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A Data-Independent Mass Spectrometry Approach for Screening and Identification of DNA Adducts

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Abstract

Long-term exposures to environmental toxicants and endogenous electrophiles are causative factors for human diseases including cancer. DNA adducts reflect the internal exposure to genotoxicants and can serve as biomarkers for risk assessment. Liquid chromatography-multistage mass spectrometry (LC-MSⁿ) is the most common method for biomonitoring DNA adducts, generally targeting single exposures and measuring up to several adducts. However, the data often provide limited evidence for a role of a chemical in the etiology of cancer. An “untargeted” method is required that captures global exposures to chemicals, by simultaneously detecting their DNA adducts in the genome; some of which may induce cancer-causing mutations. We established a wide selected ion monitoring tandem mass spectrometry (Wide-SIM/MS²) screening method utilizing ultra-performance-LC nanoelectrospray ionization Orbitrap MSⁿ with on-line trapping to enrich bulky, non-polar adducts. Wide-SIM scan events are followed by MS² scans to screen for modified nucleosides by co-eluting peaks containing precursor and fragment ions differing by -116.0473 Da, attributed to the neutral loss of deoxyribose. Wide-SIM/MS² was shown to be superior in sensitivity, specificity, and breadth of adduct coverage to other tested adductomic methods with detection possible at adduct levels as low as 4 per 10⁹ nucleotides. Wide-SIM/MS² data can be analyzed in a “targeted” fashion by generation of extracted ion chromatograms or in an “untargeted” fashion where a chromatographic peak-picking algorithm can be used to detect putative DNA adducts. Wide-SIM/MS² successfully detected DNA adducts, derived from

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*Supporting Information

The Supporting Information is available free of charge on the ACS Publications website

Notes

The authors declare no competing financial interest.

Additional text reporting the DNA adduct data base, chemical structures and references are provided in SI-1. The preparation of DNA adduct standards, DNA digestion conditions, chemical structures of reference DNA adduct standards, and extracted ion chromatograms for Wide-SIM/MS², tCNL-MS³, pseudo-CNLS (SRM), and MS³ spectra of dG-C8-PhIP and several endogenous adducts of lipid peroxides present in human prostate, and dA-AL-I in human renal cortex tissue, analyzed by the targeted-MS³, are reported in SI-2.

chemicals in the diet and traditional medicines and from lipid peroxidation products, in human prostate and renal specimens.

Graphical Abstract

INTRODUCTION

Genome-environment interactions show that chemical exposures and life-style factors are important risk factors in the etiology of chronic diseases.^{1,2} The concept of the “exposome”, the individual exposure to the environmental chemicals and factors (air and water pollution, diet, medicines, radiation, infections, and lifestyle factors) and endogenous sources (inflammation, oxidative stress, and gut flora metabolites) are believed to be important contributing factors to diseases, including cancer.^{1,3} However, the understanding of such complex systematic changes at the molecular level demands tools that are capable of monitoring both known and unknown exposures, and their impact on biological effects.

DNA adducts are internal dosimeters to measure chemical exposures from exogenous and endogenous sources. The adduction of such chemicals or their reactive metabolites to DNA, alters DNA structure and can impede DNA/DNA and DNA/protein interactions.^{4,5} DNA adducts that escape repair can disrupt cell division, or induce mutations which can lead to downstream cellular dysfunctions.^{6–8} DNA adducts of genotoxicants have been employed as biomarkers in molecular epidemiology studies designed to assess the role of chemical exposures in the etiology of cancer.^{9–11} Epidemiologic studies combined with the mechanistic information have firmly established causative linkages between exposures to aflatoxin B₁ and liver cancer in Asia and Sub-Saharan Africa, and exposure to aristolochic acid (AA) and upper urothelial tract cancer in Asia and the Balkans.^{12,13} However, these populations were exposed to unusually high amounts of these carcinogens. In contrast, other populations in developed countries throughout the world are chronically exposed to much lower levels of an array of potentially harmful chemicals, some of which may contribute to the etiology of cancer.

Over the past three decades, ³²P-postlabeling, immunohistochemistry (IHC) and gas/liquid chromatography (GC/LC)-mass spectrometry (MS) have served as the major methods to

measure DNA adducts. The advantages and limitations of each technology have been discussed.^{14–18} LC-MS has emerged as the most versatile technique to measure many classes of DNA adducts.^{16,19–21} We and other laboratories have developed targeted LC-MS methods to qualitatively and quantitatively analyze DNA adducts for a number of human carcinogens.^{20,22,23} However, to broadly screen for DNA adducts of multiple classes of carcinogens, an “adductomics” approach is required where robust and universal methods of sample preparation and unbiased MS scanning features can be employed to characterize an array of DNA adducts. The recent improvements in MS scanning acquisition capabilities and instrument sensitivity have set the stage for the use of LC-MS in high-throughput applications in DNA adductomics.

The basis of DNA adductomics is largely centered on the observation that almost all chemically stable modified 2'-deoxyribonucleosides lose the neutral fragment of the deoxyribose (dR) moiety (116 Da or 116.0473 Da in high resolution accurate mass (HRAM) MS), when subjected to collision-induced dissociation (CID).²⁴ The first example of this analysis was performed by Chaereboundt and coworkers using fast atom bombardment in combination with constant neutral loss (CNL) scanning to characterize the DNA adducts formed with 2,3-epoxypropylphenyl ether.²⁵ The Matsuda laboratory was the first to use the term “adductome” and used a QqQ-MS to monitor 374 continuous selected reaction monitoring (SRM) transitions of precursors ($[M+H]^+$) to aglycones ($[M+H-116]^+$, or $[AH_2]^+$) ions, covering the mass range of most putative adducted deoxynucleosides from m/z 228.8 to 602.8.²⁶ (Figure 1, A). This “pseudo-CNL” methodology was used to characterize lipid peroxidation DNA adducts in human tissues.^{27–29} Numerous signals were detected; however, it is not known whether these analytes are DNA adducts or simply other compounds in the DNA digest matrix that lose 116 Da upon CID. The Turesky laboratory laid the groundwork for the linear ion trap (LIT)-MS based adductomics method.³⁰ This technique is based on data-dependent acquisition (DDA) by CNL scanning followed by MS³ scan stage (CNL-MS³). The loss of deoxyribose moiety from the precursor ions (MS²) triggers the acquisition of the MS³ product ion scan of the aglycone ions.^{30,31} The CNL-MS³ scan mode can detect DNA adducts by untargeted scanning or by the use of a targeted mass list (tCNL-MS³) (Figure 1, B). Compared to the QqQ-MS approach, CNL-MS³ provides rich spectral data about the adduct structures with MS² and MS³ fragmentation data. Balbo and Villalta extended CNL-MS³ scanning to HRAMS using the Orbitrap MS, which provides greater specificity by monitoring the loss of the neutral dR fragment at high mass accuracy.^{32,33}

In 2012, a proteomics data-independent acquisition (DIA) methodology was introduced by Aebersold,³⁴ which involves sequential window acquisition of all theoretical fragment-ion spectra (SWATH). Employing a quadrupole-Time-of-Flight (QqTOF) MS, each cycle contains an MS survey scan and sequential MS² of all precursor ions across a specified mass range in evenly-divided or variable mass windows. In contrast to DDA, where MS² fragmentation ions are chosen in real-time based upon the survey full scan, the DIA method performs MS² fragmentation on all ions present, albeit with larger isolation widths, and with no prior knowledge about the identity of the analytes. SWATH has been successfully applied to qualitative and quantitative analyses of proteomics.^{35,36} and metabolomics.^{37,38}

In this study, we adapted the SWATH approach to establish an unbiased, untargeted DIA approach, termed Wide-SIM/MS², to screen for stable DNA adducts derived from many chemical sources using the Orbitrap Fusion™ MS. Multiple wide-SIM scans are performed, and each scan is followed by high-energy collision-induced dissociation (HCD)-MS² of all ions detected in the previous wide-SIM scan (Figure 1, C). The resulting data is screened for DNA adducts through extraction of ion chromatograms from the wide-SIM and MS² data. The detection of the presumed DNA adduct is determined by cochromatography of the precursor ion peak from the wide-SIM scan event, and the corresponding aglycone [M + H-116.0473]⁺ peak from the MS² scan event, using a mass tolerance of 5 parts-per-million (ppm). The detected adducts can be further characterized by MS³ data acquisition in a subsequent analysis.

To optimize the methodology, we have examined the individual commercial nuclease solutions for extraneous compounds which may negatively impact the successful analyses of DNA adducts.³⁹ We have also optimized the MS parameters for detection of a variety of DNA adducts formed with ubiquitous carcinogens that include aromatic amines, heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, that originate from tobacco, herbal remedies, food sources, environment, and α,β -unsaturated alkenals, which are cellular metabolic by-products of lipid peroxidation. We have compiled a list of 100 known bulky hydrophobic DNA adducts reported in the literature,^{20,23} whose structures, formulae and monoisotopic mass of precursors and aglycones are listed in Supplementary Information (SI), Table S1.

The performance of the optimized Wide-SIM/MS² method was compared to a tCNL-MS³ method, both using the same Orbitrap MS, and a pseudo-CNL method using QqQ-MS. Wide-SIM/MS² scanning had the greatest breadth in DNA adduct coverage and, in contrast to the tCNL-MS³ method, the search for unknown DNA adducts can be done retrospectively. We demonstrate the potential of Wide-SIM/MS² in DNA adductomics, by identifying DNA adducts derived from some of these exogenous and endogenously produced chemicals in human prostate and renal specimens (Figure S1).⁴⁰⁻⁴²

EXPERIMENT

Materials

Calf thymus DNA (CT DNA), DNase I (Type IV, bovine pancreas), Benzonase nuclease ultrapure, alkaline phosphatase (*Escherichia coli*), nuclease P1 (from *Penicillium citrinum*) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphodiesterase I (*Crotalus adamanteus* venom) was purchased from Worthington Biochemicals Corp. (Newark, NJ). Optima™ LC-MS grade NH₄CH₃CO₂, HCO₂H, CH₃CN, CH₃OH and H₂O, were purchased from Fisher Chemical (Pittsburgh, PA). *O*⁶-[4-oxo-4-(3-pyridyl)-butyl]-2'-deoxyguanosine (*O*⁶-POB-dG), [pyridine-2H₄]-*O*⁶-POB-dG; *N*-(deoxyguanosin-8-yl)-4-aminobiphenol (dG-C8-4-ABP), [¹³C₁₀]-dG-C8-4-ABP; *N*-(deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-AaC), [¹³C₁₀]-dG-C8-AaC; *N*-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (dG-C8-MeIQx), dG-C8-[²H₃C]-MeIQx; *N*-(deoxyguanosin-8-yl)-2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (dG-C8-IQ), [¹³C₁₀]-dG-C8-IQ; *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

(dG-C8-PhIP), dG-C8-[²H₃C]-PhIP, [¹³C₁₀]-dG-C8-PhIP; 10-(deoxyguanosin-*N*²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo-*a*]pyrene (dG-*N*²-B[*a*]PDE), [¹³C₁₀]-dG-*N*²-B[*a*]PDE; 6-(1-hydroxyhexanyl)-8-hydroxy-1,*N*²-propano-deoxyguanosine (HNE-dG) and [²H₁₁]-HNE-dG were synthesized as described.^{30,43–47} B[*a*]P-, 4-ABP- and PhIP-modified CT DNA were kindly provided by Dr. Frederick A. Beland from the National Center for Toxicology Research/US FDA.^{44,48,49} Dr. Francis Johnson and Dr. Radha Bonala, Stony Brook University, generously provided 7-(deoxyguanosin-*N*²-yl)-aristolactam-II (dG-AL-II) and 7-(deoxyadenosin-*N*⁶-yl) aristolactam II (dA-AL-II), 7-(deoxyguanosin-*N*²-yl)-aristolactam-I (dG-AL-I), and 7-(deoxyadenosin-*N*⁶-yl)-aristolactam I (dA-AL-I).⁵⁰ Dr. Pramod Upadhyaya from the University of Minnesota, and Dr. Ian A. Blair from University of Pennsylvania, kindly provided the [pyridine-²H₄]-*O*⁶-POB-dG, and 4-oxo-(2E)-nonenal (ONE) adducts ONE-dA and ONE-dG standards, respectively.

DNA from Human Prostate and Renal Cortex

The research protocol was approved by the Institutional Review Board at University of Minnesota. De-identified human prostate non-tumor-adjacent specimens were obtained during necessary surgery at the University of Minnesota.⁵¹ De-identified renal cortex surgical specimens were from subjects exposed to AA and were obtained from Dr. Chung-Hsin Chen, Department of Urology, National Taiwan University Hospital, Taipei, Taiwan.¹³ The procedure for DNA isolation has been previously published.⁵²

Optimized Enzymatic Digestion of DNA

CT DNA or human DNA (20 µg) were spiked with adduct standards and subjected to enzymatic digestion. Non-modified CT DNA was spiked with labeled and unlabeled adduct standards (0.4 - 8.0 adducts per 10⁸ nucleotides, SI S2-1), and a mixture of B[*a*]P-, PhIP- and 4-ABP-modified CT DNA of known adduct levels were used to optimize the digestion conditions (SI S2-2). DNA from human tissues were spiked with isotope labeled standards at 4 - 8 per 10⁸ nucleotides level. DNA samples were digested at 37 °C with Benzonase (300 U) and nuclease P1 (0.1 U) for 3.5 h, followed by digestion with phosphodiesterase 1 (3.2 mU) and alkaline phosphatase (40 mU) overnight at 37 °C. The solutions were dried by vacuum centrifugation and reconstituted in 50% DMSO (50 µL), sonicated, centrifuged at 21,000 xg for 5 min and transferred to a silylated borosilicate glass inserts for LC-MS analysis.

NanoUPLC Chromatography Conditions and MS parameters

All analyses were performed with identical UPLC systems and chromatographic conditions. A Dionex UltiMate 3000 RSLC nanoUHPLC System was interfaced either to an Orbitrap Fusion™ Tribrid™ MS (Thermo Fisher Scientific, San Jose, CA) for the Wide-SIM/MS² and tCNL-MS³ analyses, or a TSQ Quantiva™ MS (Thermo Fisher Scientific, San Jose, CA) for the pseudo-CNL and CNL analysis. A Nanospray Flex™ ion source was used from Thermo Fisher Scientific. The emitters (75 µm ID x 200 mm, 15 µm orifice) were from New Objective (Woburn, MA). and custom-packed with Luna C18 (Phenomenex Corp. Torrance, CA) stationary phase (5 µm, 120 Å). An Acclaim PepMap trap cartridge RP C18 (0.3 × 5 mm, 5 µm, 100 Å, Thermo Fisher Scientific) was employed for online DNA adduct enrichment. The LC solvents were (A) 0.05% HCO₂H in H₂O and (B) 0.05% HCO₂H in

95% CH₃CN. The DNA digests (5 μ L) were injected onto the trap column and washed with solvent A for 4 min at a flow rate of 12 μ L/min by the loading pump, to remove the polar, non-modified nucleosides. After trapping, the adducts were back-flushed onto the analytical column and a linear gradient commenced at 1% B and reached 30% B over 4 min periods, at a flow rate of 0.6 μ L/min. Then, the flow rate was decreased to 0.2 μ L/min and the gradient went from 30% to 99% B over 30 min. The flow rate was switched back to 0.6 μ L/min for the sequential column washing and equilibration. The MS was operated in the positive ionization mode with a 2200 V spray voltage and 300 °C ion transfer tube temperature.

The wide-SIM/MS² method contained a total of 20 scan events, where odd numbered events were wide-SIM scans and the even numbered events were MS². Each wide-SIM event had an isolation width of 30 m/z , in contrast to a single m/z being monitored in the traditional SIM scan, and the sequential MS² scan event fragmented all ions detected in the previous wide-SIM, and the product ion scan range was m/z 100 to 550. Ions in both events were detected using the Orbitrap to achieve high mass accuracy measurements. In this way, a mass range of m/z 330–630 was covered for both the wide-SIM and MS² data acquisition. The MS parameters were: isolation mode, quadrupole; isolation width, 34 m/z (for edge overlapping); RF-lens, 90%; resolution, 60,000 (full width of the peak at its half maximum, fwhm, at 200 m/z); maximum injection time, 100 ms; data type, profile; AGC, 5×10^4 for wide-SIM and 1×10^5 for MS²; MS² scan mode (collision energy), HCD (25%). The ion source conditions and MS parameters of the targeted-MS³ and tCNL-MS³ methods in Orbitrap-MS, and the pseudo-CNL, and CNL methods in QqQ-MS are described in SI S2-3.

Data Acquisition and Analysis

Xcalibur™ version 3.0.63 (Thermo Scientific) was used for data acquisition and analysis. Theoretical m/z of precursors, aglycones, and fragments were generated with ChemBioDraw Ultra version 13.0.2. The extracted ion chromatograms (EIC) were manually generated with a 5 ppm mass tolerance using the Qual browser module of Xcalibur.

RESULTS AND DISCUSSION

DNA adductomics analysis using Wide-SIM/MS² and tCNL-MS³ with HRAM detection

There are several major challenges to address for the establishment of robust MS-based DNA adductomic methods. DNA adducts occur in humans at levels that are more than six-orders of magnitude lower than the non-modified nucleosides. The non-modified nucleosides can overload the HPLC column and/or rapidly fill the IT or Orbitrap, resulting in extremely short trap fill times (injection times), which dramatically reduces the method sensitivity.^{53,54} Thus, enrichment procedures are required to remove non-modified nucleosides and reliably measure DNA adducts. In addition, the DNA digest matrix is complex and isobaric interferences due to contaminants originating from the nuclease preparations or buffers can lead to false-positive or false-negative detection,^{55,56} depending upon the resolving power and the size of the extracted mass tolerance. Furthermore, components in the matrix can cause ion suppression effects or shorten injection times, which adversely affect the performance of the method.¹⁷ We have employed the Orbitrap Fusion™ MS with MSⁿ scanning capabilities and HRAM detection, to tackle these surmountable

analytical challenges. The Orbitrap Fusion™ contains a front-end quadrupole mass filter, and it has both linear ion trap and Orbitrap mass analyzers. These features allow HRAM data acquisition permitting CNL-MS³ and Wide-SIM/MS² analysis for the characterization of DNA adducts. The mass list of adducts in Table S1 served as the targeted mass inclusion list in the tCNL-MS³ and is also used for manual inspection of adduct precursor and aglycone ions in the Wide-SIM/MS² data analysis. However, polar adducts, such as those formed with reactive oxygen species cannot be enriched by our online-trapping, and thus were excluded in this study.

In tCNL-MS³, ions from the MS survey scan are detected in the Orbitrap with an isolation window from m/z 330 to 630.^{20,23} If precursor ions of DNA adducts on the mass list are detected within 10 ppm of the theoretical value, the putative adducts are fragmented. The product ions with the neutral loss of $116.0473 \text{ Da} \pm 20 \text{ ppm (dR)}$ or $121.0641 \pm 20 \text{ ppm (}[^{13}\text{C}_5\text{]}\text{-dR)}$ undergo MS³ fragmentation for further characterization. In the Wide-SIM/MS² method, packets of ions are injected into the Orbitrap using 20 sequential scan events, where all odd events are wide-SIM scans of 30 m/z windows, and ensuing even scan events are the MS² fragmentation scans of the ion range of the previous wide-SIM event. In this scanning method, all precursor ions that undergo a neutral loss of dR can be detected.

The Orbitrap Fusion™ MS utilizes automatic gain control (AGC) to regulate the number of ions entering the Orbitrap to avoid space-charging effects,^{21,53} allowing for maximum injection time for detection of low abundance ions while eliminating overfilling of the detector when species of higher concentration are present.⁵⁴ The employment of 10 consecutive wide-SIM scan events (m/z 330–360, 360 – 390, etc.) reduces the number of different ions entering the Orbitrap per scan event, enhancing sensitivity of detection of low level DNA adducts through longer injection times, when compared to a single MS survey scan covering the same mass range (m/z 330 – 630) with significantly shorter injection times. The instrument settings for the mass resolution, AGC, and maximum injection time are the major parameters that determine the scan speed for both tCNL-MS³ and Wide-SIM/MS² methods. Increasing the AGC allows more ions to enter the trap, thus increasing the likelihood to detect adducts present at low levels; however, large AGC and maximum injection time values can increase the injection times until there are insufficient data points for extraction of the chromatographic peaks. Similarly, the rate of data acquisition is inversely proportional to the Orbitrap resolution setting and therefore care must be taken to assure the resolution setting allows for a sufficient data acquisition rate.

Optimization of DNA digestion conditions

Our enzymatic digestion conditions are robust and routinely used for targeted MS analyses of many classes of DNA adducts.^{52,57,58} However, upon scanning for precursor ions of DNA adducts in the MS survey scan, we discovered that isobaric interferences and ion suppressive components are present in the DNA digestion matrix, and other components contribute to background ions that can significantly reduce the injection fill times and adversely affect the performance of the method. Moreover, many isobaric interferences and ion suppressive components are present in the DNA digestion matrix. Hence, we adapted the methodology developed by the Vouros laboratory,³⁹ and lowered the amount of DNase I, which was the

primary source of background contamination; alternatively, DNase I was replaced with a recombinant endonuclease — Benzonase. 4-ABP-, B[a]P- and PhIP-modified CT DNA with known levels of adducts were used as controls to ensure that the enzymatic digestion efficiency and the recoveries of adducts were quantitative. The optimization parameters for enzyme digestion are described in the SI, S2-2.

Isobaric interferences and limits of detection with Wide-SIM/MS²

The EIC traces of dG-C8-4-ABP are used as an example to show the performance of the Wide-SIM/MS² method, and the challenges associated with the complexity of DNA digest matrix that impact measurements. Peaks for the precursor and aglycone ions of dG-C8-4-ABP and its internal standard [¹³C₁₀]-dG-C8-4-ABP co-elute in the corresponding wide-SIM and MS² scan events extracted with a 5 ppm mass tolerance (Figure 2). The signal of dG-C8-4-ABP in the DNA digestion matrix is about 60% (both in wide-SIM and MS²) of the signal when the same amount of pure standard was injected on-column. The decrease in signal is attributed to losses of the adduct during sample preparation, online trapping, or ion suppression effects of the matrix. The signals of the precursor ion in wide-SIM and the aglycone in MS² increase proportionally as a function of the level of spiking. The average area ratio between precursor and aglycone was consistently at 0.7 for both the pure standard and the DNA adduct in the digestion matrix, indicating isobaric interferences were negligible for dG-C8-4-ABP. However, at the lowest spiking level (0.4 adducts per 10⁸ nucleotides), signals of the precursor peak of dG-C8-4-ABP in wide-SIM dropped out at 20% maximum peak height, indicating the limit of detection was reached.

There were relatively fewer precursor ion scans observed across the chromatographic peak of [¹³C₁₀]-dG-C8-4-ABP at all levels of spiking, particularly in the DNA digest matrix. In fact, no signal was detected in the DNA digest matrix at the lowest spiking level. Nevertheless, the aglycone ion of [¹³C₁₀]-dG-4-ABP was detected with more scans across the chromatographic peak in the MS² scan event for all levels of spiking. More scans of the precursor [¹³C₁₀]-dG-C8-4-ABP were detected after relaxing the mass tolerance to 10 ppm, but the chromatographic peak was asymmetric (Figure 3). This observation could be due to the “ion coalescence” phenomenon⁵⁹ occasionally observed with Fourier-transform ion cyclotron resonance and Orbitrap mass spectrometry, whereby an abundant co-elution background ion with a very close *m/z* value to [¹³C₁₀]-dG-C8-4-ABP may have interfered with its detection. An inspection of the data indicated that this was not the case.

Alternatively, the resolving power of 60,000 (FWHM) at *m/z* 200 may not have been sufficient to fully separate the parent ion of [¹³C₁₀]-dG-C8-4-ABP with isobaric interferences. To address this possibility, we increased the resolution to 500,000, the maximum resolving power of the Orbitrap Fusion™ MS, and employed single wide-SIM and MS² scan events to detect [¹³C₁₀]-dG-C8-4-ABP both with and without the presence of the DNA digestion matrix (level of spiking 4 adducts per 10⁹ nucleotides). At a resolving power of 60,000, the mass spectrum extracted from wide-SIM of the peak at *t_R* 19.0 ± 0.3 min, in CT DNA digest, showed one signal at *m/z* 445.2080, which is 7.0 ppm away from the theoretical *m/z* value of [¹³C₁₀]-dG-C8-4-ABP (*m/z* 445.2111). However, at a resolution of 500,000, an additional signal appeared at *m/z* 445.2043, which required a minimum resolution of 200,000 (FWHM) to achieve 10% valley separation from the detected [¹³C₁₀]-

dG-C8-4-ABP ion (m/z 445.2096, 3.4 ppm from the theoretical m/z).⁶⁰ Thus, a resolving power of 60,000 was insufficient to isolate the m/z of [$^{13}\text{C}_{10}$]-dG-C8-4-ABP, resulting in a shift of the observed m/z beyond the 5 ppm mass tolerance.

The EICs of the precursor and aglycone ions for the adduct standards spiked in the CT DNA digestion matrix are shown in Figure S5. All spiked adducts were detected at 1.2 – 2.4 adducts per 10^8 nucleotides, for 20 μg DNA digestion. This amount of DNA, while often obtainable from tissue biopsy samples, is larger than the amount necessary to perform some targeted DNA adduct quantitative assays where measurements can be made with 1 – 10 μg DNA.²¹ Note, the precursor ions of some DNA adducts at the lowest level of spiking (4 adducts per 10^9 nucleosides) were prone to false-negative detection in wide-SIM in contrast to the aglycones detected with the MS^2 scan event. Although the co-elution of the precursor ions and their aglycones is the first criteria that we have established for the identification of a DNA adduct by Wide-SIM/ MS^2 , in some cases the aglycones may serve as the more robust candidates for screening of DNA adducts.

Wide-SIM/ MS^2 is unbiased towards detecting adducts, however, the ability to detect each individual adduct is limited by different analytical considerations. For some adducts, isobaric interferences were present within the 5 ppm mass tolerance window (*vide supra*). For other adducts, such as dG-C8-IQ and [$^{13}\text{C}_{10}$]-dG-C8-IQ, the FWHM was only 0.1 min compared to an average value of 0.3 min for most of the other adducts. As a result, dG-C8-IQ signal was present above the 10% maximum peak height in only two wide-SIM and MS^2 scans. In addition, for ([pyridine- $^2\text{H}_4$)]- O^6 -POB-dG and ([$^{13}\text{C}_{10}$)]-dG- N^2 -B[a]PDE, the universal HCD MS^2 collision energy used produced predominant fragment ions other than the aglycones, and the ions attributed to the aglycones were barely visible (Figure 4). In fact, the quantitation of O^6 -POB-dG, by QqQ-MS, was done by monitoring the charged pyridyloxobutyl moiety.⁴³ However, the versatility of the Wide-SIM/ MS^2 scanning method provides the opportunity to probe for other neutral losses or product ions for certain classes of DNA adducts in the post-acquisition data processing.

Adductomics method comparison: Wide-SIM/ MS^2 vs tCNL- MS^3 vs pseudo-CNL

We analyzed the same samples by tCNL- MS^3 with the Orbitrap FusionTM and by the pseudo-CNL method with the QqQ-MS. To minimize the detection of false positives and redundant MS^n scanning events, a targeted mass inclusion list of 100 adducts was used, along with a dynamic exclusion list to eliminate continual fragmentation of the putative adducts for 60 sec if three scans occurred within 30 sec. Representative EIC from tCNL- MS^3 are shown in Figure S6. The profiles of some precursor ions from the MS survey scan displayed some irregular chromatographic peaks due to the incomplete resolution of isobaric interferences. However, these interferences were removed at the MS^2 scan stage, and the quality of the spectra acquired at the MS^3 scan stage were similar to those acquired upon targeted- MS^3 analysis. The reliability of the scanning methods and the number of adducts detected by Wide-SIM/ MS^2 method and those triggered the MS^3 scans in tCNL- MS^3 method are reported in Table 1. Based on the 15 spiked adducts, where the aglycone is the predominant product ion at the MS^2 scan stage, 12 and 7 adducts were detected in Wide-SIM/ MS^2 and tCNL- MS^3 methods, respectively, at the lowest spiking level. All 15 adducts

were detected in Wide-SIM/MS² at the intermediate spiking level, compared to 12 detected by tCNL-MS³. Thus, Wide-SIM/MS² scanning is the more robust method for screening adducts.

The TSQ Quantiva™ MS was used to perform the pseudo-CNL method reported by Kanaly.²⁶ A method was made using three-hundred mass transitions of $[M+H]^+ \rightarrow [M+H-116]^+$ from m/z 330 to 630 and four transitions of $[M+H]^+ \rightarrow [M+H-121]^+$ ($[^{13}C_{10}]$ -dG-C8 adduct of 4-ABP, AαC, IQ, PhIP). To compare the robustness of the detection, we built two additional targeted methods containing large numbers of SRMs by monitoring either 19 transitions corresponding to the 20 spiked adduct standards ($[^2H_{11}]$ -HNE-dG and dG-C8-4-ABP have the same nominal mass) or 100 transitions of other potential adducts reported in the literature (Table S1). Representative EICs of the adduct standards spiked into DNA at 4–8 per 10⁸ nucleotides are shown in Figure S7; however, none of the adducts were detected when spiked at the lowest level in the digestion matrix (data not shown). In the pseudo-CNL, the spiked adducts of dG-C8-MeIQx, dA-Al-II, dG-AL-II and labeled and unlabeled dG-C8 adducts of 4-ABP, AαC, IQ, were detected with signal-to-noise (S/N) ratio larger than three. Note, dG-*N*²-B[a]PDE and *O*⁶-POB-dG were excluded from this SRM analysis because product ions other than the aglycone were the predominant features. We chose three sec cycle time for the pseudo-CNL method to ensure the number of scans across the peak above 10% relative abundance was at least six scans, except for the extremely narrow peaks of dG-C8-IQ and $[^{13}C_{10}]$ -dG-C8-IQ, where ~ four scans were acquired. Since the dwell time is inversely proportional to the number of transitions used for a given cycle time, decreasing the number of transitions resulted in the acquisition of more scans with smoother chromatographic peaks and lower background signals. Overall, the sensitivity of this method decreased following the order of 19 transitions > 100 transitions > 300 transitions. In comparison to the traditional CNL approach by QqQ-MS, our data showed a higher sensitivity in the pseudo-CNL method.

Wide-SIM/MS² screening with human samples

Wide-SIM/MS² was applied to screen for DNA adducts in human prostate and renal tissues. Figure 5 shows the detection of the DNA adduct dG-C8-PhIP in prostate; PhIP is a rodent and potential human prostate carcinogen formed in cooked meat.⁶¹ The DNA adduct dA-AL-I, formed by the human renal carcinogen AA, a chemical naturally occurring in some traditional herbal medicines,¹³ was detected in kidney tissue of renal cancer patients. The product ion spectra acquired at the MS³ scan stage (Figure S8) are consistent with the mass spectra of these adducts previously reported by the targeted UHPLC-MS³ methods.^{51,58}

Putative endogenous DNA adducts derived from lipid peroxidation products were also detected in human tissues by Wide-SIM/MS², and also observed using tCNL-MS³ method and targeted MS³ detection. Proposed adducts include but are not limited to *trans*-4-hydroxy-2-nonenal (HNE) adducts HNE-dG (precursors $[M+H]^+$ m/z 422.2034), HNE-dA (m/z 406.2085), HNE-dC (m/z 382.1973); 4-oxo-(2*E*)-nonenal (ONE) adducts ONE-dG (m/z 404.1929), ONE-dA (m/z 388.1079), and ONE-dC (m/z 364.1867).⁴¹ The product ion spectra support the proposed structures based on the published spectral data,⁴¹ or by the proposed fragmentation pathways (Figure S8).

In contrast to Wide-SIM/MS², pseudo-CNL analysis (Figure S9) detected multiple peaks across the EIC for the mass transitions of the spiked adduct standards and endogenous adducts in human prostate DNA. Some of these peaks appeared to be matrix sample components which were not DNA adducts, as they were not detected by the more sensitive and stringent Wide-SIM/MS² scanning method. In the absence of internal standards and with an inability to acquire MS³ spectra, the identity of these endogenous adducts cannot be confirmed by QqQ-MS. It is reasonable to propose that pseudo-CNL tends to generate false-positive results.

CONCLUSIONS

We report an untargeted and unbiased screening method, Wide-SIM/MS², to detect bulky hydrophobic DNA adducts formed with multiple classes of genotoxins. The co-elution of precursor ions in wide-SIM and their aglycones in the corresponding MS² fragmentation event, employing accurate mass detection with a 5 ppm mass tolerance, provides robust chromatographic and mass spectral evidence to support the occurrence of a DNA adduct. However, a targeted-MS³ scan, is required to confirm the identity of the adduct against a database of the synthetic DNA adduct standards. Since the DNA adducts are identified by post-acquisition data mining, adducts with common neutral losses other than 116.0473 Da can also be data-mined, and broaden the search criteria for different classes of DNA adducts, such as for dG-N²-B[a]PDE and other PAHs,⁶² and pyridyloxobutyl adducts, such as *O*⁶-POB-dG, formed from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.⁴³ HRAM detection by the Orbitrap Fusion MS greatly improves the selectivity, sensitivity and breadth of the assay compared to the previous adductomic methods using low resolution QqQ-MS or LIT-MS.

A recently developed Orbitrap-HRAM method employing untargeted wide-SIM was used to screen for more than 30 putative DNA adducts in human colon and other DNA samples.⁶³ The criteria for DNA adduct identification included a minimal signal intensity of 10,000 counts, a maximum mass tolerance of 10 ppm, a stable retention time of the DNA adduct, and a ¹²C/¹³C isotope ratio approaching the natural ratio of 99:1. The screening method targeted aglycones liberated from DNA after acid hydrolysis. Thus, monitoring the loss of the neutral dR moiety, by CID, could not be performed. There are no current guidelines for the validation of analytical methods for the detection of DNA adducts in biological matrices. The reliability in the assignment of an adduct structure based on mass accuracy with a 10 ppm tolerance, without confirmatory MSⁿ product ion spectral data, to our experience, is often not of sufficient stringency to identify DNA adducts. Precursor ions combined with co-eluting characteristic product ions at the MS² or MS³ scan stages provides stronger support for the assigned structure (*vide supra*). Ultimately, the matching of chromatographic retention time and the mass spectra of the aglycones must be compared to synthetic standards as proof of adduct identity.

Our methods utilize on-line sample enrichment, and the screening of adducts is currently restricted to bulky hydrophobic DNA adducts that bind to the trapping column. The trapping procedure removes salts and polar components present in the enzymatic DNA digestion matrix. However, isobaric components from digestion matrix may still adversely affect the

detection of some adducts, especially in the wide-SIM scan mode. Moreover, certain DNA adducts present at low levels remain difficult to detect because ions resulting from highly abundant components of the DNA digest matrix lead to short injection times, which limits the sensitivity of the Orbitrap detection.

We are pursuing approaches to improve the cleanliness of our DNA samples and further reduce background signals to screen for DNA adducts of carcinogens with a wide range of chemical properties. We are also developing software tools to streamline the data mining, by automated screening for precursor ions and their common neutral losses, such as 116.0473 Da or other neutral fragments lost during HCD, which will facilitate data analyses and required to achieve unbiased DNA adductomic screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schemes for the DNA adductomic methods compared in this paper. Adducts in the m/z range 330–630 were analyzed. (A) Depicts the pseudo-CNL, where 300 SRM transitions of $[M+H]^+ \rightarrow [M+H-116]^+$ were monitored in the QqQ-MS. (B) Describes the logic used for MS² ion selection (observation of an ion, from the mass list, in the full scan spectrum) and MS³ fragmentation (following the loss of 116.0473 Da upon MS² fragmentation) for the targeted-CNL/MS³ method. (C) Illustrates the adduct detection, first by Wide-SIM/MS², then its structural confirmation by targeted-MS³ analysis. Both (B) and (C) spectral data were detected in the high resolution Orbitrap MS.

Figure 2.

The extracted ion chromatograms of the precursors and aglycones of dG-C8-4-ABP and $[^{13}\text{C}_{10}]$ -dG-C8-4-ABP analyzed by Wide-SIM/MS², with a resolution of 60,000 and a 5 ppm mass tolerance. Samples shown here were at two levels either with pure standards, or standards spiked into CT DNA prior to the digestion. The amount of standards injected online are specified at the top of the figure.

Figure 3.

EIC and the mass spectra of precursor $[^{13}\text{C}_{10}]$ -dG-C8-4-ABP standard or $[^{13}\text{C}_{10}]$ -dG-C8-4-ABP spiked into CT DNA digest analyzed by Wide-SIM/MS² at resolutions of 60,000 or 500,000. Precursors were extracted at 5 and 10 ppm mass tolerances. Inserts of the zoomed-in spectra between m/z 445.20 to 445.22 show the location of the theoretical m/z 445.2111 (red dash line), the mass range of the 5 ppm mass tolerance (teal box) and that of the 10 ppm tolerance (lavender box).

Figure 4.

EIC of dG- N^2 -B[a]PDE and O^6 -POB-dG standards analyzed by Wide-SIM/MS². In MS² scans, the signals of the extracted ions of the aglycones were minor compared to fragment ions derived from the B[a]P triol and the pyridyloxobutyl moieties. Adduct structures and the fragmentation mechanisms are shown. X is a compound from the matrix that co-eluted with O^6 -POB-dG and undergoes CID to form an ion at m/z 152.05669.

Figure 5.

Application of Wide-SIM/MS² to human DNA samples: dG-C8-PhIP and some endogenous lipid peroxide adducts in prostate, and dA-AL-I in renal cortex. Adduct structures were confirmed by their MS³ spectra (SI Figure S8) and consistent with the published data.^{41,51,58}

Table 1Number of spiked adduct standards detected by Wide-SIM/MS² and tCNL-MS³ methods

Spiked DNA adduct Standards, per 20 µg DNA	Total number of adduct standards	Number of standards detected with Wide-SIM/MS ² ^b	Number of standards detected with tCNL-MS ³ ^c
0.4–0.8 per 10 ⁸ nucleotides	15 ^a	12	7
1.2–2.4 per 10 ⁸ nucleotides	15 ^a	15	12
4–8 per 10 ⁸ nucleotides	15 ^a	15	15

^aTotally 20 adducts were spiked, but *O*⁶-POB-dG, [pyridine-²H₄]-*O*⁶-POB-dG, dG-*N*²-B[*a*]PDE, [¹³C₁₀]-dG-*N*²-B[*a*]PDE and [²H₁₁]-HNE-dG were not reported because the aglycones were minor product ions.

^bAn adduct is successfully detected, by Wide-SIM/MS², when the number of scans across the peak is greater than 3 (10% maximum peak height). Such criterion was dropped to 2 scans for dG-C8-IQ and [¹³C₁₀]-dG-C8-IQ, whose peak widths were exceptionally narrow.

^cAn adduct is successfully detected, by tCNL-MS³, when at least 2 scans were triggered in both MS² and MS³ events.