Assessment of the Medicines Lidocaine, Prilocaine, and Their Metabolites, 2,6-Dimethylaniline and 2-Methylaniline, for DNA Adduct Formation in Rat Tissues

Jian-Dong Duan, Alan M. Jeffrey, and Gary M. Williams

Department of Pathology, New York Medical College, Valhalla, New York

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ABSTRACT:

The local anesthetics lidocaine (lido) and prilocaine (prilo) are metabolized to their constituent aromatic amines 2,6-dimethylaniline (DMA, 2,6-xylidine) and 2-methylaniline (MA, o-toluidine), respectively, which are both tumorigenic in rats. The capacity of lido and prilo to form DNA adducts was assessed in major target tissues for aromatic amines in male F344 rats in comparison to equimolar doses of DMA and MA using the 32P-postlabeling assay. Direct reaction of putative DNA-reactive metabolites N-hydroxy-DMA and N-hydroxy-MA with isolated DNA yielded reference adducts. Rats were dosed by p.o. gavage with 0.5 mmol/kg b.wt. of each test substance or the vehicle either once or daily for 7 days. After repeat administrations of either prilo or lido, DNA adducts were detected in the liver and nasal mucosa. Urinary bladder DNA adducts were detected only in lido and DMA repeat dosed rats.

Groups dosed with DMA or MA showed adducts in both single- and multiple-dose groups, except for the single-dose DMA liver and urinary bladder samples, which were below the level of detection. No DNA adducts were detected in any of the white blood cell samples, which were below the level of detection.

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The metabolic data DMA and MA are carcinogenic in rats (Beland et al., 1997; Haseman and Hailey, 1997; http://monographs.iarc.fr/ENG/Monographs/allmonos90.pdf). DMA increased the incidence of adenomas and carcinomas of the nasal cavity in Charles River CD rats when fed up to 3000 ppm (http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr278.pdf) (∼300 mg/kg b.wt./day). In addition, increased incidences of s.c. fibromas and fibrosarcomas in male and female rats and an increased incidence of neoplastic nodules of the liver in female rats may have been related to dosing. Administration of MA·HCl at 3000 or 6000 ppm in the diet to F344 rats induced sarcomas of the spleen, probably as a result of methemoglobin formation and splenic congestion, sarcomas in other organs, mesotheliomas of the abdominal cavity or scrotum in males, and transitional-cell carcinomas of the urinary bladder and splenic sarcomas in females (http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr153.pdf). The International Agency for Research on Cancer (IARC) found no human carcinogenicity data on DMA, although there was sufficient evidence of its carcinogenicity in rats. Several epidemiological studies on workers with potential exposure to MA revealed greater incidences of bladder cancer, although exposures to other aromatic amines also occurred (http://

ABBREVIATIONS: lido, lidocaine; prilo, prilocaine; DMA, 2,6-dimethylaniline; MA, 2-methylaniline; IARC, International Agency for Research on Cancer; NM, nasal mucosa; UBE, urinary bladder epithelium; NPL, nucleotide 32P-postlabeling; CT-DNA, calf thymus 2'-deoxyribonucleic acid; TLC, thin-layer chromatography; HLB, hydrophilic-lipophilic balance.
Lido and prilo have not been tested for carcinogenicity. Prilo, as of October 2007, is undergoing micronucleus testing by the National Toxicology Program (http://ntp.ncbi.nlm.nih.gov/?objectid=BD463EC1-123F-7908-7B3495234B051AF).

The major mechanism of carcinogenicity of aromatic amines such as DMA and MA is via metabolism by cytochromes P450 to the N-hydroxyl derivatives (Beland and Kadlubar, 1985). These products may be further metabolized by conjugation to yield reactive metabolites. DNA binding has been reported for DMA (Short et al., 1989; Gonçalves et al., 2001; Skipper et al., 2006) but not for MA (Richter et al., 2008). Alteration of DNA is recognized as a possible mechanism by which compounds such as these exert some of their carcinogenic effects (Preston and Williams, 2005).

Lido and prilo have been shown to form hemoglobin adducts in humans (Bryant et al., 1994; Gaber et al., 2007). Because hemoglobin adducts are considered to be informative surrogate markers for potential DNA reactivity of the ultimate carcinogenic metabolites of aromatic amines (Richter et al., 2008), this suggested that lido and prilo could possibly form DNA adducts in vivo.

In the present investigation, the ability of lido and prilo to form DNA adducts in rat target tissues of aromatic amines, the liver, nasal mucosa (NM), and urinary bladder epithelium (UBE), was assessed using the nucleotide 32P-postlabeling assay (NPL) developed by Randerath and Randerath (1994). With a cumulative dose of 3.5 mmol to rats, both compounds produced DNA adducts in the liver and NM, whereas lido, but not prilo, produced adducts in the UBE. The adducts corresponded to those produced by their arylation metabolites. With a single dose of 0.5 mmol neither produced measurable DNA adduct levels with a detection limit estimate of about 1 adduct in 108 normal cells.

Materials and Methods

Chemicals. DMA (99%), MA (99%), 2-nitro-m-xylene, calf thymus 2’-deoxyribonucleic acid (CT-DNA), micrococcoc nucleic, spleen phosphodiesterase, and nuclease P1 were obtained from Sigma Chemical Co. (St. Louis, MO). Lido·HCl and prilo·HCl (melting points 75–77°C and 166.2–167.1°C, respectively) were obtained from MP Biomedicals, Inc. (Solon, OH). 2-Nitrotoluene was obtained from Acros Organics (Morris Plains, NJ), T4 polynucleotide kinase was from USB Corp. (Cleveland, OH), adenosine (γ32P) triphosphate (NEG035C) was from PerkinElmer Life (Waltham, MA), and PEI cellulose thin-layer chromatography (TLC) plates (JT473-4) were from VWR Scientific Corp (Bridgeport, NJ).

Animals. Male Fischer F344 rats, about 6 to 8 weeks of age (Taconic Farms, Hudson, NY), were maintained in the New York Medical College animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The 30 rats were randomly divided into 10 groups (Table 1). After 1 week of acclimatization, groups 1 through 5 were administered single doses by gavage of the test compounds and killed 24 h later for tissue collection. Groups 6 through 10 were administered 1 dose/day for 7 days, and the rats were killed 24 h after the final gavage. These dosages were chosen to be lower than those used in the carcinogenicity studies of DMA and MA (i.e., up to 300 and 600 mg/kg/day, respectively) (http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr153.pdf; http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr278.pdf).

Blood samples, taken in EDTA-containing tubes, were mixed by inversion and kept in ice for isolation of nuclei with lysis buffer (Qiagen Inc., Valencia, CA). The livers, NM, and UBE were immediately removed and frozen with liquid nitrogen and stored at −80°C until DNA isolation.

DNA Isolation. DNA isolation used Qiagen Genomic 100G tips following the manufacturer’s protocol (Qiagen Inc.). DNA was redissolved in water, and its purity was estimated from 230:260:280 ratio of the UV spectra in 10 mM Tris HCl buffer, pH 7.2.

Synthesis of N-Hydroxyamines. These were prepared according to modifications of methods previously described (Beland et al., 1997; Jeffrey et al., 2002). Briefly, to 20 mg of ammonium chloride in 1 ml of 60% ethanol/water on ice, saturated with inert gas, was added 50 mg of 2-nitro-m-xylene or 45 mg of 2-nitrotoluene. Zinc powder (118 mg) was then added in small portions. After addition of the first portion, the reaction started. The reaction mixture was kept at 10 to 15°C, and additional zinc powder was slowly added. After 1 h, the excess zinc was removed by centrifugation, and the supernatant was transferred to another tube. The ethanol was removed under reduced pressure, leaving about 300 µl of water, which was extracted with 500 µl of ethyl acetate. After centrifugation, the upper layer was transferred to a new tube, dried with anhydrous sodium sulfate, and reduced under vacuum to 100 µl. Slowly adding 500 µl of cold hexane allowed crystallization. The crystals were washed with cold hexane, dried, and stored at −70°C until use. The mp were: N-hydroxy-DMA obs 92 to 95°C, lit 97 to 99°C (Bamberger and Rising, 1901b; Marques et al., 1997), and N-hydroxy-MA obs 40 to 42°C, lit 42 to 44°C (Bamberger and Rising, 1901a; Marques et al., 1996).

In Vitro Modification of DNA. CT-DNA (0.5 mg) dissolved in 500 µl of 0.1 M sodium phosphate buffer, pH 7.2, was incubated with N-hydroxy-DMA or N-hydroxy-MA (18 µg/20 µl of 30% ethanol) at 37°C for 12 h. Half the volume of 7.5 M ammonium acetate was added, followed by 2 volumes of ethanol to precipitate the DNA. Cold ethanol (70%) was used to wash the DNA, which was redissolved in water.

NPL of DNA Adducts. The procedures were conducted as previously described (Jeffrey et al., 2002). The DNA samples (10 µg) were enzymatically digested to 2’-deoxyribonucleoside 3’-phosphates using micrococcal nuclease and spleen phosphodiesterase. The digestion mixtures were then enriched for DNA-modified bases using nuclease P1 digestion (Reddy and Randerath, 1986) or Oasis hydrophilic-lipophilic balance (HLB) (Waters, Milford, MA) (Jeffrey et al., 2002) enrichment methods. The DNA-modified bases were then labeled using adenosine (γ32P) triphosphate and T4 polynucleotide kinase. The labeled modified bases were resolved using two-dimension TLC. D1 direction used 2 M sodium phosphate buffer, pH 5.6, run from bottom to top with wick for 16 h. D2 direction used 0.28 M ammonium sulfate/50 mM Tris HCl and prilo HCl and prilo.
sodium phosphate buffer, pH 6.6, on plates rotated by 90° counterclockwise and run with wick for 5 h. The 32P-labeled modified bases were detected using a GE Healthcare (Little Chalfont, Buckinghamshire, UK) Storm system and quantified using ImageQuant (GE Healthcare) and Peakfit (SPSS, Inc., Chicago, IL) software.

Results

All the rats tolerated the dosings well and showed no visible adverse effects from the treatments. Estimated DNA adduct levels are summarized in Tables 2 and 3.

**NPL Assays in Vitro.** N-Hydroxy-DMA and N-hydroxy-MA were incubated with CT-DNA, and adducts were assessed by the NPL assay. With N-hydroxy-DMA one major spot was observed together with several minor ones (Fig. 2a), and N-hydroxy-MA–treated DNA showed several spots of similar intensities (Fig. 3a) at ~10-fold lower levels than the N-hydroxy-DMA major spot. No spots were detected in control CT-DNA incubation group (Figs. 2d, 3d).

Table: DNA Adduct Levels in Various Tissues

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<tr>
<th>Adduct Level (± S.D.)</th>
<th>Ratio L/NM</th>
<th>Adduct Level (± S.D.)</th>
<th>Ratio A/C</th>
<th>Adduct Level (± S.D.)</th>
<th>Ratio L/NM</th>
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| Control BD | BD         | Prilo 0.56 ± 0.17      | 0.66 ± 0.06 | MA 7.73 ± 1.24      | 3.26 ± 1.47 | 7normal nucleotides, single dose

**Discussion**

The present investigation revealed that the local anesthetics lido and prilo and their aromatic amine metabolites, DMA and MA, respectively, formed DNA adducts in several tissues in the male F344 rat detected by the sensitive NPL technique. The chromatographic conditions used were established to be optimal for identification of the most likely DNA adducts by showing that they identified DNA adducts resulting from reaction of synthetically prepared N-hydroxy derivatives of DMA and MA. Our findings with DMA are in agreement with earlier reports of DNA binding in rat liver and NM (Short et al., 1989). In the mouse, binding was reported highest in the bladder and liver, but the NM was not studied (Skipper et al., 2006). Interestingly, we found no positive in vivo report of binding of MA to DNA. The dose levels used in our studies were at least 5 times less than those used in the positive bladder carcinogenicity studies, but the fact that adduct levels were below detection in the UBE does not support the hypothesis that DNA adducts are involved in bladder carcinogenesis.
the IARC conclusion (Baan et al., 2008) that it causes human bladder cancer at still much lower exposures.

Metabolic activation of DMA and MA is thought to involve $N$-hydroxylation and possible subsequent esterification with sulfate. These esters are highly reactive intermediates (Marques et al., 1996; Beland et al., 1997). In the present studies, the detected DNA adducts formed in rats and those obtained by reaction of the synthetic $N$-hydroxy derivatives with CT-DNA in vitro were chromatographically identical. Moreover, DNA adducts formed in lido- or prilo-dosed rats were also chromatographically indistinguishable from those formed in vitro from $N$-hydroxy-DMA and $N$-hydroxy-MA, respectively, establishing that these adducts were formed via the aromatic amine metabolites, not the whole molecules.

Multiple doses of lido and prilo each produced one major, but chromatographically different, DNA adduct in both liver and NM. Because DNA adduct formation is recognized as a basis of carcinogenicity (Preston and Williams, 2005), this suggests that lido and prilo are potentially carcinogenic to rats if administered at dosages comparable with those used in the present studies. However, although all the chemicals were dosed in equimolar amounts, the DNA adduct levels were lower with lido and prilo compared with the corresponding free arylamines, indicating less bioavailability of the anesthetic-derived arylamine metabolites (Tables 2 and 3).

The efficiency of DNA adduct formation appeared to be different for different target organs, although it must be kept in mind that the levels of DNA adducts estimated by NPL are minimum values because different DNA adducts have different efficiencies of digestion, enrichment, and phosphorylation by the polynucleotide kinase. Given this caveat, the highest level of DNA adducts was found in the NM of DMA multidose-treated rats. We previously reported, as did Short et al. (1989), that DMA formed DNA adducts in the NM, the major target organ for its carcinogenicity, at higher levels than in the liver or testes (Jeffrey et al., 2002). Either DMA is concentrated in NM, readily activated there, or both, as shown for other chemicals of this type (reviewed in Jeffrey et al., 2006). Lido similarly formed DNA adducts in the NM, although to a lower level than DMA and with less
tissue specificity than with DMA, the NM/liver DNA adduct ratio being approximately only 1:1 for lido compared with 10:1 for DMA. It may be that lido, being slowly metabolically converted to DMA, is less specifically metabolized in F344 rats’ NM, even though equal molar quantities of lido and DMA were administered.

In the MA-dosed group, there was somewhat greater DNA adduct level in the liver than in the NM, although neither was a major site of tumor formation (http://ntp.niehs.nih.govntp/htdocs/LT_rpts/tr153.pdf). This indicates, as have other studies (Jeffrey et al., 2006), that formation of adducts is insufficient for carcinogenicity. Prilo formed a relatively high level of MA-derived DNA adduct in the liver, but no adduct was detected in UBE DNA in prilo- or MA-dosed or multidose groups, suggesting that MA is mainly bioactivated in the liver. The enzyme activities for both cytochrome P450 and sulfotransferase are the highest in the liver (DeBaun et al., 1970; Guengerich and Liebler, 1985), which is consistent with the relatively high adduct yield at this site (Gonçalves et al., 2001; Jeffrey et al., 2002, 2006). The absence of MA adducts in the NM may indicate that the tissue concentration or metabolism of MA is different from DMA in the NM.

Regarding the organ specificity of DMA and MA, although it is clear from the National Toxicology Program study (http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr278.pdf) that DMA forms nasal tumors, they reported “that there were no clinical signs,” implying that the nasal tumors were only detected by pathologic examination. In the case of MA (http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr153.pdf), nasal pathology was not assessed, and so it is uncertain whether nasal tumors might have been formed.

DNA adducts are a sensitive biomarker of compound activation to potentially carcinogenic metabolites. Our study characterized formation of lido- and prilo-derived DNA adducts in rats, and these findings could facilitate the development of methods to monitor similar effects in humans. The desirability of biomonitoring for effects of these medicines is supported by the report of Skipper et al. (2006) of association of 14C-DMA with DNA detected by accelerator mass spectrometry in mice at a dose 3 orders of magnitude lower (i.e., 100 µg/kg b.w.t.) than used in the present study. Because they reported bladder adducts, study of exfoliated UBE might shed light on the discrepancy between the proposed classification of MA as a human bladder carcinogen (Baan et al., 2008) and our finding of no detectable adduct formation in the rat UBE. Nevertheless, because aromatic amines belong to the class of chemical carcinogens with cancer-initiating properties, which may be expressed even at low doses (Williams et al., 1999), the use of large doses, such as 122 mg/kg/24 h of lido in treatment of seizures (Malingre et al., 2006), a dose that is close to that used in this study (144 mg/kg), should be carefully considered. Other anesthetics that also contain the DMA moiety, such as bupivacaine, mepivacaine, and ropivacaine, are still in current use, although ropivacaine does not seem to be metabolized to DMA in humans and etidocaine has been withdrawn.

References


FIG. 10. Rat liver and NM DNA adducts formed by single or multiple (seven daily) gavage doses.

DNA Adducts Level in 10^6 Normal Nucleotides

Liver
Nasal mucosa

Control Lido Prilo MA DMA

Multiple doses

Single dose

157–164.
129: 3–11.
13–19.
167: 184–186.
14: 827–852.


Address correspondence to: G. M. Williams, Department of Pathology, New York Medical College, Valhalla, NY 10595. E-mail: gary_williams@nymc.edu