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Detection of benzylic adducts in DNA and nucleotides from 7-sulfooxymethyl-12-methylbenz[*a*]anthracene and related compounds by ³²P-postlabeling using new TLC systems

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Abstract

7,12-Dimethylbenz[*a*]anthracene (DMBA) is a highly potent experimental carcinogen, that must be transformed to its ultimate carcinogenic form *in vivo*. The *meso*-region theory of aromatic hydrocarbon carcinogenesis predicts that 7-hydroxymethyl sulfate (7-HMBA) ester plays a major role in the metabolic activation, benzylic DNA adduct formation and complete carcinogenicity of HMBA and DMBA. This study was undertaken to detect highly lipophilic benzylic DNA adducts resulting from the reaction between 7-hydroxymethyl sulfate ester of HMBA (7-SMBA) and DNA as well as determine their DNA base selectivity. Synthetic 7-SMBA was incubated with DNA (800 µg/ml) and individual deoxynucleoside 3'-monophosphates (600 µg/ml) and benzylic adducts were analyzed by ³²P-postlabeling/TLC following their enrichment with butanol extraction. Dilute ammonium hydroxide-based solvents were developed to detect the highly lipophilic aralkyl adducts. The reaction with DNA, dGp and dAp gave rise to multiple adducts; dCp and dTp showed no significant adducts. Chromatographic comparison revealed that the major DNA adduct was derived from dG. The methodology developed was also found applicable for highly lipophilic adducts resulting from sulfate esters of structurally-related metabolites of DMBA.

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Keywords: DMBA; Aralkylation; ³²P-Postlabeling; Benzylic DNA adduct formation

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1. Introduction

DNA adduct formation is postulated to be a major step by which polyaromatic hydrocarbons (PAHs) induce mutations and cancer in target organs [1]. Most of the PAHs need to be metabolically activated to electrophilic forms to exert their deleterious effect [2]. 7,12-Dimethylbenz[*a*]anthracene (DMBA), a potent mammary carcinogen, also requires metabolic activation before it elicits its biologic response [3].

The carcinogenic property of DMBA is unambiguously accepted, though the exact nature of the pathway to ultimate carcinogenic metabolite is still debated. Activation of DMBA to its ultimate carcinogen has been proposed to involve several different oxidative mechanisms. Four major mechanisms have been proposed for the activation of the DMBA: (i) the bay-region diol-epoxide pathway [4]; (ii) the one-electron oxidation pathway [5]; (iii) the M-region quinone pathway [6]; and (iv) the *meso*-region benzylic ester pathway [6,7]. The first pathway involves the epoxidation of the two bonds of the angular ring, catalyzed by the cytochrome P450, leading to metabolites, which include phenols, diols, diol-epoxides and tetrols [8]. The second pathway involves one-electron oxidation to produce a *meso*-region radical cation intermediate [5,9]. The third pathway involves dihydrodiol dehydrogenase(s) which oxidize M-region PAH trans-dihydrodiol, formed during the bay-region pathway, to the corresponding *o*-quinone [6]. The fourth pathway, formulated by Flesher and Syn-dor [7], proposes that a necessary first step in carcinogenesis for most alkyl substituted PAHs is biotransformation to a *meso*-anthracenic hydroxyalkyl metabolite. PAHs lacking alkyl substituent first undergo a bioalkylation substitution reaction in the *meso*-anthracenic positions(s) or L-region [10]. We have previously demonstrated the presence of aralkyl pathway in vivo for benzo[*a*]pyrene [11].

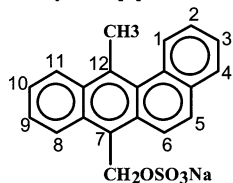
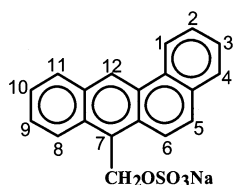
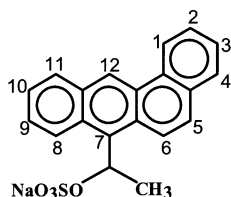
Following the unified hypothesis for the bioactivation of DMBA, hydroxylation of the 7-methyl group is the first step in carcinogenesis. The second step is the metabolic formation of a benzylic derivative bearing a good leaving group (i.e. sulfate, phosphate, acetate esters, and/or

secondarily generated halides) to generate a highly reactive carbonium ion intermediate. This carbocation would be expected to react with critical cellular nucleophiles.

The *meso*-region theory may not be the major pathway for tumorigenesis but may play a significant role in the complete carcinogenesis process [12]. The *meso*-region theory predicts the carcinogenicity of a PAH based on the presence or absence of a *meso*-carbon atom. Substitution of methyl groups in the *meso*-carbon increases the carcinogenicity of the compound. The parent compound of DMBA, benzantracene is a poor carcinogen, while the DMBA, where both the *meso*-carbons are substituted with methyl groups is a strong carcinogen. According to the theory, the first step of biochemical activation should be the hydroxylation of the methyl substitutions in the *meso*-region. Previous studies have established that DMBA is metabolized to 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA) in rat mammary gland [13] and also to induce sarcoma at the site of injection [14]. The structure activity relationships, according to the *meso*-region theory are described in detail by Flesher et al. [15].

Most of the ultimate carcinogens react with DNA forming adducts, which when left unrepaired or undergoing error-prone repair before the onset of DNA replication lead to permanent genetic changes in critical genes to the initiation and progression of cancer. Thus, the ability of the ultimate carcinogen to form DNA adducts is generally accepted as an essential step in the development of pronounced carcinogenic properties.

The sulfate ester of DMBA, 7-sulfooxymethyl-12-methylbenz[*a*]anthracene (7-SMBA) along with three other structurally related sulfate esters, namely, 7-sulfooxymethylbenz[*a*]anthracene (7-SBA), 7- α -sulfooxyethylbenz[*a*]anthracene (7- α -SEBA) and 1-sulfooxymethylpyrene (1-SMP) (Fig. 1) were synthesized. These electrophilic metabolites were tested (i) for their capacity to interact with DNA and individual nucleotides and (ii) to identify the DNA base(s) involved in the interaction. Benzylic DNA adducts were analyzed by ³²P-postlabeling as described [16], except that new chromatographic separation systems were

7-sulfooxymethyl-12-methylbenz[*a*]anthracene (7-SMBA)7-sulfooxymethylbenz[*a*]anthracene (7-SBA)7- α -sulfoxyethylbenz[*a*]anthracene (7 α -SEBA)

1-sulfooxymethylpyrene (1-SMP)

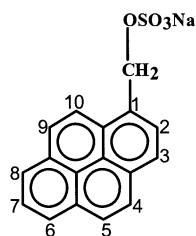


Fig. 1. Structures of sulfate esters of different polycyclic aromatic hydrocarbons tested for DNA adduction.

developed to accommodate the high lipophilicity of the expected aralkyl adducts.

2. Materials and methods

2.1. Chemicals

DMBA was purchased from Sigma Chemicals Co. (St. Louis, MO). Calf thymus DNA, obtained

from Sigma Chemical Co., was further purified by treatment with RNases A and T1, and proteinase K, followed by solvent extractions and precipitation with ethanol. Other chemicals and TLC solvents used for ^{32}P -postlabeling were as described elsewhere [16].

2.2. Synthesis of sulfoxy metabolites

7-HMBA was synthesized from 7,12-benzanthraquinone, via the intermediary formation of 7-iodomethyl-12-methylbenz[*a*]anthracene [17,18]. 7-Hydroxymethylbenz[*a*]anthracene (7-HBA) was synthesized from benz[*a*]anthracene, via the intermediary formation of the 7-formylbenz[*a*]anthracene [19,20]. 1-Hydroxymethylpyrene (1-HMP) was prepared from 1-pyrenecarboxaldehyde in a manner similar to the preparation of 7-HBA from 7-formylbenz[*a*]anthracene [21]. 7- α -Hydroxyethylbenz[*a*]anthracene (7-HEBA) was synthesized following published procedures [22,23] from benz[*a*]anthracene via the intermediary formation of 7-acetylbenz[*a*]anthracene.

The sulfate esters, 7-SMBA, 7-sulfooxymethylbenz[*a*]anthracene (7-SBA), 1-sulfooxymethylpyrene (1-SMP), and 7 α -SEBA were all synthesized from the respective benzylic hydroxymethyl derivatives, following the published procedure [24].

The synthesized sulfate esters were initially checked by TLC on a Whatman reverse-phase KC-18 plates in 9:1 methanol:water. This was followed by reverse-phase HPLC on a Microsorb C-18 column using isocratic elution with 100% methanol to reconfirm purity. The structures of the sulfate esters were confirmed using direct injection MS/MS analysis using a Micromass Quattro-II using negative ionization spray coupled to an HPLC.

2.3. Modification of DNA and deoxynucleoside 3'-monophosphates (dNp) *in vitro*

Calf thymus DNA (800 $\mu\text{g/ml}$) and dAp, dGp, dCp and dTp (600 $\mu\text{g/ml}$) were incubated with the sulfate esters of DMBA and other structurally related compounds (400 μM) at 37 $^\circ\text{C}$ for 4 h. The DNA was purified by precipitation with ethanol. The modified dNp were extracted with ethyl

acetate to remove the unreacted metabolites and the aqueous phase was dried under vacuum. The DNA and dNp were dissolved at a concentration of $\sim 1 \mu\text{g}/\mu\text{l}$ in H_2O .

2.4. Analysis of adducts by ^{32}P -postlabeling

DNA and nucleotide samples were analyzed by ^{32}P -postlabeling, after enrichment of adducts with butanol extraction [16]. Briefly, DNA (5 μg) was digested with a mixture of micrococcal nuclease and spleen phosphodiesterase (enzyme/substrate, 1:5, w/w, 5 h, 37 °C). Adducts were enriched by butanol extraction. Enriched adducts were labeled by T4 polynucleotide kinase (0.2 U/ μl)-catalyzed phosphorylation in the presence of [γ - ^{32}P]ATP ($< 2 \mu\text{M}$; $\sim 3000 \text{ Ci}/\text{mmol}$). Adducts derived from the reaction of dNp were analyzed without the enzymatic step. Labeled adducts were resolved by multidirectional PEI-cellulose TLC (D1 in D3 template) using the following solvent system: D1 = 1 M sodium phosphate, pH 5.8 onto 5 cm Whatman number 17 wick; D3 = 0.5 M ammonium hydroxide till top; D4 = *iso*propanol:4 M ammonium hydroxide (1.5:1) till top; D5 = 1 M sodium phosphate, pH 5.8 onto 5 cm Whatman number 1 wick. To evaluate adduct levels, aliquots of the DNA digest and the individual dNp (2 ng) were also ^{32}P -labeled in parallel with adducts. Labeled normal nucleotides were resolved by 1-directional PEI-cellulose TLC in 0.5 M acetic acid/2 M formic acid, following conversion to 5'-monophosphates by treatment with nuclease P1 [25]. Adducts were visualized and quantified by Packard Instant Imager (Packard Instrument Co. Inc., Downers Grove, IL). The relative adduct labeling was calculated as: $\text{RAL} = [\text{cpm of adducts}/\text{cpm of total nucleotides}] \times 1/\text{dilution factor}$. The adduct levels are expressed as modified nucleotides/ 10^3 nucleotides.

2.5. Co-chromatography of adducts derived from DNA and modified dNp

DNA adducts were identified by co-chromatography with adducts derived from individual dNp for base selectivity. Aliquots of the labeled DNA digest and nucleotides were mixed such that

adduct radioactivity in the DNA and the dNp samples were comparable. Adducts were resolved by multi-directional PEI-cellulose TLC using four different solvent systems (Table 1 below).

3. Results and discussion

The aralkyl adducts are so highly lipophilic that the conventional high-salt, high-urea solvents used in PEI-cellulose TLC [26] were found incapable of eluting adducts from the origin effectively (not shown). Hence a new solvent system had to be developed. By using dilute ammonium hydroxide-based solvents both in D3 and D4, the PEI-cellulose TLC behaved more like a partition chromatography instead of an affinity chromatography. This helped resolve these adducts better than the conventional affinity [26] or semi-partition [16] system used for diol-epoxide type adducts. In the partition system, the diol-epoxide adducts were faster and clustered at the top of the chromatogram. The aralkyl adducts, being more lipophilic than the diolepoxy-related adducts, were slower and were well resolved (not shown).

The 7-SMBA formed one major (#6) and nine minor adducts when reacted with DNA (Fig. 2A). Adduct patterns were less complex when the reactions were carried out with individual nucleotides, with dAp and dGp, forming three and two adducts, respectively (Fig. 2B and C); dCp and dTp showed no appreciable adduct formation (not shown). Co-chromatography of adducts derived from DNA and individual nucleotides revealed that the major and one minor adduct (#1) in the DNA were derived from dG (Fig. 2E), while one minor adduct (#8) was derived from dA (Fig. 2D). These assignments were confirmed in three additional chromatography solvents (Fig. 2, Systems 2–4). Notably, the System 1 resulted in best separation, resolving all ten adducts. The major adduct accounted for nearly 45% of the total adducts. There were several minor adducts that were unaccounted for by co-chromatography, which could either represent undigested dinucleotides and/or different isomeric forms of adducts.

7-SMBA, being highly electrophilic, reacted readily with DNA bases. Even though 7-SMBA

Table 1
Solvents used in the multi-directional PEI-cellulose TLC for the four different systems used

	D1	D3	D4
System 1	1 M Sodium phosphate, pH 6.0	0.7 M Ammonium hydroxide	1.5:1 <i>Iso</i> propanol:4 M ammonium hydroxide
System 2	1 M Sodium phosphate, pH 6.0	0.7 M Ammonium hydroxide	0.6 M Sodium bicarbonate, 7 M urea, pH 10
System 3	1 M Sodium phosphate, pH 6.0	0.7 M Ammonium hydroxide	4 M Lithium formate, 7 M urea, pH 3.5
System 4	1 M Sodium phosphate, pH 6.0	0.6 M Sodium bicarbonate, 7 M urea, pH 10	1.5:1 <i>Iso</i> propanol:4 M ammonium hydroxide

is a metabolite of 7-HMBA formed in vivo, its binding with DNA bases has not been reported for lack of a suitable methodology. Using this methodology, our preliminary experiments have revealed the presence of benzylic adducts in rats treated with DMBA and 7-HMBA (to be published).

The new separation system was also found applicable for resolving DNA adducts formed in vitro with sulfoxyl derivatives of structurally similar compounds, i.e. 7-SBA, 7- α SEBA and 1-SMP. Each compound resulted in the formation of

one major and 7–9 minor adducts (Fig. 3). However, reaction of these metabolites with individual nucleotides, dAp and dGp showed only up to three adducts each (Fig. 3); dCp and dTp showed no appreciable adduct formation (not shown). Co-chromatographic revealed that the major adduct formed in each case was derived from dG and one or two minor adducts arose by interaction with dA (Fig. 3). These conclusions are consistent with the DNA base selectivity observed with 7-SMBA. The major adduct formed from 7-SBA and 1-SMP each accounted for 70% of the

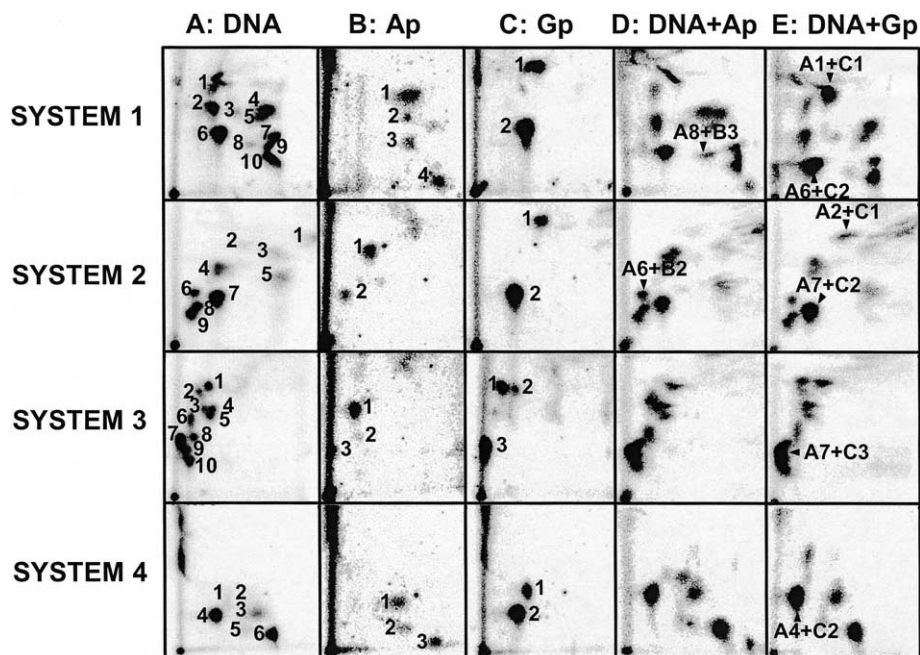


Fig. 2. Representative maps of ^{32}P -adducts formed by in vitro interaction of 7-SMBA with DNA and indicated deoxynucleoside 3'-monophosphates (Columns A–C). Co-chromatography analyses of adducts derived from DNA and individual nucleotides are shown in Column D and E. The four solvent systems in which the DNA adducts were separated are described in Table 1.

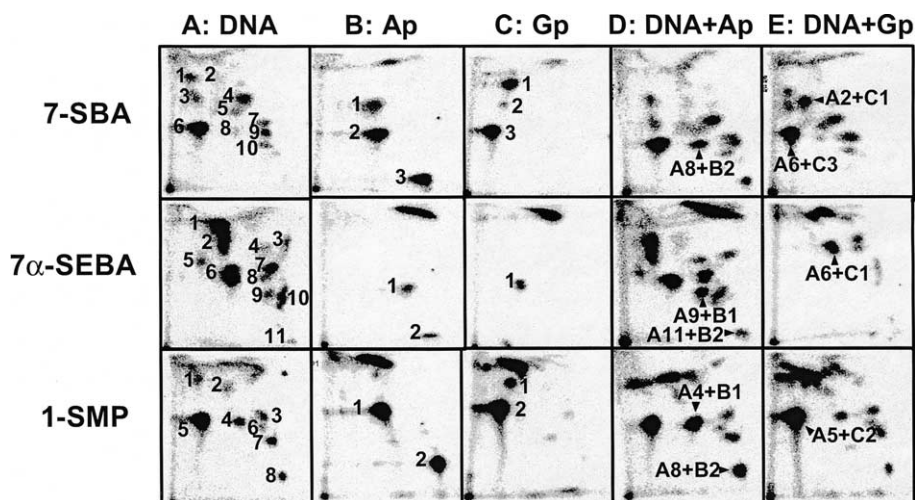


Fig. 3. Representative maps of ^{32}P -adducts formed by in vitro interaction of 7-SBA, 7α -SEBA and 1-SMP with DNA and indicated deoxynucleoside 3'-monophosphates (Columns A–C). Co-chromatography analyses of adducts derived from DNA and individual nucleotides are shown in Column D and E. Adducts were separated using the solvent system 1 (Table 1).

total DNA adducts, while it accounted for 40% of the total adduction from 7α -SEBA.

The levels of adducts from 7-SMBA, 7-SBA and 7α -SEBA were higher with DNA than with individual nucleotides, except for 1-SMP which showed a higher preference for nucleotides (Fig. 4). The binding preference of 1-SMP with nucleotides is consistent with similar preferential binding for 6-sulfoxymethylbenzo[*a*]pyrene (K. Stansbury and R. Gupta, unpublished data)—a

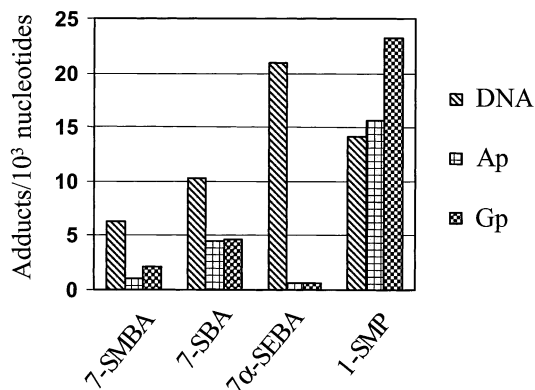


Fig. 4. Levels of adducts formed in vitro by interaction of 7-SMBA, 7-SBA, 7α -SEBA and 1-SMP with DNA and dNp. The values are mean of five replicates and the levels are expressed as adducts/10³ nucleotides.

compound which also has a fused-benzene ring structure.

The new chromatography solvents have identified specific DNA adducts formed by 7-SMBA and structurally similar compounds with DNA. The high adduction of the sulfoxymethyl metabolites shows their high electrophilicity. The new chromatography system has provided a better resolution of adducts even at very low levels overcoming the difficulty of detecting adducts without the interference of the high levels of more polar diepoxide-related adducts. Experiments are underway to determine the relative preponderance and tissue distribution of benzylic and diol-epoxide DNA adducts in vivo.

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