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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Running Title Page

Running title: Assessment of Prilocaine and Lidocaine DNA Adducts in the Rat

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Abbreviations: 2-methylaniline MA
2,6-dimethylaniline DMA
lidocaine lido
nasal mucosa NM
nucleotide $^{32}$P-postlabeling NPL
prilocaine prilo
thin-layer chromatography TLC
urinary bladder epithelium UBE
ABSTRACT

The local anesthetics lidocaine (lido, 2,6-xylidine) and prilocaine (prilo, o-toluidine) are metabolized to their constituent aromatic amines 2,6-dimethylaniline (DMA) and 2-methylaniline (MA), 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 quantities 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 oral gavage with 0.5 mmole/kg bw 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 multi-dose treated 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 cells 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) (Figure 1) are commonly used local anesthetics (FDA, 2005; PDR, 2007) 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 (Dentsply Pharmaceuticals, 2007). 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 (Figure 1). The literature on this has been extensively reviewed (Carson, 2000). Lido undergoes N-dealkylation to ethylglycinexylidide and glycinexylidide, which is mainly mediated by CYP3A4. These metabolites are hydrolyzed to DMA (Dentsply Pharmaceuticals, 2007). Both lido and prilo increase formation of methaemoglobin (Vasters et al., 2006; Weiss et al., 1987) 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 (AstraZeneca LP, 2006; Beland et al., 1997; Haseman and Hailey, 1997; International Agency for Research on Cancer, 2008). DMA increased the incidence of adenomas and carcinomas of the nasal cavity in
Charles River CD rats when fed up to 3000 ppm (National Toxicology Program, 1990) (~300mg/kg bw/day). In addition, increased incidences of subcutaneous 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 and mesotheliomas of the abdominal cavity or scrotum in males and transitional-cell carcinomas of the urinary bladder and splenic sarcomas in females (National Toxicology Program, 1979). IARC found no human carcinogenicity data on DMA although sufficient evidence of its carcinogenicity in rats. Several epidemiological studies on workers with potential exposure to MA revealed increased rates of bladder cancer, although exposures to other aromatic amines also occurred (International Agency for Research on Cancer, 2008). Nevertheless, in light of several positive rodent studies and hemoglobin adducts in humans, IARC concluded MA to be carcinogenic to humans (Baan et al., 2008).

Lido and prilo have not been tested for carcinogenicity. Prilo, as of October 2007, is undergoing micronucleus testing by the NTP (National Toxicology Program, 2007).

The major mechanism of carcinogenicity of aromatic amines such as DMA and MA is via metabolism by cytochrome P450 to the N-hydroxyl derivative (Beland and Kadlubar, 1985). These products may be further metabolized by conjugation to yield reactive metabolites. DNA binding has been reported for DMA (Gonçalves et al., 2001; Short et
al., 1989; 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). Since 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 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 the Randeraths (Randerath and Randerath, 1994). With a cumulative dose of 3.5 mmole 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 arylamine metabolites. With a single dose of 0.5 mmole neither produced measurable DNA adduct levels with a detection limit estimate of about 1 adduct in 10^8 normal nucleotides. It is, however, well recognized that with NPL some adducts are significantly underestimated.
Materials and Methods

Chemicals: DMA (99%), MA (99%), 2-nitro-\textit{m}-xylene, calf thymus 2'-deoxyribonucleic acid (CT-DNA), micrococcal nuclease, spleen phosphodiesterase and nuclease P₁ were obtained from Sigma Chemical Co., St. Louis, MO. Lido•HCl and prilo•HCl (appropriate 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 from USB Corp, Cleveland OH, adenosine (γ³²P) triphosphate (NEG035C) from PerkinElmer Life Waltham, MA and PEI Cellulose thin-layer chromatography (TLC) plates (JT4473-4) through VWR Scientific Corp, Bridgeport NJ.

Animals: Male F344 rats, about 6-8 weeks of age (Taconic Farms, Hudson, NY), were maintained in the NYMC AAALAC-accredited animal facilities. The 30 rats were randomly divided into ten groups (Table 1). After a week of acclimatization, groups 1-5 were administered single doses of the test compounds and killed 24 hours later for tissue collection. Groups 6-10 were administered 1 dose/per day for 7 days by gavage and the rats were killed 24 hours 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 (National Toxicology Program, 1979; National Toxicology Program, 1990).

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 100/G tips following the manufacturer’s protocol (Qiagen Inc, Valencia CA). DNA was redissolved in water and its purity 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 2-nitro-m-xylene or 45 mg 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 10-15°C and additional zinc powder was slowly added. After 1 hour, the excess zinc was removed by centrifugation and the supernatant transferred to another tube. The ethanol was removed under reduce pressure leaving about 300 µl water which was extracted with 500 µl ethyl acetate. After centrifugation, the upper layer was transferred to a new tube, dried with anhydrous sodium sulfate and reduced under vacuum to 100ul. Slowly adding 500 µl cold hexane allowed crystallization. The crystals were washed with cold hexane, dried and stored at –70°C until use. N– hydroxy-DMA mp obs 92-95°C, lit 97-99°C (Bamberger and Rising, 1901b; Marques et al., 1997) and N–
hydroxy-MA  mp obs 40-42°C, lit 42-44°C (Bamberger and Rising, 1901a; Marques et al., 1996).

**In vitro modification of DNA:** CT-DNA (0.5 mg) dissolved in 500 µl of 0.1M 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 hours. 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 re-dissolved in water.

**Nucleotide ³²P-postlabeling (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 HLB (Jeffrey et al., 2002) enrichment methods. The DNA modified bases were then labeled using adenosine (γ-³²P) triphosphate and T4 polynucleotide kinase. The labeled modified bases were resolved using two-direction TLC. D1 direction used 2 M sodium phosphate, pH 5.6 buffer run from bottom to top with wick for 16 hours. D2 direction used 0.28 M ammonium sulfate/50mM sodium phosphate buffer, pH 6.6 on plates rotated by 90° counter-clockwise and run with wick for five hours. The ³²P-labeled modified bases were detected using a Molecular Dynamics Storm system and quantified using Imagequant and Peakfit software.
RESULTS

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

**NPL Assays *in vitro***:

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

Enrichments of *in vitro* DNA adducts by OASIS HLB column and NP₁ methods both yielded DNA adducts (Figure 2a, 2b). 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-, rather than the usual three-, directional TLC system since the *N*-hydroxylamine-DNA adduct spots moved in sodium phosphate buffer normally used to elute the residual normal 2'-'deoxyribonucleotides, ATP and inorganic phosphate into the wick.

In light of these findings, only the NP₁ 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 (Figure 4d) and in the NM of both MA- and DMA-dosed groups (Figure 5d, 5e). 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 (Figures 2 & 3), although the intensities of the spots were more than thirty 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 (Table 2 and Figures 3-5).

In multi-dose groups, liver and NM DNA adducts were found in all dosed groups, and in lido- and DMA-dosed groups, similar adducts were found in the UBE (Table 2 and Figures 6, 7 & 8). The control and white blood cells (Figure 9) groups were negative. In the DMA-dosed groups, the total NM DNA adducts were 10 times higher than the liver adducts while in the MA-dosed groups, liver DNA adducts were 2.4 higher than NM adducts (Table 2). Thus, except for white blood cells, DNA adducts were found in at least some of the groups from both the single and multiple dosages.
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 shown to be optimal for identification of the most likely DNA adducts by demonstrating 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 five time 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 (Beland et al., 1997; Marques et al., 1996). 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. Since 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 to those used in the present studies. However, although all chemicals were dosed in equimolar amounts, the DNA adduct levels were lower with lido and prilo compared to the corresponding free arylamines, indicating less bioavailability of the anesthetic-derived arylamine metabolites (Table 2).

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 multi-dose 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 demonstrated 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 to liver DNA...
adduct ratio being approximately only 1:1 for lido compared to 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 (National Toxicology Program, 1979). This indicates, as have other studies (Jeffrey et al., 2006), that formation of adducts is insufficient for carcinogenicity. Prilo formed relatively high MA-derived DNA adduct in the liver, but no adduct was detected in UBE DNA in prilo- or MA-dosed multi-dose groups, suggesting that MA is mainly bioactivated in the liver. The enzyme activities for both cytochrome P-450 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; Jeffrey et al., 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, while it is clear from the NTP study (National Toxicology Program, 1990) 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 (National Toxicology Program, 1979), 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. (Skipper et al., 2006) of association of 14C-DMA with DNA detected by accelerator mass spectrometry in mice at a dose three orders of magnitude lower (i.e. 100 µg/kg bw) than used in the present study. Since 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, since 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/24hr of lido 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.
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FDA. EMLA Cream & Patch (2005)


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National Toxicology Program. Toxicology and Carcinogenesis Studies of 2,6-Xyldine (2,6-Dimethylaniline) (CAS No.87-62-7) in Charles River CD Rats (Feed Studies) (1990)

http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr278.pdf 278 Research Triangle Park, NC 27709, National Institute of Environmental Health Sciences


Legends for Figures

Figure 1  Structures of Test Compounds

Figure 2  In vitro reacted calf thymus DNA adducts from enrichment by NP₁ (a) or OASIS HLB chromatography (b) compared to in vivo nasal mucosa adducts after NP₁ enrichment (c) and control DNA (d).

Figure 3  DNA adducts formed from synthetic standard and single 2-methylaniline dosed groups

Figure 4  Liver DNA adducts in single dose treatment groups

Figure 5  Nasal mucosa DNA adducts in single dose treatment groups

Figure 6  Liver DNA adducts in multi-dose treatment groups

Figure 7  Nasal mucosa DNA adducts in multi-dose treatment groups

Figure 8  Urinary bladder epithelium DNA adducts in multi-dose treatment groups

Figure 9  White blood cell DNA adducts in multi-dose treatment groups
Figure 10  Rat liver and nasal mucosa DNA adducts formed by single or multiple (7 daily) gavage doses.
### Table 1. Rat Groups and Doses

<table>
<thead>
<tr>
<th>Compound</th>
<th>Group ID</th>
<th>mg/kg</th>
<th>Group ID</th>
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<td>Control</td>
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<td>6</td>
<td>0.0</td>
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<td>Prilo•HCl</td>
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<td>7</td>
<td>770.4</td>
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<td>8</td>
<td>866.4</td>
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<td>53.6</td>
<td>9</td>
<td>321.6</td>
</tr>
<tr>
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<td>5</td>
<td>60.6</td>
<td>10</td>
<td>363.6</td>
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</table>

*Three male F344 rats in each group, group 1-5 dosed for 1 day (0.5 mmole), group 6-10 dosed for 7 days (3.5 mmole).*
Table 2. Estimates of DNA Adducts Level in $10^{-7}$ Normal Nucleotides

Single Dose

<table>
<thead>
<tr>
<th>Liver</th>
<th>Nasal Mucosa</th>
<th>UBE</th>
<th>White Blood Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Adduct Levels ($\pm$ SD)</td>
<td>Adduct Levels ($\pm$ SD)</td>
<td>Ratio $^a$</td>
</tr>
<tr>
<td>Control BD</td>
<td>Control BD</td>
<td>Control BD</td>
<td>L/NM</td>
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<tr>
<td>Prilo BD</td>
<td>Prilo BD</td>
<td>Prilo BD</td>
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<tr>
<td>Lido BD</td>
<td>Lido BD</td>
<td>Lido BD</td>
<td></td>
</tr>
<tr>
<td>MA 1.16 ± 0.25</td>
<td>MA 0.55 ± 0.03</td>
<td>DMA 1.12 ± 0.23</td>
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<td>DMA BD</td>
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Multi-Dose

<table>
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<tr>
<th>Liver</th>
<th>Nasal Mucosa</th>
<th>Urinary Bladder Epithelium</th>
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<tr>
<td></td>
<td>Adduct Levels ($\pm$ SD)</td>
<td>Adduct Levels ($\pm$ SD)</td>
</tr>
<tr>
<td>Control BD</td>
<td>Control BD</td>
<td>Control BD</td>
</tr>
<tr>
<td>Prilo 0.64 ± 0.13</td>
<td>Prilo 0.46 ± 0.29</td>
<td>Prilo BD</td>
</tr>
<tr>
<td>Lido 0.56 ± 0.17</td>
<td>Lido 0.66 ± 0.06</td>
<td>Lido BD</td>
</tr>
<tr>
<td>MA 7.73 ± 1.24</td>
<td>MA 3.26 ± 1.47</td>
<td>MA BD</td>
</tr>
<tr>
<td>DMA 2.33 ± 0.09</td>
<td>DMA 23.10 ± 4.28</td>
<td>DMA BD</td>
</tr>
</tbody>
</table>

$N$-Hydroxy-DMA adducts level in vitro is estimated as 248 in $10^{-7}$ normal nucleotides

$N$-Hydroxy-MA adducts level in vitro is estimated as 36 in $10^{-7}$ normal nucleotides

$^a$ Ratio A/C of free amine DNA adduct level/corresponding drug DNA adduct level;

Ratio L/NM of DNA adduct in liver to NM (If not indicted, the values were not statistically different)

$^b$ BD, Below limit of detection
Lidocaine
2-(diethylamino)-N-(2,6-dimethylphenyl) acetamide

Prilocaine
N-(2-methylphenyl)-2-(propylamino)-propanamide

2,6-Dimethylaniline
(2,6-xylidine)

2-Methylaniline
(o-toluidine)

Figure 1
Figure 2

a $N$-hydroxy-DMA (NP1)

b $N$-hydroxy-DMA (OASIS HLB)

c Nasal Mucosa DMA DNA

d Control DNA
Figure 3
Figure 4
Figure 5
Figure 6

a  Control  b  Prilo  c  Lido  d  MA  e  DMA
Figure 7

a Control  b Prilo  c Lido  d MA  e DMA
Figure 8
Figure 9
Figure 10