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

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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 $^{32}$P-postlabeling assay. Direct reaction of putative DNA-reactive metabolites 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 under either dosing regimen. The lido- and prilo-DNA adducts detected were chromatographically indistinguishable from those formed either in DMA- or MA-dosed rats, respectively, or by chemical reaction of the corresponding N-hydroxy derivatives with DNA. Thus, lido and prilo can generate DNA adducts in rats via their aromatic amine metabolites, although at lower levels than equal molar quantities of their amine metabolites.

The aromatic amine-containing medicines lidocaine (lido) and prilocaine (prilo) (Fig. 1) are commonly used local anesthetics (Physicians’ Desk Reference, 2007; http://www.fda.gov/MedWatch/SAFETY/2005/Dec_PI/Emla_PI.pdf) either by injection for dental work or often in the form of topical patches. In addition, lido has been used extensively as an antiarrhythmic agent (Cattererall and Mackie, 2006), in the tumescent technique for liposuction (Nordström and Stånge, 2005), and in the treatment of neonatal seizures (Malingre et al., 2006).

Lido and prilo are mainly metabolized in the liver but not by plasma amidases (http://www.dentsply.com/assets/DFU/oraqix_pi.pdf). After amide ester hydrolysis, lido and prilo release the monocyclic aromatic amines 2,6-dimethylaniline (DMA, 2,6-xylidine) and 2-methylaniline (MA, o-toluidine), respectively (Fig. 1). The literature on this has been extensively reviewed (http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Anesthetics.pdf). Lido undergoes N-dealkylation to ethylglycinexylidide and glycinexylidide, which is mainly mediated by CYP3A4. These metabolites are hydrolyzed to DMA (http://www.dentsply.com/assets/DFU/oraqix_pi.pdf). Both lido and prilo increase formation of methemoglobin (Weiss et al., 1987; Vasters et al., 2006) probably via the corresponding N-hydroxyarylamines (Weisburger and Weisburger, 1973). This can be an acute toxicological problem associated with the use (Buckley and Benfield, 1993) and misuse (Balit et al., 2006) of these drugs and may be the basis for some effects in rats at high dosages.

The metabolites DMA and MA are carcinogenic in rats (Beland et al., 1997; Haseman and Hailey, 1997; http://monographs.iarc.fr/ENG/ Monographs/allmono90.php; http://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?id=989&type=dis). 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 $^{32}$P-postlabeling; CT-DNA, calf thymus 2’-deoxyribonucleic acid; TLC, thin-layer chromatography; HLB, hydrophilic-lipophilic balance.
Toluene was obtained from Acros Organics (Morris Plains, NJ), T4 polynucleotide kinase (G) were enzymatically labeled using adenosine (\(\gamma^32\)P) triphosphate and T4 polynucleotide kinase. DNA isolation used Qiagen Genomic 100/g tips following 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 \(\mu\)l of water, which was extracted with 500 \(\mu\)l 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 \(\mu\)l. Slowly adding 500 \(\mu\)l of cold hexane allowed crystallization. The crystals were washed with cold hexane, dried, and stored at \(-70^\circ\)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 \(\mu\)l of 0.1 M sodium phosphate buffer, pH 7.2, was incubated with \(N\)-hydroxy-DMA or \(N\)-hydroxy-MA (18 \(\mu\)g/20 \(\mu\)l 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 \(\mu\)g) 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 (\(\gamma^32\)P) triphosphate and T4 polynucleotide kinase. The labeled modified bases were resolved using two-direction 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 0.2 M ammonium sulfate/50 mM sodium phosphate buffer, pH 7.2, with wick for 16 h. D2 direction used 2 M sodium phosphate buffer, pH 5.6, run from bottom to top with wick for 16 h. D2 direction used 2 M sodium phosphate buffer, pH 5.6, run from bottom to top with wick for 16 h.
### 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).

Enrichments of in vitro DNA adducts by Oasis HLB column and NP_{1} methods both yielded DNA adducts (Fig. 2, a and b). However, about 90% of DNA adducts were lost in the Oasis HLB column method because they were inadequately hydrophobic to be fully retained, and thus the majority of the DNA adducts were in the loading and 5% methanol washing eluates of the column. The more hydrophilic nature of these DNA adducts also necessitated the development of a two-directional, rather than the usual three-directional, TLC system because the N-hydroxyamine-DNA adduct spots moved in sodium phosphate buffer normally used to elute the residual normal nucleotides, ATP, and inorganic phosphate into the wick.

In light of these findings, only the NP_{1} method was used for the NPL analysis of the in vivo samples.

**DNA Adducts Formed in Vivo.** In the single-dose groups, DNA adducts were found in the livers of MA-dosed group (Fig. 4d) and in the NM of both MA- and DMA-dosed groups (Fig. 5, d and e). The chromatographic properties of the major spots were very similar to those of the in vitro N-hydroxy-DMA- and N-hydroxy-MA-CT-DNA samples, respectively (Figs. 2 and 3), although the intensities of the spots were more than 30 times stronger for the chemically reacted samples than those observed in vivo. No similar spot was detected in other groups at these levels of sensitivity (Tables 2 and 3; Figs. 3–5).

In multidose groups, liver and NM DNA adducts were found in all the dosed groups, and in lido- and DMA-dosed groups, similar adducts were found in the UBE (Tables 2 and 3; Figs. 6–8). The control and white blood cell (Fig. 9) groups were negative. In the DMA-dosed groups, the total NM DNA adducts were 10 times higher than the liver adducts, whereas in the MA-dosed groups, liver DNA adducts were 2.4 higher than NM adducts (Tables 2 and 3). Thus, except for white blood cells, DNA adducts were found in at least some of the groups from both the single and multiple dosages. These data are summarized graphically in Fig. 10.

### 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). Surprisingly, 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 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 a 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.gov/ntp/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.wt.) 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 dosages, 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, meptivacaine, 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


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