Regulatory Toxicology and Pharmacology 119 (2021) 104838

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

A weight of evidence assessment of the genotoxicity of 2,6-xylidine based on existing and new data, with relevance to safety of lidocaine exposure

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ARTICLE INFO

Keywords:
Genotoxicity
2,6-Xylidine
Weight of evidence assessment

ABSTRACT

Lidocaine has not been associated with cancer in humans despite 8 decades of therapeutic use. Its metabolite, 2,6-xylidine, is a rat carcinogen, believed to induce genotoxicity via N-hydroxylation and DNA adduct formation, a non-threshold mechanism of action. To better understand this dichotomy, we review literature pertaining to metabolic activation and genotoxicity of 2,6-xylidine, identifying that it appears resistant to N-hydroxylation and instead metabolises almost exclusively to DMAP (an aminophenol). At high exposures (sufficient to saturate phase 2 metabolism), this may undergo metabolic threshold-dependent activation to a quinone-imine with potential to redox cycle producing ROS, inducing cytotoxicity and genotoxicity. A new rat study found no evidence of genotoxicity *in vivo* based on micronuclei in bone marrow, comets in nasal tissue or female liver, despite high level exposure to 2,6-xylidine (including metabolites). In male liver, weak dose-related comet increases, within the historical control range, were associated with metabolic overload and acute systemic toxicity. Benchmark dose analysis confirmed a non-linear dose response. The weight of evidence indicates 2,6-xylidine is a non-direct acting (metabolic threshold-dependent) genotoxin, and is not genotoxic *in vivo* in rats in the absence of acute systemic toxic effects, which occur at levels 35 × beyond lidocaine-related exposure in humans.

1. Introduction and background

Lidocaine, widely used in human and veterinary medicine for over 8 decades, is on the World Health Organisation list of essential medicines (WHO, 2019) as one of mankind's safest, most effective local anaesthetic agents. However, 2,6-xylidine (2,6-dimethylaniline, CAS No. 87-62-7), a metabolite of lidocaine in humans (Parker et al., 1996) and food producing species (Thuesen and Friis, 2012; Hoogenboom et al., 2015) has been identified as a rat carcinogen by the U.S. National Toxicology Program (NTP, 1990), raising questions regarding safety of exposure. 2, 6-Xylidine (2,6-dimethylaniline, CAS No. 87-62-7) is an aromatic amine. Its structure and pathways of metabolism are shown in Fig. 1.

In the NTP study, 2,6-xylidine was administered to rats in diet for 2 years at doses from 300 to 3000 ppm (estimated to equate to 15–150 mg/kg/day), and increased incidences of nasal tumours in both sexes, and neoplastic nodules in livers of females, were reported in high dose groups (NTP, 1990). In 1993, the International Agency for Research on Cancer (IARC) concluded that 2,6-xylidine is possibly carcinogenic to humans i.e. group 2B (IARC, 1993). When lidocaine is administered to humans at maximum daily recommended levels of 300–400 mg/day, (which amounts to 50% 2,6-xylidine by molar weight) the maximum 2, 6-xylidine exposure would be 2.5–3.5 mg/kg/day, i.e. well below the lowest dose used in the NTP study. Lidocaine has given consistent negative results in a variety of standard *in vitro* and *in vivo* genotoxicity tests (see Supplementary Table 1), and clinical monitoring over 80 years of therapeutic use in humans has not identified evidence to suggest a causal association between lidocaine use and cancer formation (Holdcroft and Nouette-Gaulain, 2014; Friedman and Ury, 1983; Selby et al., 1989; Fuzier et al., 2009). To better understand this dichotomy there is a clear need to examine the genotoxicity and carcinogenicity of 2,6-xylidine including pathways of potential metabolic activation and evidence of a potential threshold for these effects.

2,6-xylidine is included in the SIDS database (SIDS, 2012), and a number of reports have been published reviewing its carcinogenicity and genotoxicity. The most recent review was conducted by the Health Council of the Netherlands (2015) who concluded that "2,6-xylidine is suspected to be carcinogenic to man, and recommends classifying the

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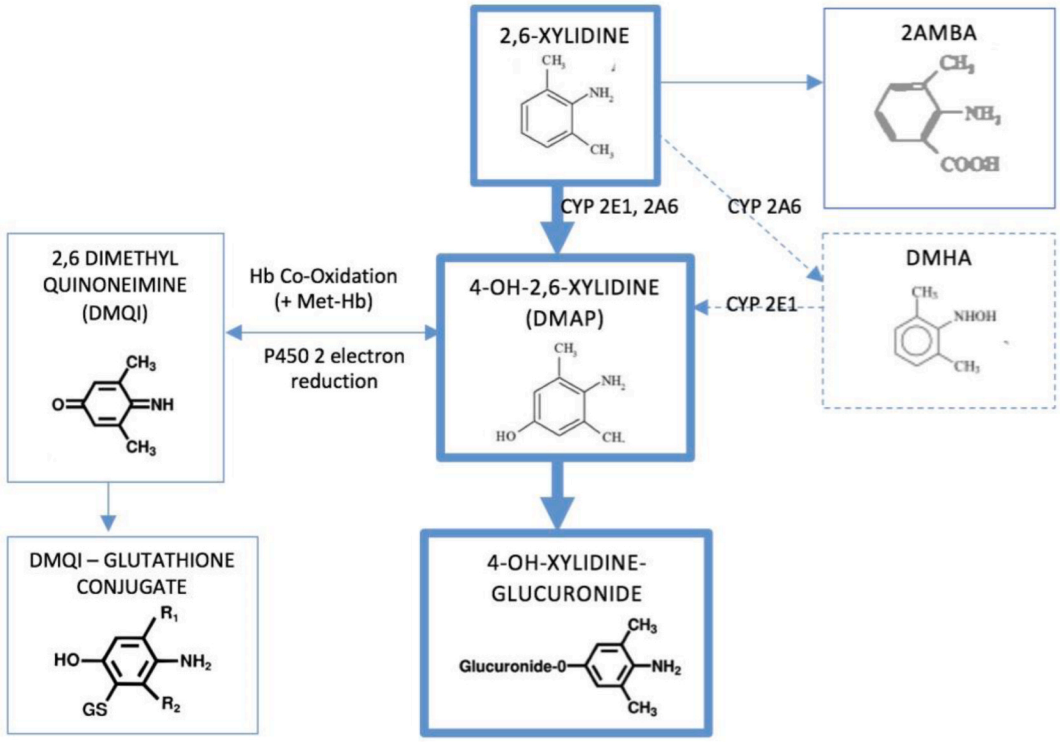
https://doi.org/10.1016/j.yrtph.2020.104838
Received 7 August 2020; Received in revised form 31 October 2020; Accepted 3 December 2020
Available online 7 December 2020

Abbreviations:

2-AMBA	2-amino-3-methylbenzoic acid
8-OH-dG	8-hydroxy-deoxyguanosine
Aprt	adenine phosphoribosyltransferase gene
BMD	benchmark dose
BWL	bodyweight loss
CA	chromosomal aberrations
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
dG	deoxyguanosine
DMHA	dimethylphenyl N-hydroxylamine
DMAP	4-hydroxy-2,6-xylidine or 3,5-dimethyl-4-aminophenol
DMQI	2,6 dimethylxylidine quinoneimine
EMS	ethyl methanesulfonate
GLP	good laboratory practice
Gpt	bacterial guanine-hypoxanthine phosphoribosyltransferase gene
Hb	haemoglobin
Hprt	hypoxanthine-guanine phosphoribosyltransferase gene
IARC	International Agency for Research on Cancer
met-Hb	met-haemoglobin
LOQ	level of quantification
MF	mutant frequency
MN	micronucleus or micronuclei
MTD	maximum tolerated dose
NCE	normochromatic erythrocytes
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NO(A)EL	no-observed adverse effect level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocytes
PoD	point of departure
ROS	reactive oxygen species
TK	toxicokinetic
Tk	thymidine kinase locus
UDS	unscheduled DNA synthesis
WoE	weight of evidence

compound in category 2". The majority of these reports however, summarise results of genotoxicity studies without assessment of the biological relevance of each individual study. Furthermore, in these assessments, N-hydroxylation of 2,6-xylidine to form dimethylphenyl N-hydroxylamine (DMHA, Fig. 1), with subsequent esterification and DNA adduct formation, has been presumed as the putative mechanism of carcinogenicity in the rat, in line with that of carcinogenic polycyclic aromatic amines (Beland and Kadlubar, 1985). This is generally



Solid Blue line = major metabolic pathway documented in vitro (Gan et al., 2001; Parker et al., 1996) and in vivo (Lindstrom et al., 1963; Hardy, 1986; Short et al., 1989).
Thin Blue line = minor metabolic pathway documented in vivo (rats/mice) (Lindstrom et al., 1963; Jefferies et al., 1998)
Dotted Blue line = very minor metabolic pathway, only evident in vitro (Kirkland et al., 2012; Gan et al., 2001), or in vivo (humans) using indirect techniques (Nelson et al., 1978).

Fig. 1. 2,6-Xylidine metabolism – major and minor pathways.

considered a non-threshold, direct genotoxic mechanism of action. Ultimately, however (as detailed below), there is little *in vivo* evidence to support N-hydroxylation of 2,6-xylidine as a putative model of carcinogenicity in the rat. Importantly, metabolic studies identify that, by virtue of its structure, 2,6-xylidine is resistant to N-hydroxylation and instead is metabolised almost exclusively via 4-hydroxylation to an aminophenol (2,6-dimethylaminophenol or DMAP). Aminophenols generate pathology via different metabolic activation pathways (characteristically threshold affected) as compared with N-hydroxylamines. This has important implications that have generally not been considered in previous reviews.

In this paper we review potential pathways of metabolic activation and genotoxicity of 2,6-xylidine and perform a critical review of the published literature pertaining to its genotoxic potential, including the biological relevance of individual studies. Additionally, a new *in vivo* study has been performed to investigate genotoxic dose response in rats and evidence for a metabolic threshold for genotoxic effects, and also to address some identified deficiencies in the database. The data are then assessed in terms of weight of evidence (WoE) for the genotoxic potential of 2,6-xylidine.

2. Review of potential pathways of metabolic activation and genotoxicity of 2,6-xylidine

2.1. N-hydroxylation (formation of DMHA)

N-hydroxylation of polycyclic aromatic amines is the primary step in a well described pathway of metabolic activation, which may result in mutagenesis and carcinogenesis. Following *N*-hydroxylation *in vivo,* co-oxidation reactions with haemoglobin (Hb) result in met-haemoglobin (Met-Hb) and nitrenium ion formation (Fig. 1). Nitrenium ions are "hard" electrophiles which (via esterification) have a propensity to form irreversible covalent bonds with Hb and DNA resulting in adducts. DNA adducts of carcinogenic polycyclic aromatic amines are typically formed via covalent linkage of the amide nitrogen to the C8 of 2′-deoxyguanosine (dG), leading to C8-dG adducts. These are bulky and possess conformational heterogeneity, resulting in a variety of mutagenic outcomes. The most common involve frameshift mutations and base substitutions, predominantly G → T, and also G → C transversions (Kozack et al., 2000). This is a direct, non-threshold mechanism of DNA damage. Monocyclic aromatic amines may also be susceptible to N-hydroxylation predisposing to nitrenium ion, and Hb or DNA adduct formation. There is less evidence however, to indicate these smaller adducts are similarly mutagenic (Neumann, 2005). However, It should be noted that adducts are generally considered a biomarker of exposure rather than effect (Jarabek et al., 2009); they may be rapidly cleared or repaired and do not necessarily lead to persistent genetic damage or mutations.

Nevertheless, in support of the hypothesis that 2,6-xylidine induces genotoxicity via this mechanism: DMHA has been identified as a metabolite of 2,6-xylidine *in vitro* following incubation with rat hepatic microsomes (Kirkland et al., 2012): Synthesized *N*-acetoxy metabolites do form C8-dG adducts when reacted with DNA *in vitro* (Marques et al., 1997); Hb and DNA adduct formation (particularly prominent in ethmoid tissue) has been documented in rats following 2,6-xylidine administration (Bryant et al., 1994; Duan et al., 2008; Short et al., 1989b; Jeffrey et al., 2002), and synthesized DMHA is also reported to be mutagenic in Ames tests (Nohmi et al., 1984; Marques et al., 1997; Jeffrey et al., 2002). Relevance to humans has been concluded by finding DMHA as a metabolite of lidocaine in urine (albeit at extremely minor levels constituting <1% of the dose (Nelson et al., 1978)). Additionally, an "association" between bladder cancer and 2,6-xylidine exposure has been reported (Gan et al., 2004; Tao et al., 2013). This, however, was based on finding elevated 2,6-xylidine haemoglobin (Hb) adducts in bladder cancer patients after diagnosis (and hence many months or years after cancer initiation). As Hb-adducts are short-lived, a causative association cannot be concluded. Critically these studies failed to examine post-diagnosis lidocaine exposure as a confounding variable. Lidocaine is frequently used for pain mitigation in bladder cancer patients, and may cause raised 2,6-xylidine Hb-adducts (Bryant et al., 1994). As such, it is arguably the most likely aetiology of increased Hb-adducts in bladder cancer patients.

Ultimately however, despite the *in vitro* findings above, DMHA has not been detectable as a metabolite of 2,6-xylidine *in vivo* in rats. Metabolic studies (both *in vitro* and *in vivo*) identify that 2,6-xylidine is overwhelmingly metabolized via 4-hydroxylation to DMAP, rather than N-hydroxylation to DMHA. Whilst minor DMHA production has been detectable *in vitro* (Kirkland et al., 2012; Gan et al., 2001), there has been no success detecting any evidence of DMHA production and/or esterification to *N*-acetoxy derivatives *in vivo* in the rat (Lindstrom et al., 1963; Hardy, 1986; Short et al., 1989a). In addition, 2,6-xylidine does not share the same propensity for Hb and DNA adduct formation as other aromatic amines. Sabbioni (1992) identified that when fed to rats orally, 2,6-xylidine did not share the Hb binding capacity of other aromatic amines, noting that the methyl groups in the 2 and 6 positions (either side of the amine) near abolished Hb binding, and appeared to cause steric interference, impeding N-hydroxylation and subsequent formation of reactive nitrenium ions responsible for protein reactivity through acetoxy or sulphate esterification. Fishbein and McClelland (1987) similarly reported a poor yield of nitrenium ion from DMHA in aqueous conditions. Consistent with this, rat studies of DNA adduct formation identified that 2,6-xylidine only induces very weak adduct formation that has generally only been detectable following high dose administration (>60 mg/kg; Duan et al., 2008) and/or following 7–9 days of repeated high repeated dose administration (262.5–310 mg/kg/day; Short et al., 1989b; Jeffrey et al., 2002). Furthermore, there is little evidence to support N-hydroxylation as the putative mechanism of 2,6-xylidine DNA adduct formation. Jones and Sabbioni (2003), for example, did not find evidence of DNA adduct formation in the livers of rats treated orally with high dose DMHA, and DNA adduct characterization studies found a different pattern of adduct formation *in vitro* from reacting DNA with synthesized *N*-acetoxy-2,6-xylidine, than is characteristic of the carcinogenic polycyclic aromatic amines. Although C8dG and C8dA adducts were present, these were in minor proportions, and ring-as opposed to *N*-derived adducts were found to predominate (Gonçalves et al., 2001; Jones and Sabbioni, 2003; Marques et al., 1997). Additionally, in a C57BL/6 mouse study with 2,6-xylidine administered at 83 μg/kg by intraperitoneal dosing, Skipper et al. (2006) reported that DNA adducts, although detectable at low levels in urinary bladder and liver 4 h following administration, were considerably depleted by additional DNA purification (suggesting the possibility of adduct formation with intranuclear proteins rather than DNA), and also showed rapid further depletion over time. DNA adducts were cleared from the bladder with a half-life of 8 h, and from the liver with a half-life of 10 h, inconsistent with long-lasting irreversible covalent DNA binding. Although rapid clearance of DNA adducts does not necessarily imply a lower risk for mutation or cancer, for most identified carcinogens, adducts persist over a period of weeks. As commented by Bieler et al. (2007) "several studies have found a positive correlation between carcinogen–DNA adduct levels and persistence in relation to target organ specificity for tumour formation, suggesting the critical importance of persistent carcinogen–DNA adducts". Finally, although synthesized DMHA (at a concentration of 10 nM (i.e. 1.37 μg)/plate) was found to induce mutagenicity in the Ames test (Nohmi et al., 1984), 2,6-xylidine was not similarly mutagenic when incubated with rat hepatic microsomes, despite the presence of DMHA in biologically produced concentrations (Kirkland et al., 2012). Nelson et al. (1978) furthermore reported that "Preliminary carcinogenicity testing of 2,6-di-methylphenylhydroxylamine through the National Cancer Institute testing program has produced no tumors in Swiss albino mice". Together these findings thus throw considerable doubt on N-hydroxylation as the putative mechanism of genotoxicity and carcinogenicity of 2,6-xylidine in the rat.

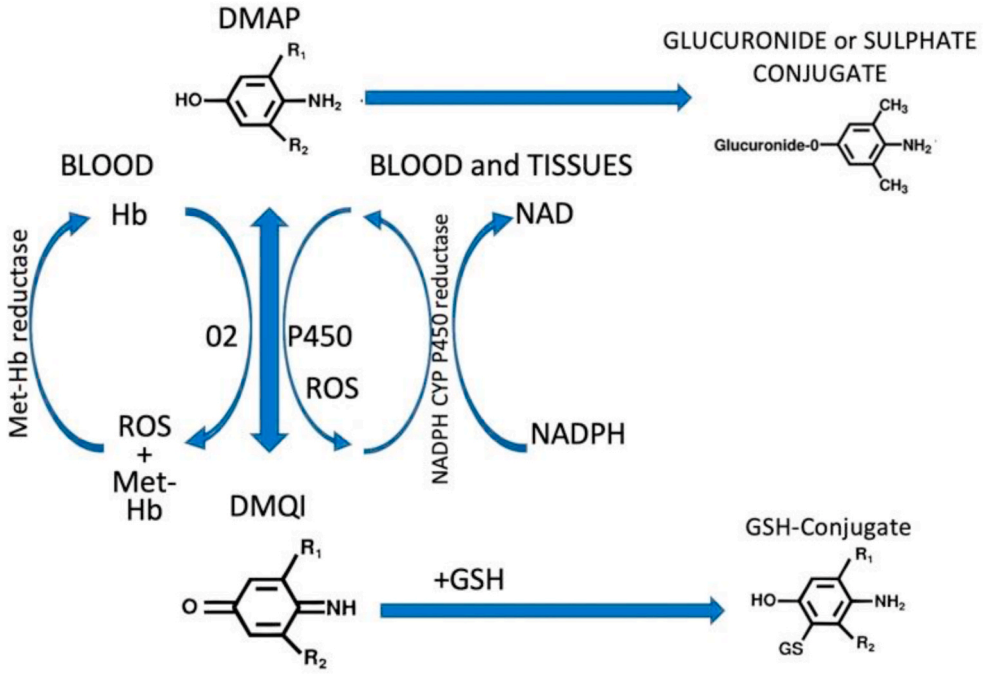


Fig. 2. Pathways of quinone-imine formation from DMAP

2.2. 4-Hydroxylation (formation of DMAP)

An alternate potential mechanism of pathology is via metabolic activation of DMAP to form 2,6-dimethylquinoneimine (DMQI, Figs. 1 and 2). DMAP, the major metabolite of 2,6-xylidine, is an aminophenol, with structural similarity to acetaminophen (also known as 4-acetoxy-*p*-aminophenol or paracetamol). Aminophenols are electrophilic and highly protein reactive. In biological systems this promotes their rapid conjugation with glucuronide and/or sulphate and elimination in the urine, which is one of the major pathways of detoxification and elimination of xenobiotic substances (Bray et al., 1952). At high levels of production however, phase 2 metabolism may saturate. In this situation, aminophenols (in their free state) are subject to co-oxidization reactions (such as with Hb) forming quinone-imines, which are reactive electrophiles and oxidizing agents (Dahlin et al., 1984; Guengerich et al., 1985). Met-Hb and reactive oxygen species (ROS) are concomitantly produced in this reaction, which may be further exacerbated by redox cycling resulting in oxidative stress (Kiese, 1966; Nakajima and Kusumoto, 1963). Quinone-imine formation may also occur in tissues via cytochrome p450 two electron oxidation reactions, similarly resulting in ROS production and redox cycling, albeit without met-Hb formation (Porubek et al., 1986). Oxygen radicals are cytotoxic and are also known to cause DNA damage (G:C → A:T transitions and G:C → T:A transversions), induce C8 DNA adducts and can be a cause of spontaneous mutations (Hsie et al., 1986; Kreutzer and Essigmann, 1998; Suzuki and Kamiya, 2017). Chronic cytotoxicity and/or oxidative injury are well known pathological mechanisms that predispose to carcinogenicity (Okada 2002, 2014; Strzelczyk and Wiczkowski, 2012). The body however has strong anti-oxidant defence mechanisms that prevent and/or rapidly repair oxidative damage (Davies, 2001; Lan et al., 2004).

Unlike nitrenium ions, quinone-imines are "soft" electrophiles. They are Michael acceptors and readily react with soft nucleophiles like sulfhydryl residues on cysteine in glutathione and proteins. Reactions with nitrogen nucleophiles such as lysine and histidine are much slower compared to that of sulfur nucleophilic additions, although they have been reported (Bolton et al., 2017; Ravindra et al., 2016). High affinity for glutathione acts to detoxify quinone-imines by reduction or conjugation and elimination via the mercaptopurine pathway, and prevents redox cycling and associated ROS production. In overproduction scenarios, however, glutathione depletion may occur, resulting in unfettered redox cycling and ROS production sufficient to overwhelm cellular antioxidant defence, leading to secondary cytotoxic and genotoxic effects (Athersuch et al., 2018; Hinson et al., 2010). Amino-phenols thus characteristically act as threshold cytotoxins and secondary genotoxins. For example, activation of acetaminophen to N-acetyl-*p*-quinone-imine (NAPQI) via this pathway is believed to be the putative mechanism of cytotoxicity and DNA injury following paracetamol overdose. Cytotoxic and clastogenic effects may, however, occur at much lower concentrations *in vitro*, since *in vitro* systems do not contain co-factors necessary for phase 2 metabolism and may be deficient in glutathione, methaemoglobin reductase and/or other anti-oxidant substances.

There is considerable evidence to suggest that DMAP may undergo metabolic activation and induce pathology via this pathway *in vitro*, and *in vivo* in rats following high dose 2,6-xylidine administration sufficient to saturate phase 2 metabolism. *In vitro*, in its free state, DMAP is reported to be an unstable molecule that rapidly oxidises non-enzymatically to quinone-imine derivatives (Abdel-Rehim et al., 2006; Tam et al., 1990). In biological systems *in vitro*, in the absence of phase 2 metabolites, DMAP is haem-reactive and a potent inducer of met-Hb (Hartman et al., 2017) as well as intracellular ROS production with associated clastogenic effects (Chao et al., 2012). *In vivo* however, DMAP undergoes extensive and rapid glucuronide and/or sulfur conjugation, such that there is negligible availability of free compound to participate in haem co-oxidation reactions. Conjugation greatly reduces protein reactivity, and promotes its excretion in the urine (Skipper et al., 2010). In rats, following 2,6-xylidine administration, DMAP is found in urine almost exclusively (>99%) in conjugated form (Lindstrom et al., 1963). This is also the case in humans following lidocaine administration, (Keenaghan and Boyes, 1972; Tam et al., 1987; Nelson et al., 1977). Following high dose 2,6-xylidine administration to rats however, there is evidence to suggest that phase 2 metabolism of DMAP may saturate, such that haem co-oxidation may occur, resulting in met-Hb and DMQI formation. DMQI-glutathione conjugates have been detected in urine and hepatic tissue of rats following intraperitoneal doses of 90 mg/kg in rats (Jeffries et al., 1998; Rosen et al., 1984) and a statistical increase in met-Hb formation is seen following 80 mg/kg intravenous (i.v.) doses of

2,6-xylidine in rats (Lindstrom et al., 1969). Repeat dose toxicity studies identify an oral dose of 50 mg/kg 2,6-xylidine as a no-effect level (NOEL) for met-Hb production in rats (Yamaguchi et al., 2005). Chronic dosing at, or acute dosing significantly above these threshold levels therefore has the potential to saturate glutathione binding and deplete glutathione, leading to cytotoxic and genotoxic (most likely clastogenic) effects. In this regard, it is notable that 2,6-xylidine Hb and DNA adducts have only been detected in rats following high and/or prolonged dosing, seemingly at or above levels associated with saturation of phase 2 metabolism of DMAP and evidence of DMQI/Met-Hb production. Bryant et al. (1994) reported 2,6-xylidine adduct formation in rats follows intraperitoneal doses of 900 mg/kg. As quinone-imines may bind to Hb via thioether bonds, 2,6-xylidine Hb adducts detected following such high dose administration may indicate DMQI- rather than DMHA-derived adducts, as previously presumed. Similarly, given the lack of significant formation of DMHA *in vivo*, and that ring-, instead of N-derived DNA adducts predominate, and DMQI – histone binding is reported (Ravindra et al., 2016), it is possible that these could derive from DMQI reaction with intranuclear proteins (as contaminants), and/or with DNA, although evidence of direct DNA binding currently remains elusive (Skipper et al., 2010).

3. Review of published data pertaining to the genotoxicity of 2,6-xylidine

Given the reported carcinogenicity of 2,6-xylidine in rats, and possible modes of action, a critical review of its genotoxic potential is important. 2,6-Xylidine has been tested in a number of *in vitro* and *in vivo* test systems for gene mutations, chromosomal damage and DNA damage. Some tests have also been performed on DMHA and DMAP. The various published *in vitro* and *in vivo* genotoxicity studies (as of May 2020) are summarised in Supplementary Table 2. Comments on the strengths and weaknesses of these studies, and their biological relevance, are given below.

3.1. Bacterial reverse mutation (Ames) tests

3.1.1. With 2,6-xylidine

Standard Ames tests conducted prior to the NTP carcinogenicity study in 1990 were predominantly negative. Hartman et al. (1979), Nelson et al. (1978), Zimmer et al. (1980), and Florin et al. (1980) all reported negative results in Ames tests. Nohmi et al. (1984) also reported no mutagenic activity for 2,6-xylidine in the absence of S9, however noted a low mutagenic activity (39 revertants/μM) in *S. typhimurium* strain TA100 in the presence of 30% rat liver S9. (The metabolic activation conditions were described in Nohmi et al., 1981). No conclusions of biological relevance can be drawn in view of the weakness of the response and the high S9 concentration. However, it should be noted that the range of strains used in all of the above referenced studies did not meet current OECD guideline recommendations (OECD, 1997), in that strains to detect chemicals acting via oxidative mechanisms, inducing cross-links or inducing mutations at A-T-rich regions of the DNA (such as *S. typhimurium* TA102, or *E. coli* WP2 strains) were not included.

Ames tests were performed and reported as part of the NTP carcinogenicity study report (NTP, 1990), but with no detail on methodology. *S. typhimurium* strains TA100, TA1535, TA1537, TA98 were studied with and without rat and hamster S9. All were clearly negative, but again neither TA102 nor an *E. coli* strain was included. Additional NTP studies were subsequently undertaken and reported by Zeiger et al. (1988). Studies were undertaken in 3 different laboratories with *S. typhimurium* strains TA97 (one laboratory), TA98, TA100, TA1535 (all three laboratories) and TA1537 (one laboratory), with and without 3 different concentrations (5, 10 and 30%) of hamster and rat liver S9. Again, TA102 or an *E. coli* strain were not included. Modified standard reporting was used and included categories of "equivocal" (increase less than 2× control and sporadic, not reproducible or not dose dependent) and "weakly positive" (increase less than 2 × control, dose dependent, reproducible), which the authors noted to be highly subjective and to vary between laboratory sites. Using this new terminology, they concluded 2,6-xylidine was non-mutagenic when tested with 10% S-9 at one laboratory (TA98, TA100, TA1535), and weakly mutagenic (when tested with 30% S-9) at the other 2 sites. (A more detailed review of the data on the NTP website and in Zeiger et al. (1988) is given in Supplementary Document 1).

In addition, a "weakly positive" response (<1.5-fold increases over concurrent controls) was subsequently reported for 2,6-xylidine by Kugler-Steigmeier et al. (1989), in *S. typhimurium* strain TA100 with 20% rat S9. Results were negative in strain TA98. Weakly positive results (<2 × control) in TA100 and (<3 × control) in TA1535 with metabolic activation (rat S9), were also reported by Masumori et al. (2005a), in studies undertaken for the Japanese Chemical Evaluation Database under OECD Guideline 471 (OECD, 1997).

It is usually accepted that 2-fold increases in TA100 revertants, or 3-fold increases in TA1535 revertants, constitute biologically meaningful positive responses, but reproducible increases of less than these levels, particularly if accompanied by evidence of a dose response, cannot be considered clearly negative. Thus, in laboratories that used 30% hamster S9 in the studies mentioned above and further detailed in Supplementary Document 1, biologically meaningful responses in TA100 revertants were seen. In other experiments and other laboratories, with other strains and with different activation conditions, there were weaker or equivocal responses, but overall, the data do indicate weak (<2 × control), but reproducible mutagenic responses for 2,6-xylidine in TA100 under non-standard metabolic activation conditions (20–30% S9).

In the most recent and comprehensive study, undertaken to comply with OECD (1997) guidelines, Kirkland et al. (2012) obtained clearly negative results in Ames tests using all of the currently required strains (TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*) in the absence of S9, and in the presence of 10 and 30% rat S9 or 30% human S9. This study included bacterial strains over-expressing N-acetyl transferase (YG1024 and YG1029), and DMHA was documented to be metabolically produced and present in the media.

Hamster S9, which had given some weakly positive responses in the NTP studies (Zeiger et al., 1988) was not used in the studies by Kirkland et al. (2012), since this is not recommended for routine regulatory testing. Furthermore, the metabolism of 2,6-xylidine may vary considerably between species. Rats and humans are known to metabolise 2, 6-xylidine almost exclusively via 4-hydroxylation and/or phase 2 conjugation. Dogs however, produce a wider range of metabolites *in vivo*, including 2-amino-3-methylbenzoic acid (2-AMBA), along with trace amounts of 2,6-dimethylnitrosobenzene (Hardy, 1986; Short et al., 1989a). Guinea pigs have reduced capacity for 4-hydroxylation but increased *N*-conjugation (Keenaghan and Boyes, 1972). The metabolism of 2,6-xylidine in hamsters has not been examined, and may not reflect rat or human metabolism or be relevant for assessment of human mutagenic risk. Therefore, the recent robust negative results of Kirkland et al. (2012) with human and rat S9 (even at elevated levels such as 30%) should be seen as more relevant than the weak positive results with hamster S9.

3.1.2. With the N-hydroxy metabolite (DMHA)

Nelson et al. (1978) reported no genotoxicity for DMHA in *S. typhimurium* strain TA1538 ± S9, however this may not be the most sensitive strain. DMHA was found to be mutagenic in *S. typhimurium* TA100 (Nohmi et al., 1984; Marques et al., 1997; Jeffrey et al., 2002), however all 3 studies used synthesized DMHA at concentrations that may not be achievable when formed in biological systems by metabolism of 2,6-xylidine, as discussed in Kirkland et al. (2012). Marques et al. (1997) reported that synthesized DMHA was the most mutagenic of several mono and di-substituted anilines in *S. typhimurium* TA100, but

that this did not result from a higher efficiency of adduct formation; DNA adduct efficiency of *N*-acetoxy-2,6-xylidine was 2% versus up to 9% in less or non-mutagenic arylamines. Similarly, Nohmi et al. (1984) reported strong (100-10,000-fold increases) genotoxicity for DMHA in strain TA100 with or without S9. In the same set of studies, however, DMHA did not cause reductions in the activity of *Bacillus subtilis* transforming DNA. The authors concluded that while other phenylhydroxylamine derivatives caused DNA damage through the generation of active molecular species, such as nitrenium ions, without any enzymatic activation, DMHA was an exception, and appeared to require further metabolic activation by bacterial enzymes to stimulate mutagenesis. A possibility in this regard, is conversion to DMAP via "Bamberger re-arrangement" (the process of chemical re-arrangement of phenylhydroxylamines to aminophenols). DMHA is known to be rapidly converted to DMAP in the presence of liver microsomes and CYP 2E1 (Vmax 6.9 nmol/min/mg, Km 500 μM) (Gan et al., 2001). Bamberger rearrangement also occurs in bacterial cells catalyzed by lyase and mutase bacterial enzymes, as detailed by Nadeau et al. (2003).

The recent studies of Kirkland et al. (2012) investigated biological production of DMHA from 2,6-xylidine *in vitro* and also included tests with bacterial strains that over-express N-acetyl transferase. These authors showed that DMHA was formed from high concentrations of 2, 6-xylidine by rat and human S9, (predominantly 30% S9), and analysed the P450 enzymes involved and the Michaelis Menten kinetics of the reactions. The results indicated very low production levels of DMHA from 2,6-xylidine, with a Km of 46 and a Vmax of 0.04 nmol/min/mg. However, despite the presence of DMHA, mutagenic responses were not observed in strain TA100, or in the YG1024 and YG1029 strains overexpressing N-acetyltransferase. This is clearly in contrast to the findings from Nohmi et al. (1984), Marques et al. (1997), and Jeffrey et al. (2002), despite all the experiments in Kirkland et al. (2012) including pre-incubation methodology, which should have ensured the bioavailability of any metabolites.

An explanation may revolve around the disparity in concentration of DMHA (per plate) employed in the trials, based on whether it was formed "biologically" or added "extraneously" (see Supplementary Table 2 for comparison of concentrations). The kinetics of DMHA formation in biological systems, in contrast to instant addition of DMHA, may also be a factor. Whilst adduct-related genotoxicity is generally regarded as a non-threshold process, genotoxicity secondary to ROS production will only occur at concentrations which overwhelm the cellular anti-oxidant defence capabilities. ROS production may not occur from low levels of DMHA produced from biological systems (such as used by Kirkland et al., 2012) and/or may be insufficient to overwhelm anti-oxidant processes in the cultured cells and hence may not result in genotoxic effects. By contrast, ROS production from rapid exposure to higher concentrations of DMHA, either added directly or through conversion to DMAP, may rapidly overwhelm the anti-oxidant capacity of the *in vitro* system and result in oxidative effects.

3.2. Studies in mammalian cells in vitro

3.2.1. Gene mutation tests in mammalian cells

3.2.1.1. With 2,6-xylidine. A mouse lymphoma *Tk* mutation study, initially reported in an abstract by Rudd et al. (1983) was subsequently reported in more detail in a retrospective review by Mitchell et al. (1997), where somewhat more detail was given. At the highest concentration tested in the absence of S9 (0.8 μL/mL), the response was considered "inconclusive or inadequately tested", most likely indicating insufficient concentrations were tested over the critical range of 10–20% relative total growth (RTG) to be able to evaluate the results as clearly positive or negative. A "limited positive" result was concluded in the presence of induced S9 but only at the top concentration, 0.25 μL/mL (approximately 250 μg/mL), and this was defined as a concentration-related increase in mutant frequency (MF), where the spontaneous mutant frequency was $>20 \times 10^{-6}$, and an induced MF of $>70 \times 10^{-6}$ was observed at RTG >10% for at least one concentration. This study was performed prior to current recommendations (OECD, 2016a), and before it was common practice to "size" the mutant colonies. However, it appears (from the Rudd et al., 1983 abstract) that small colony mutants were predominantly induced indicating a clastogenic mode of action. It is not known how much cytotoxicity was induced at the top concentration of 2,6-xylidine, and therefore it is not known whether the results would be considered biologically relevant when judged using current criteria.

3.2.1.2. With 2,6-xylidine and/or metabolites DMHA, DMAP. Chao et al. (2012) tested 2,6-xylidine and 3,5-xylidine (0–1000 μM), as well as their aminophenol and *N*-hydroxy metabolites (5–100 μM and 5–250 μM respectively) in the presence of a human S9 mix, for cell survival and mutation in Chinese hamster ovary (CHO) cells at the *Aprt* locus in AA8 and UV5 CHO cells, and at the *Gpt* locus in AS52 cells. ROS production and DNA damage were also examined in AS52 cells in a comet assay in the absence and presence of ascorbate and *N*-acetyl cysteine (NAC), a readily absorbed glutathione precursor. Since preliminary studies showed "no material differences" between 2,6- and 3,5-xylidine, the more detailed studies, and much of the data presented in the paper, are from the latter. Unfortunately, the authors do not describe what they consider to be "material differences". Furthermore, the potential for *N*- versus 4-hydroxylation will differ between these 2 isomers, with N-hydroxylation being more likely with the 3,5-isomer due to less steric hindrance from the positions of the methyl groups. Steric factors may also affect glutathione affinity (Rosen et al., 1984), such that mutagenic activity of 3,5-xylidine may exceed that of 2,6-xylidine. No mutant frequency data were presented for 2,6-xylidine, however characterization of the nature of mutations induced by 2,6-xylidine (with S9) was undertaken by sequencing some *gpt* mutant colonies from the CHO AS52 cells (presumably from a preliminary experiment). There was a significant increase in G:C to A:T transitions, which is the most common base substitution induced by oxidative stress (Kreutzer and Essigmann, 1998). It is not known under what conditions (concentration, cytotoxicity) these mutants were induced, or the magnitude of any mutagenic responses. It was reported that 2,6-xylidine (0–100 μM) in the presence of human S9 did not induce ROS production, but no details were given. DMAP and DMHA, however, induced prominent ROS production over the same dose range (further detailed below). Since AS52 cells can detect both clastogens and gene mutagens, it is not known what mode of action may have led to any mutagenic responses seen with 2,6-xylidine.

The aminophenols were far more toxic and induced mutations at lower concentrations than the N-hydroxylamines, and both hydroxy derivatives were more toxic than the anilines activated by exogenous S9. DMAP and DMHA induced intracellular ROS production in a dose dependent manner over the dose range studied (up to 250 μM). The aminophenols of both isomers exhibited potent ROS production at concentrations up to 25 μM (3.4 μg/mL), exceeding by several fold the ROS induced by higher concentrations (100 μM; 13.7 μg/mL) of the N-hydroxylamines. Similarly, using the comet assay, DMAP induced a statistically significant increase in DNA strand breaks at 50 μM (6.8 μg/mL, the highest level tested). Comet induction doubled, (but did not reach statistical significance) for DMHA at 250 μM (34 μg/mL). Ascorbate and NAC were protective against strand breaks in both cases. There was thus a strong association between ROS production and mutagenicity in the CHO AS52 cells. It is relevant to note that AS52 cells are known to be sensitive to oxidative injury (Kim et al., 2003), a finding that was corroborated by the trials above and identified to be secondary to ascorbate deficiency. Mutagenic effects seen in CHO AS52 cells were not seen in AA8 or UV5 cells, unless first depleted of ascorbate in this series of studies. Together with the findings from cell lines treated with 3, 5-xylidine and its metabolites, (including absence of evidence of

increased mutagenicity of the *N*-hydroxy metabolite in cells lines over-expressing *N*-acetyl transferase 2 (NAT2)), the authors concluded *"Comparative evaluation of the results indicates that the principal mechanism of mutagenic action is likely to be through redox cycling of intracellularly bound aminophenol/quinone imine structures to generate ROS rather than through formation of covalent DNA adducts"*.

Jang et al. (2006) examined various metabolites 2,6- and 3,5-xylidine for induction of cytotoxicity and *Hprt* mutations in human lymphoblastoid TK6 cells and HCT116 colon cancer cells. Further details were reported by Skipper et al. (2010). All metabolites induced dose and time dependent cytotoxicity, but aminophenols were more cytotoxic than *N*-hydroxy metabolites, and 3,5- were more cytotoxic than 2,6-xylidine metabolites. When tested for *Hprt* mutagenicity, Skipper et al. (2010) reported that both the aminophenol and *N*-hydroxy metabolites of 3,5-xylidine were clearly mutagenic but that DMHA was not mutagenic over the same dose range. (Cytotoxicity and mutagenicity of 2, 6-xylidine and DMHA in AS52 cells was noted in brief, as presumably later reported in detail in Chao et al. (2012) as discussed above).

3.2.2. Chromosomal aberration (CA) tests in mammalian cells

There are only 2 publications investigating induction of chromosomal aberrations (CA) in mammalian cells *in vitro*, both with 2,6-xylidine. Masumori et al. (2005b) treated Chinese hamster lung (CHL/IU) cells for 6 h with 2,6-xylidine at concentrations of 0, 303, 606 and 1212 μg/mL in the absence of S9, and at 0, 633, 744 and 876 μg/mL in the presence of induced rat liver S9. According to the Japanese Toxicity Testing of Environmental Chemicals Data Base, (also referenced in the SIDS report, 2012), the study was undertaken under GLP standards to OECD Guideline 473 (OECD, 1997b), but did not comply fully because it only included short term (6 h) exposures. Reduction in cell growth to 50% was seen at around 1212 μg/mL in the absence of S9 and somewhere between 744 and 876 μg/mL in the presence of S9. A dose-dependent increase in structural CA was observed, reaching highly significant levels at the top concentration in the absence of S9, and at the top 2 concentrations in the presence of S9. The aberrations consisted principally of chromatid breaks and exchanges.

Galloway et al. (1987) tested 2,6-xylidine (+/-S9) for induction of CA in CHO cells as part of the NTP trials (NTP, 1990). The concentrations tested ranged from 900 to 1200 μg/mL and from 1200 to 1400 μg/mL in the absence and presence of induced rat liver S9 respectively. Precipitate was present at all concentrations tested, with and without S9. The latest revisions to OECD guidelines recommend that, for mammalian cell tests with insoluble substances, the highest concentration tested should be the lowest concentration exhibiting precipitate at the end of treatment, but at the time these studies were conducted this limitation was not specified in the OECD guideline (OECD, 1997b). In this respect the results may be questionable. Whilst a slight increase in CA frequency (to 8% cells with CA, excluding gaps and endoreduplication) was seen at 1000 μg/mL in the absence of S9, this was compared to a very low solvent control frequency (1% cells with CA), and frequencies as high as 8% had been seen in solvent control cultures in other experiments reported in the same publication. Therefore, this mid-dose level response may well be considered not to be biologically relevant. The most significant increase in CA frequency (-S9) was at 1200 μg/mL (19% cells with CA, excluding gaps and endoreduplication), but only 16 cells (out of a target of 100 cells) could be scored, and since there was no concurrent measure of cytotoxicity, it is highly likely that significant cell killing occurred at this concentration, which could indirectly lead to increased chromosomal breakage. In the presence of S9, significant increases in % cells with CA, excluding gaps and endoreduplication (19–88%) were seen at all 3 concentrations analysed. However, in the absence of a concurrent measure of cytotoxicity, the possibility of chromosomal damage at some of the concentrations tested being an indirect consequence of high levels of cell killing cannot be ruled out.

3.3. In vivo studies

All published *in vivo* studies were performed with 2,6-xylidine, and none were performed with the metabolites.

3.3.1. Mouse micronucleus (MN) tests in bone marrow or peripheral blood

Parton et al. reported on 2 different study designs to investigate the induction of MN in mouse bone marrow by 2,6-xylidine. In the first study (Parton et al., 1988) male ICR mice were dosed once by oral gavage with 87.5, 175 and 350 mg/kg, the top dose being approximately 50% of the oral LD_{50}. Animals were sacrificed 24, 48 and 72 h later, smear slides made and stained with Wright's Giemsa stain, and 1000 polychromatic erythrocytes (PCE) per animal scored for MN. This is much lower than the 4000 PCE/animal currently recommended (OECD, 2016b). Bone marrow toxicity was determined as the ratio of PCE to normochromatic erythrocytes (NCE). MN frequencies in vehicle control animals were normal and were significantly increased by positive control treatment (100 mg/kg cyclophosphamide, sampled at 24 h). There was no evidence of bone marrow toxicity from 2,6-xylidine treatment based on PCE:NCE ratio, and no plasma samples were taken for determination of the presence of 2,6-xylidine, so exposure of the bone marrow was not verified. MN frequencies in 2,6-xylidine-treated animals were generally similar to vehicle controls at all doses and sampling times. Although a 2.2-fold increase in MN frequency was seen at the top dose at the 48-hr. sampling time, it was not significantly different, and the authors concluded the result was negative.

In the second study Parton et al. (1990) dosed male ICR mice by oral gavage on 1, 2 or 3 consecutive days with 2,6-xylidine at 75 or 375 mg/kg/day. Animals were sacrificed 24 h after the last dose, smear slides made and stained with Wright's Giemsa, and 2000 PCE/animal scored for MN. Although numbers of PCE scored are an improvement on the earlier study, this is still less than currently recommended (OECD, 2016b). No bone marrow toxicity was evident according to PCE:NCE ratios, and no plasma samples were taken for determination of the presence of 2,6-xylidine, so exposure of the bone marrow was not verified. MN frequencies in vehicle control animals were a little higher than in the previous study, but still considered normal, and were significantly increased by positive control treatment (100 mg/kg cyclophosphamide given once only and sampled at 24 h). MN frequencies in 2,6-xylidine-treated mice were similar to controls at both dose levels and after 1, 2 or 3 doses, and there were no significant differences.

Kohara et al. (2018) also reported negative results from a mouse bone marrow micronucleus test. Male ddY mice (the same animals as used in a comet assay, discussed below) were administered 200 mg/kg 2, 6-xylidine by oral gavage once per week for 4 weeks. Bone marrow cells were collected 24 h after the final dose. There were 3 males per group and no concurrent positive control. Only 1000 PCE/animal were scored for MN, which is much lower than the 4000 PCE/animal currently recommended (OECD, 2016b). No bone marrow toxicity was evident, and no plasma samples were taken for determination of the presence of 2, 6-xylidine, so exposure of the bone marrow was not verified. There was no increase in MN PCE following treatment with 2,6-xylidine.

Kohara et al. (2018) also reported negative results in a mouse MN test in peripheral blood PCE (reticulocytes) of transgenic Muta^{TM}Mouse. Mice received 2,6-xylidine by oral gavage on a single occasion at a dose of 100 mg/kg. Cells were examined for MN in the peripheral blood 48 h following treatment. There were 5 animals per group, with positive and negative control groups, although the positive control data were not reported. It is not clear how many cells were scored, or whether they were scored manually or by flow cytometry. There was no increase in MN frequency above concurrent control.

In the latest version of the OECD guideline (OECD, 2016b) it is stressed that negative results can only be considered meaningful if there is evidence of bone marrow exposure. None of the above studies included any measurement of test chemical in plasma, and, as stated

above, there was no bone marrow toxicity. There were no 2,6-xyidine absorption data reported in these mouse studies, however, 2,6-xylidine is rapidly and completely absorbed following oral dosing in rats (IARC, 1993). Rapid absorption with widespread tissue distribution, (including to bone marrow) has been documented in rats fed 63 mg/kg radio-labelled 2,6-xylidine by gavage (NTP, 1990), and doses of 226 mg/kg/day by gavage have been documented to result in plasma levels of 0.36 μg/mL in rats (Yasuhara et al., 2000), and therefore it is reasonable to assume that absorption from the GI tract into the systemic circulation does occur following oral dosing in rodents. Thus, the bone marrow would have been exposed during these *in vivo* mouse MN studies.

3.3.2. *In vivo/in vitro unscheduled DNA synthesis (UDS) test in rat liver*

The *in vivo/in vitro* rat liver UDS test became popular in the late 1980s to early 1990s because it provided an additional tissue (other than bone marrow or blood) in which to detect genotoxic effects in rodents, and was particularly useful at detecting hepatocarcinogens that produced only weak or no effects on MN induction in peripheral tissue such as bone marrow. However, its sensitivity has been questioned (see Kirkland and Speit, 2008; Hardy et al., 2017), and it is less widely used today, having been superseded by new tests in regulatory strategies, such as rodent transgenic mutation assays and the comet assay, which provide improved sensitivity and opportunities to sample a much wider range of tissues. Nonetheless, a UDS test on 2,6-xylidine could be considered relevant since the liver will be a primary site of metabolism of 2,6-xylidine, particularly in rats (the only species in which carcinogenicity has been documented).

Mirsalis et al. (1989) dosed Fischer 344 rats on a single occasion by oral gavage with 2,6-xylidine at 40, 200 and 850 mg/kg. Animals were sampled 2 and 12 h later, livers were perfused, suspensions of hepatocytes seeded on to coverslips, and incubated with ^{3}H-thymidine to be incorporated into any replicating DNA. UDS was scored autoradiographically, counting the numbers of silver grains over the nucleus and over 2 comparable areas of cytoplasm for a total of 150 cells per animal. Net nuclear grain counts (nucleus minus cytoplasm) in vehicle control animals were <zero (which is normal), and significant responses (high net nuclear grain counts) were induced by the positive control chemicals (dimethylnitrosamine and 2-acetaylaminofluorene). Net nuclear grain counts in all groups of 2,6-xylidine-treated animals were <zero and therefore similar to vehicle controls. Although a negative result was correctly concluded, and, as discussed above, it is clear that exposure of the liver would have occurred following oral dosing, negative results with the UDS assay should be viewed with caution.

3.3.3. *Transgenic mouse gene mutation assay*

Kohara et al. (2018) dosed male Muta^{TM}Mice with 100 mg/kg 2, 6-xylidine given weekly for 4 weeks by oral gavage. There were 4–5 mice per group. Positive controls received benzo(a)pyrene and negative controls received the vehicle (olive oil). The DNA was extracted from the nasal tissues, bone marrow, and liver on day 7 after the last dosing. This treatment and sampling regime is somewhat different from that recommended in OECD guideline 488 (OECD, 2013). Lambda phage containing the *LacZ* transgene were prepared by the *ex vivo* packaging method, and appropriate strains of *E. coli* C were infected with the reconstituted lambda phage. *LacZ* mutations were measured in *galE*$^{-}$ bacteria, and *cII* mutations were measured in G1225 (*hfl*$^{-}$) bacteria. Numbers of total plaques and numbers of mutant plaques were counted, and the mutation frequency (MF = number of mutants/total number of plaques) was calculated. In addition, the *cII* sequence was analyzed by an ABI 310 Genetic Analyzer. There was no increase in the frequency of *LacZ* or *cII* gene mutations in the liver or bone marrow (n = 5 mice), however there was a marginal increase in mutation frequency of both genes in the nasal tissue (4 out of 5 treated mice provided acceptable data), reaching 2× the control value (3 out of 5 control mice provided acceptable data), although this did not reach statistical significance for the *cII* gene. The mutations were predominantly A:T to G:C transitions and G:C to T:A transversions. It should be noted that no historical control mutant frequency data were presented, so the biological relevance of this increase is unclear.

3.3.4. *Comet assays in multiple tissues of mice*

Kohara et al. (2018) dosed male ddY mice with 2,6-xylidine by oral gavage at 200 mg/kg (10 mL/kg) on a single occasion and bone marrow, liver, lung and kidneys were isolated 3 and 24 h after the last dosing for detection of DNA damage (the single cell gel/comet assay). There were 3 males per group, but no positive control. The numbers of cells scored was given as 500 cells/group, and the comets were categorized into 5 different classes, dependent on extent of DNA migration, rather than the more conventional %tail DNA or tail moment measures. Positive results were obtained in lung, kidney, and liver at 3 h But were not observed at 24 h after the last dosing. With so little detail it is difficult to assess whether the study would comply with the OECD Guideline 489 (OECD, 2016c) that was subsequently developed and adopted.

Sasaki et al. (1999) tested 2,6-xylidine for induction of DNA strand breaks (comet assay) in 8 different mouse tissues. Male ddY Mice (n = 4 per group) were dosed orally, by gavage, with a single dose (the maximum tolerated dose, 350 mg/kg, which is 50% of the LD_{50}), and tissues were sampled 3, 8 and 24 h later. Animals dosed with 2,6-xylidine showed reddish staining of urine, which may be indicative of haemolysis. Overt cyanosis due to met-haemoglobinaemia occurred in aniline-treated mice dosed at this level in the same publication, and it is thus likely that sub-clinical met-Hb formation was also induced in 2, 6-xylidine-treated animals (this dose level is 7× the no-effect level for met-Hb formation in rats). Even low levels of met-Hb can cause the oxygen dissociation curve to shift towards the left, which can result in tissue anoxia. Furthermore, the presence of met-haemoglobinaemia indicates the co-production of ROS during haem co-oxidation reactions. Untreated animals were used as a zero-time control. Isolated nuclei (50/tissue) were scored for comets. This is slightly unusual since most practitioners use whole cells, but, as stated in OECD guideline 489 (OECD, 2016c), both isolated nuclei and whole cells are considered equally acceptable. The authors express comet results as tail length, whereas the recommended parameters, % tail DNA or tail moment, take into account the amount of DNA in the tail as well as its length. The results with 2,6-xylidine were unusual. Statistically significant increases in comet tail length were seen in stomach and bladder at the 8-h sampling time only – but were negative at 3 and 24 h, which are the recommended sampling times, according to OECD guideline 489 (OECD, 2016c). It is difficult to understand the biological relevance of a DNA strand breakage result only seen at a non-standard sampling time. Significant increases in comet tail length were seen at 3 and 8 h (but not 24 h) in brain, and at 8 and 24 h in lung tissue. Although the tail lengths were greater than were seen in untreated animals (zero-time controls), and must therefore be considered positive responses, the DNA strand breaks were only seen in tissues that did not correspond with known tumour risk in rats, (although nasal tissue was not examined). In mice 2, 6-xylidine had no tumour modifying effect in NNK-induced pulmonary proliferative lesions in rasH2 transgenic and non-transgenic CB6F1 mice, despite exposure to doses of 3000 ppm for 26 weeks, or 2000 ppm for 53 weeks, (Takahashi et al., 2003), although it did promote inflammation, metaplasia and hyperplasia in the nasal tissue of NNK-treated rasH2 mice, and proliferation of Bowmans glands in rasH2 and non-transgenic mice (Minemura et al., 2003). In the paper by Sasaki et al. (1999), the comet assay was positive with all chemicals that form met-Hb, and may therefore have been a marker of acute oxidative stress. The unusual time pattern of responses seen with 2,6-xylidine could be indicative of an acute oxidative injury event which was subsequently resolved. Sections of each tissue were fixed, embedded and sectioned for histopathological examination, but no microscopic signs were observed following any treatment, implying that there was no significant induction of necrotic/apoptotic or degenerated cells, i.e. the comet responses

could not be attributed to general cytotoxicity. However, the comet assay is an indicator test and, like the UDS assay, detects DNA damage which might be effectively repaired or be lethal, and does not necessarily indicate an ability to induce stable genetic alterations (such as mutations).

3.4. Summary and conclusions from critical review of the literature

2,6-xylidine showed weakly positive responses in TA100 (and once in TA1535) in the presence of non-standard metabolic conditions (hamster S9; 20 or 30% rat S9) in some trials, while in other tests, including the most recent study carried out to current OECD guidelines, the results were clearly negative. 2,6-xylidine induced chromosomal aberrations in hamster ovary and lung cells *in vitro* at high concentrations or under cytotoxic conditions, and induced gene mutations in eukaryotic test systems *in vitro*, associated with evidence of ROS production and oxidative injury. *In vivo*, 2,6-xylidine was considered non-clastogenic in micronucleus assays, in the peripheral blood and bone marrow of mice after oral doses up to a dose of 375 mg/kg, however, no measurement of test chemical in plasma was made in these studies. An oral *in vivo* UDS assay in rat liver was clearly negative up to a dose of 850 mg/kg, however, exposure of the liver was not checked. A transgenic rodent gene mutation assay was weakly positive (2× control) in nasal tissue after 4× weekly oral doses of 100 mg/kg, but bone marrow and liver were negative. Although AT to GC transitions and GC to TA transversions were observed, the result is of questionable biological relevance because of the absence of historical control mutant frequency data. Two *in vivo* comet assays with 2,6-xylidine were reported to be positive, but the tissues that responded and the timings of the responses were inconsistent and difficult to interpret. The doses used may have induced acute systemic toxicity.

In vitro studies with DMHA showed that genotoxic effects are present in prokaryotic and eukaryotic test systems using synthesized DMHA, but not when it was generated biologically within the test system. DMAP was shown to be more toxic and more mutagenic than DMHA, and both hydroxy derivatives were more toxic than metabolically-activated 2,6-xylidine. In an *in vitro* comet assay with DMHA and DMAP, the positive responses were attributed to redox cycling of intracellularly bound aminophenol/quinone imine structures to generate ROS.

Overall, there is evidence that 2,6-xylidine has weak genotoxic characteristics *in vitro* and is able to induce DNA damage *in vivo*, following high dose administration, however, there are no clear positive findings that 2,6-xylidine exposure results in lasting genotoxic effects due to stable genetic damage. The results would support a threshold-dependent metabolic activation pathway, such as may occur secondary to metabolic activation of DMAP to DMQI with redox cycling and ROS production, only evident at doses or concentrations sufficient to overwhelm phase 2 metabolism of DMAP and glutathione detoxification of DMQI. Furthermore *in vivo*, anti-oxidant defence mechanisms exist to prevent, and/or rapidly repair oxidative DNA injury (Davies, 2001; Lan et al., 2004) and therefore any cytotoxic and/or genotoxic effects resulting from ROS production would also be expected to exhibit a threshold.

Given the confusing pattern of *in vivo* results with 2,6-xylidine, the use of methods that do not comply with the most current guidelines, and the absence of systemic exposure data, it was felt important to conduct a new, robust *in vivo* study.

4. New *in vivo* micronucleus and comet study in Sprague-Dawley rats

In view of the above comments, the aim of this study was to examine the genotoxic dose response of 2,6-xylidine, associated toxicokinetic parameters and markers of metabolic-related oxidative stress *in vivo* in rats, to better elucidate the potential presence and nature of a metabolic threshold for genotoxic effects. The study was performed using Sprague Dawley rats, as this is currently the only animal species to have shown carcinogenic effects. Bone marrow was sampled for MN since the published MN studies had only been conducted in mice, and the comet assay was performed in liver, since this is a primary site of metabolism, and in nasal tissue since this is the primary site for tumours in rats. The study was conducted at Integrated Laboratory systems, Inc. (ILS) in August 2019 in accordance with United States Environmental Protection Agency's (U.S. EPA) Good Laboratory Practice (GLP) Regulations (40 CFR Part 160) and OECD guidelines TG474 (OECD, 2016b) and TG489 (OECD, 2016c). All data underwent quality control review. All procedures were in compliance with the Animal Welfare Act Regulations, 9 CFR 1–4 and animals were handled and treated according to the Guide for the Care and Use of Laboratory Animals.

4.1. Test chemicals

2,6-Xylidine (99.4% pure; batch no. STBH6422) and the vehicle (corn oil) in which it was formulated, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The positive control chemical, ethyl methanesulfonate (EMS) was also purchased from Sigma-Aldrich, and was formulated in sterile filtered 0.9% saline.

4.2. Animals

Hsd:Sprague Dawley® rats (strain Harlan; SD), aged 8–10 weeks, were purchased from Envigo Laboratories (Frederick, MD, USA). The numbers of animals were 38 males plus 38 females for the MN/comet/bioanalytical phases, 23 males plus 23 females for histopathology/urinalysis, and 20 males plus 20 females for toxicokinetic (TK) analysis. They were housed singly or 2–3 per cage in polycarbonate cages with micro-isolator tops, with absorbent heat-treated hardwood bedding (Northeastern Products Corp., Warrensburg, NY). Animal rooms were maintained at a temperature of 20.5–23.1 °C, and humidity of 39.0–74.8%, with lighting on a 12/12-h light/dark cycle. Certified Purina Pico Chow No. 5002 (Ralston Purina Co., St. Louis, MO) and reverse osmosis treated tap water (City of Durham, NC) were provided *ad libitum*. Rats were acclimated for 7 days prior to the start of treatment for the main study, or for 2 days prior to dosing for TK analysis. Each animal was uniquely identified by ear punch prior to the start of the study. The weight ranges of the animals at the start of treatment were 240.7–290.3 g for the males, and 191.3–221.3 g for the females.

4.3. Basic study design

The basic design was as described by Bowen et al. (2011). Groups of male and female rats were dosed by oral gavage either with 2,6-xylidine (5 dose levels per sex), or the vehicle (corn oil), or EMS, daily for 3 consecutive days at approximately 0, 24, and 45 h. At 48 h. (i.e. 3 hrs. after the final dose) bone marrow was sampled for MN analysis; liver and nasal tissues were sampled for comet analysis and for the analysis of 2,6-xylidine and metabolite concentrations, and markers of oxidative stress (8-OH-dG and glutathione levels). Additional groups of rats were dosed similarly, with harvest of liver and nasal tissues for potential histopathological analysis. Separately, groups of satellite rats were similarly dosed with 2,6-xylidine or vehicle, and blood was collected via a jugular vein catheter 1, 2, 4, 8, and 24 h post final dose (±1 min) for toxicokinetic analysis.

4.4. Selection of dose levels

The dose levels for 2,6-xylidine were selected following the conduct of a range finder study in which all animals of both sexes were administered the test substance via oral gavage at 0, 24 and 45 h. Animals given 2,6-xylidine at 400 mg/kg (females) and 500 mg/kg (males) exhibited some clinical signs of toxicity symptoms but survived to the terminal necropsy (3 h following the final dose). Animals at higher dose

levels exhibited severe toxic signs and did not survive to the terminal necropsy. Therefore, these dose levels (400 mg/kg/day for females; 500 mg/kg/day for males) were considered to be the maximum tolerated doses (MTDs). Four additional, lower, dose levels were included in the main study.

The EMS dose level (150 mg/kg/day) was selected based on previous usage as a positive control. Similar dosing regimens had historically resulted in no adverse clinical signs but resulted in significant MN and comet responses.

4.5. Group allocation

Animals were assigned to a dose group using a procedure that stratifies animals across groups by body weight such that mean body weight of each group was not statistically different from any other group using analysis of variance (ANOVA) (Statistical Analysis System version 9.2, SAS Institute, Cary, NC). The group designations are shown in Supplementary Tables 3 and 4

4.6. Dose preparation and administration

2,6-Xylidine was prepared at ILS in corn oil at concentrations of 1.5, 5, 10, 12.5, 20, 25, 40 and 50 mg/mL. Formulations were prepared once and stored at room temperature, protected from light, until used. Dose formulation samples were prepared and analysed at ILS in accordance with GLP regulations. Since 2,6-xylidine and the vehicle were miscible liquids, no homogeneity testing took place. At each dose level, 2 samples were collected on the day of preparation, one for immediate analysis and one to serve as a backup sample. The final diluted concentration of each sample fell within the analytical range of the method, namely each formulation was ±15% of nominal and a %RSD (Relative Standard Deviation) was ≤10.0%.

The positive control, EMS, was prepared daily (room temperature, protected from light) at ILS in 0.9% saline at a concentration of 15 mg/mL.

Dose formulations were administered via oral gavage at a dose volume of 10 mL/kg body weight for 3 consecutive days at 0, 24 h (±30 min) and 45 h (±30 min). Dose volumes were based on individual animal daily body weight. Dose formulations were placed on a stir plate at least 30 min prior to dosing and continuously stirred. The nasal tissue from the male rats (groups 1–6, Supplementary Table 3) were not frozen as per protocol and fresh animals were dosed to collect these tissues.

4.7. Clinical observations

Mortality/moribundity were checked twice daily on weekdays, once daily on weekends. Rats were observed within 2 days of arrival (for allocation of animals to dose groups), prior to daily administration, and (except TK groups 15–26) at termination. Cage-side observations were performed approximately 1 h after dose administration each day (groups 1–14). Body weights were determined within 2 days of arrival (for allocation of animals to dose groups), prior to daily administration, and (except TK groups 15–26) at termination.

4.8. Tissue sampling and processing

At 3 h (±30 min) following the final dose administration the animals were humanely euthanized using CO_2 asphyxia confirmed by exsanguination. Tissue sampling and processing for MN, comets, histopathology and bioanalysis followed standard methods, in accordance with GLP Regulations (40 CFR Part 160) and OECD guidelines TG474 (OECD, 2016b) and TG489 (OECD, 2016c). Bioanalytical samples were processed using HPLC/MS/MS (for 2,6-xylidine, conjugated-DMAP and glutathione) and GC/MS/MS (for DMHA) using validated procedures in accordance with FDA 2018 Guidance for Industry on Validation of Bioanalytical Methods. Blood and tissue sampling and processing methods are described in detail in Supplementary Document 2. Animal allocation to bioanalytic, and toxicokinetic groups, and reference standards used in analysis are shown in accompanying Supplementary Tables 3, 4 and 5, respectively.

4.9. Statistical analysis

Individual animal data and group means (with standard deviations) were calculated and reported. For final body weight, body weight gain (or loss) measurements, MN-PCE and PCE frequency data, and comets (% tail DNA), all data were analyzed using Statistical Analysis System version 9.2 (SAS Institute, Cary, NC). Homogeneity of variance was analyzed using Levene's test and normality was assessed using the Shapiro-Wilk test on the control data. Homogenous data was then analyzed using a one-way analysis of variance (ANOVA), and test article-administered groups were compared to the appropriate control group using a one-tailed Dunnett's test. Dose-dependent changes were evaluated using a linear regression model. Data that were not homogeneous and normally distributed were analyzed using the appropriate non-parametric one-tailed Dunn's test. Dose-dependent changes were evaluated using an appropriate non-parametric trend test. Positive control data were analyzed by an appropriate *t*-test. P-values of <0.05 were considered significant.

4.10. Results

4.10.1. Body weights, clinical observations and maximum tolerated dose

Mean body weight changes are shown Table 1. It can be seen that there was a statistically significant and dose dependent decrease in final body weight, and therefore also in body weight gain, in male rats administered 2,6-xylidine. Males exposed to 250 and 500 mg/kg/day actually experienced significant body weight loss (4% and 7% respectively over the 48 h of the study) compared to control animals, and this loss correlated strongly with dose ($R^2 = 0.9$, $p = 0.003$). There was a statistically significant dose dependent linear trend for loss of weight in

Table 1
Summary of body weight changes.

A: Male Rats (Groups 1–7)			
Dose Level (mg/kg/day)	Initial Group Mean Body Weight (g) ± SD	Final Group Mean Body Weight (g) ± SD	Mean Body Weight Gain (g) ± SD[c]
0	274.60 ± 6.19	282.76 ± 8.34	7.1 ± 3.6
15	270.56 ± 13.72	277.74 ± 8.43	4.5 ± 13.5
50	276.92 ± 7.82	270.48 ± 7.48	−3.0 ± 2.3
125	271.66 ± 8.43	275.70 ± 7.02	2.2 ± 6.5
250	271.12 ± 4.69	**266.08 ± 11.90**[a]	**−9.7 ± 6.2**[a]
500	275.30 ± 6.99	**259.43 ± 4.51**[a b]	**−19.7 ± 6.1**[a b]
EMS-150	274.84 ± 6.16	**249.46 ± 7.77**[d]	**−26.6 ± 3.4**[d]
B: Female Rats (groups 8–15)			
Dose Level (mg/kg/day)	Initial Group Mean Body Weight (g) ± SD	Final Group Mean Body Weight (g) ± SD	Mean Body Weight Gain (g) ± SD[c]
0	207.22 ± 7.88	209.20 ± 8.29	1.4 ± 3.2
15	201.84 ± 5.92	206.10 ± 7.97	0.9 ± 3.2
50	204.16 ± 6.60	200.74 ± 8.13	−1.1 ± 7.5
100	200.92 ± 9.03	199.06 ± 8.65	−3.5 ± 6.3
200	205.90 ± 4.51	201.86 ± 7.33	−0.4 ± 3.8
400	205.51 ± 7.43	197.00 ± 6.45	**−6.0 ± 5.2**[b]
EMS-150	208.50 ± 5.63	**187.96 ± 8.20**[d]	**−22.7 ± 5.8**[d]

EMS = ethyl methanesulfonate.
SD = standard deviation.
[a] Statistically significant compared to the concurrent control mean (Dunnett's test).
[b] Dose dependent change (linear trend).
[c] Calculated from individual animal data.
[d] Statistically significant compared to the concurrent control mean (*t*-test).

female rats, however final body weight did not differ significantly from controls in any of the 2,6-xylidine treatment groups.

The rapid (4–7.1%) body weight loss over 2 days in the male 250 and 500 mg/kg/day groups approached the maximum limit of 10% body-weight loss (BWL) for dosing over 7 days recommended to define the MTD for short-term rat toxicity studies (Chapman et al., 2013). These authors noted that a marked BWL which occurs over a shorter time period is less acceptable than the same BWL over a longer time period. A reason for this is that rapid weight loss may indicate significant dehydration due to inappetence and/or fluid loss, which may be associated with a myriad of secondary physiological consequences. Chapman et al. (2013) proposed a decision tree for determination of MTD based on body weight loss and clinical factors, which, particularly if present in combination, were a sign that the MTD had been reached, or even exceeded, and that a lower dose should be examined.

Clinical signs were noted in all groups of male rats administered 2,6-xylidine, and in females at doses of 100, 200, and 400 mg/kg/day. Signs of toxicity were dose dependent and ranged from mild (rough coat), through to severe in the high dose groups, including decreased movement, piloerection, ungroomed, uncoordinated movement, lethargy, cold to touch, rales, hunched, and abnormal breathing. In groups 1–14, all animals survived to the scheduled sacrifice (i.e. to 3 h after the last dose), however none of the male rats in the 500 mg/kg/day group in the TK part of the study survived to 24 h after the last dose. Based on these findings, the top dose of 500 mg/kg/day was considered to have exceeded the MTD for male rats in this study. Clinical effects were of lesser magnitude in male rats in the 250 mg/kg/day group than in rats dosed at 500 mg/kg/day, as they did not include breathing anomalies, and affected only 5 of 8 animals. In females dosed at 200 and 400 mg/kg/day, 2/8, and 13/13 (respectively) exhibited behavioural abnormalities indicative of toxicity from mild-substantial degree. These data are considered to indicate that doses of 250 mg/kg/day in males and 400 mg/kg/day in females met the OECD TG 489 criteria for an acceptable MTD for comet assay studies (OECD, 2016c).

4.10.2. Micronucleus (MN) results

Mean frequencies of bone marrow PCE and MN-PCE are presented in Table 2. MN-PCE frequencies in all vehicle control animals were within the laboratory's historical negative control range (95% confidence intervals are 0–0.31% for males and 0–0.28% for females). Administration of EMS, the positive control, resulted in statistically significant increase in % MN-PCE that clearly exceeded the historical negative control ranges, accompanied by significant decreases in % PCE, in both male and female rats. The study was therefore considered valid. It can be seen from Table 2 that no increases in the frequencies of MN-PCE were observed for male or female rats administered 2,6-xylidine, and all MN-PCE frequencies fell within the above-mentioned historical negative control ranges. Although there was a dose-dependent decrease in % MN-PCE and a significantly lower % MN-PCE in females exposed to 200 mg/kg/day 2,6-xylidine, these changes are not considered to have any biological relevance. There was therefore no evidence of induction of MN in rat bone marrow of rats dosed with 2,6-xylidine up to, or above, the MTD.

Table 2
Summary of MN frequencies in rats administered 2,6-xylidine.

A: Males		
Dose Level (mg/kg/day)	Mean % PCE ± SEM	Mean % MN-PCE ± SEM (4000 PCE/animal scored)
0	56.2 ± 0.7	0.12 ± 0.03
15	57.8 ± 0.7	0.08 ± 0.02
50	53.8 ± 1.7	0.13 ± 0.03
125	55.4 ± 1.2	0.09 ± 0.03
250	58.0 ± 0.8	0.09 ± 0.01
500	54.6 ± 2.0	0.09 ± 0.01
EMS-150	**49.8 ± 0.2****	**0.72 ± 0.06****
B: Females		
Dose Level (mg/kg/day)	Mean % PCE ± SEM	Mean % MN-PCE ± SEM (4000 PCE/animal scored)
0	52.6 ± 1.7	0.14 ± 0.02
15	57.4 ± 0.6	0.08 ± 0.01
50	54.2 ± 1.6	0.09 ± 0.02
100	54.8 ± 1.4	0.08 ± 0.03
200	56.0 ± 1.4	**0.05 ± 0.02***
400	54.2 ± 2.1	0.07 ± 0.02[a]
EMS-150	**46.2 ± 1.1****	**0.51 ± 0.08****

EMS = ethyl methanesulfonate.
MN-PCE = micronucleated polychromatic erythrocytes.
SEM = standard error of the mean.
*Statistically significant compared to the concurrent control mean (Dunnett's test).
**Statistically significant compared to the concurrent control mean (*t*-test).
[a] Dose dependent change (downward linear trend).

4.10.3. Comet results

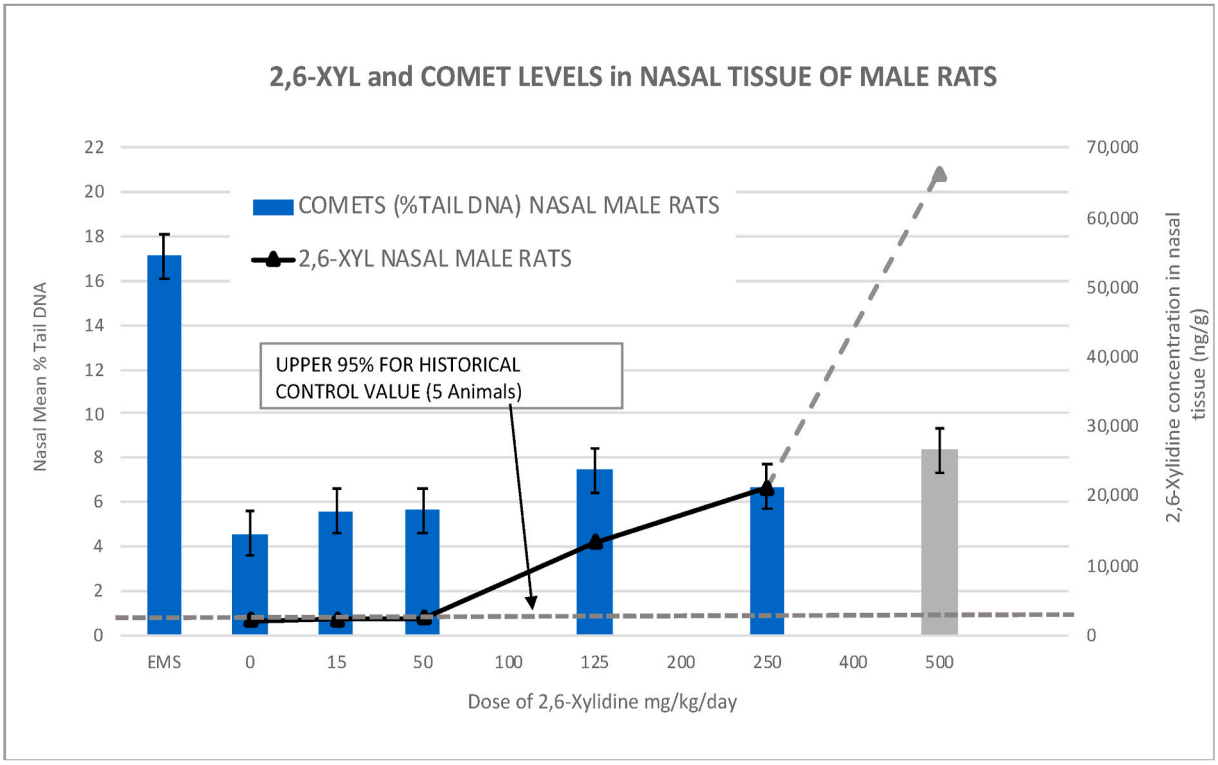
The group mean values for the % tail DNA for nasal tissue and liver of rats administered 2,6-xylidine or the positive control are shown in Table 3, and presented graphically in Figs. 3 and 4, together with 2,6-xylidine concentrations (see below). It should be noted that 2 animals (one male in the vehicle control group for nasal tissue, and 1 male in the 15 mg/kg/day group for liver tissue) were removed from the calculation of means, and therefore excluded from further statistical analysis, as outliers (abnormally high % tail DNA values), and 1 female from the EMS group for nasal tissue was also excluded due to insufficient cells scored.

For nasal tissue, the % tail DNA values for vehicle control animals exceeded the historical negative control ranges (95% confidence intervals are 0–1.5% for males and 0–1.8% for females). However, the historical negative control data for rat nasal tissue was based on a very limited data set of only 5 animals. When compared with % tail DNA values in liver (see below) the concurrent vehicle control values obtained in this study (see Table 3) are considered normal for a high turnover tissue. Moreover, the positive control chemical (EMS) induced increases in % tail DNA that were > 2× control values and significantly different. Thus, the comet evaluations in nasal tissue were considered valid. It can be seen from Table 3 and Fig. 3 that, although % tail DNA

Table 3
Summary of comet assay results in liver and nasal tissue.

A: Males		
Dose Level (mg/kg/day)	Liver Mean % Tail DNA ± SEM	Nasal Mean % Tail DNA ± SEM
0	1.44 ± 0.31	4.59 ± 2.43[b]
15	1.15 ± 0.20[b]	5.59 ± 2.19
50	3.42 ± 1.60	5.62 ± 1.35
125	2.60 ± 0.90	7.45 ± 3.08
250	4.32 ± 1.10	6.70 ± 1.59
500	4.59 ± 1.15[a]	8.35 ± 1.37
EMS-150	**20.76 ± 1.25***	**17.13 ± 1.16***
B: Females		
Dose Level (mg/kg/day)	Liver Mean % Tail DNA ± SEM	Nasal Mean % Tail DNA ± SEM
0	2.03 ± 0.69	3.81 ± 1.24
15	1.74 ± 0.42	1.41 ± 0.43
50	1.55 ± 0.70	2.03 ± 0.92
100	2.09 ± 0.30	1.07 ± 0.50
200	1.79 ± 0.41	4.58 ± 2.76
400	2.10 ± 0.49	2.31 ± 0.54
EMS-150	**14.83 ± 1.24***	**7.74 ± 1.07***[b]

EMS = ethyl methanesulfonate.
SEM = standard error of the mean.
*Statistically significant compared to the concurrent vehicle control (*t*-test).
[a] Dose dependent change (upward linear trend).
[b] Based on 4 animals – see text for details.



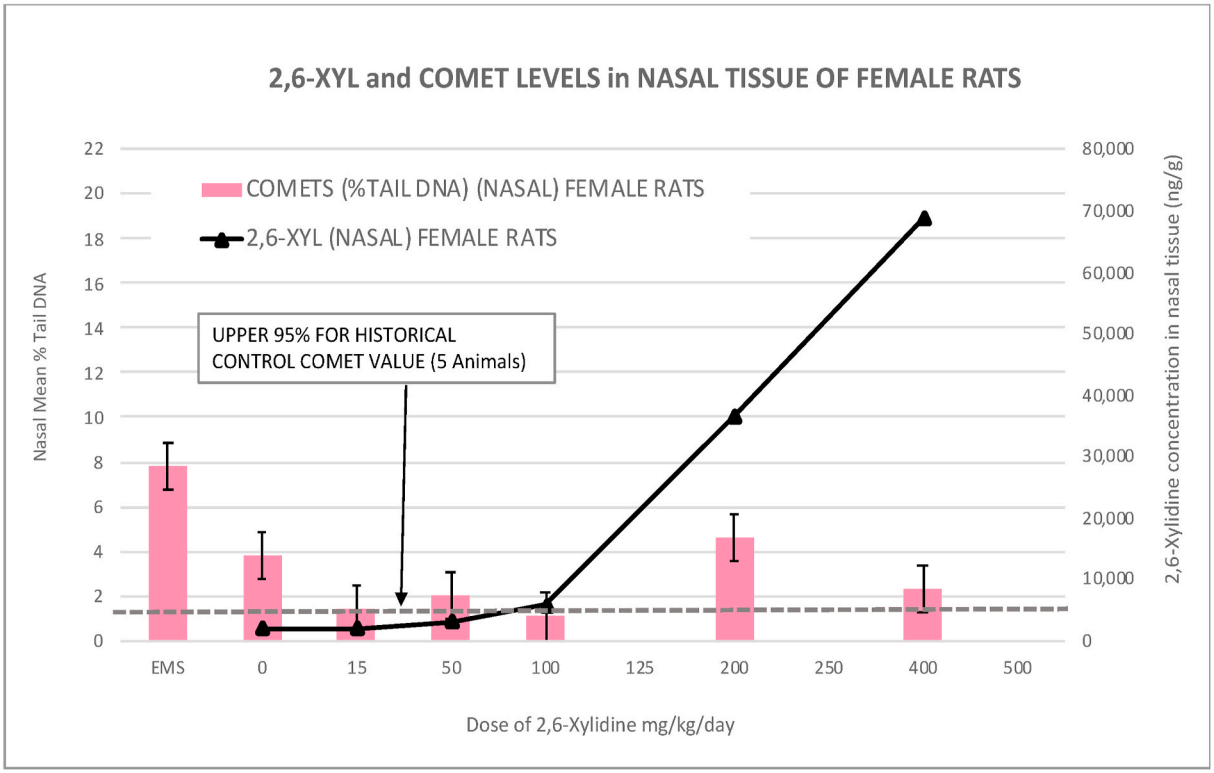
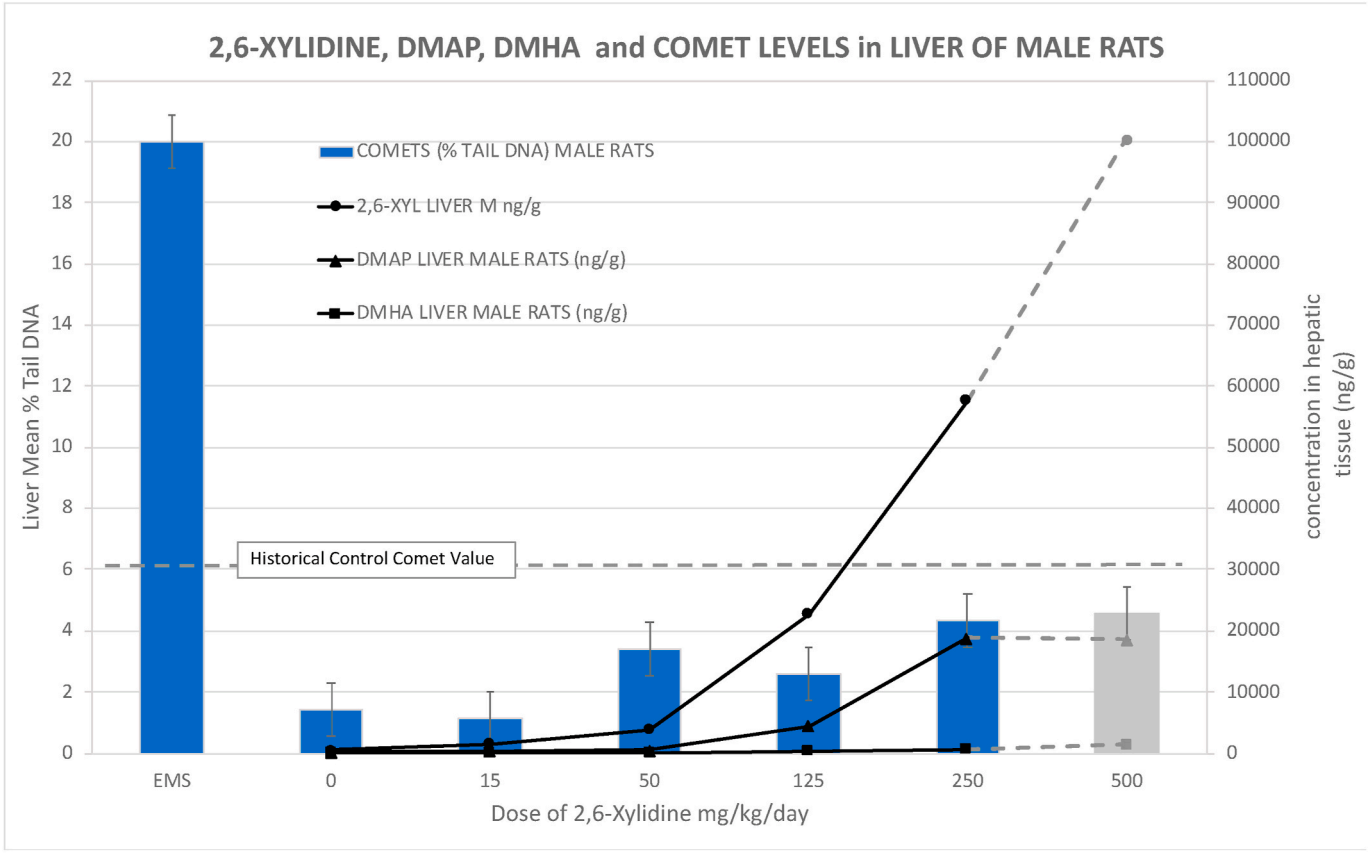


Fig. 3. Comet responses and 2,6-xylidine levels in nasal tissue.

values exceeded the very limited historical negative control range, there were no significant increases in % tail DNA as compared with the concurrent vehicle control groups in either female or male rats exposed to 2,6-xylidine. It was therefore concluded that 2,6-xylidine did not induce DNA damage in nasal tissue of rats, dosed up to, or above, the MTD.

For liver tissue, the % tail DNA values for vehicle control animals all fell within the historical negative control ranges (95% confidence intervals for the most recent studies are 0–8.8% for males and 0–7.2% for females), and the positive control chemical (EMS) induced significant increases in % tail DNA. Thus, the comet evaluations in liver tissue were considered valid. It can be seen from Table 3 and Fig. 4 that there were no significant increases in % tail DNA in either female or male rats



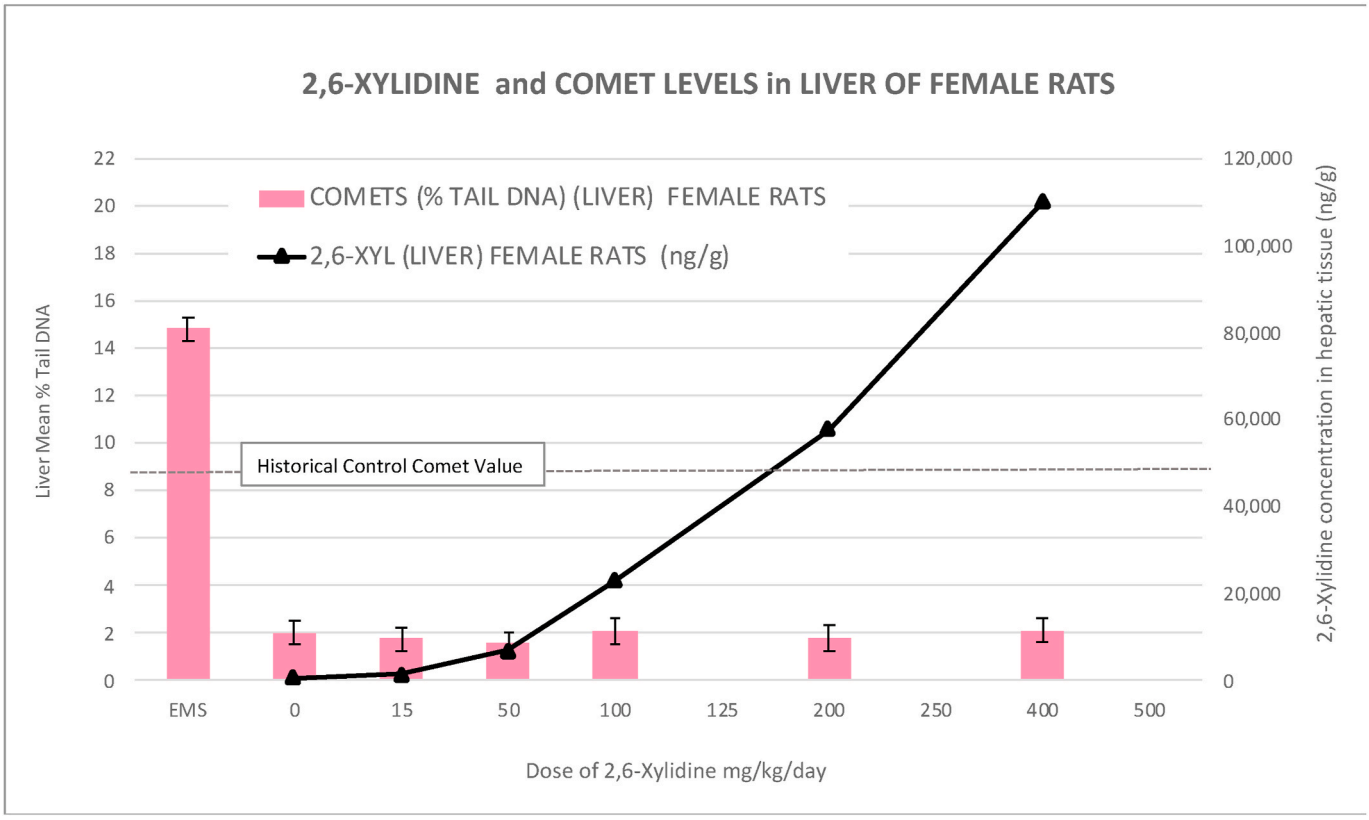


Fig. 4. Comet responses and 2,6-xylidine, DMHA and DMAP levels in liver.

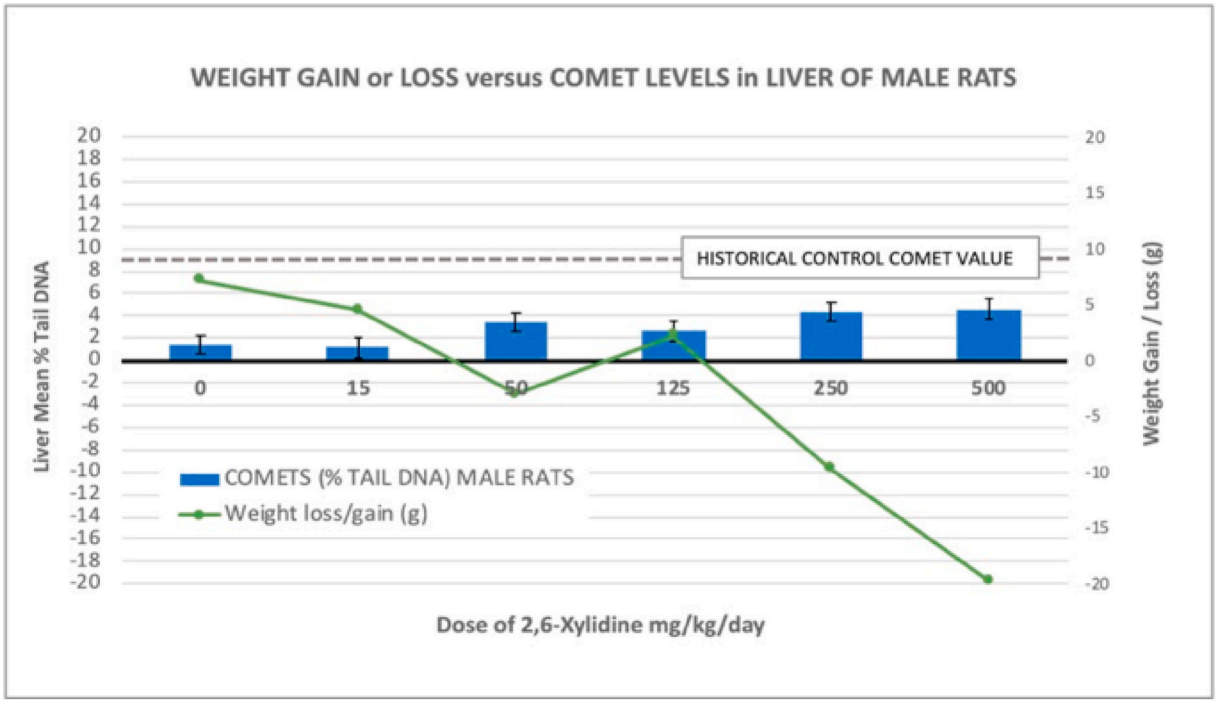


Fig. 5. % Tail DNA for male rat livers versus weight gain/loss. Treatment groups showing the greatest numeric increase above negative control values were also those that exhibited the greatest weight loss.

exposed to 2,6-xylidine as compared with concurrent controls, all values were within the historical negative control range, and below the OECD recommended limit of 6% for hepatic tissues (OECD, 2016c). In male rat livers however, there was a low magnitude but statistically significant dose response. It was therefore concluded that 2,6-xylidine did not induce DNA damage in livers of female rats, but there was an equivocal response in livers of male rats.

Fig. 5 shows that there was a strong correlation between dose, weight loss and comet responses in male rat liver. Additional analyses were therefore performed on the comet dose response findings in male liver and potential for co-linearity with weight loss. This identified (Fig. 6) that there was a strong correlation between weight loss in male rats and comet values in male liver ($R^2 = 0.85$, $p = 0.008$). Weight loss rather than dose provided the stronger correlation with comet values using multiple linear regression. There was no significant dose response trend if results from animals suffering acute toxicity with significant weight loss (i.e. the 250 and 500 mg/kg/day groups) were excluded from the analysis. Therefore, in male rats dosed up to and including 125 mg/kg/

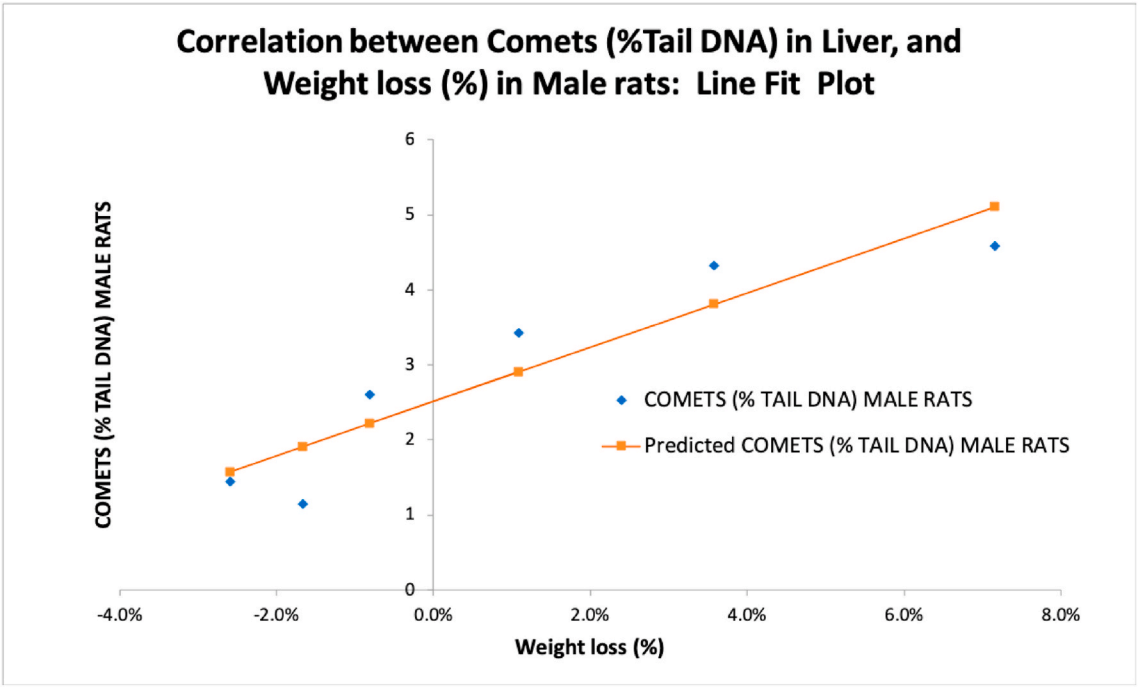


Fig. 6. Correlation between comets (%tail DNA) in liver and weight loss in male 2,6-xylidine treated rats - line fit plot.

Table 4
Summary of 2,6-xylidine concentrations in plasma and tissues sampled 3 h after the last dose.

A: Males				
Dose Level (mg/kg/day)	Liver (ng/g) ± SD	Nasal (ng/g) ± SD	Plasma (ng/mL) ± SD (MN/comet subset)	Plasma (ng/mL) ± SD (Histopathology subset)
0	LLOQ (<500)	LLOQ (<2000)	LLOQ (<1000)	LLOQ (<1000)
15	1508.0 ± 692.2	2206.0 ± 339a	LLOQ (<1000)	LLOQ (<1000)
50	3806.0 ± 1366.4	2374.0 ± NA[b a]	10166.0 ± 15085.0[a]	2972.0 ± 1371.4
125	22742.0 ± 29422.7a	13310.0 ± 4682.5	12440.0 ± 16101.0[a]	13416.0 ± 2830.2
250	57660.0 ± 25365.2	21100.0 ± 8102.5	25840.0 ± 11146.9	34140.0 ± 10228.0
500	100271.4 ± 74382.3	66240.0 ± 26034.7	61340.0 ± 53166.3	67960.0 ± 27940.0

B: Females			
Dose Level (mg/kg/day)	Liver (ng/g) ± SD	Nasal (ng/g) ± SD	Plasma (ng/mL) ± SD
0	LLOQ (<500)	LLOQ (<2000)	LLOQ (<1000)
15	1403.2 ± 1411.6[a]	LLOQ (<2000)	LLOQ (<1000)
50	6792.0 ± 2418.1	3050.0 ± NA[b a]	3292.0 ± 811.3
100	23060.0 ± 3431.2	5880.0 ± 6157.0[a]	11838.0 ± 3572.1
200	57880.0 ± 14315.3	36720.0 ± 10158.8	28380.0 ± 4387.7
400	110166.7 ± 55189.2	68816.7 ± 27360.2	61200.0 ± 33369.4

LLOQ = lower limit of quantitation.
SD = standard deviation.
[a] Calculated from individual animal data using the LOQ value for values < LLOQ.
[b] Data from a single animal above the LOQ.

day, the comet results in liver met all 3 criteria for a clearly negative result as specified by OECD (OECD, 2016c), namely not significantly different from concurrent controls, within the historical negative control range, and no dose response.

4.10.4. Bioanalysis

Group mean 2,6-xylidine concentrations in plasma, liver and nasal tissue sampled at sacrifice for the comet/MN analyses (i.e. 3 hrs. after the final dose) are presented in Table 4. At 15 mg/kg/day 2,6-xylidine concentrations were below the limits of quantitation in plasma and nasal tissue of female rats, however it was detectable in the liver of both sexes and nasal tissue of males. At dose levels of 50–500 mg/kg/day, 2,6-xylidine was detectable in plasma, liver and nasal tissue of all rats (male and female), and increased in a dose-responsive manner. These data confirm systemic, as well as nasal and hepatic tissue exposure to 2,6-xylidine in male and female rats.

Group mean data for 2,6-xylidine concentrations in plasma for the TK timepoints are presented for males in Table 5. The plasma levels of 2,6-xylidine increased in a dose-dependent manner in both sexes for each timepoint except for the 24-h timepoint at which all samples were below the limit of quantitation. Additionally, the lowest dose level (15 mg/kg/day) was below the limit of quantitation with the exception of a single male at the 1-h timepoint. Since the bone marrow is a well-perfused tissue, these data also confirm systemic (and therefore bone marrow) exposure to 2,6-xylidine in male and female rats.

4.10.5. Additional analyses of male rat liver

Based on the "equivocal" result from a positive trend test for comets in the male liver, additional analyses for concentrations of DMHA,

Table 5
Summary of 2,6-xylidine concentrations in rat plasma[a] (from TK analysis).

A: Males					
Dose Level (mg/kg/day)	1-h Plasma (ng/g) ± SD	2-h Plasma (ng/g) ± SD	4-h Plasma (ng/g) ± SD	8-h Plasma (ng/g) ± SD	24-h Plasma (ng/g) ± SD
0	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)
15	1190.0 ± NA[b]	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)
50	3500 ± 1017	2530 ± 545	1673 ± 529	2340 ± NA[b]	<LLOQ (1000)
125	55333 ± 71614	12767 ± 1002	11500 ± 608	7293 ± 920	<LLOQ (1000)
250	25433 ± 2479	27033 ± 4148	30067 ± 6921	27500 ± 3551	<LLOQ (1000)
500	131200 ± 101749	177000 ± 18682	197667 ± 32083	217333 ± 31214	NA[c]

B: Females					
Dose Level (mg/kg/day)	1-h Plasma (ng/g) ± SD	2-h Plasma (ng/g) ± SD	4-h Plasma (ng/g) ± SD	8-h Plasma (ng/g) ± SD	24-h Plasma (ng/g) ± SD
0	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)
15	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)	<LLOQ (1000)
50	6055 ± 1563	4960 ± 2797	5115 ± 2595	2257 ± 131	<LLOQ (1000)
100	10317 ± 3991	11767 ± 1686	10840 ± 3265	9413 ± 3455	<LLOQ (1000)
200	6567 ± 2775	22067 ± 2470	26767 ± 2776	18100 ± 1808	<LLOQ (1000)
400	84267 ± 41850	97767 ± 19989	88767 ± 24830	73333 ± 20814	<LLOQ (1000)

SD = standard deviation.
LLOQ = lower limit of quantitation.
[a] Calculated from individual animal data using data above the limit of detection.
[b] Data from a single animal above the limit of detection.
[c] No males survived in this group to 24 h post dose.

Table 6
Summary of DMAP-conjugate, DMHA, and glutathione concentrations in male rat liver[a].

Dose Level (mg/kg/day)	DMAP-Conjugates (ng/g) ± SD	DMHA (ng/g) ± SD	L-Glutathione (ng/g) ± SD
0	LLOQ (<200)	LLOQ (<50)	607400.0 ± 126638.5
1	308.0 ± 95.3	LLOQ (<50)	646400.0 ± 129666.1
50	555.0 ± 234.5	LLOQ (<50)	831600.0 ± 105632.9
125	4420.0 ± 4717.2[a]	318.4 ± 420.0[a]	735400.0 ± 114500.2
250	18920.0 ± 4680.5	671.0 ± 382.7	764800.0 ± 183626.5
500	18617.1 ± 6979.0	1458.9 ± 1078.4	753714.3 ± 315266.4

SD = standard deviation.
LLOQ = lower limit of quantitation.
[a] Calculated from individual animal data using the LLOQ value for values < LLOQ.

DMAP-conjugate and glutathione were performed on male liver tissue only, to investigate metabolic parameters and indications of oxidative stress. The data are tabulated in Table 6, and presented graphically for DMAP-conjugate and DMHA in Fig. 3. DMAP-conjugate was detectable at 15 mg/kg/day and increased with dose up to 250 mg/kg/day, with

the levels exhibiting similar levels (i.e. a plateau) at 250 and 500 mg/kg/day, suggesting that 4-hydroxylation of 2,6-xylidine became saturated in male rat liver at doses >125 mg/kg/day. DMHA was not detectable in quantifiable levels in rats dosed at 15 or 50 mg/kg/day, but was present in relatively minor, but increasing concentrations in rats dosed at 125–500 mg/kg/day. Therefore, male rat livers were exposed to DMHA and DMAP at doses that did not lead to increased comets. Glutathione levels were not significantly different from vehicle control levels in any of the 2,6-xylidine-dosed groups.

4.10.5.1. Histopathology male rat liver. When liver samples were evaluated for histopathology, microscopic findings were evident in the 500 mg/kg/day group including minimal increases in centrilobular hepatocellular hypertrophy (3/5 animals), diffuse periportal hepatocellular vacuolation (2/5 animals), the number of hepatocellular mitoses (2/5 animals) and inflammation (1 animal). A minimal increase in periportal hepatocellular vacuolation and mitosis was also seen in 1/3 animals treated at 250 mg/kg/day, but not in any animals treated at lower doses.

4.10.5.2. Benchmark dose analysis of comet response in male rat liver. Benchmark dose analysis was also conducted using the weak dose response in comet % tail DNA in male rat liver tissue. The benchmark dose (BMD) approach is considered the most suitable for use in calculating point of departure (PoD) metrics from continuous genetic toxicity data, and is more conservative than measures such as no-observed genotoxic effect level (NOGEL) or slope transition dose (Johnson et al., 2014; MacGregor et al., 2015). It has been recently shown that a critical effect size (CES) of 50% is suitable for use in calculating BMD confidence intervals (BMD CI) (Zeller et al., 2017). Furthermore, increased precision in the BMD confidence interval (CI) calculation can be obtained by using the covariate BMD approach (Slob and Setzer, 2014; Wills et al., 2016). The $BMDL_{50}$ is considered the most appropriately conservative PoD metric for use in defining human exposure limits. It should be noted that since the comet assay detects early events, it is therefore more conservative as an endpoint, and usually provides lower PoDs, than intermediate and apical endpoints such as chromosomal aberrations, micronuclei or gene mutations.

BMD analysis was conducted using PROAST, the dose–response modelling software developed at the National Institute for Public Health and the Environment (RIVM) in the Netherlands [http://www.proast.nl; version 65.5] (Slob, 2002). The default assumptions from PROAST were used for the BMD analysis. The nested set of models used included the non-linear exponential and Hill models that are recommended by the European Food Safety Authority (EFSA, 2009). The CES 50% was used to define the BMD values and the lowest BMD_{50} and $BMDL_{50}$ from both models are included in Table 7, along with the highest $BMDU_{50}$. Due to the weak dose response in the *in vivo* liver comet data, standard BMD analysis showed no dose response, therefore increased precision was required through the covariate BMD approach, as described by Wills et al. (2016), using liver comet data from 2 structural related chemicals, *o*-anisidine and *p*-chloroaniline which had been tested using a similar protocol. Using this approach, the Hill and exponential models provided a suitable fit with 2,6-xylidine and showed the liver comet response was non-linear. The $BMDL_{50}$ metric for comet was calculated to be 97 mg/kg/day (Table 7), i.e. lower than NOGEL of 125 mg/kg/day, as expected. Detailed data and the BMD modelling are shown in Table 8 and Fig. 7.

Table 7
BMD confidence intervals for male rat liver comet results (% tail DNA).

	p-chloroaniline (mg/kg)	2,6-xylidine (mg/kg)	*o*-anisidine (mg/kg)
BMD_{50}	36	322	391
$BMDL_{50}$	11	97	141
$BMDU_{50}$	54	512	572

The values are calculated from BMD modelling shown in Fig. 7 (raw data shown in Table 8). The lowest BMD_{50} and $BMDL_{50}$, and the highest $BMDU_{50}$ from the non-linear exponential and Hill models, are presented.
p-chloroaniline data from Barfield and Burlinson (2015).
o-anisidine data from Hobbs et al. (2015).

Table 8
Summary of % tail DNA data in rat liver for 2,6-xylidine and the 2 related substances used for covariate analysis. In each case n = 5. SEM = standard error of means.

Dose (mg/kg/day)	% Tail DNA; mean of medians	% Tail DNA: SEM (means of medians)	Test substance	Reference
0	1.44	0.31	2,6-xylidine	Current ILS study
15	1.15	0.2	2,6-xylidine	Current ILS study
50	3.42	1.6	2,6-xylidine	Current ILS study
125	2.6	0.9	2,6-xylidine	Current ILS study
250	4.32	1.1	2,6-xylidine	Current ILS study
500	4.59	1.15	2,6-xylidine	Current ILS study
0	0.3	0.06	o-anisidine	Hobbs et al. (2015)
150	0.4	0.11	o-anisidine	Hobbs et al. (2015)
300	0.4	0.15	o-anisidine	Hobbs et al. (2015)
600	0.9	0.22	o-anisidine	Hobbs et al. (2015)
0	0.9	0.41	p-chloroaniline	Barfield and Burlinson (2015)
37.5	0.66	0.1	p-chloroaniline	Barfield and Burlinson (2015)
75	3.47	0.76	p-chloroaniline	Barfield and Burlinson (2015)
150	7.43	1.65	p-chloroaniline	Barfield and Burlinson (2015)

4.11. Discussion of new MN/comet study results

4.11.1. Metabolic and toxicokinetic findings

Administered at a dose of 15 mg/kg/day, to rats, 2,6-xylidine was present in quantifiable levels in hepatic tissue, but not quantifiable in plasma or nasal tissue in the majority of rats in this study. This suggests that, at this dose, it is efficiently cleared via intrahepatic metabolism, consistent with findings previously reported by Lindstrom et al. (1963). These authors investigated 2,6-xylidine metabolism and elimination in Osborne-Mendel rats, and reported that at low doses (up to 50 ppm in feed) 2,6-xylidine was virtually completely metabolised, with little unmetabolized 2,6-xylidine detectable in urine. Over 90% of the dose was recovered in urine as conjugated-DMAP, with small amounts of unmetabolized 2,6-xylidine and no other metabolites detected. This study identified 4-hydroxylation as the major pathway of metabolism and elimination of 2,6-xylidine *in vivo* in rats, which is further confirmed by our finding of conjugated-DMAP, but not DMHA in livers of male rats dosed at 15–50 mg/kg/day in the current study. Additionally, we did not find evidence of clinical toxicity in male or female rats, nor hepatotoxicity in male rats dosed at these levels, consistent with previous reports identifying 20 mg/kg/day as a NO(A)EL for repeated dose toxic effects of 2,6-xylidine in CD rats (NTP 1990). Yasuhara et al. (2000) similarly reported that 2,6-xylidine was below levels of detection in plasma, and did not induce cytotoxic effects in nasal tissue based on histopathological analysis, when fed to rats at doses of 300 ppm in diet (estimated

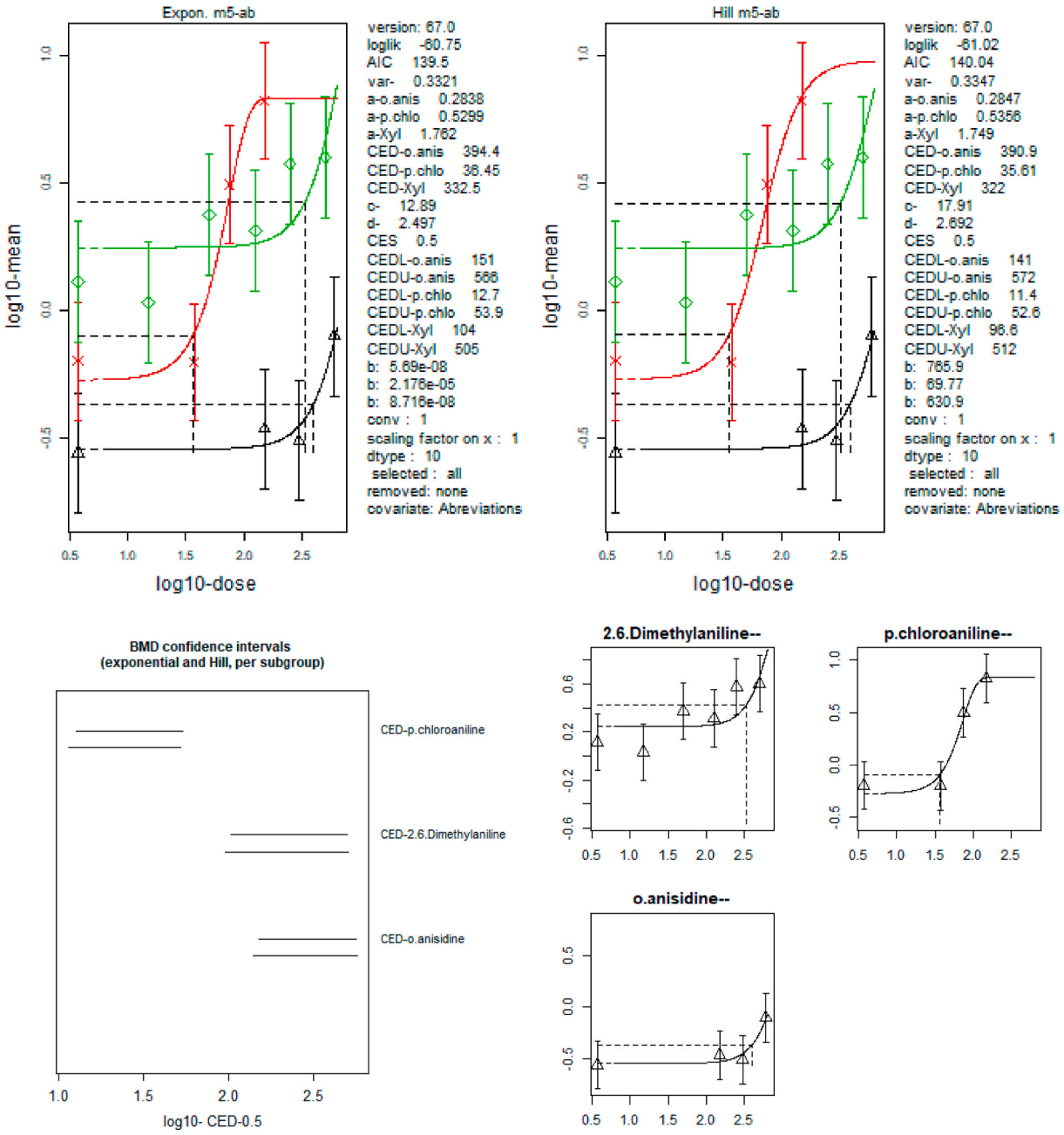


Fig. 7. BMD_{50} outputs (PROAST v67) defined for % tail DNA dose response data from male rat livers exposed to 2,6-xylidine.

intake 27 mg/kg/day) for one week. Together these data support the conclusion that when administered at doses up to 15–20 mg/kg/day, 2, 6-xylidine is effectively detoxified via 4-hydroxylation and phase 2 metabolism, and eliminated in the urine without pathological effect.

Higher doses appear to be associated with saturation of 4-hydroxylation, disturbed metabolism and onset of systemic toxic effects. Administered at higher doses to rats in the most recent study (described above), 2,6-xylidine became detectable in plasma and nasal tissues (as well as liver), and increased in a dose dependent manner. DMAP-conjugate concentrations in the livers of male rats appeared to saturate at doses >125 mg/kg/day. This was associated with the onset of clinical signs indicative of systemic toxicity, including significant weight loss of 4–7%, which also became evident in male rats at doses >125 mg/kg/day. These findings are also consistent with previous reports. Lindstrom et al. (1963) reported that at high doses (200 mg/kg/day by gavage for 8 days), in addition to conjugated-DMAP, unmetabolized 2, 6-xylidine appeared in the urine in significant amounts indicating saturation of metabolic clearance via 4-hydroxylation. Discolouration of the urine was noted following 8 days of dosing, considered to be indicative of significant disturbance of normal metabolism. In addition to conjugated-DMAP and 2,6-xylidine, trace amounts of 2-AMBA were detected in urine, suggesting that low level metabolism may occur via alternative pathways at these high dose levels. Hardy (1986), and Short et al., 1989a reported similarly. These authors used Gas Chromatography (GC) and GC/Mass Spectrometry to examine urinary metabolites in male Fischer 344 rats administered 2,6-xylidine by gavage at 262.5 mg/kg/day for one or 10 days. DMAP-conjugate was the predominant urinary metabolite identified in urine, constituting up to 66% of the dose with the bulk of the remainder (up to 33%) excreted unmetabolized. Trace levels of *N*-2,6-trimethylaniline were detected (<LOQ), however no other metabolites were present in detectable level; metabolites for which standards were prepared, but which were not detected included;

DMHA, N-acetyl-2,6-DMA, 2,6-dimethyl-nitrobenzene and 2-AMBA.

N-oxidation (to DMHA) has, however, been identified as a very minor intra-hepatic pathway of metabolism, (evident only following high doses of 125 mg/kg/day and above) in male rats in the current study. This finding is in contrast to Lindstrom et al. (1963) and Hardy (1986) who did not identify DMHA (or other N-oxidised metabolites) in urine of rats. It is, however, consistent with *in vitro* reports. Kirkland et al. (2012) detected DMHA as a minor metabolite of 2,6-xylidine *in vitro* using high concentrations of 2,6-xylidine incubated with rat hepatic microsomes. The reaction had a Km of 45 and Vmax of 0.04, indicative of very low production. Gan et al. (2001) examined 2,6-xylidine metabolism following incubation with human hepatic microsomes. Low level DMHA production (constituting 2–3% of metabolic product) was detected following incubation with 2,6-xylidine at nanomolar concentrations (however DMAP was the only metabolite present following incubation with 2,6-xylidine at higher concentrations). These authors furthermore reported that, when present, DMHA was rapidly re-arranged to DMAP via Bamberger arrangement; rapid re-arrangement of DMHA to DMAP, could explain why DMHA is detectable in hepatic tissue, but not in rat urine *in vivo*.

Together these findings are considered to support the conclusions of previous authors that, by virtue of the two methyl groups either side of the amine moiety, 2,6-xylidine is highly resistant to N-hydroxylation and preferentially metabolised via 4-hydroxylation and phase 2 conjugation. At oral doses <15–20 mg/kg/day it is rapidly and extensively metabolised via this process and eliminated in urine without pathological effect. At doses >125 mg/kg/day however, there is evidence of metabolic overload with saturation of production of conjugated-DMAP associated with acute systemic toxic effects. DMHA appears to be a minor intermediate intrahepatic metabolite *in vivo* in male Sprague-Dawley rats only following high dose oral 2,6-xylidine administration.

4.11.2. Clinical and toxicological findings

In this study, mild clinical signs of toxicity became evident at a dose of 50 mg/kg/day and increased in severity with dose, such that all rats in high dose male and female groups displayed signs of moderate to severe toxicity, including reduced and/or uncoordinated movement, and rales or moderate to severe abnormalities of breathing. Weight loss was also a feature. This was particularly evident in male rats dosed at 250 and 500 mg/kg/day, where 4 and 7% bodyweight loss (respectively) occurred over the 48 h of the study, and thus 500 mg/kg/day was determined to have exceeded the MTD for this study.

Clinical findings in the current study concorded with previous published reports of acute and repeated dose systemic toxic effects of 2,6-xylidine in rats. These findings identified weight loss, lethargy, cyanosis, breathing abnormalities, and circulatory collapse as acute toxic effects. On bioanalysis, haemolysis and met-Hb formation, high urine output with low urinary specific gravity (indicative of diuresis) along with renal and hepato-toxicity were reported. In addition, male rats are reported to be more sensitive to acute systemic toxicity than female rats (Jacobson, 1983; SIDS, 2012; NTP, 1990).

It is notable that the acute systemic toxic effects of 2,6-xylidine are likely to be associated with ROS production, oxidative stress and/or oxidative DNA injury as have been reported in other settings, thereby potentially confounding assessment of *in vivo* genotoxicity. Rapid weight loss, for example, is generally indicative of acute dehydration due to inappetence and/or fluid loss. Dehydration results in reduced tissue perfusion and tissue anoxia resulting in oxidative stress (França et al., 2007). Dehydration resulting in loss of 3% bodyweight in human volunteers, for example, has been shown to induce oxidative stress, as evidenced by a statistically significant increase in malondialdehyde (MDA) levels, total antioxidant status (TAS), and DNA damage as assessed via lymphocyte comet assay (Paik et al., 2009). Similarly, rales and breathing anomalies may signify the presence of hypoxia. Paradoxically, hypoxic cells increase their mitochondrial production of reactive oxygen species (ROS) leading to oxidative stress. High levels of lipid, protein, and DNA oxidative damage have been measured in humans after exposure to hypoxia (Møller et al., 2001). Similarly, met-Hb impedes tissue oxygen transfer, contributing to tissue hypoxia, and has been shown to induce oxidative stress and related DNA injury (Albuquerque et al., 2015). Although similar studies are not available in rats, these data identify the potential for the observations in *in vivo* genotoxicity tests to be confounded by systemic toxicity-induced oxidative stress. *In vivo*, oxidative stress may be short-lived, and/or oxidative damage may be rapidly repaired. The comet assay is a highly sensitive marker of DNA injury and repair (Speit and Rothfuss, 2012), and may be more sensitive to short-lived oxidative stress than other markers such as 8-OH-dG and MN (Hartmann et al., 1998). Systemic toxic effects such as these are thus more likely to influence an "indicator" endpoint such as comets than an "apical" endpoint such as MN.

4.11.3. Micronucleus study findings

In the current study, there was no evidence of a genotoxic response to 2,6-xylidine *in vivo* based on MN findings in rats dosed up to (females) and above (males) the MTD. Systemic 2,6-xylidine exposure (plasma and tissues) was clearly evident. These data support previous negative MN findings in bone marrow and in peripheral blood of mice.

4.11.4. Comet study findings

In the current study, there was no evidence of a genotoxic response to 2,6-xylidine in rat nasal tissues or hepatic tissue of female rats when dosed up to the MTD, or in male rats at non-toxic doses (i.e. up to 125 mg/kg/day), based on comet results.

In male rat hepatic tissue an "equivocal" response was evident which prompted further investigation. The equivocal response was recorded by virtue of finding a low magnitude positive dose response, although all % tail DNA levels fell well within historical control ranges (<8.8% tail DNA), as well as below the OECD recommended upper limit of 6% (OECD, 2016c), and none were significantly different from concurrent controls. Results from all treatment groups were therefore within the normal variability of the test.

The low magnitude dose response was driven both by low % tail DNA values for male liver in the vehicle control and 15 mg/kg/groups, and by a relative increase in % tail DNA in the 250 and 500 mg/kg/day groups. Higher doses of 2,6-xylidine were administered to male than female rats in the high dose groups, however hepatic tissue concentrations were comparable (see Table 4), such that this did not explain the relative increase in comets in the hepatic tissue of male rats. Male rats did, however, exhibit greater systemic toxicity (including weight loss) than female rats. Systemic toxic effects in male rats dosed at 250 and 500 mg/kg/day were such as may have induced oxidative stress (such as secondary to dehydration and/or tissue hypoxia, as detailed above), potentially impacting comet results. In support of this, there was strong co-linearity between weight loss and dose, and weight loss rather than dose showed the stronger correlation with % tail DNA response in hepatic tissue in male rats (see Fig. 6). Bioanalysis also revealed evidence of saturation of 4-hydroxylation (the normal pathway of metabolic detoxification an elimination) in hepatic tissue of male rats at doses > 125 mg/kg/day. Glutathione levels however were not depleted (Table 6). This would argue against high levels of DMQI production and suggest that a threshold for significant ROS production secondary to redox cycling of DMQI (if present) was not reached prior to the onset of acute systemic toxicity. In addition, there was evidence of early hepatotoxicity in male rats in these two groups.

The nature of histopathological findings are probably not sufficiently severe to indicate that comets in male rat liver were increased as an indirect consequence of frank liver cytotoxicity, however, as noted in the JaCVAM validation trial (OECD, 2014) "no definitive list of histopathological changes that are always associated with increased DNA migration is available and increased comet values in the presence of histopathological changes should be interpreted with caution and may warrant repeating the assay at lower doses". Moreover, it should be

considered that more severe histopathological changes to liver may have been seen at later sampling times, and that only 2 days after the start of dosing may have been too early to observe such changes.

The equivocal dose response was therefore potentially impacted by the presence of metabolic overload and excessive systemic toxicity in rats dosed at 250 and 500 mg/kg/day, (the latter of which is considered to have exceeded the MTD for this trial). OECD guideline 489 (OECD, 2016c) recommends dose levels used for both acute and sub-acute versions of the comet assay should cover a range from the maximum to one producing little or no toxicity. In this study, a range of lower doses (15–125 mg/kg/day) were used that did not induce significant systemic toxicity, weight loss, or hepatotoxicity in male rats. Examined over this ("non-toxic") dose range, there was no significant dose response and the comet results in male rat liver were clearly negative. Additionally, 2, 6-xylidine, DMAP-conjugate and DMHA were present in hepatic tissue over this dose range (see Fig. 3 and Tables 4 and 6), such that lack of exposure to 2,6-xylidine or metabolites is not an explanation of lack of genotoxic response at "non-toxic" doses.

Therefore, there was no clear indication of biologically relevant genotoxic activity in male or female rats at doses up to those inducing significant weight loss or signs of acute systemic toxicity, which in females was 400 mg/kg/day, and in males was 125 mg/kg/day. The male liver comet dose response result may therefore be considered not biologically relevant (all data within historical control range) or it may be considered that there is a threshold defined by acute systemic toxicity. Since there was no induction either for MN in bone marrow, or comets in nasal tissue or liver, at acceptable levels of toxicity, the no-effect doses are concluded to be 125 mg/kg/day in males and 400 mg/kg/day in females.

These results are considered to highlight the importance of using a range of doses for comet assay studies that include doses that produce little or no toxicity in accordance with OECD guidance GL 489. Previous studies testing 2,6-xylidine for induction of DNA strand breaks via the comet assay in mice (Kohara et al., 2018; Sasaki et al., 1999 above) reported increased comets in various tissues, but the sampling times and tissues affected were inconsistent across the 2 studies. Moreover, both these investigators used only a single dose level (200 mg/kg and 350 mg/kg via gavage respectively). These doses were thus similar to, or exceeded, dose levels which induced metabolic overload, significant weight loss and systemic toxic effects in rats in the current study. There are currently no data regarding 2,6-xylidine metabolism, nor the nature of acute toxic effects in mice, however the LD_{50} of 2,6-xylidine in mice (710 mg/kg), is well below that reported in rats (1230 mg/kg; Vernot et al., 1977), suggesting that mice may be more sensitive to 2,6-xylidine toxicity than rats. Neither authors reported on body weight or other clinical effects, however Sasaki et al. (1999) did report evidence of urine discoloration in mice. This is also reported as an acute toxic effect in rats associated with disturbed normal metabolism, met-Hb formation and haemolysis (NTP, 1990; Lindstrom et al., 1969). It is therefore possible that systemic toxic effects may have contributed to comet results in mice in these studies.

5. Overall discussion of published and new data

5.1. 2,6-Xylidine carcinogenicity: mechanistic considerations

A possible mode of action for rat nasal tumours induced by 2,6-xylidine (NTP, 1990) is one in which large 2,6-xylidine dietary exposures overwhelm normal hepatic metabolism and detoxification (via 4-hydroxylation and conjugation) delivering high concentrations of 2,6-xylidine via the systemic circulation to nasal tissue, which is known to have high metabolic capacity (Yasuhara et al., 2000; Koujitani et al., 1999, 2001). One hypothesis is that it may then be metabolised via N-hydroxylation, resulting in DMHA formation and genotoxicity via DNA adduct formation. According to current thinking, unless a specific DNA repair pathway can be shown to produce a threshold, this would be a non-threshold mechanism. An alternative hypothesis is that it may be metabolised to DMAP with potential to undergo threshold-affected metabolic activation to DMQI. In this situation, cytotoxicity and genotoxicity secondary to DMQI redox cycling and ROS production would be expected only if present in concentrations sufficient to overwhelm phase 2 conjugation of DMAP and glutathione detoxification of DMQI. Additionally, *in vivo*, anti-oxidant defence mechanisms exist to prevent, and/or rapidly repair oxidative DNA injury (Davies, 2001; Lan et al., 2004) such that any cytotoxic and/or genotoxic effects resulting from ROS production may also exhibit a threshold.

The metabolism of 2,6-xylidine in rat nasal tissue has not been studied, however the metabolism of 2,6-diethylaniline (a close structural analogue that also induces nasal tumours in rats) has been extensively studied. These studies have identified that 4-hydroxylation of the 2,6-dialkylalinine (forming 2,6-diethyl-aminophenol) occurs at a 7- fold greater rate in rat nasal tissue as compared with the liver, due to a high concentration of site- and species-specific p450 CYP2A3 (Feng et al., 1990; Green et al., 2000). Detoxifying enzymes important in 2,6-xylidine metabolism (including UTP-glucuronide transferase, and organic sulphates/PAS sulphatases, GSH and GSH-transferase), although present in rat nasal tissue, are, however, at lower levels than in the liver in Sprague Dawley rats (Longo et al., 1988; Heydens et al., 1999). This, (in conjunction with the relatively high pO2 environment), predisposes to quinone-imine formation in rat nasal tissues. Quinone-imine protein and glutathione thioether binding result in nasal tissue persistence, and (at high production levels), cytotoxicity and a proliferative response (ROS production has not been measured). This is considered the primary mechanism predisposing to 2,6-diethylaniline tumour formation in the nasal tissue of rats (Heydens et al., 1999). It should be noted that 2, 6-diethylaniline does not undergo N-oxidation in rat hepatic or nasal tissue, consistent with the postulate that alkyl groups in the 2,6-position impede N-hydroxylation of dialkylanilines (Feng et al., 1990; Green et al., 2000). These findings are highly consistent with, and similar to those of Tydén (2004), who reported tissue persistence of 2,6-xylidine 24 h post administration in rats occurs most prominently in olfactory tissue and Bowman's glands, and is associated with a markedly increased capacity of rat olfactory tissue to activate 2,6-xylidine to a protein-binding metabolite *in vitro*, occurring at a rate 5–12 times greater than other tissues including hepatic tissue. Together these data suggest that intra-nasal metabolic activation of 2,6-xylidine may occur secondary to excessive production of DMAP, (sufficient to overwhelm phase 2 metabolism and result in DMQI-formation) rather than production of DMHA and activation to nitrenium ions as previously presumed.

To further investigate these hypotheses, it was intended in the current study to undertake assessment of DMAP, DMHA, glutathione and 8-OH-dG levels in tissues exhibiting clearly positive genotoxicity. In the event, however, there was no evidence of clearly positive genotoxicity in any of the tissues. Additional investigations were therefore undertaken only in male rat hepatic tissue by virtue of finding an equivocal comet response in this tissue. Nevertheless, some observations can be made, as discussed below.

5.2. Nasal tissue findings

In overview, results of the current study are considered to support the conclusion that at low doses (15–20 mg/kg/day), 2,6-xylidine is efficiently cleared by hepatic metabolism so that there is negligible systemic or nasal tissue bioavailability such as to induce toxic, cytotoxic or genotoxic effects. Despite being detectable in liver, 2,6-xylidine concentrations were below quantifiable levels in nasal tissue in the majority of rats administered 2,6-xylidine at 15 mg/kg/day in this study, and there was no increase in comet levels. Yasuhara et al. (2000) previously documented lack of nasal cytotoxicity, and undetectable levels of 2,6-xylidine in plasma of rats fed doses of 30 ppm in diet for 1 week (estimated intake 26 mg/kg/day). It is notable as well, that there was no

significant increase in nasal, or other tumours in rats fed 15 mg/kg/day 2,6-xylidine in the NTP (1990) rat carcinogenicity study.

At higher doses, 2,6-xylidine is bioavailable in nasal tissues in both male and female rats. Despite this finding however, we did not find evidence of any biologically relevant DNA damage in rat nasal tissue in this study. This argues against any direct genotoxic mechanism of action of 2,6-xylidine in nasal tissue (such as due to N-hydroxylation to DHMA, with nitrenium ion and mutagenic DNA adduct formation). It also suggests that if 2,6-xylidine induces DNA damage via a threshold mechanism, (such as due to DMAP-DMQI redox cycling and ROS-related oxidation injury) that such a threshold (sufficient to overwhelm nasal phase 2 metabolism and anti-oxidant capacity) was not reached. This does not preclude the possibility, however, that such a threshold may be reached with more prolonged dosing at levels sufficient to deplete phase 2 metabolites, glutathione and/or anti-oxidant capacity over time. Yasuhara et al. (2000) reported that cytotoxicity became evident in rat nasal tissue, particularly in Bowmans glands, following 4 weeks administration of 2,6-xylidine at doses of 256.2 mg/kg/day.

5.3. Role of oxidative injury secondary to DMAP-DMQI redox cycling

The low magnitude increase in comets seen in the current study in hepatic tissue of male rats exhibiting acute systemic toxicity, was not associated with glutathione depletion. This suggests that DMQI and ROS could only have been formed (if at all) at low concentrations, insufficient to overwhelm anti-oxidant capacity, which is consistent with lack of clearly positive comet results. Furthermore, any potential effect of low-grade oxidative stress on comet results via this mechanism would be difficult to distinguish from that potentially induced secondary to acute systemic effects such as dehydration, met-Hb and/or tissue hypoxia, which, amongst other factors, may directly impact production of ROS, (França et al., 2007; Pastukh et al., 2015). In the absence of clearly positive comet responses there was no point to measure markers of oxidative DNA damage (such as 8-OH-dG) since levels of this marker would also be expected to be within the normal range. Overall, results of this study are taken to indicate that acute systemic toxicity occurs prior to a threshold for glutathione depletion and the potential for oxidative DNA injury secondary to DMAP-DMQI-redox cycling in rats.

5.4. Implications for role of DMHA

In the current study we have shown there is minor intrahepatic DMHA production *in vivo* in male Sprague-Dawley rats fed high doses of 2,6-xylidine of 125 mg/kg/day or greater. It is noted however that (in the absence of weight loss and systemic toxicity) DMHA production was not associated with a significant increase in comet levels above concurrent or historical control values such as in male rats fed 125 mg/kg/day, suggesting lack of direct association with genotoxicity. This is consistent with findings reported by Kirkland et al. (2012) who did not find evidence of a positive response in the Ames test using rat liver S9 despite biological DMHA production. This is considered to further argue against a putative role for DMHA in the genotoxicity and carcinogenicity of 2,6-xylidine in rats.

5.5. Relevance to humans

The maximum potential human 2,6-xylidine exposure deriving from approved use of lidocaine is 3.5 mg/kg/day. This is 35 × lower than the dose (>125 mg/kg/day) that induced metabolic overload and acute systemic toxicity in rats in this study, and which is identified as a no-effect level for potential genotoxic effects.

Studies of lidocaine metabolism in humans (Keenaghan and Boyes, 1972; Nelson et al., 1977; Tam et al., 1990), identify that a large part of the dose (63.5–80%) undergoes intrahepatic amide hydrolysis (a rate-limited process confined to the liver, catalysed by hepatic carboxylesterase1A (Higuchi et al., 2013)) and is ultimately eliminated in the urine as conjugated DMAP. 2,6-xylidine itself, however, is generally undetectable or present at very low levels only in plasma, and constitutes < 1–2% of the dose eliminated in the urine, despite high dose lidocaine infusions, such as for cardiac arrhythmia prevention or epidural infusion (Tam et al., 1987; Blankenbaker et al., 1975). Other potential metabolites of 2,6-xylidine (such as DMHA) have only been detected in trace amounts in urine (<1% of the dose) (Nelson et al., 1974, 1978). These findings indicate that 2,6-xylidine, (produced as an intrahepatic metabolite of lidocaine), is rapidly and extensively converted to DMAP, conjugated, and eliminated in the urine via a process that does not saturate, (such as to result in significant systemic bioavailability of 2,6-xylidine), despite high dose lidocaine infusions. Low level met-Hb formation (statistically significant but not biologically relevant) (Filipiak-Strzecka et al., 2015; Weiss et al., 1987; Hjelm and Holmdahl, 1965), as well as 2,6-xylidine-Hb-adduct formation (Bryant et al., 1994), have been reported following therapeutic lidocaine administration. Together these data suggest that 2,6-xylidine exposure following therapeutic lidocaine in humans is sufficient to result in low level haem oxidation of DMAP, (forming Hb-adducts), however (other than extremely rare cases with predisposing factors, detailed below) it is insufficient to overwhelm phase 2 metabolism of DMAP, glutathione detoxification of DMQI and cellular anti-oxidant defences, such as to reach a threshold for redox cycling and significant met-Hb production (with associated DMQI and ROS formation and potential cytotoxic or genotoxic effects). This is consistent with lack of evident genotoxicity of lidocaine and lack of any evident causative association with carcinogenicity over 8 decades of extensive human use.

Clinically significant met-haemoglobinaemia, although a well-documented complication of amide local anaesthetics such as benzocaine and prilocaine, is only extremely rarely reported in association with lidocaine administration (Chowdary et al., 2013; Guay, 2009; Vallurupalli and Manchanda., 2011; Gutenberg et al., 2013). This difference is attributed to the relatively low *in vivo* capacity of DMAP (the primary met-Hb forming metabolite of lidocaine, Higuchi et al., 2013) to form met-Hb, which, *in vivo*, requires threshold-affected metabolic activation. The N-hydroxylamine metabolites of the other local anaesthetics may induce met-Hb formation more directly (Hartman et al., 2014, 2017). Nevertheless, rare cases of significant met-haemoglobinaemia are reported in settings in which large doses of lidocaine have been administered in combination with other oxidising agents (Karim et al., 2001), and/or in the presence of other factors that reduce or deplete glutathione, intracellular anti-oxidant or met-Hb reduction capacity. Neuhaeuser et al. (2008) for example, reported met-haemoglobinaemia following administration of lidocaine ($13 \pm \underline{3}$ mg/kg) to infants undergoing cranio-facial surgery, during which they also required major blood transfusion. A number of factors may have predisposed the infants to met-haemoglobinaemia in this setting. In addition to the high dose, young infants may have immaturity of; phase 2 metabolism (Lu and Rosenbaum, 2014); erythrocyte met-haemoglobin reductase activity (Nilsson et al., 1990), and cellular uptake of cysteine, (which affects glutathione levels) (Lavoie et al., 2002). Furthermore, stored blood may be depleted in glutathione and have reduced glutathione synthetase activity (Whillier et al., 2011). This highlights the importance of considering risk factors for threshold-affected metabolic activation of DMAP in patients with such risk factors.

6. Summary and conclusions

In summary, 2,6-xylidine appears resistant to N-hydroxylation and/or activation to nitrenium ions. Instead, it predominantly metabolises via 4-hydroxylation to DMAP which, at high doses that are sufficient to saturate phase 2 metabolism, may undergo a threshold affected metabolic activation pathway forming DMQI, with potential to redox cycle producing ROS, met-Hb and inducing secondary cytotoxic and genotoxic effects. A review of the literature (above) identified evidence that 2,6-xylidine has weak genotoxic characteristics *in vitro* and is able to

induce DNA damage *in vivo*, however, there were no clear positive findings that 2,6-xylidine exposure results in lasting genotoxic effects due to stable genetic damage. The results would support a threshold-dependent metabolic activation pathway, with cytotoxic and genotoxic effects secondary to DMQI redox cycling and ROS production, only evident at doses or concentrations sufficient to overwhelm phase 2 metabolism of DMAP, glutathione detoxification of DMQI and cellular anti-oxidant defences.

A new combined comet and MN study identified lack of *in vivo* genotoxicity in rats at non-toxic doses. A weak dose-related response for comets in male rat livers was considered not biologically relevant since all % tail DNA values fell within the historical negative control range, ("background noise") and the low magnitude dose response was associated with significant weight loss and systemic toxicity. Systemic toxic effects were of a nature as may have induced low-grade oxidative stress and contributed to the marginal comet dose response. Metabolic analyses confirmed DMAP as the predominant metabolite of 2,6-xylidine. Saturation of conjugated-DMAP production, associated with signs of acute systemic toxicity (indicative of metabolic overload), occurred at doses >125 mg/kg/day. DMHA was identified as a minor *in vivo* metabolite in hepatic tissue of male rats administered doses of 125 mg/kg/day or greater, however it was not associated with increased comets at the 125 mg/kg/day (non-toxic) dose. These analyses confirmed that rat tissues were exposed to high levels of 2,6-xylidine, and to the metabolites DMAP and DMHA. A mechanistic assessment was not possible in the absence of clearly positive genotoxic effects at non-toxic doses. Benchmark dose analysis of these data confirmed a non-linear dose response and allowed a conservative estimate of the PoD to be determined (97 mg/kg/day). Since there was no induction either for MN in bone marrow, or comets in nasal tissue or liver, at acceptable levels of toxicity, the no-effect doses for *in vivo* genotoxicity are concluded to be 125 mg/kg/day in males and 400 mg/kg/day in females.

In conclusion, there is no clear evidence that 2,6-xylidine or its metabolites induce genotoxic damage *in vivo* at doses that do not cause metabolic overload and acute systemic toxicity. 2,6-Xylidine appears resistant to metabolic activation via N-hydroxylation, and instead metabolizes predominantly to DMAP, which (at doses sufficient to overwhelm phase 2 metabolism) may undergo threshold-affected metabolic activation to DMQI, with potential to redox cycle producing ROS. Cytotoxic and genotoxic effects due to DMQI redox cycling and ROS production may be evident *in vitro* or *in vivo* where phase 2 metabolism of DMAP and glutathione detoxification of DMQI and/or cellular anti-oxidant defence systems are overwhelmed. However, in the latest study *in vivo*, no clearly positive genotoxic effects were seen, including in nasal tissue, despite dosing to the MTD. In liver, glutathione levels were not depleted prior to a threshold for metabolic overload (saturation of conjugated DMAP production with acute systemic toxicity) being reached. The weight of evidence therefore indicates that 2,6-xylidine is not a direct acting genotoxin or mutagen. It exhibits a PoD based on threshold-dependent metabolic activation, such that it is not genotoxic *in vivo* in the absence of acute systemic toxic effects. Exposure levels that maximally occur through approved therapeutic use of lidocaine in humans are at least 35 × lower than those that induce metabolic overload and systemic toxic effects in rats, consistent with lack of evident association between lidocaine use and cancer despite 8 decades of human therapeutic use.

Funding body information

The new experimental work described in this paper was funded by Animal Ethics Pty, Yarra Glen, Victoria, Australia. Michael Streicker was Study director for that work. Animal Ethics also funded David Kirkland to review the published data and draft the manuscript, and also funded George Johnson to conduct the benchmark dose calculations. Meredith Sheil is a founding director and indirect shareholder of Animal Ethics Pty Ltd.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: D. Kirkland received funding from Animal Ethics Pty Ltd. to review the data and co-ordinate the preparation of the manuscript. G. Johnson received funding from Animal Ethics Pty Ltd. To conduct the BMD analysis of the published bone marrow micronucleus data and the new liver comet data. The new study was sponsored by Animal Ethics Pty Ltd, oversighted by Triveritas, a contract research provider, and carried out by independent research company Integrated Laboratory Systems, LLC to GLP and VICH standards, to address national and international veterinary medicines regulatory approval requirements. Dr Sheil is an inventor of Tri-Solfen®, a founding director and indirect shareholder of Animal Ethics Pty Ltd.

Acknowledgements

We would like to acknowledge the contributions of Andrew Hewitt and Donna Matson (Triveritas) in helping organise and manage the new comet/MN study at ILS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2020.104838.

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