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Recent technical and biological development in the analysis of biomarker *N*-deoxyguanosine-C8-4-aminobiphenyl

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Abstract

4-Aminobiphenyl (4-ABP) which is primarily formed during tobacco combustion and overheated meat is a major carcinogen responsible for various cancers. Its adducted form, *N*-deoxyguanosine-C8-4-aminobiphenyl (dG-C8-4-ABP), has long been employed as a biomarker for assessment of the risk for cancer. In this review, the metabolism and carcinogenicity of 4-ABP will be discussed, followed by a discussion of the current common approaches of analyzing dG-C8-4-ABP. The major part of this review will be on the history and recent development of key methods for detection and quantitation of dG-C8-4-ABP in complex biological samples and their biological applications, from the traditional ³²P-postlabelling and immunoassay methods to modern liquid chromatography-mass spectrometry (LC-MS) with the latter as the focus. Many vital biological discoveries based on dG-C8-4-ABP have been published by using the nanoLC-MS with column switching platform in our laboratory, which has also been adopted and further improved by many other researchers. We hope this review can provide a perspective of the challenges that had to be addressed in reaching our present goals and possibly bring new ideas for those who are still working on the frontline of DNA adducts area.

Keywords

4-Aminobiphenyl; DNA adducts; dG-C8-4-ABP; LC-MS; Bladder cancer

1. Introduction of 4-ABP

Humans are constantly exposed to chemicals which could bind with our DNA and form carcinogenic DNA adducts. The formation of DNA adducts is typically from two sources. External exposures which include environmental pollutants from air and water, radiation damage, lifestyle factors, diet and drugs, and infectious agents. Some examples are polycyclic aromatic hydrocarbons (PAHs), *N*-nitroso compounds (NOCs) and aromatic amines (AAs). Internal exposures which include reactive metabolites related to oxidative stress, inflammation, bacterial and viral infection, endogenous toxic chemicals etc. [1–3]. In

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this review we will be discussing 4-ABP, an external carcinogen linked to various cancers such as bladder, liver and breast, and has been the focus of carcinogen research in our laboratory for the past several years.

4-ABP is one of the AAs which exists in industrial production process, such as the use of dye intermediate, rubber antioxidant and fertilizer. However, today the major source of 4-ABP is from occupational exposure, tobacco combustion [4] and overheated meat [5]. The well-studied DNA adduct for this carcinogen is dG-C8-4-ABP which is a well-confirmed biomarker for bladder cancer and has been used to address the puzzle of “Gender Disparity” in human bladder cancer where the incidence is about 3–4 times higher in men than in women [6]. Therefore, this review will discuss the biological relationship of dG-C8-4-ABP with various cancers and the recent technical developments for the analysis of this DNA adduct. The structures of 4-ABP and dG-C8-4-ABP are shown in Fig. 1.

2. Metabolism of 4-ABP and the development of cancer

4-ABP is carcinogenic in different animal species such as human, mice, rats, rabbits and dogs. However, it has been shown that 4-ABP has quite different target organs for each species. For example, in mice, the primary target organ for 4-ABP is liver, then bladder. However, in humans and dogs, the target organ tends to be bladder. For other species, 4-ABP could be carcinogenic for other organs such as kidney and ovarian [7]. As a consequence, the metabolism of 4-ABP can be significantly different in species and organs. Below we will be discussing the general and commonly accepted metabolism pathways that implicate liver and bladder as the major organs targeted by 4-ABP.

It has been proposed that carcinogenesis begins with the formation of DNA adducts [8–12]. In order to form DNA adducts, carcinogens either directly react with DNA or undergo metabolic activation to electrophilic species such as epoxides, quinone methides, diazonium ions, and nitrenium ions which then bind with DNA and form DNA adducts [13]. 4-ABP belongs to the latter category, as it is not genotoxic and mutagenic until activated by certain enzymes to nitrenium ion. However, metabolic activation of 4-ABP is still not fully understood to date and appears to be species- and organs-dependent. Therefore, only a general and commonly accepted metabolism route [7] is shown in Fig. 2.

Briefly, upon exposure to 4-ABP, this procarcinogen is first activated primarily in the liver by enzyme cytochrome P450 1A2 (CYP1A2) and related enzymes to form *N*-hydroxy-4-ABP (N-OH-4-ABP). CYP1A2 enzyme has long been recognized as the primary enzyme in this activating process. However, it was later discovered that it may not be the enzyme or at least not the primary enzyme for activating 4-ABP. This assumption was supported by the finding that CYP1A2 knockout mice does not significantly impact DNA adduct levels in bladder and liver [14].

N-OH-ABP, once generated, will either remain in the liver or transported to other organs. The metabolite fraction may undergo further metabolic transformation in liver and elsewhere, which can convert N-OH-ABP to non-active or reactive conjugates. These reactions include sulfation, acetylation and glucuronidation, which are catalyzed by Phase 2

enzymes. Initially, N-OH-ABP can be catalyzed by *N*-acetyltransferase (NAT) to form *N*-hydroxy-4-acetyl-aminobiphenyl (*N*-OH-AABP) and *N*-acetoxy-4-acetylaminobiphenyl through O- or *N*-acetylation. Then the products, N-OH-AABP and *N*-acetoxy-4-acetylaminobiphenyl, can be converted to *N*-acetoxy-4-aminobiphenyl by an enzyme called *N*, *O*-acetyltransferase [15,16]. The pH of urine is normally below 7 and the acetyl group of *N*-acetoxy-4-aminobiphenyl can form the carcinogenetic nitrenium ion in bladder through oxidation under this acidic condition [16,17].

The sulfation in the liver has been shown to be related with the formation of DNA adducts in liver, as the metabolites may be highly labile and reactive [18] and may cause liver cancer. In contrast, glucuronidation in the liver may lead to bladder cancer, as the metabolites, once excreted in the urine, may be labile especially in acidic urine and may deliver carcinogenic species to the bladder epithelium [19,20].

4-ABP binds primarily to the C-8 site of guanine and forms dG-C8-4-ABP to cause what is referred to as nucleobase lesions on the DNA sequence. All living cells have a self-protect/repair system to repair such lesions but there is no guarantee that 100% of these lesions can be repaired, especially if there are too many lesions. Even a single unrepaired nucleobase lesion can possibly induce base mispairing during DNA replication and thus cause gene mutation, which may lead to cancer development. Therefore, the presence of DNA adducts can be a potential indicator for the evaluation of the development of cancer [13].

3. Steps of analyzing DNA adducts

The steps of analyzing DNA adducts are summarized in Fig. 3. Typically, DNA samples can be obtained from a variety of biological sources such as tissues, blood, urine, cells, saliva, even hairs. The sources of DNA may also vary according to species. For animals, DNA can be easily isolated from organs such as liver, bladder, kidney and brain [21–24] where their persistence and repair has been studied. However, for human, the typical way of obtaining DNA samples is through easily accessible sources or non-invasive approaches such as blood, urine, oral buccal cells, saliva and urinary epithelial cells [25,26]. For example, researchers already showed that analyzing nucleosides in human urine is feasible using high performance liquid chromatography (HPLC)-electrospray ionization (ESI)-MS/MS [27–31]. Recently, with the development of LC-MS technology, analyzing DNA samples in formalin-fixed paraffin-embedded human tissues has become possible as well, the significance of which will be discussed in the method section.

All cell or tissue samples first need to be homogenized to release the DNA. There are several homogenization methods including Potter-Elvehjem, French Press, freeze-thaw, sonication, detergent or organic solvents lysis. Tough tissues such as bladder and muscle tissue are normally homogenized by Potter-Elvehjem, whereas soft tissues such as liver and brain are homogenized by sonication. Samples such as blood cells and bacteria cells can be homogenized with osmotic and detergent lysis methods [32].

After homogenization, the released DNA has to be extracted from the complex biological matrices to obtain relatively purified DNA sample for digestion. The methods used for this

step are typically liquid-liquid extraction, solid phase extraction (SPE), off-line HPLC, size exclusion, etc. The extracted and purified DNA then needs to be digested to release the DNA adducts. For digestion, a cocktail which is comprised of a combination of nucleases (cleave the phosphodiester bond of nucleic acids including DNA and RNA), DNases (cleave the phosphodiester bond of DNA only), phosphodiesterase (break any phosphodiester bond) and alkaline phosphatase (removes phosphate group from nucleic acids) is normally used [33]. During digestion, the DNA is normally broken down into mononucleotides dA, dG, dC, dT along with the adducted mononucleotides such as dG-C8-4-ABP. In general, LC-MS analysis is conducted by converting the nucleotides into the corresponding nucleosides.

After DNA digestion, the adducted mononucleotides need to be separated from the unmodified/normal nucleotides, residue proteins, lipids and inorganic salts to achieve better detection and quantitation results. There are several methods that can be used for this process, such as liquid-liquid extraction, SPE, ultrafiltration, immunoaffinity purification, and off-line HPLC [34,35]. Liquid-liquid extraction is typically used for hydrophobic adducts which can be extracted into an organic phase, a relatively simple and rapid method, but lacks specificity. SPE has much broader applicability and can be used for isolation of all kinds of adducts including hydrophobic, hydrophilic and charged adducts. This is due to the availability of many different modes of SPE which include reverse phase, ion exchange, (hydrophilic interaction liquid chromatography) HILIC and mixed mode. By comparison to liquid-liquid extraction, SPE has much higher specificity and can greatly reduce the sample complexity by removing the unmodified nucleotides and salts which is especially beneficial for LC-ESI-MS analysis because it reduces most of the matrix interference and increases ionization efficiency [36]. Immunoaffinity requires a specific monoclonal or polyclonal antibody to capture the targeted DNA adducts which can be very challenging if there are many different types of adducts.

A limitation of all the above methods is that they are all essentially offline clean-up approaches which can be very time-consuming and may be susceptible to sample losses during handling. Online clean-up on the other hand has been shown to greatly reduce the time and sample cost, matrix effect and ion suppression for MS analysis, which will lead to rapid and more sensitive analysis. The enriched and purified DNA adducts will then be analyzed using all kinds of analytical methods which are summarized in the next section. Again, the discussion of all methods will be focused on dG-C8-4-ABP. It is worth mentioning that the level of DNA adducts in both animal and human tissues may be only a few adducts per 10^8 or 10^9 unmodified nucleotides [21] or even lower. Therefore, the analytical methods used for detecting and quantifying DNA adducts must be very sensitive, accurate, and specific. Analyzing DNA adducts by different methods, especially by MS, has been thoroughly reviewed by other groups [13,37–39]. In this review, we present a more historical and developmental perspective beginning with the classical ^{32}P -postlabelling and immunoassay methods and ending with the most recent and widely used nanoLC-MS and ultra-performance liquid chromatography (UPLC)-MS platforms as they relate to dG-C8-4-ABP. The discussion is not just an overview of all the methods but rather is focused on how these methods evolved with the intention of providing the direction of the development of such methods. The development of most of the methods for analyzing DNA adducts is summarized in Fig. 4.

4. Methods of analyzing DNA adduct dG-C8-4-ABP

4.1. ^{32}P -postlabelling

^{32}P -postlabelling was the very first method used for analyzing DNA adducts. It has very high sensitivity and very low sample requirement compared to other methods. It is also compatible with all kinds of DNA adducts which are induced by > 100 chemicals including aromatic amines, azo compounds, heterocyclic aromatic amines, nitro aromatics, polycyclic aromatic hydrocarbons and other genotoxicants [40–48].

^{32}P -postlabelling was first introduced in 1982 by Gupta [49]. According to its original protocol, the DNA samples were first enzymatically digested to release the adducted mononucleotides; the normal and adducted mononucleotides were then labelled with [γ - ^{32}P] ATP in the presence of T4 polynucleotide kinase; the labelled normal and adducted mononucleotides were separated by thin layer chromatography (TLC), then detected and quantified by autoradiography [49]. The sensitivity of this method was about one adduct per 10^7 – 10^8 nucleotides, and the sample requirement was only 1 μg of DNA. Three years later, in 1985, the sensitivity of this method was further improved by the same group to one adduct per 10^9 – 10^{10} nucleotides and using only 1–10 μg DNA sample [50,51]. Different versions of this method have been developed to further increase the sensitivity [52].

Given its high sensitivity and low sample requirement, ^{32}P -post-labelling has been successfully employed for screening DNA adducts in numerous organs, tissues and cells. It also has been used for various studies such as investigating the relationship between smoking and formation of DNA adducts [37] and the detection of 4-ABP DNA adducts in exfoliated urothelial cells in urine from dogs [53]. In 2014, Lee et al. [54] discovered that acrolein-DNA adduct level is 10- to 30-fold higher in normal human urothelial mucosa and bladder tumor tissues than 4-ABP-DNA adducts. As for the acrolein-DNA adduct level alone, it is 2-fold higher in bladder tumor tissues than in normal human urothelial mucosa. The same investigators later found that acrolein-DNA adduct has 5-fold higher mutagenicity than 4-ABP-DNA adduct. In this new study, they used the same ^{32}P -postlabelling method but together with an immunochemical method [55,56] and they concluded that acrolein is one of the major bladder carcinogens in tobacco smoke. To overcome some of the time-consuming and labor-intensive drawbacks associated with the traditional ^{32}P -postlabelling method which relied on the use of TLC plates, some alternative methods have been explored. Terashima, et al., devised a ^{32}P -postlabeling method that uses non-denaturing 30% polyacrylamide gel electrophoresis (PAGE) to resolve adducted nucleotides (^{32}P -postlabelling/PAGE analysis) [57]. To expedite the process, the combination of ^{32}P -postlabelling with HPLC separations has also been successfully employed [58,59].

Notwithstanding its high sensitivity and any recent advances, the ^{32}P -postlabelling method has many disadvantages. It is labor-intensive and relies on the use of radioactive ^{32}P which could be potentially harmful to the researchers [60]. It employs no internal standards for the measurement of potential sample losses, adducts recovery and labelling efficiency [61–64]. It provides no information about the structural identities of the adducts, and the assay conditions are adducts-dependent [65,66]. Therefore, other methods were developed later to overcome those issues and challenges as discussed next.

4.2. Immunoassay

Immunoassay is another traditional method for DNA adducts analysis, which is based on specific binding of an antibody to DNA adducts. The same approach is used in protein qualitative and quantitative analysis as well. The method development phase is time consuming (potentially lasting several months) although it may offer high sample throughput when the method development is completed. In addition, the method sensitivity is limited by the specificity and affinity of the antibodies [67].

In 1986, Roberts et al. [68] developed an avidin-biotin amplified ELISA assay employing polyclonal rabbit anti-KLH-(rG-C8-ABP). The detection limit for dG-C8-ABP was 18 fmol/well. In 1988, the same group improved this method using a streptavidin-biotin amplified ELISA assay and the detection limit was improved to 2 adducts per 10^8 nucleotides using 25 μ g DNA. This study was conducted on dogs but the researchers believed that it could also be used for human target-tissue dosimetry of dG-C8-4-ABP [69]. In 2012, Yoon et al. [70] used a mouse monoclonal 4C11 antibody which is highly specific for dG-C8-4-ABP and confirmed that 4-ABP has a major mutagenicity in mice bladder than kidney and liver. The method utilized only 0.5 μ g of DNA. Most recently, in 2016, Duval et al. [71] used the same method developed by Yoon et al. to assess the formation of 4-ABP adducts of cellular DNA from MCF7 cells. They showed the chemopreventive activity of isothiocyanates (ITCs) by direct inhibition of Arylamine *N*-acetyl-transferases (NAT) enzymes. Again, the immunoassay method is also time-consuming and provides no structure information.

4.3. GC-MS

Gas chromatography (GC)-MS using electrophoric derivatives and negative ion chemical ionization was introduced by the Giese group and was noted for its exceptional sensitivity in the analysis of DNA adducts [72]. In addition to good sensitivity GC-MS has, good resolution, and comprehensive library/database for adduct structure identification. However, many adducts are thermally unstable, therefore, GC-MS can only be used primarily for specific/limited classes of DNA adducts. Moreover, sample throughput of this method is low due to many factors such as the time spent on derivatization, hydrolysis of DNA, and the long temperature gradient which is similar to the issue of solvent gradient for LC-MS. In addition, GC-MS suffers from the use of a large amount of DNA [73] which could largely limit its general applicability. On account of these limitations, the application of GC-MS on DNA adducts analysis is not as widely used as the 32 P-postlabelling and immunoassay methods or the relatively new LC-MS methods which are discussed next. One notable recent application of GC-MS is in the measurement of DNA adducts of *o*-toluidine and 4-ABP in human bladder [74]. In this study, the authors found that 4 and 11 of 12 tumor samples contained adducts of 4-ABP and *o*-toluidine, respectively, in epithelial and submucosal bladder tissues of sudden death victims. This study showed the carcinogenicity of 4-ABP and *o*-toluidine in the human bladder.

4.4. LC-MS

LC-MS is currently the primary and gold standard method of analyzing DNA adducts. All the methods mentioned above, either require a large amount of sample or fail to provide definitive structural identification. LC-MS, however, especially when operated at the

nanoscale level, has been shown to fulfill the desired criteria of high sensitivity, low sample requirement and structural information. LC-MS has been used for the analysis of DNA adducts since early 1990s [38,39]. LC is the most widely used separation technique coupled to MS for DNA adducts analysis and has been comprehensively reviewed [75–78]. In the following paragraphs, different LC techniques will be discussed with focus on their sensitivity, sample requirement and also consideration of time requirement (sample throughput). A typical example of separating and detecting dG-C8-4-ABP using LC-MS is shown in Fig. 5.

Conventional/analytical LC-MS which has a flow rate ranging from 100 to 200 $\mu\text{L}/\text{min}$ to as high as 1 mL/min , despite its high robustness and low risk for clogging of transfer lines, suffers from relatively low sensitivity due to the higher flow rate and requirement of relatively high DNA quantity. One recent application of the conventional LC-MS approach is that of Yao et al. [79] for the non-targeted/global screening of DNA adducts in follicular cells from isolated ovarian follicles that were exposed to benzo[a]pyrene (B[a]P) and cigarette smoke condensate. The study used an Agilent 1200 HPLC-AB Sciex 4000 QTRAP system at flow rate of 0.22 mL/min , and reported a sensitivity of 1.3 adducts per 10^7 nucleosides using only 9 μg DNA. They found three main DNA adducts: benzo[a]pyrene-7,8-dihydrodiol-9,10-ep-oxide-dG (BPDE-dG), phenanthrene 1,2-quinone-dG (PheQ-dG) and B[a]P-7,8-quinone-dG (BPQ-dG). dG-C8-4-ABP was also identified in the follicular cells but not as dominant as the other three.

With the advances of LC technologies, and the use of micro (capillary) LC (5–10 $\mu\text{L}/\text{min}$) and more recently nanoLC (< 1 $\mu\text{L}/\text{min}$), the analysis of DNA adducts has reached much higher sensitivity using much lower sample quantity. It is worth-mentioning that higher sensitivity and lower sample requirement are extremely important especially for in-vivo and trace analysis. For instance, typically, only a few micrograms of DNA can be obtained from a mouse bladder which greatly limits adducts analysis using other less sensitive methods. One of the earliest applications of micro LC approach to the analysis of DNA adducts relates to another carcinogen *N*-acetylaminofluorene (AAF), reported by Wolf and Vouros [80] using a flow rate was 4 $\mu\text{L}/\text{min}$. However, this approach suffered from very low sensitivity and high sample requirement, 1 adduct in 10^6 nucleotides using 1 mg of DNA. Investigators from the same group [81] later used capillary LC-MS to detect and quantify dG-C8-4-ABP in human pancreatic tissues and achieved a detection limit of 5 adducts per 10^9 nucleosides using a 320 μm i.d. capillary column and a flow rate of 10 $\mu\text{L}/\text{min}$. However, the method still required 300 μg DNA for analysis. The same group of investigators later did a comparison study of LC-MS and ^{32}P -postlabelling [82] and showed that both techniques had fairly similar detection limits of about 1 adduct in 10^9 nucleosides/nucleotides, demonstrating their applicability to in-vivo studies. However, in order to achieve the same sensitivity, the amount of DNA required for LC-MS was still very high (500 μg) compared to ^{32}P -postlabelling (30 μg) [82]. The requirement for the large amount of sample quantity was at least in part due to the extensive offline cleanup [83] which involved multiple steps such as SPE, liquid-liquid extraction, and protein precipitation [80,84–88]. Nevertheless, the advances made with the use of micro-LC mentioned above inspired the incorporation of online cleanup/online SPE methods in combination with column switching which mitigated the sample requirement

problem dramatically by reducing sample losses. The online SPE technique will be discussed next.

The use of column switching to introduce analytes trapped on an SPE column as a plug injection into the analytical column was a common approach to improve the mass sensitivity in LC-MS analysis. In an early example related to the analysis of ABP adducts, Zayas et al. [89] used a column switching and conventional LC/MS to detect and quantify 4-ABP adducts in DNA from bladder cancer patients and achieved a detection limit as low as 1 adduct per 10^9 nucleotides. However, the sample requirement was still quite high (100–200 μg DNA), suggesting that the column switching technique in combination with online sample cleanup should be coupled with nanoLC rather than conventional or micro LC in order to ensure both high sensitivity and low sample requirement.

In 1997, Vanhoutte et al. [90] showed that mass sensitivity can be improved by a factor of 3300 by using a nanoflow (200 nL/min) LC/MS compared to the traditional capillary (40 $\mu\text{L}/\text{min}$) LC/MS with column-switching injections. Doerge et al. [91] used nano bore LC-MS and column switching to detect 4-ABP adducts and the mass sensitivity was 0.7 adducts in 10^7 nucleotides using 100 μg DNA. They demonstrated the exceptional cleanup efficiency by online column switching and faster overall analysis time. Moreover, the selectivity was further improved by using different cleanup strategies. Along with the rapid development of the online column switching technique, the sample requirement became progressively lower and this general approach has made LC-MS more compatible with human studies.

The development of nanoLC-MS on a chip with a full integration of sample cleanup and injection via column switching has now advanced the analysis of DNA adducts to a level that is fully comparable with ^{32}P - postlabelling and compatible with in vivo and human applications. Most recently, Randall et al. [87] in Vouros group has improved the detection limit and sample requirement by using an Agilent microfluidics HPLC chip (shown in Fig. 6) which combines online SPE and nanoLC and gave extraordinary performance. They were able to achieve a detection limit as low as 5 adducts per 10^9 nucleotides for dG-C8-4-ABP using only 1.25 μg DNA. In many respects, this has made LC-MS even better than ^{32}P -Postlabelling in human studies due to the additional feature of structure information provided by LC-MS. Since then, this platform has been adopted in a series of studies exploring the relationship between 4-ABP and bladder cancer (this will be discussed in another section). It should be noted that, other than the Agilent Chip, related chip-based technologies are also available through Thermo Scientific EASY-Spray source, New Objective PicoChip source, and Bruker Daltonic CaptiveSpray source. All of them share the same principle and thus potentially can all be used in the super high sensitivity in-vivo study of DNA adducts.

So far, as discussed above, nanoLC with the column switching technique has provided the highest performance in terms of sensitivity and sample requirement. However, this comes with a great cost which is the extremely long nanoLC running time, typically 20 min per run. Moreover, with the addition of several blank runs to remove all potential carryover, the time consumed for a nanoLC-MS analysis can be as long as 80 min [87] which greatly limits the sample throughput. This problem was partially solved by the new ultra-performance LC

(UPLC) system which is used in all kinds of applications both for small and large molecules due to its much faster analysis time (5–10 min) and much higher chromatographic resolving power.

UPLC-MS was first introduced in 2004, but only in recent years has it been used for DNA adducts analysis. In 2011, Nauwelaers et al. [92] used μ UPLC- μ ESI-LC-MS/MS and offline SPE to study the formation of 4-ABP and several heterocyclic aromatic amines (HAAs) adducts in rat and human hepatocytes. The LOQ was 1 adduct per 10^7 nucleosides using only 10 μ g DNA. In 2012, Herrmann et al. [93] used isotope dilution UPLC-MS/MS to achieve a LOD of 2–6 adducts per 10^8 nucleosides using only 12.5 μ g DNA. In 2013, Nauwelaers et al. [94] in Turesky's group used UPLC/ESI in conjunction with MS³, as opposed to the traditional MS/MS method, and discovered two additional carcinogens 2-Amino-9H-pyrido[2,3-b]indole (A α C) and 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and confirmed their carcinogenic potency in human hepatocytes using 4-ABP and dG-C8-4-ABP as a comparison. This study suggested that A α C which is formed in mainstream tobacco smoke can cause DNA damage and may induce hepatocellular cancer in smokers. In 2016, Guo et al. [95] in Turesky's group used UPLC/ESI-MS³ in the study of multiclass carcinogenic DNA adduct (including dG-C8-4-ABP) quantification in formalin-fixed paraffin tissues. Their study showed that archived pathology samples can be analyzed to help assess the causal role of exposure to hazardous carcinogenic chemicals. Moreover, the employment of DNA adducts as biomarkers in human studies, especially in human tissues such as liver, lung, bladder, and pancreas using formalin-fixed paraffin tissues, which has become possible using their developed platform, is significant, considering that the fresh-frozen human tissues are typically not available [95]. In the same year, the Turesky group [96] also used this UPLC-ESI-MS³ platform and found that the 4-ABP DNA adducts formed in human T lymphocytes were below the limit of detection which was 3 adducts per 10^9 nucleotides, and for HAA 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), it was 9 adducts per 10^9 nucleotides. In contrast, another HAA 2-amino-9H-pyrido[2,3-b] indole (A α C) was found to induce a much higher level of DNA adducts (16 adducts per 10^9 nucleotides). Their results showed that human T lymphocytes is more efficient in bioactivating A α C to form DNA adducts than other HAA carcinogens and 4-ABP.

Despite the much faster separation time and high chromatographic resolution provided by UPLC, the chromatographic separation time is still not fast enough considering the potential high-throughput requirement for the analysis of DNA adducts in the future and the demand posed by some large-scale analyses in other application areas such as those associated with radiation damage which could potentially lead to hundreds of thousands of people getting infected or exposed. Some newer and faster analytical separation techniques may have to be employed for such applications. The technique of differential mobility spectrometry (DMS)-MS, which has been developed and gained attention in the past ten years or so, holds promise in this regard, as discussed next.

4.5. DMS-MS

In recent years, DMS-MS has emerged as a rapid gas phase separation technology which has been used for many applications such as drug metabolites [97] and radiation biomarkers [98]. As opposed to LC-MS which separates compounds in minutes and even longer, the separation time for DMS-MS is only milliseconds which is well suited for rapid and high-throughput detection and quantitation. Besides, DMS-MS is capable of separating isobaric/isomeric compounds, reducing background noise, and increasing compound selectivity. The Vouros group was the first to use this platform for analysis of DNA adducts. In 2013, Kafle et al. [99] developed a DMS-MS/MS platform for the analysis of dG-C8-4-ABP and achieved a detection limit of one adduct per 10^6 nucleotides using only 2 μ g DNA. The detection limit can be greatly improved by using a more sensitive MS instrument as opposed to a prototype DMS system coupled to a 12-year old SCIEX 3000 triple quadrupole. The most noticeable advantages of this platform are the background removal by DMS as shown in Fig. 7 and much faster analysis time, typically a 15- to 30-fold higher throughput than LC-MS [98].

4.6. Other methods

In addition to the methods discussed above, there are other new methods that could potentially be used for DNA adducts analysis, such as MS^n which gives much higher selectivity. One example is in a 2010 publication by Turesky's group where they used a technique called linear quadrupole ion trap/multistage tandem mass spectrometry (LC-ESI/MS/MSⁿ) [100] for the analysis of DNA adducts. Another method is high resolution MS method based on the use of Orbitrap mass analyzer which has very high mass accuracy (1–2 ppm), high resolving power (up to 200,000 amu), large dynamic range (around 5000 amu) and also comparable sensitivity to triple quadrupole. However, the application of orbitrap in DNA adduct analysis area has not been reported as much as other methods, which is possibly due to the relatively short time since its introduction (first introduced in 2010), high price and high maintenance costs [67]. However, based on these recent reports we believe that Orbitrap is the future direction of DNA analysis, especially if this high-resolution MS can be coupled with nanoLC and column switching. Another technology is the accelerator mass spectrometry (AMS) which has incredibly high sensitivity, one adduct per 10^{11} – 10^{12} nucleotides [101–103], which is the best among all other mass spectrometry techniques. However, its application for measurement of dG-C8-4-ABP has not been reported, since this technique was designed only for quantitation of radioactive isotopes and cannot provide structural information for DNA adducts. In certain respects, this is very similar to the disadvantage of ^{32}P -postlabelling method. Finally, laser-induced fluorescence (LIF) method can potentially provide incredibly yoctomolar detection limits but its sensitivity is limited by the specificity and efficiency of the derivatization reaction between adducts and the fluorophore [104].

4.7. Summary of dG-C8-4-ABP detection methods

The major analytical methods of analyzing DNA adduct dG-C8-4-ABP are summarized in Table 1. Clearly, nanoLC and UPLC with the column switching technique provide the

highest sensitivity and lowest sample requirement and will certainly be the most popular and promising platforms in the future.

5. The analysis of 4-ABP adducted oligonucleotides

So far, researchers have focused on adducted mononucleotide/ mononucleoside analysis, which does not provide any information about adducted sites on DNA sequence. The adducts may have site-selectivity or sequence-selectivity, which has been shown by several groups [105–111]. The mutagenic effects of a carcinogen can be potentially linked with the location of the adducts on the sequence [39]. Therefore, it is important to locate the adducted sites, which can only be accomplished using MS based technologies since the traditional polymerase chain reaction (PCR) sequencing technology provides no information for the adducts [112,113]. One example in mapping the adducted site is shown by Chowdhury etc. [111]. In this study, DNA alkylation and two adducts BPDE and 4-ABP were detected and mapped by using a UPLC-MS platform. Another example of determining the site-selectivity of 4-ABP was shown by Vaneet Sharma [106] that ABP has a very clear site selectivity for adduction on a 14-mer oligonucleotide ACCCG(1)CG(2)TCCG(3)CG(4)C. In this example, he used nanoLC-MS/ MS and achieved baseline separation of four ABP adducted sequence isomers. The site selectivity was determined by comparing the peak areas of four isomers and in the order: (G4) > (G2) > (G1) > (G3) [106]. Separation of the same sequence with different adducted sites is important and challenging due to the similar biochemical characteristics of the isomers. LC-MS has good performance on separating those adducted sequence isomers but DMS-MS can potentially have even greater efficiency for the application of separating the DNA sequence adducts due to its capability of separating isobaric/isomeric compounds.

6. Investigation of 4-ABP induced bladder cancer

Bladder cancer is one of the major cancers associated with 4-ABP, and it has been studied for many years in Vouros' group in a collaboration with Zhang's group [6,21,87,114]. Two major research directions have been undergoing in both laboratories: one is to understand the role of 4-ABP in the development of bladder cancer; another is to develop prevention strategies for bladder cancer. Recently, Zhang's group showed the chemopreventative effects of sulforaphane on dG-C8-4-ABP formation in mouse bladder cells [21]. In this study, they showed that a well-known chemopreventive phytochemical sulforaphane (SF) which exists in broccoli and other cruciferous vegetables can inhibit ABP-induced DNA damage by activating the NF-E2 related factor-2 (Nrf2)-regulated cytoprotective signaling pathway, and suggested that consumption of broccoli can potentially reduce bladder cancer risk and mortality. In the following year, in the same group and using the same method, Paonessa et al. [115] showed that while Nrf2 in bladder cells protects the cells against ABP in vitro, surprisingly, in vivo, Nrf2 in mouse liver promotes 4-ABP DNA adduct formation in the bladder of the mice by stimulating liver Phase 2 enzymes that metabolize 4-ABP and promote delivery of the carcinogenic metabolites to the bladder. This finding has significant implication for development of Nrf2-directed chemopreventive agents and was made possible using the highly sensitive and specific detection method for dG-C8-4-ABP. In 2015, the same group found the inverse relationship between bladder and liver in 4-ABP induced

DNA damage by showing that in mice, male bladder is more susceptible to ABP-induced carcinogenesis than female bladder and the situation is completely reversed in liver (as shown in Fig. 8). They suggested that androgen may play a key role in this gender disparity by down-regulating some metabolic enzymes which activate ABP in the liver [114]. Ongoing joint studies in the two laboratories suggest that sulfotransferase enzymes which catalyze the sulfation of 4-ABP may play key role in mediating the gender disparity described above.

7. Conclusion and future outlook

The development of analytical methodologies for detection and quantitation of DNA adducts in biological samples has opened new opportunities for investigation of the etiology of environmentally induced cancer as discussed in a recent review addressing the relationship of tobacco derived DNA adducts and lung cancer [116]. The preceding discussion, which traced the evolution of the different technologies for the analysis of dG-C8-4-ABP, has effectively demonstrated the key role played by mass spectrometry and specifically LC-MS in this effort both to-date and in the future. In assessing the value of an analytical approach, features such as sensitivity, reliability, selectivity, sample requirements and speed of analysis are major considerations. In LC-MS, mass resolution and scanning methods in addition to the chromatographic efficiency are critical in accomplishing these goals. All of these parameters have been remarkably integrated in addressing the challenges of the analysis of dG-C8-4-ABP and, for that matter, DNA adducts in general.

Early mass spectrometric studies of carcinogen modified deoxynucleosides in which the carcinogen was covalently attached to the nucleobase, identified a common fragmentation process involving the loss of the deoxyribose moiety (116 Da) upon CID. It was soon realized that the use of triple quadrupoles could capitalize on this phenomenon to screen for such adducts in biological samples using the constant neutral loss (CNL) scan mode [80,85,117]. The value of this fundamental ion fragmentation process has been incorporated into what is referred to as the new field of adductomics utilizing a broad range of additional scanning modes including high resolution accurate mass screening (monitoring for losses of 116 Da) with a variety of hybrid instruments, as discussed in a seminal review by Balbo, Turesky and Villalta [37]. The CNL scanning mode suffers from low sensitivity and provides only a preliminary indication regarding the presence of potential adducts in a sample. The data have to be further evaluated by targeting the transition: $[M + H]^+ \rightarrow [M + H - 116]^+$ of all “suspects” using a selected reaction monitoring targeted (SRM) approach for further verification. Additional CID of the $[M + H - 116]^+$ ion (MS^3) using a linear quadrupole ion trap has been shown to result in better sensitivity due to reduction of the chemical background noise as shown in the detection of DNA adducts in human hepatocytes exposed to 4-ABP [92]. The information content of the screening process itself has been greatly improved via the use of CNL- MS^3 and data dependent (DD) or data independent (DI) CNL- MS^3 utilizing quadrupole-time of flight (Q-TOF). More recently higher resolution Orbitrap instrumentation using a wide selected ion monitoring tandem MS screening method (WSIM/ MS^2) and looking at fragments differing by 116.0473 Da was shown to be superior in sensitivity, specificity, and breadth of adduct coverage with adduct detection levels as low as 4 per 10^9 nucleotides [118]. A related high resolution untargeted single ion monitoring

(SIM) approach was also used to screen for DNA adducts in human colon [119]. As in the classical CNL approach, following initial screening, targeted MS/MS or MS³ is utilized to more firmly establish the presence and/or identity of DNA adduct candidates.

Detection and quantitation of 4-ABP-induced DNA adducts in biological samples remain a challenge but recent technical advancements especially the development of super high-performance LC and more sensitive and accurate MS techniques have improved our capability in this area. However, more efficient sample preparation and clean-up methods, better separation methods, more sensitive MS instruments are still needed in order to further reduce the cost and time of analysis, to improve the detection limitation for super trace analysis, and to provide more confident identification of the DNA adducts not only for dG-C8-4-ABP but also for other DNA adducts. The breakthrough of findings in cancer research will always be inseparable from the development of the analysis platforms and methods. For example, in an interesting reversal of roles, an intriguing idea was introduced in a recent publication suggesting that monitoring the formation and level of DNA modifications induced by anticancer drugs may provide a strategy using DNA adducts from anticancer drugs as mechanism-based biomarkers of susceptibility to therapy [120]. To better enable the structural and biological evaluation of the presence and formation of DNA adducts, it has also been proposed that the biomarkers, in the specific case N5-R-FAPy, must be site specifically incorporated into synthetic DNA strands. Such approaches may help elucidate their effects on DNA replication and the all-important mechanisms of repair [121]. In this regard, the analysis of oligonucleotide adducts discussed earlier in this review may have a broader significance [105]. In conjunction with such new concepts, the demonstrated ability to detect adducts in archived pathology samples using formalin-fixed paraffin tissues, will open new opportunities to the study the role of chemical exposure in human cancer [95].

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Abbreviations

4-ABP	4-aminobiphenyl
dG-C8-4-ABP	<i>N</i> -deoxyguanosine-C8-4-aminobiphenyl
LC-MS	liquid chromatography-mass spectrometry
PAHs	polycyclic aromatic hydrocarbons
NOCs	<i>N</i> -nitroso compounds
AAs	aromatic amines
CYP1A2	cytochrome P450 1A2
N-OH-ABP	<i>N</i> -hydroxy-4-aminobiphenyl
NAT	<i>N</i> -acetyltransferase

N-OH-AABP	<i>N</i> -hydroxy-4-acetyl-aminobiphenyl
HPLC	high performance liquid chromatography
SPE	solid phase extraction
HILIC	hydrophilic interaction liquid chromatography
ESI	electrospray ionization
UPLC	ultra-performance liquid chromatography
TLC	thin layer chromatography
ITCs	isothiocyanates
GC	gas chromatography
B[a]P	benzo[<i>a</i>]pyrene
BPDE	benzo[<i>a</i>] pyrene-7,8-dihydrodiol-9,10-epoxide
PheQ	phenanthrene 1,2-quinone
BPQ	B[<i>a</i>]P-7,8-quinone
AAF	acetylaminofluorene
HAAs	aromatic amines
AaC	2-Amino-9H-pyrido[2,3- <i>b</i>] indole
MeIQx	2-Amino-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>] pyridine
DMS	differential mobility spectrometry
AMS	accelerator mass spectrometry
LIF	laser-induced fluorescence
PCR	polymerase chain reaction
Nrf2	NF-E2 related factor-2

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Fig. 1.

(a): Structure of 4-ABP; (b): Structure of dG-C8-4-ABP.

Fig. 2.
Metabolism of 4-ABP and the development of cancer.

Fig. 3.
Steps of analyzing DNA adducts.

Fig. 4.
The method development of analyzing DNA adducts.

Fig. 5.

An example of separating and detecting dG-C8-4-ABP using LC-MS. (A) The chromatogram of dG-C8-4-ABP and its isotopically labelled internal standard dG-C8-4-ABP- d_9 . (B) MS/MS spectrum of dG-C8-4-ABP. (C) MS/MS spectrum of dG-C8-4-ABP- d_9 .

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Fig. 6.
The column switching technology in the Agilent microfluidic chip.
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Fig. 7. Background removal of dG-C8-4-ABP with DMS. (A) DMS-transparent/off mode; (B) DMS-on mode, ethyl acetate was used as the gas modifier.
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Fig. 8.
dG-C8-ABP levels in ABP-treated mice.
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Table 1

Summary of major analytical methods of analyzing dG-C8-4-ABP.

Platform	Sample req. (µg DNA)	Sensitivity (adducts/ nucleotides)	Ref.	Other information
³² P-post labelling	3	1/1e8	[73]	
Immunoassay	25	2/1e8	[69]	
GC-MS	100	0.32/1e8	[122]	
microLC-MS	300	5/1e9	[86]	
UPLC-MS	10	1/1e7	[123]	MicroUPLC-MS
	5	2/1e8	[95]	With column switching
	5	1/10e9	[94]	With column switching
	3	3/10e9	[96]	
LC-MS column switching	100	0.7/1e7	[91]	
	5	5/1e9	[21]	nanoLC-MS
	100–200	1/1e9	[89]	