNA adducts detected in ctDNA treated with 100 μ M PR104A, hydrolyzed *N*7-guanine and *N*7/*N*3-adenine adducts appear to be the most

abundant (Table S1). In the future, this methodology could be used for measuring PR104A-induced DNA adducts in biological samples from cancer patients, providing personalized information about patient responses to PR104A exposure.

CONCLUSION

We developed a unique LC-MS³ DNA adductomic approach to detect anticipated (targeted analysis) and unknown (untargeted analysis) DNA adducts induced by DNA alkylating agents using accurate neutral loss of guanine (151.0494 amu), adenine (135.0545 amu), thymine (126.0429 amu), and cytosine (111.0433 amu), which were added to the neutral loss of 2'-deoxyribose (116.0474 amu) to allow for the detection of all base adducts. To our knowledge, this is the first DNA adductomic approach that involves the neutral loss of DNA bases for DNA adduct screening. The targeted method uses a list of parent ions corresponding to anticipated DNA adducts, with data dependent MS² triggered fragmentation spectra acquisition for the most intense ions in the full scan spectra from this list. The advantage of a large inclusion list, introduced here for the first time for screening DNA adducts, is increased sensitivity and selectivity of DNA adduct detection. This method was applied for detecting mono and crosslinked DNA adducts induced in purified ctDNA and in a human cancer cell line DNA by the experimental DNA alkylating agent PR104A. The new DNA adductomic approach is expected to be useful for screening for DNA adducts induced by endogenous or exogenous exposures, for developing DNA alkylating drugs, and for addressing potentially adverse drug reactions resulting from interactions with DNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Common structural features of DNA adducts and detection by neutral loss monitoring. Base, DNA nucleobase (guanine, adenine, thymine, or cytosine); M, modification; dR, 2'-deoxyribose. A) Neutral loss monitoring of dR is traditionally used for nucleoside adduct screening; B) Neutral loss monitoring of the base as a new strategy to detect base adducts.

Figure 2.

HRAM DNA adductomic approach triggering MS³ fragmentation by neutral loss monitoring of masses corresponding to DNA bases. The experiment was performed on a reference standard solution containing 25 fmol/ μ L D_4 -N7-POB-G. Panel A: Total ion count (TIC) full scan (FS) chromatogram; Panel B.1: TIC FS chromatogram expanded for the region between 15.0–16.2 min, B.2: Extracted mass FS chromatogram of m/z 303.1502 (D_4 -N7-POB-G) from the full scan data, B.3: MS² scan event of m/z 303.1502, and B.4: MS³ scan event triggered by a mass difference of 151.0494 amu between the m/z 303.1502 in the FS spectrum and the corresponding triggered MS² spectrum demonstrating the presence of an adduct; Panel C: MS³ spectrum of D_4 -N7-POB-G.

Figure 3.

Detected DNA adducts formed from the reaction of PR104A with ctDNA (in the presence of AKR1C3) and HT-29 cells. A) Extracted ion chromatograms (5 ppm) of the exact masses that triggered an MS³ fragmentation event for the ctDNA experiment (Table S1). Only DNA adduct masses detected in three independent replicate experiments are reported. The star (★) next to the peak indicates the signal that triggered the MS³ event; B) Proposed structures of the DNA adducts detected in ctDNA (Table S1) and HT-29 cells (Table 1); dG, deoxyguanosine; dA, deoxyadenosine.

Figure 4.

Correlation between PR104A concentration and adjusted peak area for selected DNA adduct masses detected by a MS^3 fragmentation event in the 100% CH_3OH fraction of HT-29 cells treated with increasing concentration of PR104A (Table 1). Error bars represent the standard deviation derived from three technical replicates.

Scheme 1.
Metabolic activation of PR104A and DNA adduct formation.

Table 1

Targeted analysis of adducts by triggered MS³ fragmentation events for DNA extracted from HT-29 cells treated with increasing concentrations of PR104A. Only DNA adduct masses that triggered an MS³ fragmentation event in all three technical replicates are reported. The “X” represents an MS³ fragmentation triggered for the corresponding [M+H]⁺.

Structures ^a	Theoretical [M+H] ⁺ ^b	Neutral loss ^c	Proposed alkylating compound	cDNA exposed to PR104A ^d					HT-29 cells exposed to PR104A				
				100 μM					50% CH ₃ OH fraction				
				50% CH ₃ OH fraction	100% CH ₃ OH fraction	100 μM	250 μM	500 μM	100 μM	500 μM	100 μM	250 μM	500 μM
A	446.1895	A	PR104M	X			X	X	X				
C	462.1844	G	PR104M	X			X	X					X
M	464.1556	A	PR104M							X	X	X	X
F	476.1637	A	PR104A		X					X	X	X	X
N	480.1505	G	PR104M									X	X
D	492.1586	G	PR104A	X		X	X	X	X	X	X	X	X
O	494.1298	A	PR104A									X	X
J	510.1247	G	PR104A		X				X	X	X	X	X
P	514.0680	C	PR104A										X
K	538.0793	A	PR104A		X						X	X	X
L	554.0742	G	PR104A		X					X	X	X	X
H	570.1361	G	PR104A		X								X
I	608.2059	dR	PR104A		X							X	X
G	741.2448	dR	PR104A		X							X	X
MS ³ events (total)				3	8	1	3	3	5	8	13		

^aProposed structures of the detected DNA adducts can be found in Figure 3B;

^bAll [M+H]⁺ indicated had a retention time greater than 10 minutes and were not present in control samples (DMSO control, buffer and enzymes used for DNA hydrolysis);

^cNeutral loss of base or dR;

^dMasses that also triggered an MS³ fragmentation event in cDNA samples treated with PR104A in the presence of AKR1C3 (Table S1).