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# $^{32}$P-Postlabelling analysis of DNA adducts of 4,4′-methylene*bis*(2-chloroaniline) in target and nontarget tissues in the dog and their implications for human risk assessment

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**4,4′-Methylene*bis*(2-chloroaniline) (MOCA) has considerable human occupational exposure and it induces urinary bladder tumors in the dog, a species that has been often used as a model for aromatic amine-induced urinary bladder carcinogenesis in humans. Metabolic activation and formation of DNA adducts are considered to be critical steps in this process; and two major C8-adenine adducts have been shown to be formed *in vitro* by reaction with the proximate carcinogenic metabolite *N*-hydroxy-MOCA. MOCA–DNA adducts have also been detected *in vivo* in treated rats and in exfoliated urothelium of a worker accidentally exposed to MOCA. Thus, the aim of this study was to detect and quantify DNA adducts in the urinary bladder of dogs exposed to MOCA and thereby provide data that could be useful for risk assessment after human exposure to MOCA. Beagle dogs were treated with single and multiple doses of MOCA and DNA adduct levels were determined in liver and bladder epithelium. After a single dose, adduct levels in the liver were 1.5-fold higher than that in the bladder epithelium. Adduct levels in these two organs increased 3- to 5-fold after 10 doses and adducts in the liver were then 2.8-fold higher than that in the bladder epithelium. The levels found in these two organs after single exposures were compared, per unit exposure dose, with that reported for other carcinogenic aromatic amines. The comparison showed that MOCA was as effective in DNA adduct formation as most other potent urinary bladder carcinogens. These results suggest that MOCA may have high carcinogenic potential in humans and are consistent with the recent classification of MOCA as a probable human carcinogen.**

## Introduction

4,4′-Methylenebis(2-chloroaniline) (MOCA*) is an important industrial chemical with considerable potential human exposure (1). MOCA is genotoxic in cell systems and is carcinogenic in experimental animals, inducing liver and lung tumors in rats and urinary bladder tumors in dogs (2). Like several other aromatic amines that are known to induce urinary bladder tumors in humans (3), MOCA has recently been upgraded by the International Agency for Research on Cancer to a 'probable human carcinogen', based on epidemiological studies, animal bioassays and mechanistic considerations (4).

Since DNA adduct formation is recognized to be important for tumor induction by many chemical carcinogens, the analyses of such reaction products can be useful for biological monitoring and for risk assessment after human exposure to these compounds (5). Using $^{32}$P-postlabelling and GC/MS techniques, DNA adducts of 4-aminobiphenyl have been positively identified in urinary bladder biopsies from cigarette smokers (6,7) and have been indicated in exfoliated urothelial cells in urine samples from smokers (8). Urinary bladder tumors are also induced in the dog by 4-aminobiphenyl and other aromatic amines; and this animal has often been used to study the carcinogenesis of these compounds (9). In this regard, pharmacokinetic studies have indicated that the primary determinants for DNA adduct formation in the dog bladder epithelium are the urinary concentration of reactive *N*-hydroxy arylamine and the relative voiding interval (10,11).

Similarly, the reaction of the *N*-hydroxy-MOCA metabolite with DNA has been demonstrated *in vitro* and two major MOCA–DNA adducts have been characterized as *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (C8-dA-1) and *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorotoluene (C8-dA-2) (12,13). These same adducts are also formed *in vivo* after administration of MOCA to rats (13). Recently, C8-dA-1 has been identified in exfoliated urothelial cells of a worker acutely exposed to MOCA (14).

A major purpose of this work was therefore to determine, by the $^{32}$P-postlabelling technique, the extent and nature of MOCA–DNA adduct formation *in vivo* in the urinary bladder epithelium of the dog. In addition, these studies were undertaken to evaluate methods to analyze MOCA–DNA adducts for possible application to human biomonitoring and risk assessment.

## Materials and methods

### *Chemicals*

[Methylene-$^{14}$C]MOCA (radiochemical purity >98%, sp. act. 57 mCi/mmol) was purchased from Chemsyn Science Laboratories, Lenexa, KS. Unlabelled MOCA was obtained from Pfaltz-Bauer, Waterbury, CT and was purified by silica gel (grade 923, 100–200 mesh from A Grace Division, Davison Chemicals, Baltimore, MD) column chromatography as described previously (13) (purity >98 %). Proteinase K, ribonuclease A, ribonuclease T1, micrococcal nuclease, nuclease P1 and Nonidet P-40 were all from Sigma Chemical Co., St Louis, MO. Spleen phosphodiesterase and enzymes used for the synthesis of [$^{32}$P]-ATP were from Boehringer, Mannheim, Germany and T4 polynucleotide kinase (PNK) was from US Biochemicals, Cleveland, OH. [$^{32}$P]-Orthophosphoric acid with a sp. act. of >5000 Ci/mmol was obtained from ICN Chemical and Radioisotope Division, Irvine, CA. $C_{18}$-Reversed phase TLC plates were from Whatman Inc., Clifton, NJ and PEI-cellulose coated TLC plates were from Merck, Darmstadt, Germany and Macherey-Nagel, Duren, Germany.

### *$^{32}$P-Postlabelling*

$^{32}$P-Labelled ATP was synthesized as modified by Gupta *et al.* (15), from the original description by Johnson and Walseth (16). The sp. act. of radiolabelled ATP was regularly determined by labelling a limited amount of 3′-dAMP (17). The specific activities obtained were 3000–4000 Ci/mmol. DNA was isolated from tissues using the procedure described by Reddy and Randerath (18). The DNA concentrations were determined spectrophotometrically using 20 $A_{260}$ units per mg/ml of DNA as a standard. DNA samples of 0.2–4 μg were hydrolysed

*Abbreviations: MOCA, 4,4′-methylene*bis*(2-chloroaniline); C8-dA-1, *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol; C8-dA-2, *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorotoluene; MN, micrococcal nuclease; PNK, polynucleotide kinase; SPD, spleen phosphodiesterase.

by 3 h of incubation at 37°C with 0.25 units of micrococcal nuclease (MN) and 2.5 μg of spleen phosphodiesterase (SPD) in 10 μl of 10 mM $CaCl_2$ and 20 mM sodium succinate buffer, pH 6.0.

Three different postlabelling assays were tested: the standard variant with excess of low sp. act. ATP in the presence of normal nucleotides (19); the adduct intensification variant with limiting amount of high sp. act. ATP in the presence of normal nucleotides (20); and the nuclease P1 variant of the assay in which normal nucleotides are dephosphorylated before labelling of adducts (17). When nuclease P1 was used, 4 μg of DNA was hydrolysed in a total volume of 7 μl and 3 μg of the enzyme in 3 μl of 0.3 mM $ZnCl_2$ and 0.25 M sodium acetate buffer (pH 5.0) was added and the mixture incubated for another 30 min at 37°C. To each sample (10 μl) was added labelling cocktail (10 μl) containing 200–300 μCi of[$^{32}$P]-ATP, 1 μl of 100 mM Bicine buffer (pH 9.6) and 1.2 units of polynucleotide kinase in 1 μl of 20 mM Bicine buffer (pH 9.6) (0.06 units/μl final concentration), 10 mM dithiothreitol, 1 mM spermidine and 10 mM $MgCl_2$. For the standard assay, 600 pmol of non-radiolabelled ATP was also added; and, for the nuclease P1 modification, the cocktail also contained 0.17 M Tris-base. The samples were incubated for 40 min at 37°C. Excess ATP was eliminated by incubation with apyrase for 40 min.

The thin-layer chromatographic techniques used were either based on separation by reversed phase silica plates as described by Randerath *et al.* (21) with minor modifications (method A) or on PEI-cellulose plates as described by Kaderlik *et al.* (14) (method B).

For the $C_{18}$ plates (method A), four samples were applied 18 mm from the edge of channelized plates (50×100 mm) that had been predeveloped overnight in 0.4 M ammonium formate buffer, pH 6.2. A paper wick (50×100 mm, Whatman no. 1) had been attached to the top of each glass plate using ordinary paper clips and a piece of used X-ray film as a back support. The plates were developed overnight in 0.4 M ammonium formate buffer (pH 6.2) inside a glass tank (200×200 mm). After drying, the plates which had been prescored 12 mm above and 6 mm below the site of sample application were broken along these cuts. Each plate was also cut vertically between the channels. The samples on these ~9×18 mm large chips were magnet-transferred individually to 100×100 mm PEI-plates (Merck) by developing for 1 h at 60°C in *n*-propanol/water (1:1) containing 1% Nonidet P-40. After the transfer, magnets and chips were removed and the PEI-plates were washed twice in water and then soaked in 0.15 M ammonium formate buffer (pH 3.5) for 10 min. The plates were dried and the adducts separated first in the same direction as the transfer using 1.8 M lithium formate, 4.25 M urea, pH 3.5, followed by 0.65 M sodium phosphate, pH 6.5 or 0.4 M LiCl, 0.25 M Tris–HCl, 4.25 M urea, pH 8.0, at a right angle to the first direction. The radioactivity was visualized by exposing X-ray film to the TLC-plates. Areas on the thin-layers corresponding to specific spots on the films were cut out and radioactivity quantitated by Cerenkov counting in a liquid scintillation counter.

The second chromatographic technique used (method B) was that previously described by Kaderlik *et al.* (14). Briefly, 3 μg of DNA was digested with MN/SPDas described above. The entire DNA digest, in a total volume of 7.5 μl, was then treated with an equal volume of nuclease P1 (1 μg/μg DNA) under standard conditions (17) and then labelled with excess carrier-free [$^{32}$P]-ATP (300 μCi) as before, except the PNK concentration was 0.15 units/μl. The labelled mixture was then analyzed by PEI-cellulose thin-layer chromatography and autoradiography as previously described (14), except that only D1 and D2 chromatography steps were performed using 1.7 M sodium phosphate buffer (pH 6.0).

*Treatment of animals*

Two female Beagle dogs (body wt, 8.2 and 9.1 kg, obtained from Marshall Lab Animals, North Rose, NY) were orally administered a single dose of 8.0 mg (30 μmol) MOCA/kg body wt. Two additional female dogs (7.5 and 8.9 kg) were given the same dose of MOCA daily (Monday–Friday) for 2 weeks. The single-dosed dogs were killed 2 days after treatment. The two multiple-dosed dogs were killed after 3 and 7 days respectively, following the last dose. Livers and urinary bladder epithelium were obtained, frozen in liquid $N_2$ and stored at −70°C until isolation of DNA.

## Results

### *Method A—reversed phase plates*

$^{32}$P-Postlabelling of *N*-hydroxy-MOCA-treated calf thymus DNA, prepared as described (13), using the standard assay with excess of ATP in relation to the amount of normal nucleotides present, resulted in two radioactive spots which were not present in untreated DNA (Figure 1A and B). The same hydrolysate prepared with [$^{14}$C]*N*-hydroxy-MOCA modified DNA was also separated on HPLC before $^{32}$P-postlabelling. Two $^{14}$C-containing peaks were also seen (Figure 2A). These peaks were

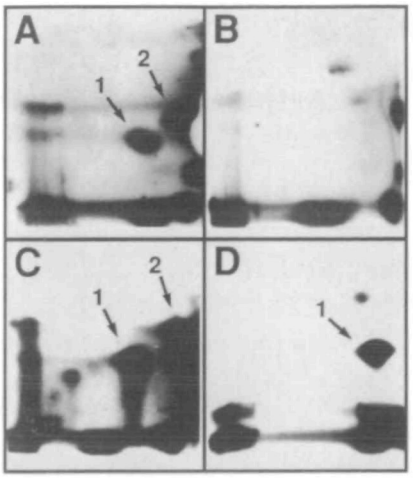


**Fig. 1.** Autoradiograms of $^{32}$P-postlabelled DNA using thin-layer chromatography method A. (**A**) *N*-hydroxy-MOCA-treated DNA using the standard procedure; (**B**) control DNA using the standard procedure; (**C**) *N*-hydroxy-MOCA-treated DNA using the adduct intensification procedure; (**D**) *N*-hydroxy-MOCA-treated DNA using nuclease P1 enhancement. C8-dA-1 and C8-dA-2 are indicated by the arrows designated 1 and 2 respectively.

each collected and treated with nuclease P1, acid and alkaline phosphatase, and then reanalyzed on HPLC (Figure 2B and C). The dephosphorylated product obtained from the first eluting peak in Figure 2A had the same retention time as C8-dA-1 from the nucleoside hydrolysate of *N*-hydroxy-MOCA treated DNA (Figure 2D). Similarly, the major portion of the dephosphorylated product from the minor peak in Figure 2A had the same retention time as C8-dA-2 (Figure 2D). Another portion of the major peak (Figure 2A) was postlabelled and found to comigrate on thin-layer chromatograms with the major spot seen when analyzing directly the *N*-hydroxy-MOCA-modified DNA (data not shown). It was thus concluded that the major adduct observed when postlabelling *N*-hydroxy-MOCA treated DNA was identical to the 3′,5′-*bis*phosphate of C8-dA-1 and the minor spot corresponded to the 3′,5′-*bis*phosphate of C8-dA-2.

When *N*-hydroxy-MOCA-treated DNA was postlabelled with limiting amount of ATP (Figure 1C), the major and the minor adducts were intensified (20) ~4- and 10-fold respectively. Dephosphorylation with nuclease P1 prior to labelling consistently resulted in the loss of the minor adduct, while the major adduct spot was essentially unchanged (Figure 1D). Instead, another adduct spot located just above the major one could be seen.

The levels determined by postlabelling were ~60% of what was found after HPLC separation and radioactivity measurement of the corresponding $^{14}$C-labelled nucleoside adducts. The highest sensitivity of the postlabelling assay was obtained with nuclease P1. However, even with this adduct enhancement procedure, the limit of detection was on the order of 1 adduct in $10^7$ nucleotides. The reason for this relatively low sensitivity was the high general background radioactivity, which originates from the use of reversed phase plates, and the presence of background spots of unknown origin, close to the right hand edge of the plates, which hampers the detection of minor radioactive spots. Subsequently, the following chromatographic system was selected for the separation of MOCA-adducts.

### *Method B—PEI-cellulose plates*

Initial efforts to use PEI-cellulose plates from Merck for D1 showed that the MOCA–DNA adduct spots moved at least 15 cm when using 0.55 or 1.1 M LiCl and 0.6 or 1 M sodium

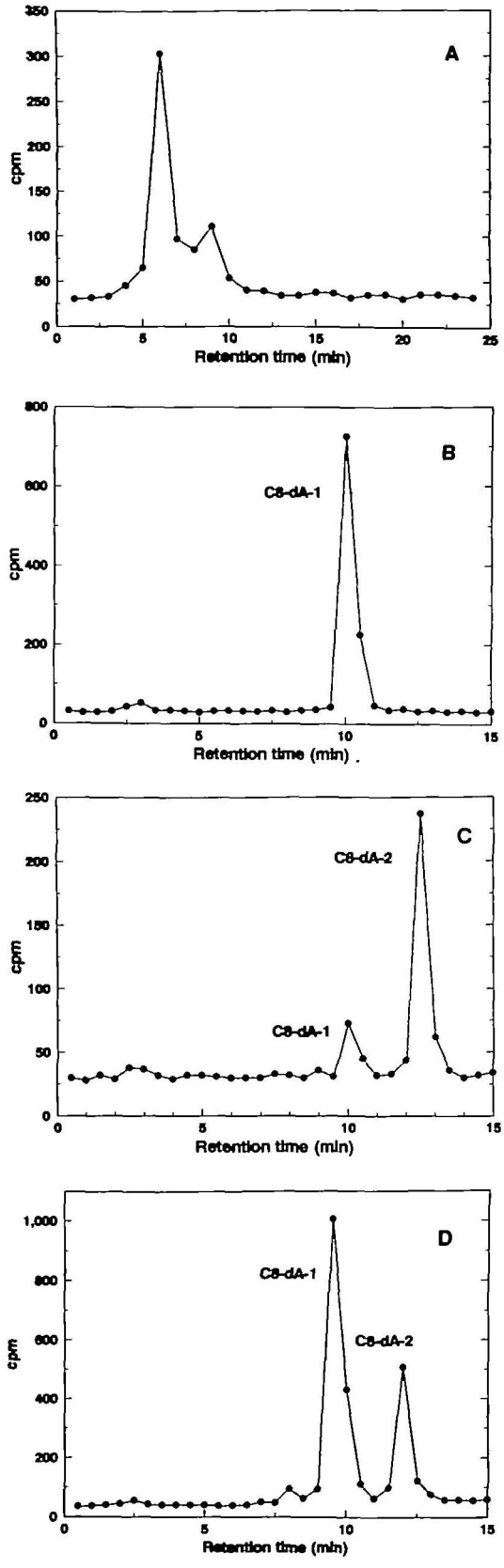


Fig. 2. HPLC separation of enzymatic hydrolysates of [$^{14}$C]*N*-hydroxy-MOCA-treated DNA. (A) After incubation with MN and SPD; (B) after major peak in A treated with nuclease P1 and acid and alkaline phosphatase; (C) after minor peak in A treated with nuclease P1 and acid and alkaline phosphatase; (D) after incubation with DNase I, nuclease P1 and acid and alkaline phosphatase. The chromatographic system used is described in ref. 13, except that the solvent gradient in A was 5–30% methanol in 20 min.

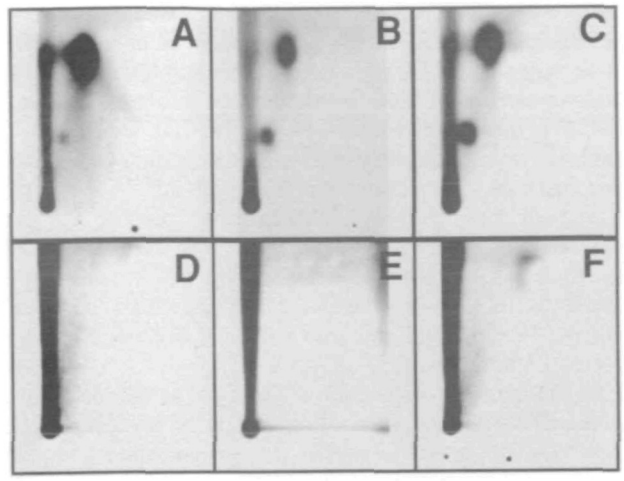


Fig. 3. Autoradiograms of $^{32}$P-postlabelled DNA using nuclease P1 enhancement and thin-layer chromatographic method B. (A) *N*-Hydroxy-MOCA-treated calf thymus DNA; (B) urinary bladder DNA and (C) liver DNA from MOCA-treated dogs; (D) untreated calf thymus DNA; and DNA from (E) urinary bladder and (F) liver of untreated dogs.

Table I. DNA adduct levels (determined by $^{32}$P-postlabelling) in the urinary bladder epithelium and the liver of dogs given 1 or 10 oral doses (8 mg/kg body wt) of MOCA and killed at different times following the last dose

| Treatment | Adducts per $10^6$ nucleotides (± SD) | |
|---|---|---|
| (*n*, time of death) | Bladder | Liver |
| 1 dose (2 dogs, 48 h) | 1.1 (0.5) | 1.7 (0.5) |
| 10 doses (1 dog, 3 days) | 3.2 (0.7) | 9.1 (1.4) |
| 10 doses (1 dog, 7 days) | 4.4 (1.0) | 8.2 (2.0) |

Table II. DNA adduct levels (per unit exposure dose) in the liver and the urinary bladder epithelium of dogs exposed to some aromatic amines

| Compound | CBI[a] | |
|---|---|---|
| | Liver | Urinary bladder |
| MOCA[b] | 56 | 35 |
| 4-Aminobiphenyl[c] | 130 | 120 |
| 2-Acetylaminofluorene[c] | 83 | 13 |
| Benzidine[c] | 5 | 3.3 |
| 2-Naphthylamine[c] | 0.7 | 3.3 |

[a]CBI (covalent binding index) is defined as the DNA binding level in μmol/mol nucleotide at a unit dose of 1 mmol/kg body wt (22).
[b]The orally administered single doses were 30 μmol/kg body wt for MOCA and 60 μmol/kg body wt for the other compounds. The animals were killed 2 days after dosing.
[c]Data calculated from refs 23,24 based on adduct level per unit exposure dose at 2 days after a single oral administration to female Beagle dogs.

phosphate pH 6.0 or 7.0 (data not shown). However, as shown by Kaderlik *et al.* (14), the MOCA–DNA adducts migrated much less when using the Macherey–Nagel plates for D1. Thus, with this method C8-dA-1 spot could be detected in *N*-hydroxy-MOCA-modified calf thymus DNA and in liver and bladder DNA of a MOCA-treated dog, but not in untreated DNA samples (Figure 3). An unknown, minor spot could sometimes be seen, migrating just above the origin. This adduct cannot be the same as adduct 2 since the later adduct has chromatographic properties virtually identical to that of adduct 1 (data not shown). The recovery of adduct 1 was 50% and the level of sensitivity was ~1 adduct per $10^8$ nucleotides. Since this method was more

sensitive than method A and the data obtained was more reproducible, all dog samples were analysed using method B.

The major MOCA–DNA adduct levels in bladder and liver of dogs treated with either 1 or 10 doses (8 mg/kg body wt) of MOCA were thus determined (Figure 3A–D). DNA from liver (nontarget organ) and urinary bladder epithelium (target organ) were analyzed (twice in duplicates) for adducts by the nuclease P1 variant of the assay, which only detects the major MOCA–DNA adduct. The major adduct and the unknown minor adduct were seen in both the bladder and the liver. As negative controls, bladder epithelial and liver DNA from an untreated dog were postlabelled and analyzed similarly (Figure 3E and F).

After a single dose, the adduct level in the liver was ~60% higher than in the urothelium (Table I), a difference which increased 2- to 3-fold after multiple treatments. After 10 doses of MOCA, the adduct levels in both organs were 3- to 5-fold higher than that following a single treatment. The data in Table I was corrected for recovery by using the *N*-hydroxy-MOCA–DNA adduct standard.

In Table II, the adduct levels in liver and bladder epithelium after a single dose of MOCA are compared at unit exposure dose (22) with published data for other aromatic amines (23,24).

## Discussion

We have found that C8-dA-1 is resistant to nuclease P1 dephosphorylation, whereas C8-dA-2 is not. Generally, adducts containing at least two aromatic rings bound to deoxyguanosine-C8 are not resistant towards the dephosphorylating effect of this enzyme (25), but little is known about adducts bound to deoxyadenosine-C8. The additional MOCA–DNA adduct spot which appears after P1 treatment (when using method A; Figure 1D) could either be a minor adduct which is highly intensified in the absence of normal nucleotides or the 5′-monophosphorylated adduct 2. If and how this adduct spot relates to the minor spot seen just above the origin when using method B (Figure 3) is not known.

The recovery of C8-dA-1 for methods A and B were 60 and 50% respectively. Since the sensitivity of method B is 10-fold greater than method A, it is more suitable for human biomonitoring (14).

The adduct pattern in bladder epithelium- and liver-DNA after treatment of dogs to MOCA appeared to be identical to the pattern seen when treating DNA *in vitro* with *N*-hydroxy-MOCA (Figure 3A, B and C). This has also been shown to be the case in the rat, but that study was performed using radiolabelled MOCA and nucleoside adduct measured as radioactive peaks after HPLC separations of DNA hydrolysates (13). In the dog, the adduct level in the liver was only slightly higher than in the bladder after a single dose of MOCA (Table I). Several other aromatic amines also give about the same adduct levels in these two tissues (Table II). After 10 treatments, the levels in the urothelium and the liver increased 3- to 5-fold, i.e. less than proportional to the administered dose. On the other hand, there was no significant difference in adduct levels between animals killed at 3 or 7 days following the last dose. The difference in adduct levels between urothelium and liver was thus more pronounced after multiple treatments. These data indicate differences in rate of repair between the two organs and a relatively fast initial repair phase followed by a much slower one. Similar kinetics for elimination of MOCA–DNA adducts has also been observed in human exfoliated urothelial cells (14), in the liver of MOCA-treated rats (13), as well as in the liver and in the bladder of dogs treated with 2-acetylaminofluorene, 4-aminobiphenyl and 2-naphthylamine (23,24).

The dog is regarded as an appropriate model for the induction of human urinary bladder cancer by aromatic amines. Moreover, DNA adduct formation is considered a necessary initial event in chemical carcinogenesis. Thus, comparison of the DNA adduct forming ability of MOCA in the bladder epithelium and the liver of the dog with corresponding published data for other aromatic amines (23,24) indicates that MOCA is considerably more potent than 2-naphthylamine, benzidine and 2-acetylaminofluorene and almost as potent as 4-aminobiphenyl. This suggests that in the dog, MOCA is as potent a genotoxin as other aromatic amine carcinogens.

However, tumor development is a multistep process and the primary role of DNA adducts is probably at the initiation step (resulting in a somatic mutation). Modulating factors may not only include differences between dog and humans with respect to metabolism and DNA repair, but there may also exist differences in adduct potencies for mutation induction and in the abilities of the carcinogens to act at later stages of tumorigenesis. In this regard, DNA adducts of aromatic amines to deoxyguanosine-C8 and deoxyadenosine-C8 have been shown to have different mutagenic potencies (23,26); and these carcinogens show marked differences in their ability to serve as tumor promoters (27,28). Nevertheless, the data presented here indicates that MOCA has high carcinogenic potency and is consistent with the re-evaluation of MOCA and its classification by IARC as a probable human carcinogen (4).

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