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Derivation of a No-significant-risk-level (NSRL) for dermal exposures to diethanolamine

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ABSTRACT

Diethanolamine (DEA) has been found to produce liver and kidney tumors in mice following lifetime dermal exposures. Data regarding the mode of action by which DEA produces these tumors were used to support a dose-response assessment that resulted in a no-significant-risk-level (NSRL) for dermal exposures to DEA. DEA and its metabolites are structural analogs to endogenous agents important to choline homeostasis. Sufficient information is available to support an epigenetic MOA involving the perturbation of choline homeostasis and hepatic methylation reactions in the formation of mouse liver tumors. This MOA may also apply to mouse kidney tumors, but direct measurements for key events in kidney are lacking. For both tumor types, dose-response data were pooled across four cancer bioassays conducted for DEA and DEA-containing condensates in order to provide a more robust characterization of the dose-response relationships. Doses were expressed in terms of dermally absorbed dose so that the dose-dependency and species differences in the dermal absorption of DEA were addressed. The resulting NSRL value of 3400 ug/day for dermal exposures to DEA is considered to be protective of human health for both tumor endpoints.

1. Introduction

Diethanolamine (DEA, CASRN: 111-42-2) is a small, water-soluble molecule that is used as a surfactant, corrosion inhibitor, and in oil refineries to remove hydrogen sulfide from natural gas. It is also used in the production of diethanolamides that serve as ingredients in cosmetics and shampoos. For these reasons, human populations may be exposed to DEA via occupational exposure in specific industries, and via use of DEA-fatty acid condensates in consumer products (e.g., cosmetics, shampoos) (CIR, 2011). Fatty acid condensates of DEA are produced by reacting fatty acids such as oleic acid, lauric acid, and coconut oil fatty acids with diethanolamine. These condensates differ widely with respect to the amount for free DEA present in the mixture: oleic acid condensate (0.19% DEA), lauric acid condensate (0.83% DEA), and coconut oil acid condensate (18.2% DEA) (NTP, 1999abc). Lifetime dermal exposures to DEA and DEA condensates dissolved in ethanol have been shown to produce liver tumors in male and female mice, and kidney tumors in male mice (NTP, 1999abc, 2001). NTP conducted a logistic regression analysis of the pooled liver tumor results from all four cancer bioassays for DEA and DEA condensates, and concluded that tumor response was primarily determined by free DEA (NTP, 2001). However, with respect to the carcinogenicity of DEA, clear species differences are apparent since no tumors were observed in similarly exposed rats (NTP, 1999abc, 2001).

The mouse liver and kidney tumor data have been used to support cancer dose-response assessments in the past. USEPA (USEPA, 2001, 2002) assumed a mutagenic mode of action and used a no threshold linear dose response model to derive dermal cancer slope factor (CSF) values from the mouse tumor data ranging from 0.0075 to 0.4 $(mg/kg\text{-}day)^{-1}$. More recently, Wang et al. (2014) used the mouse tumor data and a linear, no threshold model, to calculate a No-Significant-Risk-Level (NSRL) of 1.4 ug/day for DEA. These assessments for DEA are complicated by a number of factors, including: (1) a very high background rate for liver tumors in mice (i.e., greater than 60%); (2) the cancer bioassay for DEA (NTP, 1999a) utilized doses that are too high to provide a useful characterization of the dose-response relationship for DEA (i.e., nearly 100% tumor

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response achieved in all treatment groups), largely due to reliance on a maximum tolerated dose-based study design; (3) the dermal absorption of DEA is dose-dependent (i.e., a greater fraction of dermally applied dose is absorbed at higher doses); and (4) the dermal absorption of DEA exhibits clear species differences (Matthews et al., 1997; Sun et al., 1996) due to toxicokinetic factors that are not addressed by allometric scaling of dose (e.g., body weight raised to the ¾ power).

IARC (2013) recently reviewed the information for DEA and concluded that there is *inadequate evidence* in humans for the carcinogenicity of DEA, and that there is *sufficient evidence* in experimental animals for the carcinogenicity of DEA. IARC concluded that there was weak evidence of genotoxicity noting that DEA was not genotoxic in most in vitro test systems, and did not induce micronuclei in the mice test by NTP. Lastly, IARC concluded that there was moderate experimental support for choline deficiency as the mechanism of carcinogenesis, but noted several limitations in the database of mechanistic studies (see discussion in Section 4 below).

Although California regulations (OEHHA, 2012; Title 27, California Code of Regulations Article 7. No Significant Risk Levels § 2570) describes specific methods for calculating NSRL values, these regulations are also sufficiently flexible to permit the use of the best available science, e.g. "*Nothing in this article shall preclude a person from using evidence, standards, risk assessment methodologies, principles, assumptions or levels not described in this article to establish that a level of exposure to a listed chemical poses no significant risk.*" (ibid). The goal of this work is to conduct a dose-response assessment for DEA-induced tumor in mice based upon a consideration of the mode of action (MOA) using the best available science, data, and methods.

2. Methods

The published literature was searched (via Pubmed, Toxnet, reference list for recent reviews), and reviewed to identify key data sets for characterizing: (1) the MOA for DEA-induced mouse tumors; (2) the dose-response relationship for tumors in mice exposed to DEA via dermal application; and (3) the toxicokinetics of DEA in mice and humans. This information was summarized and used to support decisions made in the dose-response assessment.

Cancer potency values were derived for DEA using the best available science, in a manner that is consistent with available USEPA guidelines (USEPA, 2005, 2012), using the following equations:

Nonlinear Approach:

$$RfD = POD/UFt \quad (1)$$

$$NSRL = RfD^{*}BW^{*}CF \quad (2)$$

Where, RfD = Reference dose (mg/kg-day); POD = Point of departure (mg/kg-day, absorbed dose); UFt = Total uncertainty factor (unitless), which is calculated as the product of individual uncertainty factors for interspecies variation (UFa), intraspecies variation (UFh), LOAEL-to-NOAEL (UFl), subchronic-to-chronic extrapolation (UFs), and database deficiencies (UFd); NSRL = No-significant-risk-level (ug/day); BW = Body Weight (70 kg); and CF = Conversion factor (1000 ug/mg)

Linear Approach:

$$CSF = BMR/POD \quad (3)$$

$$NSRL = TR^{*}BW^{*}CF/CSF \quad (4)$$

Where, CSF = Cancer slope factor $(mg/kg\text{-}day)^{-1}$; BMR = Benchmark response rate (0.1, unitless); POD = Point of departure (mg/kg-day); NSRL = No-significant-risk-level (ug/day); TR = Target risk (1×10^{-5}); BW = Body Weight (70 kg); and CF = Conversion factor (1000 ug/mg).

This assessment relies upon the calculation of absorbed dose of DEA, which was calculated as follows:

$$MADD = MADC^{*}F_{DEA}{}^{*}ABS_{m} \quad (5)$$

Where,

MADD = Mouse absorbed DEA dose (mg/kg-day);
MADC = Mouse applied dose of DEA condensates as stated by NTP (NTP, 1999abc, 2001);
F_{DEA} = Fraction DEA present in DEA condensates; and
ABS_m = Mouse dermal absorption fraction (unitless); this value is dose-dependent, and was estimated using the relationship depicted in Fig. 3.

Subsequently, human equivalent doses were calculated and expressed in terms of applied dose using the following equation:

$$HADD = MADD/ABS_{H} \quad (6)$$

Where,

HADD = Human Applied DEA Dose (mg/kg-day)
MADD = Mouse Absorbed DEA Dose (mg/kg-day); and
ABS_H = Human dermal absorption fraction (unitless).

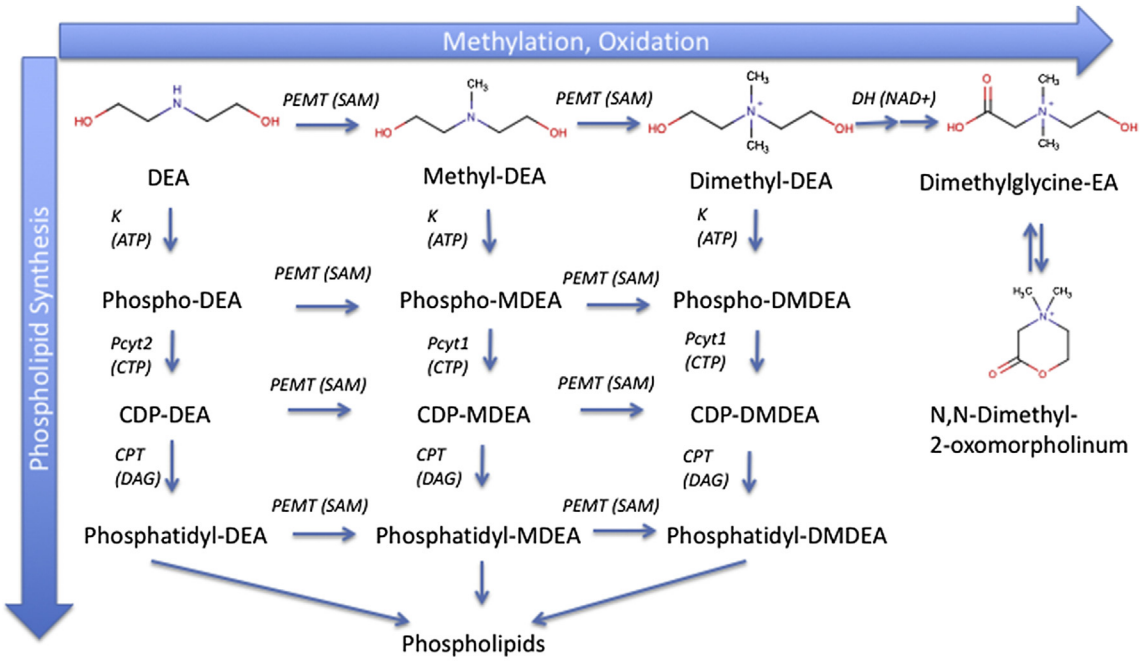
The dose-response assessment conducted for DEA includes the following steps: (1) Endpoint/data set selection, which includes consideration of data sets pooled together across several cancer bioassays; (2) Dose measure calculation, which includes the estimation of the dermal absorbed dose of DEA; (3) Dose-response model selection of the observed data based upon a consideration of model fit; (4) Point-of-departure (POD) selection, which includes calculations for determining the human equivalent dose (HED); (5) Low-dose extrapolation, which included linear and nonlinear methods; and (6) NSRL calculation. Several of these steps require a consideration of the toxicokinetics and MOA for the carcinogenic effects of DEA. For this reason, toxicokinetic and mechanistic data from the published literature were reviewed prior to conducting the dose-response assessment.

All dose-response modeling was performed using USEPA's Benchmark Dose Software (BMDS, version 2.6). A number of dichotomous models are available in this software, including gamma, logistic, log-logistic, log-Probit, multistage, Probit, Weibull, quantal-linear, and dichotomous Hill model. The best fitting model was selected based upon visual inspection, Akaike's information criterion (AIC), and chi-square goodness-of-fit p-value. The two highest dose groups (46.2 and 147 mg/kg-day, absorbed dose) in the pooled liver tumor data were dropped since a near-maximal response (97%) was observed at 16.3 mg/kg-day and dropping these two dose groups resulted in an improvement in the AIC and p-values. A benchmark response rate (BMR) of 10% was considered appropriate for the pooled data sets, consisted with USEPA guidelines (USEPA, 2012).

3. Results

3.1. Mode of action for Mouse liver and kidney tumors

MOA is defined here as a sequence of key events, including



DEA = diethanolamine; CDP = cytodine diphosphate; DAG = diacylglycerol; ATP = adenosine triphosphate; SAM = S-adenosyl methionine; PEMT = ; CPT = cytodine phosphotransferase

Fig. 1. DEA Metabolism (based on Matthews et al., 1995).

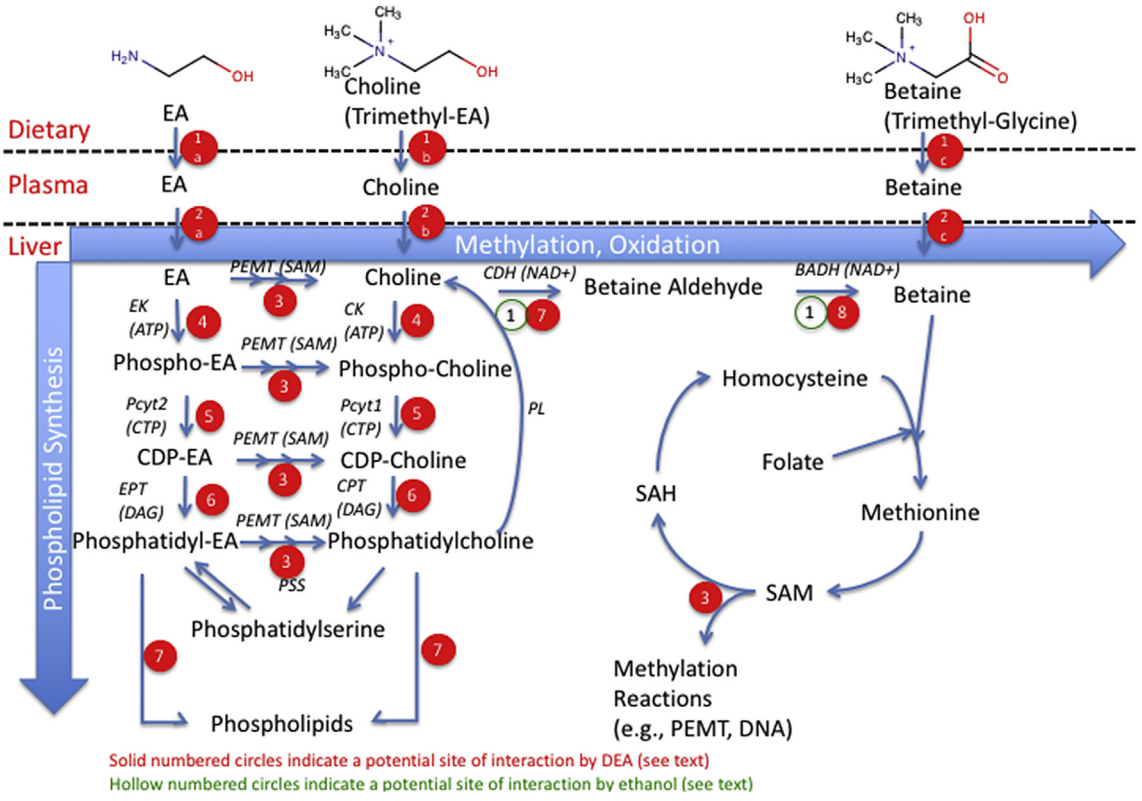


Fig. 2. Choline transport and metabolism.

toxicokinetic processes that begin with exposure to DEA, as well as the toxicodynamic processes that occur once DEA reaches the target tissue. This differs from previously used definitions for MOA, where it was defined to start with the chemical reaching the target

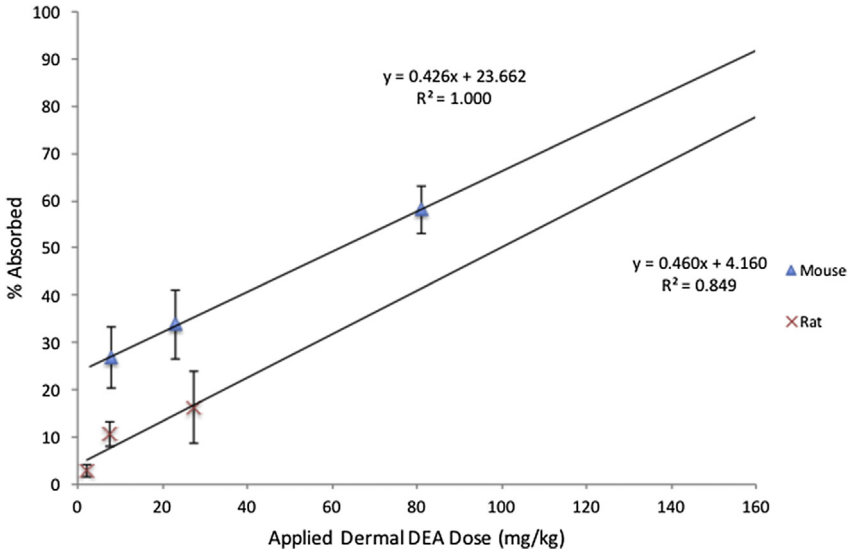


Fig. 3. Dose-Dependent Dermal Absorption of DEA in Mice and Rats in 48 h (Matthews et al., 1997).

tissue (i.e., excluding toxicokinetic processes leading up to this point) (USEPA, 2005). There is no risk without exposure, and therefore, as Borgert et al. (2015) point out, best practices in problem formulation for evaluating potential carcinogenic hazards and risks, require due consideration of dosimetry when formulating hypotheses. Although a complete evaluation of the cancer MOA for DEA within existing frameworks (USEPA, 2005; Boobis et al., 2008; Meek et al., 2014; Simon et al., 2014) is beyond the scope of this paper, data regarding DEA toxicokinetics, choline function and its perturbation by DEA and ethanol, and other potential MOAs are discussed briefly below to help guide decisions made in the dose-response assessment (Section 3.2).

3.1.1. DEA toxicokinetics

DEA is well absorbed in mice following dermal exposures, corresponding to approximately 27–58% of an administered dose (Matthews et al., 1997). In this study, single doses of 8–81 mg/kg were applied to the backs of B6C3F1 mice within 48 h. The percent of DEA absorbed exhibited clear dose-dependency, with the lowest percentage (27%) corresponding to the lowest tested dose (8 mg/kg) and the highest percentage (58%) corresponding to the highest tested dose (81 mg/kg) (Fig. 3). Dermal absorption is considerably lower (approximately 3–16%) but also clearly dose dependent for dermal absorption of DEA in F344 rats similarly exposed to 2.1–27.5 mg/kg (Matthews et al., 1997). Following exposure DEA is distributed to all tissues in rodents. In tracer studies, the highest concentrations of radiolabeled DEA are detected in the liver and kidney of rats and mice, with tissue:blood ratios exceeding a factor of 100 (Matthews et al., 1997). In rat liver and brain, the majority of the radiolabel present in tissue is in the form of the parent chemical (Matthews et al., 1995). Given that DEA is a small, water-soluble molecule, the accumulation of DEA in liver and kidney tissues is not likely to be driven by tissue partitioning. Studies in rats have shown that in the liver, DEA is metabolized to a limited extent via two general pathways (Fig. 1). DEA undergoes enzymatic methylation reactions (via transfer from S-adenosylmethionine or SAM) to form methyl- and dimethyl-DEA, which in turn can undergo enzymatic oxidation to form dimethylglycine-EA (Matthews et al., 1995, 1997). In addition, DEA and its methylated metabolites can be phosphorylated and subsequently incorporated into phospholipids. The extent to which DEA is incorporated into phospholipids is limited, and as noted above, the majority of the radiolabel detected in liver and kidney tissue was in the form of parent chemical (Matthews et al., 1995, 1997). Limited data collected using human liver tissue slices indicate that these metabolic pathways (DEA methylation, phospholipid incorporation) also occur in human tissues (Matthews et al., 1995). In rats, DEA is excreted primarily in the urine as parent chemical, however a minor ringed metabolite (N,N-dimethyl-2-oxomorpholinum) has also been detected in urine (Matthews et al., 1997).

3.1.2. Choline homeostasis

Because the proposed MOA for DEA involves perturbation of choline homeostasis (see text below), it is important to provide some background information on choline. Choline is an essential nutrient used in the synthesis of phospholipids (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin; produced in all tissues), betaine (predominantly in liver and kidney), and acetylcholine (predominantly in nerve tissue) (Zeisel, 2012; Hollenbeck, 2012). Choline can be supplied by the diet, metabolism of ethanolamine, or recycled from phospholipids. As a cation, choline is absorbed from the intestinal lumen and taken up by tissues via the organic cation transporter. Choline can be formed by the methylation of ethanolamine (using SAM as a methyl donor), which in turn can be oxidized to form betaine. Ethanolamine and choline can be phosphorylated, and further metabolized for incorporation into phospholipids (Fig. 2). Betaine can serve as methyl donor to homocysteine, ultimately contributing to the formation of SAM, which is important for methylation reactions in the cell. The liver has a higher demand than other tissues for choline since it synthesizes phospholipids that are secreted to plasma and intestinal lumen. The kidney also has a higher demand for choline since betaine serves as an important osmolyte (Obeid, 2013).

Deficient levels of choline in the diet are associated with a number of changes and adverse effects. Mice fed choline deficient diets exhibit significant reductions in hepatic choline, SAM, and phospholipid intermediate (phosphocholine, glycerolphosphocholine, phosphatidylcholine) concentrations (Lehman-McKeeman et al., 2002). In addition, choline deficient mouse hepatocytes exhibit hypomethylation of GC-rich regions of DNA similar to that observed with choline deficiency (Bachman et al., 2006), and mice exhibit increased hepatocyte proliferation (Mellert et al., 2004;

Kamendulis and Klaunig, 2005). In response to low choline levels, there is a redistribution of choline and choline-containing molecules (e.g., the liver can catabolize phosphatylcholine to recover choline) to meet the needs of critical tissues (e.g., liver, brain): (1) decreased hepatic phospholipid secretion to plasma and intestinal lumen; (2) increased mobilization of phospholipids in extrahepatic tissues and increased hepatic uptake of phospholipids from plasma (Li et al., 2007; Park et al., 2011). In the rodent liver, choline deficiency produces a prolonged state of steatosis (fatty liver), cell proliferation (Zeisel, 1996), DNA hypomethylation (Locker et al., 1986), decreased gap junction intercellular communication (GJIC; Kamendulis et al., 2004), fibrosis, necrosis, and tumors (Locker et al., 1986; Ghoshal and Farber, 1984; Zeisel et al., 1995). Hepatocellular proliferation in particular is identified as an early key event, with a 30-fold increase in DNA synthesis observed within one day of rats placed on a methyl-deficient diet (Christman et al., 1993). In the rodent kidney, prolonged choline deficiency produces necrosis and toxicity (Monserrat et al., 1969, 1972; Michael et al., 1975; Zeisel and Blusztajn, 1994). In rats, renal lesions caused by diets deficient in methionine and choline exhibited "*nuclear changes in the renal epithelium suggestive of preneoplasia …*" (Newberne et al., 1968).

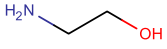
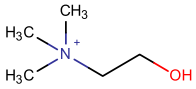
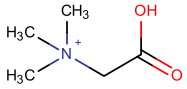
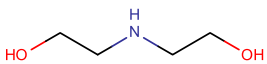
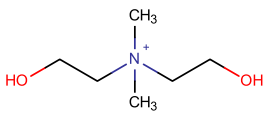
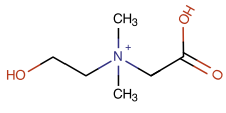
3.1.3. *Perturbation of choline homeostasis by DEA*

Inspection of Figs. 1 and 2 reveal that the metabolic pathways for DEA and for choline are very similar, and likely involve overlap of the same enzyme systems. Thus, DEA and its metabolites serve as structural analogs to endogenous agents important for choline homeostasis. Specifically DEA is a structural analog of ethanolamine (EA), dimethyl-DEA a structural analog of choline, and dimethylglycine a structural analog of betaine (Table 1). Because of the high degree of overlap in metabolic pathways, there are multiple points where DEA and its metabolites can perturb choline homeostasis (indicated by the numbered solid circles in Fig. 2).

- *Inhibition of Choline Transport (points 1 and 2 in Fig. 2)* – DEA inhibits the transport of choline across cell membranes. *In vitro* studies have shown that DEA inhibits uptake of choline in Syrian hamster embryo (SHE) and Chinese hamster ovary (CHO) cells at concentrations as low as 20 mg/L (Lehman-McKeeman and Gamsky, 1999, 2000). Significant decreases in hepatic choline concentrations have been reported in mice following short-term exposures to 80–160 mg/kg-day DEA (Stott et al., 2000; Craciunescu et al., 2009; Lehman-McKeeman et al., 2002). Potential inhibition of intestinal transport (point 1 in Fig. 2) is only relevant to oral exposures to DEA, and Stott et al. (2000) suggested that oral exposure may have occurred under the conditions of the NTP mouse bioassay. Competitive inhibition of tissue organic cation transporters would be consistent the high degree of DEA accumulation in mouse liver and kidney tissue (Matthews et al., 1997; Mendrala et al., 2001).
- *Decreased SAM Concentrations (point 3 in Fig. 2)* – Significant decreases in hepatic SAM concentrations and increased S-adenosylhomocysteine (SAH) concentrations have been reported in mice following short-term exposures to 80–160 mg/kg-day DEA (Craciunescu et al., 2009; Lehman-McKeeman et al., 2002). There are at least two ways in which DEA reduces hepatic SAM concentrations: (1) By reducing choline and betaine concentrations, there is less betaine available to donate methyl groups to homocysteine needed to regenerate SAM; and (2) SAM is likely utilized in the metabolism of DEA to methyl- and dimethyl-DEA (Fig. 1). Because dimethyl-DEA may not participate in methyl transfer reactions like its endogenous structural analog, betaine, methylated metabolites of DEA may serve as methyl sinks.
- *Decreased Phospholipid Synthesis (points 4–7 in Fig. 2)* – Significant decreases in hepatic phospholipid intermediates have been reported in mice following short-term exposures to 20–160 mg/kg-day DEA (Stott et al., 2000; Craciunescu et al., 2009; Lehman-McKeeman et al., 2002). There are at least two ways in which DEA reduces phospholipid intermediate concentrations: (1) By reducing choline concentrations, there is less available for phospholipid synthesis; and (2) In response to lower choline levels, there may be some adaptation in mice resulting in less phospholipid synthesis, as well as increased phospholipid catabolism to recover choline, as part of homeostatic mechanisms. Interestingly, the hepatic concentrations of phospholipid sphingomyelin were increased by DEA exposure. This result is best explained by an adaptive response of the mouse liver to reduce the secretion of phospholipids to blood and intestines in response to low choline levels.
- *Decreased Betaine Concentrations (Point 8 in Fig. 2)* - Significant decreases in hepatic betaine concentrations have been reported in mice following short-term exposures to 80 mg/kg-day DEA (Craciunescu et al., 2009). There are at least two ways in which DEA reduces betaine concentrations: (1) By reducing choline concentrations, there is less available for oxidation to betaine; and (2) the oxidation of dimethyl-DEA likely occurs via the same enzyme and requires the same co-factors (NAD+) as needed for choline oxidation, and therefore some degree of competitive inhibition and/or co-factor depletion is possible.

The effects of DEA on choline homeostasis are reversible upon cessation of exposure *in vivo* (Lehman-McKeeman et al., 2002; Mellert et al., 2004), and are ameliorated by choline supplementation in vitro (Lehman-McKeeman and Gamsky, 1999, 2000; Kamendulis et al., 2004). These results indicate that the weight of evidence is high for the essentiality of this key event in the

Table 1
Comparison of Chemical Structures of DEA and its Metabolites to Endogenous Agents.

Endogenous agents	Ethanolamine 	Choline 	Betaine 
Exogenous Analogs	Diethanolamine 	Dimethyl-Diethanolamine 	Dimethylglycine-EA 

pathogenesis of DEA-induced toxicity. Diets deficient in methyl donors cause depletion of SAM in liver, leading to DNA hypomethylation (Wainfan et al., 1992). These events in turn may plausibly cause altered gene expression, creating a proliferative stimulus that selectively promotes spontaneously mutated cells, resulting first in preneoplasia and eventually, if the proliferative stimulus continues, development of tumors. In studies of B6C3F1 mice, the same strain that NTP used where dermal exposure was reported to cause liver tumors, Newberne et al. (1982) reported choline deficient diet studies indicated "*the liver of the* B6C3F1 *mouse is initiated by time of weaning*", and that a choline deficient-diet acts as a tumor promotor.

Based upon the information presented above, the following events are proposed for the MOA by which DEA produces liver tumors in mice under conditions of the NTP cancer bioassays: (1) Exposure to DEA, primarily via the dermal route, but contributions from the oral route (by grooming behavior following topical application leading to oral ingestion) are also possible (Stott et al., 2000); (2) Absorption of DEA through the skin; (3) Transport of DEA from plasma to liver tissue; (4) Perturbation of choline homeostasis, decreased SAM concentrations; (5) altered DNA methylation; (6) Altered gene expression, cell proliferation, apoptosis, GJIC, and cell transformation; (7) Tumor formation/progression. The weight of evidence supporting these events, as well as potential species differences and sources of nonlinearity are summarized in Table 2. This information will be used to guide decisions made in the dose-response assessment (Section 3.2).

Less information is available regarding the potential MOA for DEA-induced kidney tumors in mice. Similar to responses observed in mouse liver, sustained increases in kidney weight and DNA synthesis were observed in male mice exposed to 160 mg/kg-day DEA for 1–13 weeks (Mellert and Bahnemann, 2001). Increased DNA synthesis was localized in the proximal tubules and the outer stripe of the outer medulla, which corresponds to the location of tumor formation. There are some additional parallels that can be made between mouse liver and kidney tissue that support a common mode of action for both tissues. The liver and kidney reflect the two tissues in mice with the highest internal exposure to DEA (Matthews et al., 1997), likely mediated by organic cation transporters. The oxidation of choline to betaine predominates in rodent liver and kidney tissue, and betaine is normally more abundant in these two tissues compared to other tissues (Kempson et al., 2013). For this reason, these two tissues may be more susceptible to perturbations of the choline/betaine/SAM pathway than other tissues. As noted above, it is plausible that mouse kidney toxicity induced by DEA could be mediated by choline deficiency, as lesions akin to preneoplasia were seen in rat kidneys following treatment with diets deficient in methionine and choline (Newberne et al., 1968). However, information currently available regarding the potential role of choline perturbation in mouse kidney tumor formation is insufficient to make any conclusions with a reasonable degree of confidence.

3.1.4. Potential vehicle effects: interaction with ethanol

Ethanol was used as the vehicle for administering DEA and DEA condensates in the NTP bioassays (NTP, 1999abc, 2001). Dermal absorption of ethanol under conditions of the NTP bioassay is fairly low (3–5%), which corresponds to absorbed ethanol doses of approximately 40–62 mg/kg-day (Leibold and van Ravenzwaay, 2003). Ethanol has been reported to enhance the dermal permeation of some agents (Heard and Screen, 2008), however this has not been specifically evaluated for DEA. Ethanol has been found to disrupt choline metabolism in perfused rat liver studies and in in vitro studies using rat liver mitochondria (Barak et al., 1973; Thompson and Reitz 1979). Dermal exposure of mice to ethanol alone, under the same conditions as the vehicle used in the NTP cancer bioassays, resulted in a significant decrease in hepatic betaine concentrations (Lehman-McKeeman et al., 2002). Both ethanol and choline oxidation share a common co-factor (NAD+). Ethanol, through its metabolism by alcohol dehydrogenase, can produce a significant change in the redox status of the liver, thereby increasing the ratio of NADH:NAD+ (Barak et al., 1973; Peters and Preedy, 1998). For this reason, one possible explanation for the interaction of ethanol on DEA carcinogenesis is via decreasing the concentration of the cofactor (NAD+) required for betaine synthesis, and subsequently decreasing hepatic SAM levels. This potential interaction is depicted by the hollow circles in Fig. 2. In addition, ethanol has been shown to promote altered cell foci and tumor formation in livers of experimental animals treated with nitrosamines (Driver and McLean 1986; Takada et al., 1986), and is widely regarded as a risk factor for cancer, including liver cancer (IARC 1988; Seitz and Stickel 2007). Together, these data indicate that the vehicle (ethanol) used by NTP in the cancer bioassays can exacerbate the perturbation of choline homeostasis, which in turn may contribute to the carcinogenic response observed in mice.

3.2. Dose-response assessment

Dose-response assessments for DEA-induced mouse liver and kidney tumors are summarized below. Information regarding the mode of action (Section 3.1) was used to guide key decisions made in the dose-response assessments.

3.2.1. Endpoint/data set selection

NTP has conducted a series of cancer bioassays for DEA and DEA-containing condensates in F344 rats and B6C3F1 mice (NTP, 1999abc; 2001). The incidence of liver tumors was increased in a dose-dependent manner in mice of both sexes, while that for kidney tumors was increased in male mice exposed to high doses (Table 3). The incidence of liver and kidney tumors was not increased in similarly exposed rats. The doses used in the DEA cancer bioassay resulted in a liver tumor response (hepatocellular adenomas, hepatocellular carcinomas, hepatoblastomas) close to 100% in all treated mice (NTP, 1999a). These data by themselves are of limited use for characterizing a POD value (*i.e.*, a 10% response falls well outside the range of observation defined by the data). However, lower tumor incidence values were reported in bioassays using test condensates with lower free DEA content levels (NTP, 1999c, 2001), and tumors were not increased in the condensate with the lowest free DEA content (NTP, 1999b). Based upon the best available science, the datasets from all four cancer bioassays (Table 3) can be combined in a manner consistent with USEPA guidelines (USEPA, 2012), which state, "*Datasets that are statistically and biologically compatible may be combined prior to dose-response modeling, resulting in increased confidence, both statistical and biological, in the calculated BMD. The simplest approach to combining datasets is to treat the data as if they were all collected simultaneously.*" In support of combining data sets, NTP conducted its own logistic regression analysis of the liver tumor data from all four cancer bioassays, and concluded that liver tumor response is primarily determined by the free DEA content of each condensate (NTP, 2001). Furthermore, visual inspection of the pooled data in Table 3 for liver tumors and kidney tumors (renal tubule adenomas and carcinomas) indicate that they define a consistent dose-response relationship. Based upon these considerations, two pooled data sets were used to characterize the cancer potency of DEA: (1) liver tumors (adenomas/carcinomas/blastomas) in male and female mice combined; and (2) kidney tumors in male mice.

In general, survival in treated animals was similar to control animals with one exception. A small (~4% at the highest dose) but

Table 2
Key events in the proposed mode of Action for DEA-Induced mouse liver tumors.

Event	Supporting evidence		Potentially inconsistent evidence	Potential implications to RIsk assessment		Sources of nonlinearity
	Animals	Humans		Vehicle interactions (ethanol)	Species differences	
1) Exposure	Mice and rats were dermally exposed to up to 160 mg/kg DEA (NTP, 1999abc, 2001)	Humans are dermally exposed to DEA in consumer products (Craciunescu et al., 2009)	No data were located	No data were located	DEA exposures to mice (up to 160 mg/kg-day; NTP, 1999abc, 2001) are orders of magnitude higher than expected in humans from consumer products (Craciunescu et al., 2009). Because of grooming behavior, dermal exposures to mice may have also included a significant oral component (Stott et al., 2000)	No data were located
2) Absorption	By the dermal route, DEA is well absorbed in mice (~27–58%), and moderately absorbed in rats (~3–16%) (Matthews et al., 1997)	Dermal absorption of DEA through human skin is low (<1%) (Brain et al., 2005; Kraeling et al., 2004)	No data were located	Ethanol has been reported to enhance the dermal permeation of some chemicals (Heard and Screen, 2008)	Clear species differences are observed, with absorption in mice>rats>humans (Matthews et al., 1997; Brain et al., 2005; Kraeling et al., 2004)	The fraction of DEA that is dermally absorbed is dose dependent (i.e., a large fraction is absorbed at higher doses; Fig. 3) (Matthews et al., 1997). Repeated dermal exposures to DEA alters the histopathology of mouse and rat skin (hyperkeratosis) in a dose-dependent manner (NTP, 1999a). However the impact on of hyperkeratosis on dermal absorption of DEA is not known.
3) Transport from plasma to liver	DEA preferentially accumulates in mouse and rat liver (Matthews et al., 1995, 1997; Mendrala et al., 2001).	DEA is taken up by human liver slices exposed to 1 mM in vitro (Matthews et al., 1995)	No data were located	No data were located	Dramatic species differences in hepatocyte DNA synthesis have been observed in vitro (mouse~rat>>human) (Klaunig and Kamendulis, 2005). These differences may be due in part to differential uptake (not specifically evaluated). Species differences in organic cation transporter affinities have been reported (Dresser et al., 2000), but have not been evaluated specifically for DEA.	Accumulation in liver tissue is likely driven by membrane transport systems (organic cation transporter) rather than partitioning/passive diffusion, and appears to be saturable (Matthews et al., 1995, 1997) in rodents. Because DEA is structurally similar to a choline precursor (ethanolamine), its uptake in to liver may be increased in a dose-dependent manner as choline deficiency becomes manifest
4) Perturbation of choline homeostasis, decreased SAM concentrations	Primary effect on choline: - Inhibiting choline uptake into cells in vitro at concentrations as low as 20 mg/L (Lehman-McKeeman et al., 1999, 2000) - Decreased hepatic choline, betaine, SAM levels in mice exposed to 40–160 mg/kg DEA (Stott et al., 2000; Craciunescu et al., 2009; Lehman-McKeeman et al., 2002)	Methylation of DEA, presumably via SAM, reported by human liver slices (Matthews et al., 1995) and in human plasma (Craciunescu et al., 2009)	Small discrepancy in dose-response concordance for SAM reduction (Lehman-McKeeman et al., 2002) and tumor formation (NTP, 1999abc, 2001). Hepatic steatosis, a hallmark of choline deficiency, was not observed in rats or mice under the conditions of the NTP bioassay. However, there are important differences between dietary choline deficiency and the proposed DEA MOA (see text in Discussion)	Ethanol oxidation requires the same cofactor (NAD+) as choline and DEA oxidation. Altering hepatic redox status (NAD+/NADH) may affect choline oxidation to betaine. Dermal exposure to ethanol results in decreased betaine concentrations (Lehman-McKeeman et al., 2002)	There are clear species differences in the oxidation of choline to form betaine, which is of major significance in rodents, but a minor pathway in humans (Lieber and Packer, 2002; Zeisel and Blusztajn, 1994). Humans rely more upon tetrahydrofolate for maintenance of SAM. Mice appear to be more sensitive than rats with respect to effects on SAM. Short-term exposures of rats to 64 mg/kg-day DEA	No data were located

(*continued on next page*)

Table 2 (*continued*)

Event	Supporting evidence		Potentially inconsistent evidence	Potential implications to RIsk assessment		Sources of nonlinearity
	Animals	Humans		Vehicle interactions (ethanol)	Species differences	
	Additional stress on methyl pool: - SAM utilized during DEA metabolism (Matthews et al., 1995) - As a betaine analog, methylated DEA metabolites may serve as methyl sinks (e.g., not available for methyl transferase activity)				failed to have an effect on hepatic SAM, while short-term exposures of mice to 80–160 mg/kg-day DEA produced a significant decrease in hepatic SAM (Stott et al., 2000; Lehman-McKeeman and Gamsky, 2000, 2002; Craciunescu et al., 2009).	
5) Altered DNA methylation	Altered DNA methylation reported in mouse hepatocytes treated with 470 mg/L DEA in vitro (Bachman et al., 2006)	No data were located	No data were located	No data were located	The B6C3F1 mouse is particularly sensitive to hypomethylation due in part to decreased capacity to maintain normal methylation status (Counts et al., 1996).	No data were located
6) Altered gene expression, DNA synthesis, apoptosis, GJIC, cell transformation	- Increased DNA synthesis and liver weights in mice exposed to 10–1250 mg/kg DEA (Mellert et al., 2004) - Sustained increased in DNA synthesis and organ weights in mouse liver and kidney following exposure to 160 mg/kg-day for 1–13 weeks (Mellert and Bahnemann, 2001) - Altered gene expression in mouse hepatocytes exposed to 500 mg/L DEA in vitro (Kamendulis and Klaunig, 2005) - Increased apoptosis in mouse neural cells following exposure to 80 mg/kg-day (Craciunescu et al., 2009) - GJIC was reduced in mouse hepatocytes exposed to up to 500 mg/L DEA (Kamendulis et al., 2004) - DEA exposure produced concentration dependent transformations in CHO cells in vitro (significant at 500 mg/L), which was ameliorated by choline supplementation (Lehman-McKeeman and Gamsky, 2000)	DNA synthesis and GJIC were not affected by DEA in human hepatocytes exposed to up to 750 mg/L in vitro (Kamendulis et al., 2004; Kamendulis and Klaunig, 2005)	No data were located	No data were located	Clear species differences (mouse~rat>>human) reported for DNA synthesis and decreased GJIC in hepatocytes exposed to DEA (Kamendulis et al., 2004; Kamendulis and Klaunig, 2005). Human hepatocytes were found to be nonresponsive for both endpoints when exposed to DEA.	No data were located
7) Tumor Formation/ Progression	Clear evidence of liver tumors in male and female mice exposed to 40–160 mg/kg-day DEA (NTP, 1999a) and DEA	No data were located	Liver tumors not observed in DEA exposed rats (NTP, 1999, 2001), despite being sensitive to choline deficiency.	No data were located	The background rate of liver tumors is much higher in mice (~60–70%) than rats (~0–2%) (NTP, 1999abc, 2001). In humans, the lifetime	No data were located

Table 2 (*continued*)

Event	Supporting evidence		Potentially inconsistent evidence	Potential implications to RIsk assessment		Sources of nonlinearity
	Animals	Humans		Vehicle interactions (ethanol)	Species differences	
	condensates (NTP, 1999b, 2001)		Importancy toxicokinetic differences (absorption) and toxicodynamic differences (sensitivity to hypomethylation) may explain this apparent inconsistency		risk of developing cancer of the liver and bile duct is low (0.9%) (SEER, 2015).	

Table 3
Dose-response data for tumors in mice dermally exposed to DEA-Containing mixtures (NTP, 1999abc, 2001).

Chemical (reference)	Free DEA Content[b]	Daily dose (mg/kg-day)[a]			Liver tumors (male and female mice combined)[d]	Kidney tumors (male mice)[e]
		Test agent administered	DEA administered	DEA absorbed[c]		
DEA (NTP, 1999a)	100%	0	0	0	72/100	3/50
		40	40	16.3	**97/100**	**7/50**
		80	80	46.2	**100/100**	**8/50**
		160	160	147	99/100	**9/50**
Coconut oil acid DEA condensate (NTP, 2001)	18.2%	0	0	0	62/100	1/50
		100	18.2	5.7	**85/100**	1/50
		200	36.4	14.3	**97/100**	**9/50**
Lauric acid DEA condensate (NTP, 1999b)	0.83%	0	0	0	58/100[f]	0/50
		100	0.83	0.20	**74/99**[f]	1/50
		200	1.7	0.40	**73/100**	1/50
Oleic acid DEA condensate (NTP, 1999c)	0.19%	0	0	0	62/99[f]	0/49
		15	0.029	0.0067	71/100[f]	0/50
		39	0.074	0.018	70/100[f]	0/50

Bolded values indicate that the incidence was significantly increased in male and/or female mice.
[a] Daily dose applied dermally 5 days/week.
[b] Based on reported free DEA content (NTP, 1999abc, 2001).
[c] Estimated based on linear regression of absorption rates as a function of dose (Fig. 3), collected by Matthews et al. (1997).
[d] Liver tumors include hepatocellular adenomas, carcinomas, and hepatoblastomas.
[e] Kidney tumors include renal tubule adenomas and carcinomas.
[f] The combined incidence of liver tumors was estimated by summing the incidence values for hepatocellular adenomas, carcinomas, and hepatoblastomas.

significant decrease was reported for the survival of female mice treated with the DEA compared to controls (NTP, 1999a). However, the survival of control animals in this study (720 days) appears to be slightly higher than reported for control female mice (684–704 days) in the other bioassays, as well as slightly higher that reported in control male mice (693–704 days) in the NTP bioassays (NTP, 1999abc, 2001). Because the effects of DEA treatment on survival were not significant or were small, incidence values for liver and kidney tumors in treated mice were not adjusted for survival.

3.2.2. Dose measure selection

In their derivation of dermal potency estimates for DEA, USEPA relied upon allometric scaling of dose. Allometric scaling of dose to body weight raised to the ¾ power serves as the recommended approach by USEPA for extrapolating doses across species following oral exposures to chemicals (USEPA, 2011). Based upon default body weights of 0.03 and 70 kg for mice and human, respectively, allometric scaling of dose to the ¾ power predicts that for a given administered oral dose, humans would achieve an internal equivalent dose that is approximately 7-fold higher than achieved in mice. Allometric scaling of oral dose primarily accounts for toxicokinetic processes, but may also include a toxicodynamic component. A brief discussion of toxicokinetic and toxicodynamic factors pertinent to dermal exposures to DEA is provided below.

- *Toxicokinetic Factors* - With respect to toxicokinetics, the factors that dictate chemical absorption by the oral route are considerably different from those for the dermal route of exposure. For example, the thickness of stratum corneum (humans>rats>mice; Monteiro-Riviere et al., 1990; ICRP, 2002), which serves as the primary barrier for dermal absorption, would suggest that for a given applied dermal dose, the internal equivalent dose achieved in humans would be lower than that achieved in rodents. This finding is consistent with relative rates across species for dermal absorption of DEA in vitro (mice>rats>humans; Sun et al., 1996) and *in vivo* (mice>rats (see Fig. 3; Matthews et al., 1997). Sun et al. (1996) determined skin permeability coefficients for undiluted DEA and aqueous solutions of DEA using skin from mice, rat, rabbits, and humans based upon cumulative penetration of DEA over 6 h. In addition, factors dictating the delivery and clearance of DEA to the target tissues are not dictated by blood flow and partitioning, but are instead driven by cell membrane transport systems (organic cation transporters, see MOA discussion), which may not behave in a manner consistent with allometric scaling predictions.
- *Toxicodynamic Factors* – The relative sensitivity of hepatocytes to the effects of DEA has been studied in mice, rats and humans (Kamendulis and Klaunig, 2005). In this study, in vitro exposure of mouse and rat hepatocytes to DEA concentrations as low as 10 μg/mL resulted in a significant increase in DNA synthesis, in a manner that is consistent with choline depletion. Conversely, no increase in DNA synthesis was observed in human hepatocytes at concentrations as high as 750 μg/mL. Treatment of the human hepatocytes with epidermal growth factor (EGF) resulted in a 2-

fold increase in DNA synthesis, demonstrating that these cells were responsive to growth stimuli. The results from this study indicate that from a toxicodynamic standpoint human hepatocytes are at least 75-fold (i.e., >750/10, based on relative LOAEL values) less sensitive than rodents to the effects of DEA. Similar results were reported mouse, rat, and human hepatocytes with respect to DEA effects on GJIC (Kamendulis et al., 2004).

For these reasons, allometric scaling of dose (using $BW^{0.75}$) was not used in this assessment to extrapolate dermal doses across species. Instead, toxicokinetic differences between species were accounted for by assessing DEA cancer potency in terms of the dermally absorbed dose of DEA. Dermally absorbed doses for each treatment group from the cancer bioassays were estimated in two steps:

(1) The administered dermal DEA dose (column 4 of Table 3) was calculated as the product of administered dose (column 3 of Table 3) by the free DEA content (column 2 of Table 3); and
(2) The administered dermal DEA doses were converted to absorbed doses by multiplying them by a dermal absorption fraction. Dermal absorption fractions for DEA were estimated using the data of Matthews et al. (1997), who assessed the dermal absorption of DEA in F344 rats and B6C3F1 mice across a range of acute DEA doses. A dose-dependent increase in absorption was reported in both species, with absorption being greater in mice than in rats (Fig. 3). The relationship between the percent absorbed and dose administered was assumed to be linear, which provided a good fit to the mouse data ($R^2 = 0.999$). To assess the absorption of DEA in the NTP cancer bioassays, this linear relationship was extrapolated to doses both below and above those evaluated by Matthews et al. (1997). The fraction of DEA absorbed in mice predicted by this linear relationship ranges from approximately 0.24 at low doses to >0.90 at the highest doses evaluated by NTP (i.e., 160 mg/kg).

Using these two steps, the dermal absorbed doses for DEA were estimated to range from 0.0069 to 147 mg/kg-day (Table 3). For this assessment, a highly health protective assumption was used, namely that mice and humans are equally sensitive to DEA on an absorbed dose basis (i.e., ignoring a potentially 75-fold difference per the in vitro data of Kamendulis and Klaunig, 2005).

3.2.3. Dose-response modeling

The dose corresponding to a 10% increase in extra risk (ED10) and its 95% lower confidence limit (LED10) were used for the POD. POD values for DEA based upon the best fitting model identified for each data set are presented in Table 4. In terms of DEA absorbed dose, the POD values were determined to be 0.39 and 4.3 mg/kg-day for liver and kidney tumors, respectively (Table 4; Figs. 4 and 5). Expressing these doses in terms of administered dose requires a quantitative estimate of the fraction of DEA absorbed through human skin. Sun et al. (1996) reported the in vitro uptake of DEA through human skin into receptor fluid ranged from a fraction of 0.0008–0.0023 (i.e., 0.08–0.23%). Although this study is considered useful for considering the relative dermal absorption of DEA across species (mice, rats, and humans), the study of Kraeling et al. (2004) was used for the quantitative estimate for human skin since this study: (1) assessed dermal absorption after 24 h of exposures, as compared to the 6 h of exposure assessed by Sun et al., 1996); and (2) assessed absorption from DEA-containing products to which human populations are exposed (shampoo, hair dye, lotion), as compared to undiluted and aqueous solutions of DEA. Kraeling et al. (2004) measured the uptake of DEA in receptor fluid after 24 h and reported dermal absorption fractions for DEA for human skin of 0.0008 for DEA in shampoo, 0.0009 for DEA in hair dye, and 0.009 for DEA in body lotion. Based on a dermal absorption fraction of 0.009 for human skin (the maximum of the range reported by Kraeling et al., 2004), these POD values correspond to human equivalent doses of 43 (0.39/0.009) and 480 (4.3/0.009) mg/kg-day for liver and kidney tumors, respectively (Table 5).

3.2.4. Low-dose extrapolation

The approach for low-dose extrapolation requires a careful consideration of the MOA by which DEA produces tumors in dermally exposed mice (Section 3.1, Table 2). Nonlinear and linear approaches were evaluated. For the low-dose nonlinear approach, a total uncertainty factor (UFt) was calculated as the product of individual components:

- *UFa (interspecies variation)* - A default value of 10 for interspecies variation is assumed to reflect the product of a toxicokinetic component ($10^{0.5}$ or ~3) and a toxicodynamic component ($10^{0.5}$ or ~3). Because the dose-response data for tumors in mice were assessed in terms of absorbed dose, which accounts for species differences in dermal absorption (absorption in humans is addressed in the exposure assessment), a value of 1 is considered appropriate for the toxicokinetic component of UFa. Similarly, since humans appear to be less sensitive than mice to the effects of DEA on cell proliferation (Kamendulis et al., 2004, 2005), a value of 1 is considered to be conservative for the toxicodynamic component of UFa for liver tumors. In the absence of data for species differences in kidney tumor formation, a value of 3 was assumed for the toxicodynamic component of UFa. Based on these considerations, UFa values of 1 (1×1) and 3 (1×3) were adopted for DEA liver and kidney tumors, respectively.
- *UFh (intraspecies variation)* – A default value of 10 was adopted for intraspecies variation for liver and kidney tumors. Based on a consideration of the proposed MOA for DEA (Section 3.1), potentially sensitive subpopulations include people with low choline intake. Chester et al. (2011) reported that based on NHANES (2007–8) data, mean intakes of choline in adult men (396 mg) and women (260 mg) are below acceptable intakes for choline (550 mg for men; 425 mg for women, but are higher for pregnancy and lactation). Variation in choline requirements may be due in part to genetic polymorphisms (Zeisel and da Costa, 2009). Single nucleotide polymorphisms (SNPs) in folate metabolizing enzymes, which may increase reliance on choline

Table 4
Summary of POD values estimated for DEA-Induced tumors in mice.

Target tissue	Sex	Dose groups	POD, absorbed dose (mg/kg-day)[a]		
			Best fitting Model	ED10	LED10
Liver	Males and Females combined	Top 2 groups dropped to improve model fit	Gamma	0.49	0.39
Kidney	Males	All	Dichotomous Hill	5.1	4.3

[a] POD values have been adjusted for discontinuous exposures (5/7 days per week) used in the cancer bioassays.

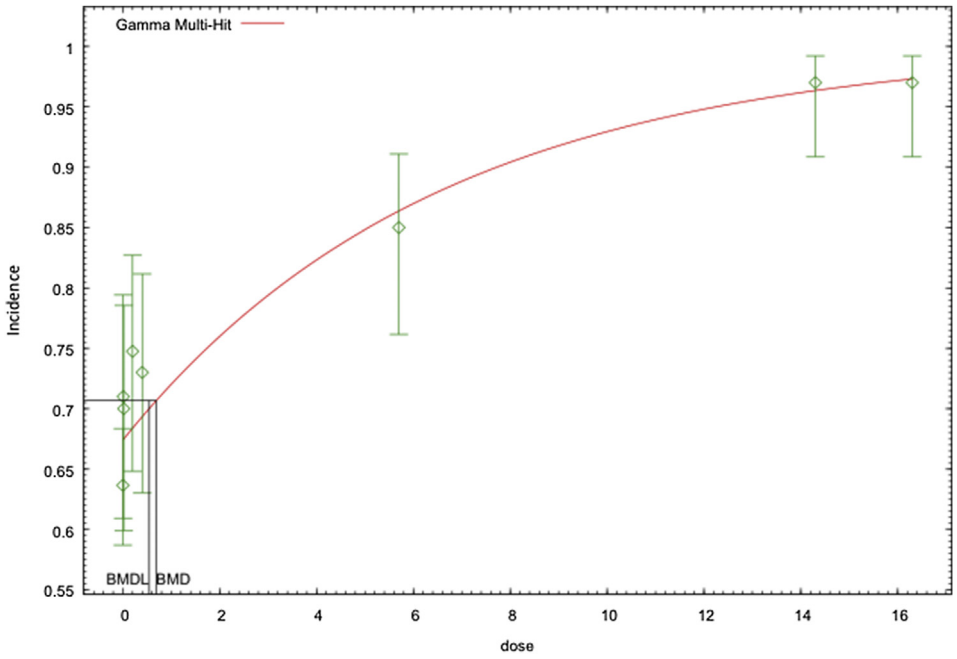


Fig. 4. Gamma Model Fit to pooled Dose-Response Data for Liver Tumors in Male and Female Mice (2 highest dose groups dropped).

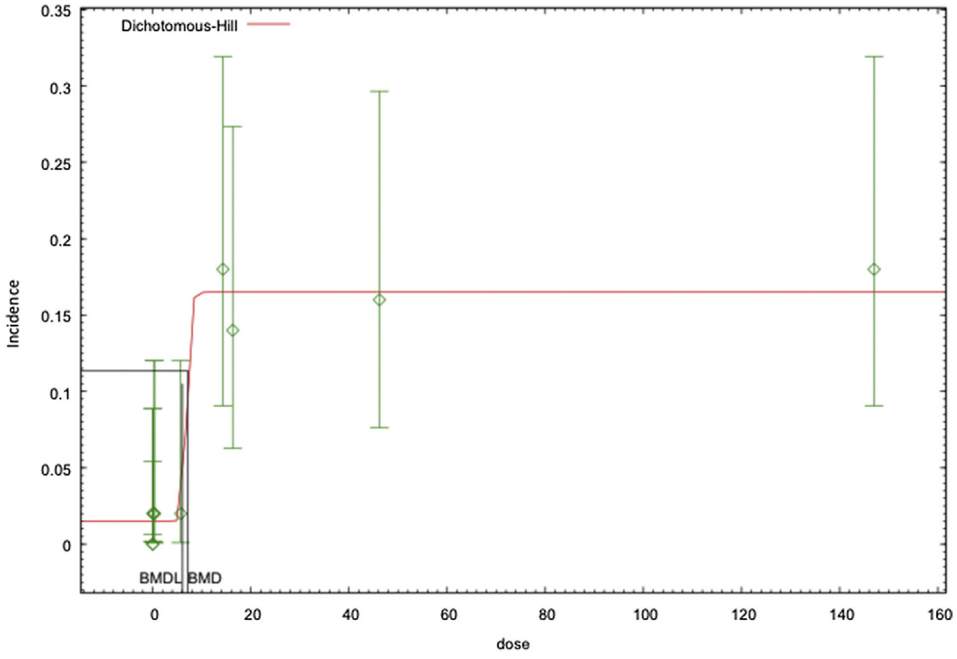


Fig. 5. Dichotomous Hill model fit to pooled dose-response data for kidney tumors in male mice.

as a source of methyl groups, as well as in PEMT have been reported to result in increased susceptibility to dietary choline deficiency (Zeisel, 2008), and therefore may also result in increased susceptibility to the effects of DEA. Nutritional demands for folate and choline are increased during pregnancy and lactation, and therefore these states may also be associated greater susceptibility to the effects of DEA. The default uncertainty factor of 10 was assumed to be protective of these subgroups.

- *UFl (LOAEL to NOAEL extrapolation)* – Although BMD methods were used to characterize the dose-response relationship for liver and kidney tumors, which would usually negate the need to include a UFl value greater than 1, it is recognized that based upon the severity of the endpoint considered, a 10% increase in the extra risk of tumors does not equate to a NOAEL value. For this reason, a value of 10 was assumed for UFl.
- *UFs (subchronic to chronic extrapolation)* – Because the cancer bioassays conducted by NTP for DEA-containing agents all include chronic exposures to DEA, a value of 1 was adopted for UFs.
- *UFd (database deficiency)* – Because the database for DEA tumors includes four cancer bioassays, each conducted in both

Table 5
Derivation of NSRL values for DEA.

Low-dose extrapolation	Mouse liver tumors		Mouse kidney tumors	
	Linear	Nonlinear	Linear	Nonlinear
Point of Departure (POD; mg absorbed/kg-day)	LED10 = 0.39		LED10 = 4.3	
Point of Departure (POD; mg administered/kg-day)[a]	LED10 = 43		LED10 = 480	
Uncertainty Factor (UF)	NA	100	NA	300
Reference Dose (RfD[b]; mg administered/kg-day)	NA	0.43	NA	1.6
Cancer Slope Factor (CSF[c]; risk per mg administered/kg-day)	0.0023	NA	0.00021	NA
NSRL (ug/day)	**300**	**30,000[d]**	**3,400[e]**	**110,000**

[a] Absorbed dose values reflect mg of DEA absorbed through the skin, while applied dose reflect mg of DEA applied to the skin assuming a conservative value of 0.9% absorption (see text). All RfD, CPF, and NSRL values are rounded to two significant figures.
[b] RfD = POD/UF.
[c] CSF = 0.1/POD.
[d] NSRL value selected for liver tumors based upon sufficient confidence in the proposed mode of action.
[e] NSRL value selected for kidney tumors based upon insufficient confidence in the proposed mode of action.

sexes in two species, it is considered robust. Therefore, a value of 1 was adopted for UFd.

Accordingly, total uncertainty factors of 100 and 300 were derived for liver and kidney tumors, respectively. Based on a POD value of 43 mg/kg-day, along with a net uncertainty factor of 100, a cancer RfD value of 0.43 mg/kg-day was derived for DEA based on mouse liver tumors. Similarly, based on a POD value of 480 mg/kg-day, along with a net uncertainty factor of 300, a cancer RfD value of 1.6 mg/kg-day was derived for DEA based on mouse kidney tumors.

For the low-dose linear approach, based on a POD value of 43 mg/kg-day and a BMR of 10%, a linear estimate of cancer potency was estimated to be 0.0023 per mg/kg-day based on DEA-induced mouse liver tumors (Table 5). Similarly, based on a POD value of 480 mg/kg-day and a BMR of 10%, a linear estimate of cancer potency was estimated to be 0.00021 per mg/kg-day based on DEA-induced mouse kidney tumors (Table 5).

3.2.5. NSRL calculations

Based upon the available information, the weight of evidence for DEA-induced liver tumors are sufficient to support a nonlinear approach for low-dose extrapolation, resulting in an NSRL of 30,000 ug/day (Table 5). An NSRL calculated assuming low-dose linearity (300 ug/day) is provided for the purposes of comparison. Although DEA-induced mouse kidney tumors likely involve a similar MOA, significant data gaps are noted in the weight of evidence. For this reason an assumption of low-dose linearity was used to derive a NSRL value of 3400 ug/day based on kidney tumors. Given that there is a biological plausible basis for assuming mouse kidney tumors could be caused by the threshold phenomena of DEA-included choline deficiency, an NSRL based on nonlinear low-dose extrapolation for kidney tumors (110,000 ug/day) is provided for the purposes of comparison. The NSRL value of 3400 ug/day for DEA is considered to be protective of both liver and kidney tumors.

4. Discussion and conclusions

Information regarding the MOA for DEA-induced mouse liver and kidney tumors were used to support dose-response assessments that resulted in derivation of NSRL values of 30,000 and 3400 ug/day, respectively. The decisions and results of dose-response assessment conducted here are compared to those for assessments conducted previously (USEPA, 2001, 2002; Wang et al., 2014). For mouse liver tumors, USEPA's linear extrapolation assumption, which essentially equates to a determination that DEA acts via a mutagenic mode of action, yielded cancer slope factors ranging from 0.1 to 0.4 $(mg/kg\text{-}day)^{-1}$. In this analysis, by using scientific data on toxicokinetics, and still assuming a no-threshold MOA for kidney tumors (which is likely not the case) linear extrapolation produced a cancer slope factor of 0.0023 $(mg/kg\text{-}day)^{-1}$ (Table 6)—a value that is approximately 50–200 fold lower than those resulting from USEPA's evaluation 15 years ago. Wang et al. (2014) derived cancer slope factors ranging from 0.081 to 0.13 (mg/kg-day)-1, which are approximately 40–60 fold higher than the value derived here (Table 6). These large differences in potency, which translate into differences in NSRLs reflect differences in the decisions made at each step of the assessment (Table 6), and highlights the importance of using knowledge of toxicokinetics and MOA data to help guide these decisions. Since there is moderate certainty, or even greater (depending upon the analysis), that mouse liver tumors arise as a result of choline homeostasis perturbation, a threshold MOA, the RfD value based on this MOA of 0.43 mg/kg-day derived here is considered to more closely represent a health protective guidance value reflecting the biological pathway perturbation threshold associated with true risk to humans. Consistent with the definition of an RfD (http://www.epa.gov/risk_assessment/glossary.htm#r), exposure to DEA at 0.43 mg/kg-day would pose no appreciable risk of deleterious effects during a lifetime.

For mouse kidney tumors, previously derived cancer slope factors, based on the assumption that the DEA MOA is identical to a mutagenic MOA, linear extrapolation calculations ranged from 0.0075 (EPA 1999, 2001) to 0.015 (Wang et al., 2014). The current analysis, although also using linear extrapolation, took into account knowledge of species differences in dermal absorption of DEA (0.009 in humans vs 0.24–0.9 in mice), and thus derived a considerably lower cancer slope factor of 0.00021 $(mg/kg\text{-}day)^{-1}$; i.e., a potency that is approximately 40–80 fold lower than previous assessments (Table 7).

Despite the fact that the cancer potency and NSRL values derived here for DEA are orders of magnitude higher than derived by USEPA (2001, 2002) and Wang et al. (2014), these values remain protective of human health due to a number of health protective assumptions made in the dose-response assessment. Sources of conservatism in this assessment include the following: (1) The potential impact of dermal effects of DEA at the site of application (hyperkeratosis, hyperplasia) observed in mice (NTP, 1999a) on the dermal absorption of DEA (i.e., increase absorption due to loss of barrier integrity) was not quantified in the NSRL; (2) The contribution of the oral pathway (Stott et al., 2000) to total dose of DEA received by mice under the conditions of the NTP bioassay were not included in the NSRL; (3) Dropping the two highest dose groups from the pooled data set to improve model fits resulted in slightly lower POD values for liver tumors; (4) The exacerbation of choline homeostasis perturbation by ethanol (Lehman-McKeeman et al.,

Table 6
Comparison of DEA assessments based on mouse liver tumors.

	This analysis	Wang et al. (2014)	USEPA (2001, 2002)
Data Set	Liver tumor (adenomas, carcinomas, hepatoblastoma) data pooled across sex and across bioassays for DEA and condensates (NTP, 1999a; NTP, 2001; NTP, 1999b; NTP, 1999c). Top two dose-groups were dropped to improve model fit.	Liver tumors (carcinomas only) in male and female mice each sex assessed separately (NTP, 1999a)	Liver (adenomas, carcinomas, hepatoblastomas) in male and female mice each sex and bioassay assessed separately (NTP, 1999a, 2001)
Dose Measure	Absorbed dose	Administered dose	Administered dose
Dose-Response Model	Best fitting based on visual inspection, AIC	Multistage	Multistage
Interspecies Extrapolation	Reliance on absorbed dose, accounting for species & product differences in absorption	Allometric scaling of administered dose (BW^3/4)	Allometric scaling of administered dose (BW^3/4)
POD	$LED10_{HED}$ = 43 mg/kg-day	$LED10_{HED}$ = 0.80 (F) 1.4 (M) mg/kg-day	NA
Low-Dose Extrapolation	Nonlinear (UF = 100)	Linear (0.1/$LED10_{HED}$)	Linear (q1*)[a]
Lifestage adjustment	No	Yes	No
Toxicity Value	RfD = 0.43 mg/kg-day	SF = 0.081–0.13 $(mg/kg\text{-}day)^{-1}$	SF = 0.10–0.40 $(mg/kg\text{-}day)^{-1}$
NSRL	30,000 ug/day	1.4 ug/day	Not calculated

[a] q1* = linear term determined for the linearized multistage model.

Table 7
Comparison of DEA assessments based on mouse kidney tumors.

	This analysis	Wang et al. (2014)	USEPA (2001, 2002)
Data Set	Kidney tumor (adenomas, carcinomas) data in males pooled across bioassays for DEA and condensates (NTP, 1999a; NTP, 2001; NTP, 1999b; NTP, 1999c)	Kidney tumor (adenomas, carcinomas) data in males using DEA bioassay (NTP, 1999a)	Kidney tumor (adenomas, carcinomas) data in males using cocamide DEA condensate bioassay (NTP, 1999a)
Dose Measure	Absorbed dose	Administered dose	Administered dose
Dose-Response Model	Best fitting model selected based on visual inspection, AIC	Multistage	Multistage
Interspecies Extrapolation	Reliance on absorbed dose, accounting for species & product differences in absorption	Allometric scaling of administered dose (BW^3/4)	Allometric scaling of administered dose (BW^3/4)
POD	$LED10_{HED}$ = 480 mg/kg-day	$LED10_{HED}$ = 6.6 mg/kg-day	NA
Low-Dose Extrapolation	Linear (0.1/$LED10_{HED}$)	Linear (0.1/$LED10_{HED}$)	Linear (q1*)[a]
Lifestage adjustment	No	Yes	No
Toxicity Value	SF = 0.00021 $(mg/kg\text{-}day)^{-1}$	SF = 0.015 $(mg/kg\text{-}day)^{-1}$	SF = 0.0075 $(mg/kg\text{-}day)^{-1}$
NSRL	3400 ug/day	NA	NA

[a] q1* = linear term determined for the linearized multistage model.

2002), used as a vehicle in the NTP bioassays, and its potential role in tumor response observed in mice were not quantified in the NSRL; (5) Toxicodynamic differences between mouse and human hepatocytes exposed to DEA (at least 75-fold) for cell proliferation and GJIC were not included in the NSRL; and (6) The maximum of the range reported for the dermal absorption fraction of DEA through human skin was used to calculate the NSRL (0.009; Kraeling et al., 2004), with dermal absorption fractions that are lower by an order of magnitude were reported for some products.

To the extent product-specific information for DEA containing products are available, a product-specific NSRL value could be calculated using the following equation:

$$NSRL_{PS} = NSRL * (0.009/ABS_{PS}) \quad (7)$$

Where $NSRL_{PS}$ = product-specific NSRL; and ABS_{PS} = product-specific dermal absorption fraction. In this way, product-specific NSRL values that are higher or lower than the value derived here (3400 ug/day) can be calculated when product-specific information is available.

Although IARC (2012) concluded there was moderate support for a choline perturbation MOA, they also identified a number of limitations, including: (1) no effect on hepatic levels of SAM was observed in mice exposed to a tumorigenic DEA dose (40 mg/kg-day) (Lehman-McKeeman et al., 2002); (2) the role of choline perturbation has not been established in mouse kidney tumors; (3) although rats are highly sensitive to choline deficiency, no tumors were observed in DEA-exposed rats following lifetime exposure (NTP, 1999). The lack of published studies on the effects of maximum tolerated doses on liver levels of choline and choline metabolites in rats creates a critical gap in the data on the proposed mechanism of choline-deficiency in the induction of liver tumors by this chemical; (4) a hallmark of dietary choline deficiency, hepatic steatosis, was not observed in rats or mice under the conditions of the NTP bioassay; and (5) Catnb mutations were reported in liver tumors from DEA-exposed mice from the NTP bioassay (Hayashi et al., 2003), and the mutation profile for mouse liver tumors from choline-deficient mice has not been characterized.

Some of these points reflect clear limitations. For example, the role of choline homeostasis in mouse kidney tumor formation has not been demonstrated, and remains an important data gap for this assessment. However, most of these points warrant additional discussion. With respect to point (1), the apparent lack of dose-response concordance between SAM and liver tumors may be attributable to differences in exposure duration (four weeks for SAM effects vs. two years for tumorigenic effects), such that the effects on SAM may become more pronounced with longer durations. In addition, differences in other factors in experimental conditions (e.g., dietary choline, folate levels) can also contribute to

differences in their observed dose-response relationships. Lastly, the control animals serving as the basis of comparison for statistical significance in Lehman-McKeeman et al. (2002) were vehicle controls (exposed to ethanol), rather than untreated controls, which may have contributed to lower SAM levels due to its effects on hepatic betaine (i.e., SAM levels in the ethanol-exposed animals may be lower than in naïve animals).

As to point (3), the absence of tumors in rats under the conditions of the NTP cancer bioassays may be due in part to the lower dermal absorption fraction reported for rats compared to that for mice (Fig. 3; Matthews et al., 1997). On an absorbed dose basis, the systemic exposures of rats to DEA were approximately 2- to 5- fold lower than corresponding mouse exposures. Because B6C3F1 mice are uniquely sensitive to hypomethylation stress (Counts et al., 1996), a factor that may contribute to their very high background rate of liver tumors, rats may require much higher doses of DEA than do mice.

With respect to point (4), there are important differences between "systemic choline deficiency" produced by feeding a choline-deficient diet and the "perturbations of choline homeostasis" produced by dermal exposures to DEA. For example, hepatic choline measures alone following DEA exposure may not reflect actual effects on cell signaling, since DEA and its metabolites are structural analogs (Table 1) may contribute to endogenous signaling processes. Craciunescu et al. (2009) reported that hepatic choline concentrations in mice were reduced from 777 nmol/g to 232 nmol/g following repeated exposure to 80 mg/kg-day DEA; however, the concentration of the structural analog dimethyl-DEA in DEA treated mice (1216 nmol/g) exceeds the control concentration of choline. In addition, mice fed choline deficient diets will develop systemic choline deficiency (i.e., low tissue concentrations throughout the body). In contrast, inhibiting choline transport from plasma to liver by DEA results in lower concentrations of DEA in the liver; however, choline levels in plasma may remain unchanged or may even be elevated due to this inhibition. For this reason, systemic signals of choline deficiency, which may factor in to systemic responses for lipid mobilization in extrahepatic tissues, may not be present in DEA-exposed mice. This is one possible explanation for why hepatic steatosis, a hallmark of choline deficiency, was not observed in mice or rats under the conditions of the NTP bioassay.

Lastly, with respect to point (5), the presence of mutations in DEA-induced mouse liver tumors is not evidence of a direct genotoxic MOA. During tumor progression, mutations or some type of genetic alteration are a necessary step for all cancers. Genetic changes or mutations that occur during the processes of cell proliferation and clonal expansion (in some cases due to genomic instability) are not the events that define a mutagenic MOA. As noted, non-genotoxic carcinogens such as oxazepam and phenobarbital, also result in increased liver tumors exhibiting Catnb mutations without H-ras mutations, which may reflect selective pressure during tumor promotion (Hayashi et al., 2003). Additionally, DNA methylation plays an important role in genomic stability, and hypomethylation can lead to genomic instability and increased mutations rates (Chen et al., 1998).

Overall, a role for choline homeostasis perturbation in the MOA for DEA-induced mouse liver tumors is well supported (Leung et al., 2005). Further research in the areas discussed above would help reduce uncertainties associated with the cancer MOA for DEA, and in turn would improve confidence in the NSRL values derived in this assessment. The NSRL values derived here are intended for DEA applied dermally, and are not considered appropriate for assessing oral exposures to DEA. The NSRL values reported here are derived in a manner that takes into account DEA absorption through human skin and can be modified when data or knowledge is available for product specific absorption characteristics.

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