Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

Research Article

Cancer Prevention Research

Organ Specificity of the Bladder Carcinogen 4-Aminobiphenyl in Inducing DNA Damage and Mutation in Mice

Jae-In Yoon, Sang-In Kim, Stella Tommasi, and Ahmad Besaratinia

Abstract

Aromatic amines are a widespread class of environmental contaminants present in various occupational settings and tobacco smoke. Exposure to aromatic amines is a major risk factor for bladder cancer development. The etiologic involvement of aromatic amines in the genesis of bladder cancer is attributable to their ability to form DNA adducts, which upon eluding repair and causing mispairing during replication, may initiate mutagenesis. We have investigated the induction of DNA adducts in relation to mutagenesis in bladder and various nontarget organs of transgenic Big Blue mice treated weekly (i.p.) with a representative aromatic amine compound, 4-aminobiphenyl (4-ABP), for six weeks, followed by a six-week recovery period. We show an organ-specificity of 4-ABP in inducing repair-resistant DNA adducts in bladder, kidney, and liver of carcinogen-treated animals, which accords with the bioactivation pathway of this chemical in the respective organs. In confirmation, we show a predominant and sustained mutagenic effect of 4-ABP in bladder, and much weaker but significant mutagenicity of 4-ABP in the kidney and liver of carcinogen-treated mice, as reflected by the elevation of background *cII* mutant frequency in the respective organs. The spectrum of mutations produced in bladder of 4-ABP–treated mice matches the known mutagenic properties of 4-ABP–DNA adducts, as verified by the preponderance of induced mutations occurring at G:C base pairs (82.9%), with the vast majority being G:C→T:A transversions (47.1%). Our data support a possible etiologic role of 4-ABP in bladder carcinogenesis and provide a mechanistic view on how DNA adduct–driven mutagenesis, specifically targeted to bladder urothelium, may account for organ-specific tumorigenicity of this chemical. *Cancer Prev Res; 5(2); 299–308. ©2011 AACR.*

Introduction

Aromatic amines and bladder cancer have been a primary focus of research since the early years of chemical carcinogenesis (1). At around the turn of the 19th century, cases of urinary bladder cancer were noted in German dye workers who were occupationally exposed to aniline and its derivatives (2, 3). Subsequent epidemiologic studies confirmed an association between exposure to chemicals of the family of aromatic amines, including 2-naphthylamine, benzidine, and 4-aminobiphenyl (4-ABP), and the incidence of urinary bladder cancer (4). These observational findings were substantiated by animal experiments in which exposure of dogs, rabbits, hamster, rats, and mice to prototype aromatic amines caused urinary bladder tumor formation (5–7). Because of the reports of epidemiologic and experimental links between exposure to aromatic amines and development of urinary bladder cancer, national and international regulatory efforts have been made to minimize occupational exposure to aromatic amines (8). Today, however, exposure to aromatic amines still occurs in a wide range of industries, including rubber, cable, and textile manufacturing, aluminum transformation, and gas, coal, pesticide, and cosmetics productions (9). In addition, nonoccupational sources of exposure to aromatic amines also exist, with tobacco smoke being the most prominent source. Both mainstream and sidestream tobacco smoke contain nanogram quantities of carcinogenic aromatic amines, such as ortho-toluidine, 2-naphthylamine, and 4-ABP, for example, mainstream smoke from a nonfiltered cigarette contains 160, 1.7, and 4.6 nanograms of the respective chemicals (10, 11). The elevated risk of bladder cancer in smokers is ascribed to their exposure to aromatic amines, with smokers of black (air cured) tobacco being at higher risk than smokers of blond (flue cured) tobacco (12–14). The latter finding accords with the richer content of aromatic amines in black tobacco products than blond tobacco products (10).

As a representative compound of the class of aromatic amines, 4-ABP has been extensively studied to elucidate the

Authors' Affiliation: Department of Cancer Biology, Beckman Research Institute of the City of Hope, Duarte, California

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

Corresponding Author: Ahmad Besaratinia, Beckman Research Institute of the City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010. Phone: 626-359-8111, ext. 65918; Fax: 626-358-7703; E-mail: ania@coh.org

doi: 10.1158/1940-6207.CAPR-11-0309

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

underlying mechanism of bladder carcinogenesis (1). Thus far, a genotoxic mode of action based on the ability of 4-ABP to induce DNA damage and mutation has been established (15, 16). *In vivo*, 4-ABP requires metabolic activation to exert its genotoxic effects (1). The biotransformation of 4-ABP consists of N-oxidation catalyzed by the cytochrome P450 enzymes, primarily CYP1A2 (17), or N-methylation or peroxidation, although to much lesser extent (18, 19). The resulting hydroxyarylamine may undergo detoxification through N-acetylation, or stay intact or in conjugation with acetate, sulfate, or glucuronate (20–22). The acetate and sulfate O-conjugates can readily interact with DNA or proteins, whereas the glucuronate O-conjugate can circulate in the body and reach the urinary tract, wherein it undergoes hydrolysis at the acidic pH of urine (21, 22). The resultant electrophilic nitrenium cation can bind directly the DNA of the urothelial cells and form covalent adducts, predominantly at the C8 position of guanine, N-(deoxyguanosine-8-yl)-4-ABP (4-ABP–DNA adduct; refs. 16, 23, 24). It is widely believed that persistent (repair resistant) 4-ABP–DNA adducts and similar adducts from the family of carcinogenic aromatic amines are etiologically involved in the genesis of human bladder cancer (1, 15, 16). To date, however, no experimental study has investigated the formation and kinetics of repair of 4-ABP–DNA adducts in relation to mutagenesis in target and nontarget organs of 4-ABP–induced carcinogenesis *in vivo*.

In this study, we have comprehensively investigated the DNA adduction and mutagenic properties of 4-ABP *in vivo* in transgenic Big Blue mice, an extensively validated model for the analysis of experimentally induced DNA damage and mutation (25). The genome of these transgenic animals contains multiple copies of a chromosomally integrated λLIZ shuttle vector, which carries 2 mutational reporter genes, including the *cII* and *lacI* that can be used for simultaneous analysis of DNA damage and mutation in any organ of interest (26). Here, we have investigated the induction of DNA adducts in relation to mutagenesis in bladder and various nontarget organs of transgenic Big Blue mice chronically exposed to 4-ABP. More specifically, we have determined the formation and kinetics of repair of 4-ABP–DNA adducts in relation to *cII* mutagenesis in target and nontarget organs of mice treated weekly with i.p. injections of 4-ABP for 6 weeks, followed by a 6-week recovery period. We have used an Immunodot blot assay with a specific antibody raised against the 4-ABP–DNA adduct (27) to evaluate DNA damage and repair in bladder and various nontarget organs of 4-ABP–treated mice. In addition, we have used the *cII* mutation detection assay (28) to assess the organ specificity of *cII* mutations, and DNA sequencing analysis to establish the type and frequency distribution of induced mutations in 4-ABP–treated animals.

Materials and Methods

Animals

Twenty male Big Blue mice (6–8 weeks old) on a C57BL/6 genetic background (Stratagene) were randomly divided into 2 groups of (1) experimental (4-ABP exposure; $n = 10$) and (2) control (sham-exposure; $n = 10$), each subdividing into 2 categories ($n = 5$), including (I) 6 weeks exposure, and (II) 6 weeks exposure + 6 weeks recovery. The mice assigned to each experimental or control group ($n = 5$) were kept in polypropylene cages in groups of 2 to 3 animals per cage, and housed in an air-conditioned animal room with ambient temperature of 21°C ± 1°C and relative humidity of 55% with 12-hours light/dark cycle. The mice had access to food (PicoLab Rodent Diet 20, PMI Nutrition International, LLC.) and water *ad libitum* throughout the study period. All experiments were conducted in the City of Hope Animal Resources Center and approved by the Institutional Animal Care and Use Committee in accordance with the recommendations of the NIH provided in the Guide for the Care and Use of Laboratory Animals.

The experimental mice received i.p. injections of 4-ABP once per week for a duration of 6 weeks using the following regimen: 1st week: 25 mg/kg body weight (bw); 2nd week: 50 mg/kg bw; 3rd week: 75 mg/kg bw; and 4th to 6th weeks: 100 mg/kg bw of 4-ABP. The specified doses of 4-ABP were prepared fresh on the day of administration by dissolving the chemical in dimethylsulfoxide (DMSO; 4-ABP and DMSO: Sigma-Aldrich Inc.). The incremental doses of 4-ABP were delivered to the mice by i.p. injection (100 μL) on the lower right or left quadrant in alternate weeks. Control mice received similar injections of solvent DMSO using the same dosing schedule as described for 4-ABP. All mice were monitored closely for development of any unusual symptoms during both the 4-ABP/sham-exposure and recovery periods. At the end of all experiments, the 4-ABP–treated and control mice were euthanized by CO2 asphyxiation, and bladder and various nontarget organs, including the lung, stomach, kidney, and liver were harvested and preserved at −80°C until further analysis.

Genomic DNA isolation

Genomic DNA from various organs of 4-ABP–treated and control mice was isolated using a standard phenol and chloroform extraction and ethanol precipitation protocol (29). The DNA was dissolved in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5), and kept at −80°C until further analysis.

Immunodot blot assay

To determine the formation and repair of 4-ABP–DNA adducts, genomic DNA of carcinogen-treated mice versus control mice was subjected to Immunodot blot assay using the mouse monoclonal 4C11 antibody (kindly provided by Dr. Regina Santella of Columbia University). The 4C11 antibody is highly specific for the 4-ABP–DNA adduct and, at the highest concentration tested, does not recognize the DNA adducts of several other aromatic amines, including 1-aminopyrene, 8-nitro-1-aminopyrene, and 6-nitro-1-aminopyrene (27). The Immunodot blot assay was conducted as described earlier with some modifications (30). Briefly, heat-denatured DNA (0.5 μg) was dot blotted onto a nitrocellulose membrane using the Convertible Filtration

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

Manifold System (Life Technologies). The membrane was laid over an absorbent paper presoaked with 0.4 N NaOH for 20 minutes at room temperature. Subsequently, the membrane was blocked by incubating in PBS plus 0.2% Tween 20 (PBS-T) containing 5% non-fat milk at 4°C overnight. After multiple washes with PBS-T, the membrane was incubated with the 4C11 antibody for 2 hours at room temperature (dilution: 1:150 in PBS-T plus NFM). The membrane was washed thoroughly with PBS-T and further incubated with an anti-mouse horseradish peroxidase–conjugated immunoglobulin (Promega) for 1 hour at room temperature (dilution: 1:10,000 in PBS-T plus NFM). To reveal peroxidase activity, the membrane was stained with the Enhanced Chemiluminescence Detection System (Amersham BioSciences UK Limited) according to the manufacturer's instructions. The stained membrane was exposed to X-ray film, and the relative intensity of luminescence was determined using the Bio-Rad Imaging Equipment applying Quantity One image analyzer (Bio-Rad Laboratories). Results are expressed as "Relative luminescence intensity," which is representative of the level of 4-ABP–DNA adducts.

cII Mutant frequency and mutation spectrum analyses

Genomic DNA of transgenic Big Blue mice contains multiple copies of the coliphage λLIZ shuttle vector, which is integrated into the murine chromosome 4 in a head to tail configuration, while harboring 2 mutational target genes, the *cII* and *lacI* (26). The *cII* mutagenesis assay is based on the recovery of the λLIZ shuttle vector from the genomic DNA of transgenic animals followed by phenotypic expression of the *cII* mutants using a commercially available bacterial expression system (Stratagene; ref. 28). The λLIZ shuttle vector is rescued enzymatically from the mouse genomic DNA and packaged into viable phage particles, which are then introduced into an indicator host *Escherichia coli* (*E.coli*). The infective λLIZ-bearing phages can multiply either lytically or lysogenically in the host *E.coli* depending on the status of cII transcription (31). The cII protein is essential for the activation of cI repressor and lambda integrase, both of which being necessary for lysogenization (28, 31). The *E. coli* indicators that carry phages with a mutated *cII* undergo lysis, thereby forming visible plaques on a special agar lawn (28). The λLIZ shuttle vector, however, harbors a *cI857* temperature sensitive (ts) mutation that makes the cI(ts) protein labile at temperatures exceeding 32°C (28). Thus, all vector-bearing phages, irrespective of the status of *cII* mutation, multiply lytically in the host *E. coli* at incubating temperatures more than 32°C (26, 28). This temperature sensitivity is the basis for the *cII* selection system in which phenotypic expression of the *cII* mutants is achieved under selective incubation condition, that is, 24°C (28). Under nonselective incubation condition, that is, 37°C, however, both wild-type and mutant *cII* are expressed (28). The ratio of plaques formed under the selective condition to those arisen under the nonselective condition is conventionally referred to as the "*cII* mutant frequency," which is representative of the frequency of induced/spontaneous mutations in the *cII* transgene (25). The phenotypically expressed *cII* mutants can be further analyzed by DNA sequencing to establish the type and distribution of mutations (mutation spectrum) in the *cII* transgene (25).

For mutant frequency determination, the λLIZ shuttle vectors containing the *cII* transgene were recovered from the genomic DNA of 4-ABP–treated and control mice and packaged into viable phage particles using the Transpack Packaging Extract Kit (Stratagene). After preadsorption of the phages to G1250 *E. coli*, the bacterial culture was grown on TB1 agar plates. To select for *cII* mutants, the plates were incubated at 24°C for 48 hours. Alternatively, the plates were incubated under nonselective condition, that is, 37°C overnight, to express both the wild-type and mutant *cII*. Verification of all putative *cII* mutants was achieved by replating under the selective condition. To determine a statistically valid mutation frequency, minimums of 3×10^5 rescued phages were screened in each experimental or control group (25). For mutation spectrometry, all verified mutant plaques were amplified by PCR using the "λ Select-*cII* sequencing primers" according to the manufacturer's recommended protocol (Stratagene). The purified PCR products were then subjected to direct DNA sequencing using the Big Dye terminator cycle sequencing Kit and ABI-3730 DNA Sequencer (ABI Prism; PE Applied BioSystems).

Statistical analysis

Given the small sizes of experimental/control groups and the intergroup variation of data, all results are expressed as medians ± 95% CIs, which give a better estimation of data distribution. Comparison of all variables between 2 separate groups was done using the Wilcoxon rank-sum test. The induced mutation spectrum produced by 4-ABP treatment was compared with the spontaneous spectrum of mutation in control (solvent treatment) by the hypergeometric test of Adams and Skopek (32). The frequencies of specific types of mutation (e.g., transitions, transversions, etc.) between 2 different groups were compared by the χ^2 test. All statistical tests were 2-sided. $P \leq 0.05$ was considered statistically significant. The S-Plus 7.0 for Windows software (Insightful Corp.) was used for all statistical analyses.

Results

Mice survival

All mice from both experimental and control groups tolerated the 4-ABP/sham-exposure regimens well, without exhibiting any sign of stress or discomfort. The survival rate of mice in the experimental and control groups was 100% at the end of both 4-ABP/sham-exposure period and the ensuing recovery time. All mice from both experimental and control groups gained steadily body weight throughout the exposure and recovery periods (data not shown).

4-ABP–DNA adduct analysis

We used an Immunodot blot assay to assess the formation and repair of 4-ABP–DNA adducts in bladder, lung, stomach, kidney, and liver of mice chronically treated with

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

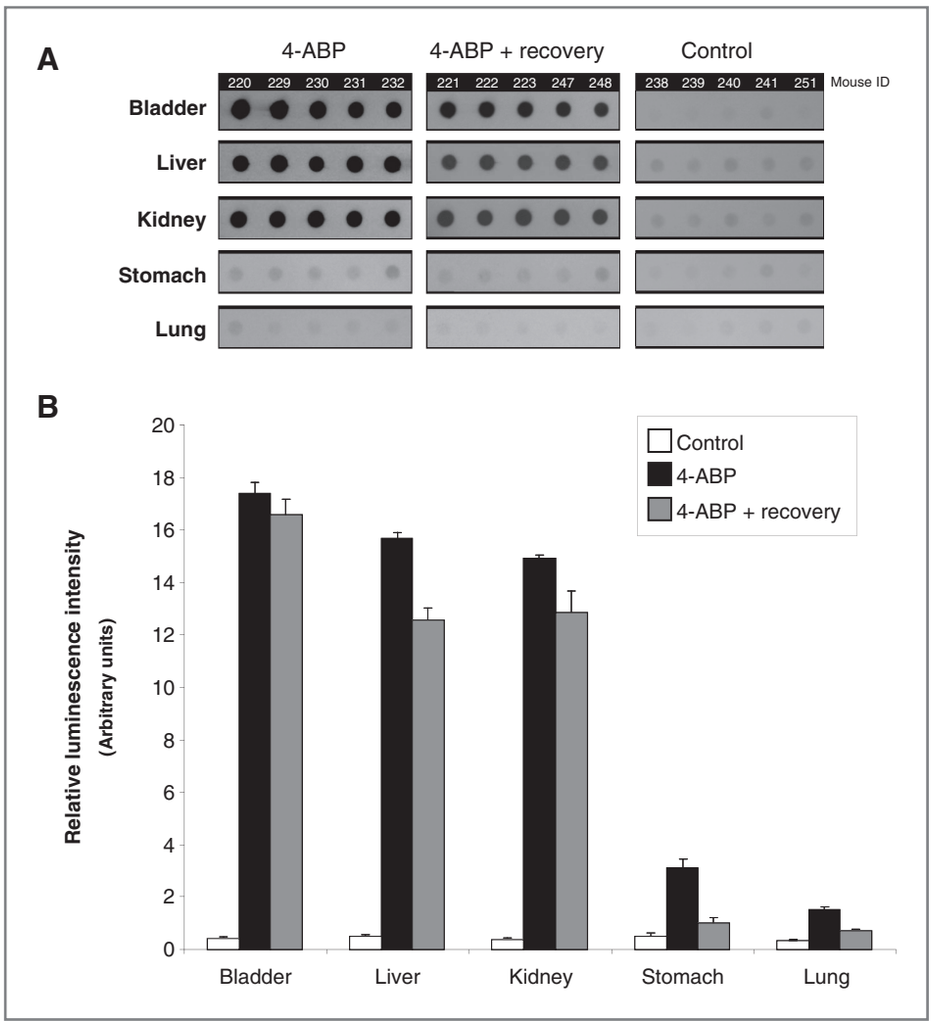

Figure 1. DNA adduction in various organs of 4-ABP–treated mice and controls. The formation and repair of 4-ABP–DNA adducts in bladder, lung, stomach, kidney, and liver of mice chronically treated with 4-ABP (6 weekly doses), before and after a 6-week recovery period, were determined using an Immunodot blot assay with the mouse monoclonal 4C11 antibody, as described in "Materials and Methods." A and B, the qualitative and quantitative results, respectively, are shown. Numbers in (A) indicate mice IDs. Results in (B) are expressed as medians (bars); error bars, 95% CIs. Note: the background immunostaining in each organ in control groups, including (I) 6 weeks sham-exposure and (II) 6 weeks sham-exposure plus 6 weeks recovery, were virtually identical (data not shown). For brevity, representative Immunodot blot assay results from the control group (II) are shown.

4-ABP (6 weekly doses), before and after a 6-week recovery period. Representative Immunodot blot assay results for the specified organs in chronically treated mice pre- and post-recovery periods are shown in Fig. 1A. Qualitatively, an intense formation of 4-ABP–DNA adducts was readily detectable in bladder, kidney, and liver of carcinogen-treated mice immediately after treatment and 6 weeks afterward; however, no appreciable formation of DNA adducts was discernable in the respective organs in control animals (Fig. 1A). Quantitatively, the highest levels of 4-ABP–DNA adducts were detectable in bladder, liver, kidney, in the order of decrease, in chemically treated mice both after the carcinogen-treatment and 6 weeks afterward (see, Fig. 1B). The background levels of 4-ABP–DNA adducts were slightly increased in the stomach and to a much lesser extent in the lung of carcinogen-treated mice immediately after carcinogen treatment.

cII Mutant frequency quantification

We determined the mutant frequencies of the *cII* transgene in bladder, lung, stomach, kidney, and liver of mice from both experimental and control groups after 6 weeks of 4-ABP/sham-exposure, and an ensuing 6-week recovery period. Because the background frequency of *cII* mutants in each organ in control groups, including (I) 6 weeks sham-exposure and (II) 6 weeks sham-exposure plus 6 weeks recovery, did not differ significantly from one another (data not shown), we used the data only from the control group (II) for all comparison purposes. As shown in Fig. 2 and Supplementary Table S1, 4-ABP was organ specifically mutagenic in chronically treated mice, with bladder being the target organ for the most pronounced mutagenic effect. The predominant mutagenicity of 4-ABP to bladder in chronically treated mice was shown by a significant increase in background *cII* mutant frequency from $2.06 \times 10^{-5} \pm 0.20 \times 10^{-5}$ in bladder of control mice to $18.86 \times 10^{-5} \pm 4.77 \times 10^{-5}$ in bladder of 4-ABP–treated mice ($P = 0.0079$). After 6 weeks of recovery, the frequency of *cII* mutants in bladder of 4-ABP–treated mice still remained significantly elevated relative to controls (17.20 ± 4.88; $P = 0.0079$). The persistent mutagenicity of 4-ABP to bladder of chronically treated mice was further confirmed by the observation that

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

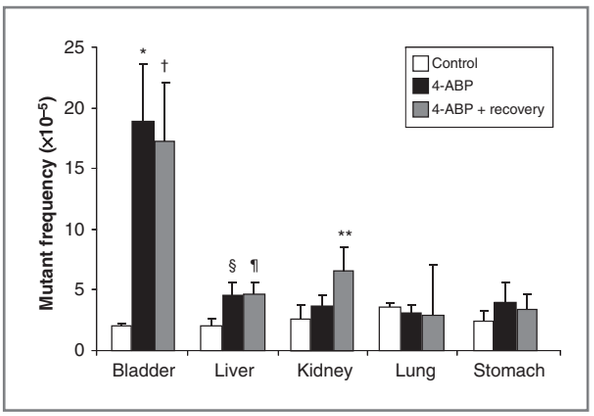

Figure 2. *cII* Mutant frequency in various organs of 4-ABP–treated mice and controls. The mutant frequencies of *cII* transgene in bladder, lung, stomach, kidney, and liver of mice chronically treated with 4-ABP (6 weekly doses), before and after a 6-week recovery period, were determined using the *cII* mutagenesis assay, as described in "Materials and Methods." *, statistically significant as compared with control; $P = 0.0079$. †, statistically significant as compared with control; $P < 0.008$. §, statistically significant as compared with control; $P = 0.0317$. ¶, statistically significant as compared with control; $P < 0.008$. **, statistically significant as compared with control; $P = 0.0465$. Results are expressed as medians (bars). Error bars, 95% CIs. Note: the background frequency of *cII* mutants in each organ in control groups, including (I) 6 weeks sham-exposure and (II) 6 weeks sham-exposure plus 6 weeks recovery, were not significantly different from one another (data not shown). Thus, we used the data only from the control group (II) for all comparison purposes.

there was no significant difference between the induced *cII* mutant frequency in bladder of 4-ABP–treated mice before and after 6 weeks recovery period ($P = 0.6905$; Fig. 2).

Furthermore, there was a slight but statistically significant mutagenic response in liver of 4-ABP–treated mice, as reflected by the elevation of background *cII* mutant frequency from $2.04 \times 10^{-5} \pm 0.56 \times 10^{-5}$ to $4.54 \times 10^{-5} \pm 1.08 \times 10^{-5}$ in this organ in carcinogen-treated animals ($P = 0.0317$). After 6 weeks of recovery, this mutagenic response was still significant, as indicated by an elevated *cII* mutant frequency of $4.62 \times 10^{-5} \pm 0.98 \times 10^{-5}$ ($P < 0.008$). In addition, there was a "delayed" mutagenic response in kidney in 4-ABP–treated mice, which manifested as a significant increase in background *cII* mutant frequency from $2.59 \times 10^{-5} \pm 1.22 \times 10^{-5}$ to $6.57 \times 10^{-5} \pm 1.94 \times 10^{-5}$ ($P = 0.0465$) in the kidney of 4-ABP–treated mice after 6 weeks of recovery. No mutagenic response was found in the lungs or stomach of 4-ABP–treated mice immediately after treatment or 6 weeks afterward (Fig. 2).

cII Mutation spectra

We established the spectra of mutations in the *cII* transgene in carcinogen-treated mice and controls by DNA sequencing of mutants from bladder genomic DNA in 4-ABP–treated mice and controls. We randomly selected 30 mutant plaques derived from the bladder DNA of each mouse from both experimental and control groups (5 mice each), and carried out DNA sequencing analysis. Detailed information on the type and frequency of mutations in the *cII* transgene in bladder DNA of 4-ABP–treated mice and controls is shown in Supplementary Tables S2 and S3. Distribution of these mutations along the nucleotide positions of the *cII* transgene is also outlined in Fig. 3 and Supplementary Fig. S1. In all cases, calculations were made both with and without the sibling mutations, which are defined as the identical mutations that occur repeatedly at the same nucleotide positions in the same sample from an individual animal, and may or may not be independent events from one another (33). As shown in

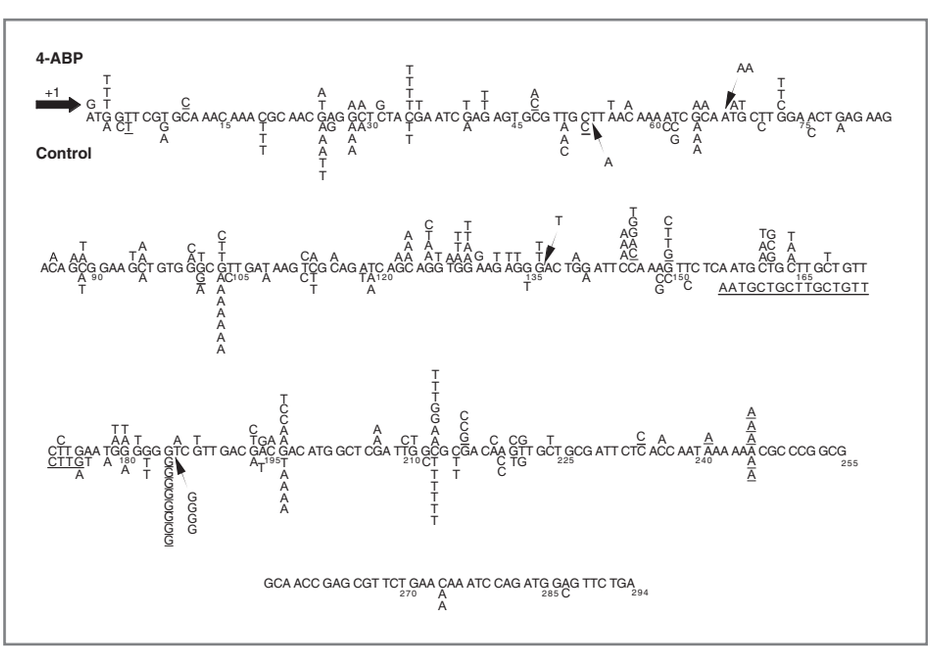

Figure 3. Detailed mutation spectra of *cII* transgene in bladder of 4-ABP–treated mice and controls. The spectra of mutations in the *cII* transgene in 4-ABP–treated mice and controls were established by DNA sequencing of mutants from bladder DNA of carcinogen-treated mice and controls, as described in "Materials and Methods." The 4-ABP–induced mutations are typed above the reference *cII* sequence, whereas the control mutations are typed below the reference *cII* sequence. Deleted bases are underlined. Inserted bases are shown with an arrow. Numbers below the bases are the nucleotide positions.

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

Supplementary Table S3, neither the spectrum of mutations produced by 4-ABP nor that of control changed significantly after the exclusion of sibling mutations. Thus, sibling mutations did not differently impact the 4-ABP–induced and control mutation spectra. Comparison of the overall spectrum of 4-ABP–induced mutation and control mutation spectrum revealed that the 2 mutation spectra were significantly different from one another both before and after the exclusion of sibling mutations ($P < 1e^{-9}$; by Adams and Skopek test). For brevity, all statistical comparisons are made after the exclusion of sibling mutations from hereon; although both mutation databases (sibling mutations included and excluded) are presented throughout.

As shown in Supplementary Table S2, single base substitutions comprised the vast majority of *cII* mutations found in bladder DNA of both 4-ABP–treated mice and controls (88.5% vs. 78.4%; $P < 0.06$). Of these, mutations occurring at G:C base pairs predominated the 4-ABP–induced mutation spectrum (80.5% vs. 56.2% in control; $P < 0.00008$), with G:C→T:A transversions being the most frequent type of mutations (44.0% vs. 11.4% in control; $P < 1e^{-7}$; Supplementary Table S3). The 4-ABP–induced mutations occurring at G:C base pairs or the specific G:C→T:A transversions were not, however, biased toward 5′-CpG–containing sequences (discussed below). Conversely, the control mutation spectrum was characterized by a preponderance of G:C→A:T transition mutations (41.9% vs. 23.1% in 4-ABP–induced mutation spectrum; $P = 0.003$), which were highly targeted to 5′-CpG dinucleotides (33.3% vs. 10.4% in 4-ABP–induced mutation spectrum; $P = 0.005$; Supplementary Table S3).

To find what specific type(s) of mutation have caused the significant increase in *cII* mutant frequency in bladder DNA of 4-ABP–treated mice relative to controls, we computed the absolute mutant frequency of each type of mutation (i.e., transitions, transversions, deletions, and insertions) in the *cII* transgene of bladder genomic DNA from both the 4-ABP–treated mice and controls. As shown in Fig. 4 and Supplementary Table S3, the absolute mutant frequencies of G:C→C:G transversions, G:C→T:A transversions, G:C→A:T transitions, A:T→T:A transversions, A:T→G:C transitions, A:T→C:G transversions, deletions, and insertions were all increased, although to different extents, in the *cII* transgene of bladder genomic DNA from 4-ABP–treated mice as compared with controls. The percentage contributions of the respective types of mutation to the overall increase in *cII* mutant frequency in bladder DNA of 4-ABP–treated mice were 12.8, 47.1, 21.3, 3.2, 5.0, 2.4, 6.9, and 1.2. Thus, mutations occurring at G:C base pairs account for 82.9% of all induced *cII* mutations in bladder DNA of 4-ABP–treated mice (Fig. 5). Of these, G:C→T:A transversion mutations, which comprise nearly half of all the induced *cII* mutations, are the main contributor to the overall increase in the *cII* mutant frequency in bladder DNA of 4-ABP–treated mice (Fig. 5).

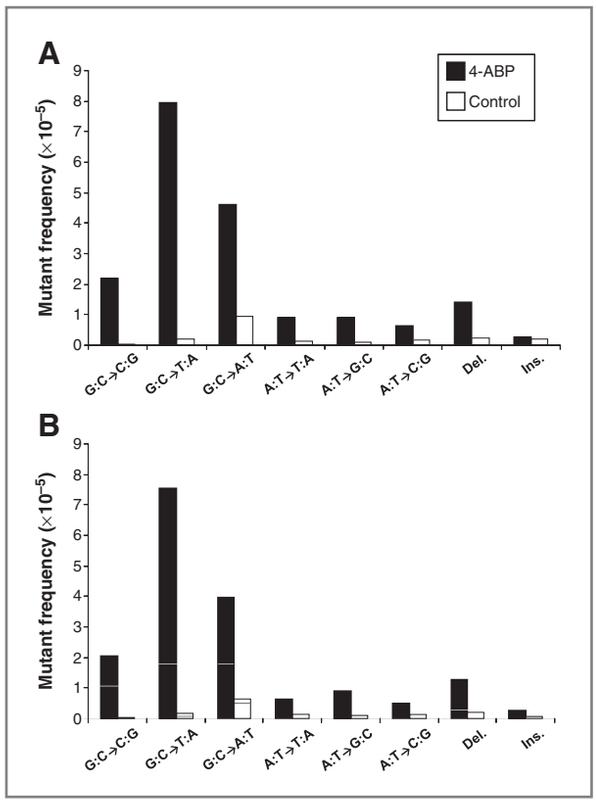

Figure 4. Spectra of *cII* mutations in bladder of 4-ABP–treated mice and controls. Absolute mutant frequency of each specific type of mutation in the *cII* transgene of bladder DNA from 4-ABP–treated mice and controls. A, sibling mutations are included (for a definition of these mutations, see text). B, sibling mutations are excluded. Horizontal lines within bars represent the contribution of mutations occurring specifically at 5′-CpG dinucleotides. Del., deletion; Ins., insertion.

Discussion

Exposure to aromatic amines is a major risk factor for bladder cancer development (1, 9, 14). The etiologic role of aromatic amines in bladder carcinogenesis revolves around their ability to form covalently bound DNA adducts, which upon eluding repair and causing mispairing during replication, may initiate mutagenesis (15, 16). To date, however, no experimental study has investigated the formation and repair of aromatic amine–DNA adducts in relation to mutagenesis in target and nontarget organs of carcinogenesis *in vivo*. In this study, we have comprehensively investigated the DNA adduction and mutagenic consequences of exposure to a representative aromatic amine compound, 4-ABP, in bladder and various nontarget organs of transgenic Big Blue mice treated weekly with 4-ABP for 6 weeks, followed by a 6-week recovery period. Our Immunodot blot analysis of 4-ABP–DNA adducts in various organs of chronically treated mice before and after the recovery period showed a repair-resistant formation of DNA adducts in bladder, kidney, and liver of carcinogen-treated animals. An intense formation of 4-ABP–DNA adducts was readily detectable in bladder, liver, and kidney of 4-ABP–treated mice

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

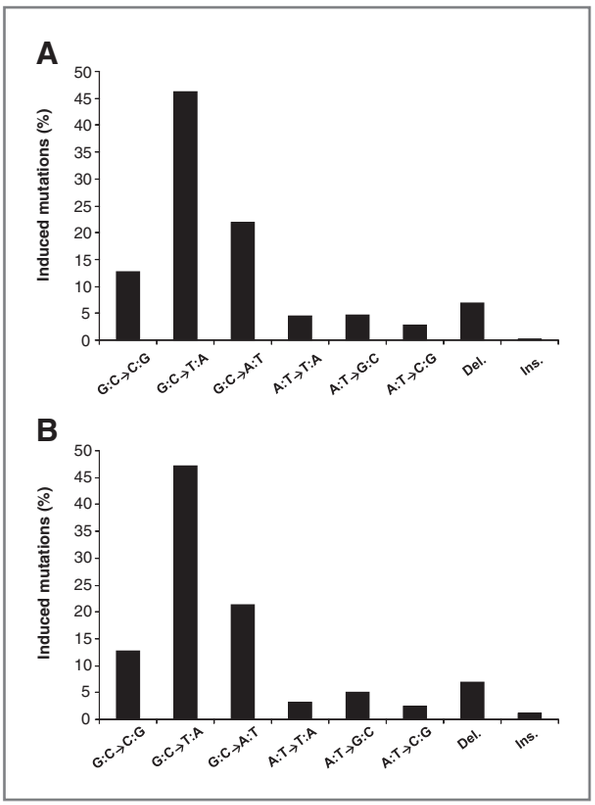

Figure 5. Spectrum of induced *cII* mutations in bladder of 4-ABP–treated mice. Percentage increase [induced mutation (%)] in frequency of each specific type of mutation in the *cII* transgene of bladder DNA from 4-ABP–treated mice relative to control. A, sibling mutations are included (for a definition of these mutations, see text). B, sibling mutations are excluded. Del., deletion; Ins., insertion.

immediately after treatment, and 6 weeks afterward (Fig. 1A). The highest levels of 4-ABP–DNA adducts were found in bladder, liver, kidney, in the order of decrease, in chemically treated mice both after the carcinogen-treatment and 6 weeks afterward (Fig. 1B). This organ specificity of 4-ABP–DNA adduction accords with its biotransformation inasmuch as reactive metabolites of 4-ABP, which are initially produced in liver, are transported to the urinary tract where they come in contact with kidney and bladder urothelial cells (17, 20–22).

Our mutagenicity analysis confirmed the above organ specificity of 4-ABP–DNA adduction and provided a mechanistic view on the known tumorigenicity of 4-ABP in mice (34, 35). As shown in Fig. 2, there was a predominant mutagenicity of 4-ABP to the bladder of chronically treated mice, which remained sustained after 6 weeks of recovery. In addition, much weaker but significant mutagenic responses were found in the liver and kidney of 4-ABP–treated mice. Although the overriding and sustainable mutagenicity of 4-ABP to the bladder of carcinogen-treated mice is consistent with its known tumorigenicity to this organ, the relatively small mutagenic response found in the liver of 4-ABP–treated mice, which were of male gender, concurs with the previous reports by others showing that 4-ABP induces far fewer liver tumors than bladder tumors in male mice [the reverse is true in female mice (see below; refs. 34, 35)]. In confirmation, Poirier and colleagues (36) and Flammang and colleagues (37) have reported higher levels of 4-ABP–DNA adducts in bladder tissues than liver tissues of male mice fed with 4-ABP via drinking water for 28 days (a reverse pattern of DNA adduction was found in the respective tissues in female mice). The organ- and gender-specific differences in 4-ABP–DNA adduction and tumorigenesis in mice may relate to tissue- and sex-specific metabolic activation of 4-ABP (e.g., O-conjugation) and hormonal status (e.g., promoting effects of testosterone), respectively (38). We note that due to low yield of DNA from mouse bladder, we only had limited amount of DNA available for simultaneous analysis of DNA adducts and mutations in this organ. Therefore, we were unable to use other quantitative techniques, such as mass spectrometry or ^{32}P-postlabeling assays, which require relatively large amount of DNA (39), for measurement of 4-ABP–DNA adducts. For example, the ^{32}P-postlabeling assay requires approximately 5 μg DNA per reaction for the detection of aromatic/hydrophobic DNA adducts (40). In this study, we obtained approximately 30 μg high molecular weight genomic DNA from the mouse bladder, of which 24 μg were needed for the *cII* mutagenesis assay (8 μg per reaction for triplicate analyses), and 1.5 μg were used for the Immunodot blot assay of 4-ABP–DNA adducts (0.5 μg per reaction for triplicate analyses).

The pronounced persistence of 4-ABP–DNA adducts in bladder relative to liver and kidney (Fig. 1B) can partially explain its highest mutagenicity found in this organ (i.e., bladder) in carcinogen-treated mice (Fig. 2). Given the small differences in the levels of 4-ABP–DNA adducts among these 3 organs; however, it is also likely that varying cellular proliferation rates, specific for each of these 3 organs, might be additionally responsible for the different mutagenic responses found in these organs in 4-ABP–treated mice. More specifically, the high proliferation capacity of the bladder urothelial cells may have contributed to the significant mutagenic response found in this organ in 4-ABP–treated animals. In this study, the mutagenic effect of 4-ABP to liver of chronically treated mice manifested immediately posttreatment and remained unchanged after an ensuing 6-week recovery period; however, the mutagenic response in the kidney of 4-ABP–treated mice was delayed, and became detectable only after 6 weeks of recovery (Fig. 2). Although the exact mechanism of this delayed mutagenic effect is currently unknown, the slowly proliferating renal cells may require prolonged time for the fixation of mutations.

In one of the early validation studies of transgenic mouse models, Fletcher and colleagues (41) have reported higher increase in the background *LacZ* mutant frequency in bladder than in liver of male adult Muta Mice treated orally with 4-ABP at a single dose (75 mg/kg bw) or at 10 daily doses (75 mg/kg bw, each). However, Chen and colleagues have shown that 4-ABP, at a single i.p. dose of 31 mg/kg bw, was

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

mutagenic only to liver of neonatal but not adult Big Blue mice in both sexes (38). The authors, however, did not investigate 4-ABP mutagenicity to bladder or any other organ of the neonatal or adult mice (38). In our preliminary experiments leading to this investigation, we refined our treatment protocol to achieve: (i) a progressively increasing dose of 4-ABP $\geq$ 420 mg/kg bw, which is tumorigenic in adult mice (35) and (ii) efficient DNA adduction and mutagenesis without causing any adverse health effects. We confirmed that our employed protocol met the above-specified criteria as we successfully showed 4-ABP–DNA adduction and mutagenesis in chronically treated mice that remained healthy until the end of all experiments.

Our mutation spectrometry analysis, which established the first comprehensive database of 4-ABP–induced mutations in mouse bladder *in vivo*, confirmed the known mutagenic potentials of 4-ABP–DNA adducts (38, 42, 43). We found a characteristic spectrum of mutations in bladder of 4-ABP–treated mice, which included a preponderance of mutations occurring at G:C base pairs, with the vast majority being G:C→T:A transversion mutations (Supplementary Table S3 and Fig. 4). Analysis of the mutation spectrum in relation to mutant frequency revealed that G:C→T:A transversion mutations accounted for approximately half of all the increase in *cII* mutant frequency found in bladder of chronically treated mice (Fig. 5). Other major types of mutations contributing to the elevation of *cII* mutant frequency in bladder of 4-ABP–treated mice were G:C→A:T transitions and G:C→C:G transversions, which comprised 23.1% and 11.9%, respectively, of all the induced mutations (Supplementary Table S3 and Fig. 5). The above targeting of mutations at G:C base pairs is consistent with the high affinity of 4-ABP to bind guanine residues in the DNA (16, 23, 24). 4-ABP or its derivatives react preferentially with the C8 position of guanine, thereby, forming a major covalent adduct, *N*-(deoxyguanosine-8-yl)-4-ABP (4-ABP–DNA adduct; refs. 16, 23, 24). Theoretical and spectroscopic analyses have shown that the 4-ABP–DNA adduct readily adopts a "*syn*" conformation around the guanine-deoxyribose linkage, and this conformational change intensifies in destabilized or unwound DNA helices, for example, during DNA replication (44, 45). The "*syn*" conformation places the O6 and N7 atoms of the modified guanine in a position to mispair with N6 and N1 atoms of an adenine or with N1 and N2 atoms of a guanine in the complementary strand, thus, resulting in G→T or G→C transversion mutations, respectively (46, 47). *In vitro* and/or *in vivo* studies in various model systems have shown that 4-ABP or its metabolites induce both G:C→T:A and G:C→C:G transversion mutations, with the former being the most prominent type of mutation (38, 42, 43, 48–50). Furthermore, mutation analysis of the *TP53* gene in human bladder tumors has shown that G:C→A:T transition mutations are the prevalent type of mutation found in the general population or specifically in individuals with known history of exposure to aromatic amines (43). Prospectively, computational prediction modeling or structural analysis will elucidate how G:C→A:T transition mutations may arise from 4-ABP–DNA adduction.

In summary, we have shown a unique organ specificity of 4-ABP in inducing persistent DNA adduction and mutagenesis in mice *in vivo*. Whereas repair-resistant 4-ABP–DNA adducts are formed in bladder, kidney, and liver of carcinogen-treated mice, which accords with the bioactivation pathway of this chemical in the respective organs (17, 20–22), a predominant and sustained mutagenic effect is found in bladder, consistent with bladder specificity of tumorigenesis known for this chemical (34, 35). In addition, 4-ABP is weakly but significantly mutagenic in the kidney and liver of carcinogen-treated mice. Of significance, the spectrum of mutations produced in the bladder of 4-ABP–treated mice perfectly reflects the known mutagenic potentials of 4-ABP–DNA adducts (38, 42, 43, 48–50). Altogether, our findings support the etiologic involvement of 4-ABP in the genesis of bladder cancer and provide a perspective on how DNA adduction leading to mutagenesis, which is specifically targeted to bladder urothelial cells, may account for bladder-tumorigenicity of this carcinogen.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank the staff and management of the City of Hope Animal Resources Center, in particular, Lauren Ratcliffe, Marie Prez, Armando Amaya, Yvonne Harper, Donna Isbell, Kenneth Golding, and Dr. Richard Ermel and specially thank Dr. Walter Tsark for helpful discussion on IACUC protocol preparation and Dr. Gerd Pfeifer for providing research support.

Grant Support

This study was supported by a grant from the American Cancer Society (RSG-11-083-01-CNE) and an "Excellence Award" from Beckman Research Institute of the City of Hope to A. Besaratinia.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 15, 2011; revised October 21, 2011; accepted November 11, 2011; published OnlineFirst November 15, 2011.

References

1. Beland FA, Kadlubar FF. Metabolic activation and DNA adducts of aromatic amines and nitroaromatic hydrocarbons. In: Cooper CS, Grover PL, editors. Chemical carcinogenesis and mutagenesis Berlin/Heidelberg: Springer-Verlag; 1990. p. 297–325.

2. Ren L. Blasengeschwülste bei Fuchsin-Arbeitern. Arch Klin Chir, 50: 588-600; 1895. Reprinted in translation: Shimkin MB (1980). Some classics of experimental oncology: 1950 selections, 1775-1965. NIH publication no 1980-2150, 1944–1951, 1895.

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

3. Leichtenstern O. Uber Harnblasenentzündung und Harnblasengeschwülste bei Arbeitern in Farbfabriken. Deut Med Wochenschr 1898;24:709–13.
4. Parkes HG, Evans AEJ. Epidemiology of aromatic amine cancers. In: Searle CE, editor. Chemical carcinogens. Washington DC: American Chemical Society; 1984. p. 277–301.
5. Huepper WC, Wiely FH, Wolfe HD, Ranta KE, Leming MF, Blood FR. Experimental production of bladder tumors in dogs by administration of β-naphthylamine. J Ind Hyg Toxicol 1938;20:46–84.
6. Spitz S, Maguigan WH, Dobriner K. The carcinogenic action of benzidine. Cancer 1950;3:789–804.
7. Walpole AL, Williams MHC, Roberts DC. Tumours of the urinary bladder in dogs after ingestion of 4-aminodiphenyl. Br J Indust Med 1954;9:105–9.
8. International Agency for Research on Cancer. Some aromatic amines, hydrazine and related substances, N-nitroso compounds and miscellaneous alkylating agents. 2-Naphthylamine. Lyon (France): 1974.
9. Steineck G, Plato N, Norell SE, Hogstedt C. Urothelial cancer and some industry-related chemicals: an evaluation of the epidemiologic literature. Am J Ind Med 1990;17:371–91.
10. Patrianakos C, Hoffman D. Chemical studies on tobacco smoke LXIV. On the analysis of aromatic amines in cigarette smoke. J Anal Chem 1979;3:150–4.
11. Besaratinia A, Pfeifer GP. Second-hand smoke and human lung cancer. Lancet Oncol 2008;9:657–66.
12. Clavel J, Cordier S, Boccon-Gibod L, Hemon D. Tobacco and bladder cancer in males: increased risk for inhalers and smokers of black tobacco. Int J Cancer 1989;44:605–10.
13. D'Avanzo B, Negri E, La Vecchia C, Gramenzi A, Bianchi C, Franceschi S, et al. Cigarette smoking and bladder cancer. Eur J Cancer 1990;26:714–8.
14. Vineis P, Esteve J, Hartge P, Hoover R, Silverman DT, Terracini B. Effects of timing and type of tobacco in cigarette-induced bladder cancer. Cancer Res 1988;48:3849–52.
15. Kadlubar FF, Beland FA, Beranek DT, Dooley KL, Heflich RH, Evans FE. Arylamine-DNA adduct formation in relation to urinary bladder carcinogenesis and Salmonella typhimurium mutagenesis. In: Sugimura T, Kondo S, Takebe H, editors. Environmental mutagens and carcinogens. New York, NY: Alan R. Liss; 1982. p. 385–96.
16. Talaska G, al-Juburi AZ, Kadlubar FF. Smoking related carcinogen-DNA adducts in biopsy samples of human urinary bladder: identification of N-(deoxyguanosin-8-yl)-4-aminobiphenyl as a major adduct. Proc Natl Acad Sci U S A 1991;88:5350–4.
17. McMahon RE, Turner JC, Whitaker GW. The N-hydroxylation and ring-hydroxylation of 4-aminobiphenyl *in vitro* by hepatic mono-oxygenases from rat, mouse, hamster, rabbit and guinea-pig. Xenobiotica 1980;10:469–81.
18. Ziegler DM, Ansher SS, Nagata T, Kadlubar FF, Jakoby WB. N-methylation: potential mechanism for metabolic activation of carcinogenic primary arylamines. Proc Natl Acad Sci U S A 1988;85:2514–7.
19. Kadlubar FF, Butler MA, Hayes BE, Beland FA, Guengerich FP. Role of microsomal cytochromes P-450 and prostaglandin H synthase in 4-aminobiphenyl-DNA adduct formation. In: Miners J, Birkett DJ, Drew R, McManus M, editors. Microsomes and drug oxidations. London, England: Taylor and Francis; 1988. p. 370–9.
20. Chou HC, Lang NP, Kadlubar FF. Metabolic activation of the N-hydroxy derivative of the carcinogen 4-aminobiphenyl by human tissue sulfotransferases. Carcinogenesis 1995;16:413–7.
21. Orzechowski A, Schrenk D, Bock-Hennig BS, Bock KW. Glucuronidation of carcinogenic arylamines and their N-hydroxy derivatives by rat and human phenol UDP-glucuronosyltransferase of the UGT1 gene complex. Carcinogenesis 1994;15:1549–53.
22. Poupko JM, Hearn WL, Radomski JL. N-Glucuronidation of N-hydroxy aromatic amines: a mechanism for their transport and bladder-specific carcinogenicity. Toxicol Appl Pharmacol 1979;50:479–84.
23. Frederickson SM, Hatcher JF, Reznikoff CA, Swaminathan S. Acetyl transferase-mediated metabolic activation of N-hydroxy-4-aminobiphenyl by human uroepithelial cells. Carcinogenesis 1992;13:955–61.
24. Swaminathan S, Reznikoff CA. Metabolism and nucleic acid binding of N-hydroxy-4-acetylaminobiphenyl and N-acetoxy-4-acetylaminobiphenyl by cultured human uroepithelial cells. Cancer Res 1992;52:3286–94.
25. Besaratinia A, Pfeifer GP. Investigating human cancer etiology by DNA lesion footprinting and mutagenicity analysis. Carcinogenesis 2006;27:1526–37.
26. Lambert IB, Singer TM, Boucher SE, Douglas GR. Detailed review of transgenic rodent mutation assays. Mutat Res 2005;590:1–280.
27. al-Atrash J, Zhang YJ, Lin D, Kadlubar FF, Santella RM. Quantitative immunohistochemical analysis of 4-aminobiphenyl-DNA in cultured cells and mice: comparison to gas chromatography/mass spectroscopy analysis. Chem Res Toxicol 1995;8:747–52.
28. Jakubczak JL, Merlino G, French JE, Muller WJ, Paul B, Adhya S, et al. Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for *in vivo* mutations in a bacteriophage lambda transgene target. Proc Natl Acad Sci U S A 1996;93:9073–8.
29. Pfeifer GP, Chen HH, Komura J, Riggs AD. Chromatin structure analysis by ligation-mediated and terminal transferase-mediated polymerase chain reaction. Methods Enzymol 1999;304:548–71.
30. Besaratinia A, Yoon JI, Schroeder C, Bradforth SE, Cockburn M, Pfeifer GP. Wavelength dependence of ultraviolet radiation-induced DNA damage as determined by laser irradiation suggests that cyclobutane pyrimidine dimers are the principal DNA lesions produced by terrestrial sunlight. FASEB J 2011;25:3079–91.
31. Herskowitz I, Hagen D. The lysis-lysogeny decision of phage lambda: explicit programming and responsiveness. Annu Rev Genet 1980;14:399–445.
32. Adams WT, Skopek TR. Statistical test for the comparison of samples from mutational spectra. J Mol Biol 1987;194:391–6.
33. Mirsalis JC, Shimon JA, Johnson A, Fairchild D, Kanazawa N, Nguyen T, et al. Evaluation of mutant frequencies of chemically induced tumors and normal tissues in lambda/cII transgenic mice. Environ Mol Mutagen 2005;45:17–35.
34. Schieferstein GJ. Carcinogenesis of 4-aminobiphenyl in mice. Jefferson, AR: National Center for Toxicological Research; 1983.
35. Schieferstein GJ, Littlefield NA, Gaylor DW, Sheldon WG, Burger GT. Carcinogenesis of 4-aminobiphenyl in BALB/cStCrlfC3Hf/Nctr mice. Eur J Cancer Clin Oncol 1985;21:865–73.
36. Poirier MC, Fullerton NF, Smith BA, Beland FA. DNA adduct formation and tumorigenesis in mice during the chronic administration of 4-aminobiphenyl at multiple dose levels. Carcinogenesis 1995;16:2917–21.
37. Flammang TJ, Couch LH, Levy GN, Weber WW, Wise CK. DNA adduct levels in congenic rapid and slow acetylator mouse strains following chronic administration of 4-aminobiphenyl. Carcinogenesis 1992;13:1887–91.
38. Chen T, Mittelstaedt RA, Beland FA, Heflich RH, Moore MM, Parsons BL. 4-Aminobiphenyl induces liver DNA adducts in both neonatal and adult mice but induces liver mutations only in neonatal mice. Int J Cancer 2005;117:182–7.
39. Farmer PB, Brown K, Tompkins E, Emms VL, Jones DJ, Singh R, et al. DNA adducts: mass spectrometry methods and future prospects. Toxicol Appl Pharmacol 2005;207:293–301.
40. Phillips DH, Arlt VM. The 32P-postlabeling assay for DNA adducts. Nat Protoc 2007;2:2772–81.
41. Fletcher K, Tinwell H, Ashby J. Mutagenicity of the human bladder carcinogen 4-aminobiphenyl to the bladder of MutaMouse transgenic mice. Mutat Res 1998;400:245–50.
42. Zayas-Rivera B, Zhang L, Grant SG, Keohavong P, Day BW. Mutational spectrum of N-hydroxy-N-acetyl-4-aminobiphenyl at exon 3 of the HPRT gene. Biomarker 2001;6:262–73.
43. Besaratinia A, Bates SE, Pfeifer GP. Mutational signature of the proximate bladder carcinogen N-hydroxy-4-acetylaminobiphenyl: inconsistency with the p53 mutational spectrum in bladder cancer. Cancer Res 2002;62:4331–8.

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

44. Broyde S, Hingerty BE, Srinivasan AR. Influence of the carcinogen 4-aminobiphenyl on DNA conformation. Carcinogenesis 1985;6: 719–25.

45. Shapiro R, Underwood GR, Zawadzka H, Broyde S, Hingerty BE. Conformation of d(CpG) modified by the carcinogen 4-aminobiphenyl: a combined experimental and theoretical analysis. Biochemistry 1986;25:2198–205.

46. Drake JW, Baltz RH. The biochemistry of mutagenesis. Annu Rev Biochem 1976;45:11–37.

47. Topal MD, Fresco JR. Complementary base pairing and the origin of substitution mutations. Nature 1976;263:285–9.

48. Manjanatha MG, Li EE, Fu PP, Heflich RH. H- and K-ras mutational profiles in chemically induced liver tumors from B6C3F1 and CD-1 mice. J Toxicol Environ Health 1996;47:195–208.

49. Melchior WB Jr, Marques MM, Beland FA. Mutations induced by aromatic amine DNA adducts in pBR322. Carcinogenesis 1994; 15:889–99.

50. Verghis SB, Essigmann JM, Kadlubar FF, Morningstar ML, Lasko DD. Specificity of mutagenesis by 4-aminobiphenyl: mutations at G residues in bacteriophage M13 DNA and G–>C transversions at a unique dG(8-ABP) lesion in single-stranded DNA. Carcinogenesis 1997;18: 2403–14.

Published OnlineFirst November 15, 2011; DOI: 10.1158/1940-6207.CAPR-11-0309

Cancer Prevention Research

Organ Specificity of the Bladder Carcinogen 4-Aminobiphenyl in Inducing DNA Damage and Mutation in Mice

Jae-In Yoon, Sang-In Kim, Stella Tommasi, et al.

Cancer Prev Res 2012;5:299-308. Published OnlineFirst November 15, 2011.