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Assessing modes of action, measures of tissue dose and human relevance of rodent toxicity endpoints with octamethylcyclotetrasiloxane (D4)

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ABSTRACT

Octamethylcyclotetrasiloxane (D4), a highly lipophilic, volatile compound with low water solubility, is metabolized to lower molecular weight, linear silanols. Toxicity has been documented in several tissues in animals following mixed vapor/aerosol exposures by inhalation at near saturating vapor concentrations or with gavage dosing in vegetable oil vehicles. These results, together with more mechanism-based studies and detailed pharmacokinetic information, were used to assess likely modes of action (MOAs) and the tissue dose measures of D4 and metabolites that would serve as key events leading to these biological responses. This MOA analysis indicates that pulmonary effects arise from direct epithelial contact with mixed vapor/aerosol atmospheres of D4; liver hypertrophy and hepatocyte proliferation arise from adaptive, rodent-specific actions of D4 with nuclear receptor signaling pathways; and, nephropathy results from a combination of chronic progresive nephropathy and silanol metabolites binding with alpha-2u globulin (a male rat specific protein). At this time, the MOAs of other liver effects – pigment accumulation and bile duct hyperplasia (BDH) preferentially observed in Sprague-Dawley (SD) rats- are not known. Hypothalamic actions of D4 delaying the rat mid-cycle gonadotrophin releasing hormone (GnRH) surge that result in reproductive effects and subsequent vaginal/uterine/ovarian tissue responses, including small increases in incidence of benign endometrial adenomas, are associated with prolongation of endogenous estrogen exposures due to delays in ovulation. Human reproduction is not controlled by a mid-cycle GnRH surge. Since the rodent-specific reproductive and the vaginal/uterine/ovarian tissue responses are not relevant for risk assessments in human populations, D4 should neither be classified as a CMR (i.e., carcinogenic, mutagenic, or toxic for reproduction) substance nor be regarded as an endocrine disruptor. Bile duct hyperplasia (BDH) and pigment accumulation in liver seen in SD rats are endpoints that could serve to define a Benchmark Dose (BMD) or No-Observed-Effect-Level (NOEL) for D4 although their human relevance remains uncertain.

1. Introduction

Octamethylcyclotetrasiloxane (D4) has a broad, comprehensive database on toxicity and pharmacokinetics (PK). In 2017, a series of papers published in a special edition of *Toxicology Letters* provided a detailed overview of D4 toxicology and an update on available studies that addressed both human health risk characterization and aspects of environmental risk assessment (Dekant et al., 2017; Domoradzki et al., 2017b; Franzen et al., 2017; Gentry et al., 2017; Jean and Plotzke, 2017; Jean et al., 2017). The goals in this paper were to consider these earlier studies and review new information to assess the most likely modes of action (MOAs) of the various toxic responses seen with D4 exposures in experimental animals and to determine the relevance of these MOAs for human risk assessment. Here, MOA refers to the identification of key, obligatory steps required to produce a biological response rather than the detailed step-by-step accounting of every molecular, cellular and tissue step leading to the biological response. Human relevance frameworks for evaluating how toxicity information could influence risk assessments were first described 20 years ago (Sonich-Mullin et al., 2001; Meek et al., 2003) and further elaborated in subsequent publications (Boobis et al., 2006,2008).

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These frameworks have been applied to both cancer and non-cancer responses in animals. In using human relevance frameworks, the first step is evaluating MOAs and key events to determine if effects observed in animals would be expected to happen in exposed humans and, only if the answer is yes or if the MOA cannot be ruled out as human relevant, would a risk assessment be performed based on any specific endpoint. Optimally, a MOA evaluation should describe both key events and the tissue dose, i.e., either parent chemical or metabolite(s), causing these effects. Tissue dose measures with D4 can be assessed based on the comprehensive suite of PK studies and physiologically based pharmacokinetic (PBPK) models for D4 (Plotzke et al., 2000; Andersen et al., 2008; McMullin et al., 2016; Campbell et al., 2017) that have documented the PK properties for D4, many of which differ substantially from organic carbon-based volatile compounds that have higher blood/air and lower fat/blood partitioning (Table 1).

When the literature review for this MOA analysis was initiated in early 2020, the work published in the 2017 was revisited and PubMed and Science Citation Index searches were pursued for publications on cyclic siloxane (D4) toxicity and cyclic siloxane (D4) pharmacokinetics. In addition, several reports of recently completed studies were made available by the Silicon Environmental Health and Safety Center (SEHC) as well as full reports from key earlier studies. All these resources are referenced here. Among published PK papers, there were several on disposition and kinetics in fish and aquatic species that were carefully evaluated but not used. The combination of high-quality toxicity studies along with detailed information on pharmacokinetics and tissue dosimetry for D4 and its silanol metabolites provided key information for MOA analyses for all relevant endpoints observed in animals.

The first section of this paper outlines the PK characteristics that determine tissue doses of D4 and its linear silanol metabolites; the second section enumerates tissue responses observed with D4 in standard toxicity studies; and a third section describes more mechanistically motivated studies. The information on dose measures and tissue responses are then used to develop hypotheses for the MOAs and key events for each of the tissue responses. The evidence supporting these hypotheses is briefly recapitulated/outlined and, where appropriate, comparisons made with alternative hypotheses.

This human relevance analysis indicates that the MOA for two of the liver responses – bile duct hyperplasia (BDH) and pigment accumulation seen preferentially in SD rats – remain unknown. However, the MOAs of all other endpoints are either not human-relevant or a consequence of using high inhalation exposures that produce aerosols with direct effects on lung epithelium. Importantly, this review indicates that the MOA for delay of the mid-cycle LH surge in the rat is due either to increasing gamma-aminobutyric acid (GABA) neuron inhibitory tone by phenobarbital-like effects of D4 in the rat CNS and/or to non-specific membrane effects of D4 on GABA receptors like those caused by inhalational anesthetics. The delay of the mid-cycle LH surge in female rats and associated delays in ovulation increases estrogenic tissue exposures. Human reproduction does not depend on a mid-cycle GnRH surge and the reproductive tissue responses and effects on reproduction in the female rat are not relevant for human risk assessment. BMD or NOELs could be derived from BDH and pigment accumulation though their human relevance remains unknown.

2. Pharmacokinetics of D4 and metabolites

After absorption into the body D4 is metabolized by methyl-group oxidation, primarily in liver, followed by sequential hydrolysis producing various linear silanols (Fig. 1). The biological effects noted in toxicology studies could arise from either D4 itself or from silanol metabolites. The determination of a MOA for each adverse response in the animal studies requires articulation of the effective compound (i.e., D4 or silanol metabolites) and the key events by which the chemical interacts in tissues to produce specific effects. The time course and achieved tissue concentrations depend on the PK properties of D4 and its silanol metabolites and the tissue responses depend on the nature of their binding with cellular components leading to the biological responses.

2.1. Pharmacokinetics

Detailed PK studies with D4 have been completed for inhalation, dermal and oral exposures. The unusual physical chemical properties of D4 – low water solubility and only moderate vapor pressure (Table 1) – pose challenges for the conduct of studies at higher exposure levels either by oral dosing in oil vehicles or by vapor inhalation. Airborne concentrations of D4 for vapor inhalation studies are limited by its vapor pressure and tendency to form aerosols. Exposures at 700 ppm (and likely down to several hundred ppm) are expected, based on physio-chemical properties, to produce mixtures of vapors and aerosol (Pauluhn, 2021) and aerosols were measured in atmospheres above 150 ppm (Krieger et al., 2021).

A rich set of plasma, tissue, exhaled breath and urinary concentrations, collected following inhalation of radiolabeled ^{14}C-D4 in rats exposed at 7, 70 and 700 ppm (Plotzke et al., 2000) was used to develop a PBPK model (Andersen et al., 2001). This PBPK model evaluated both single and multiple daily exposures for 14 days to understand the physio-chemical and biological properties that determine the time course of uptake in different tissues with

Table 1
Physical Properties of Octamethylcyclotetrasiloxane (D4).

Property	Results	Reference
Molecular Weight	296.61 Daltons	ECHA (2021)[a]
Physical state at normal temperature and pressure	Liquid	ECHA (2021)[a]
Melting point	17.7 °C	ECHA (2021)
Boiling point	175 °C	ECHA (2021)
Relative density at 25 °C	0.95 g/cm^3	ECHA (2021)
Vapor pressure	132 hPa at 25 °C	ECHA (2021)
n-Octanol-water partition coefficient (log Kow value) at 25.1 °C	6.488	ECHA (2021)
Saturation Vapor Concentration at 25 OC	1301 ppm ($\sim$ 15,800 mg/m^3)	Calculated from Vapor Pressure 132 hPa
Log (n-octanol/air) Partition coefficient	4.22 at 24 °C	(Dow Corning, 2006)
Measured Blood/Air Partition coefficient	3.66 $\pm$ 1.82	(Dobrev et al., 2008)
Measured Fat/Air Partition Coefficient	1480 $\pm$ 660	(Dobrev et al., 2008)

[a] https://www.echa.europa.eu/brief-profile/-/briefprofile/100.008.307. Accessed January 5, 2022.

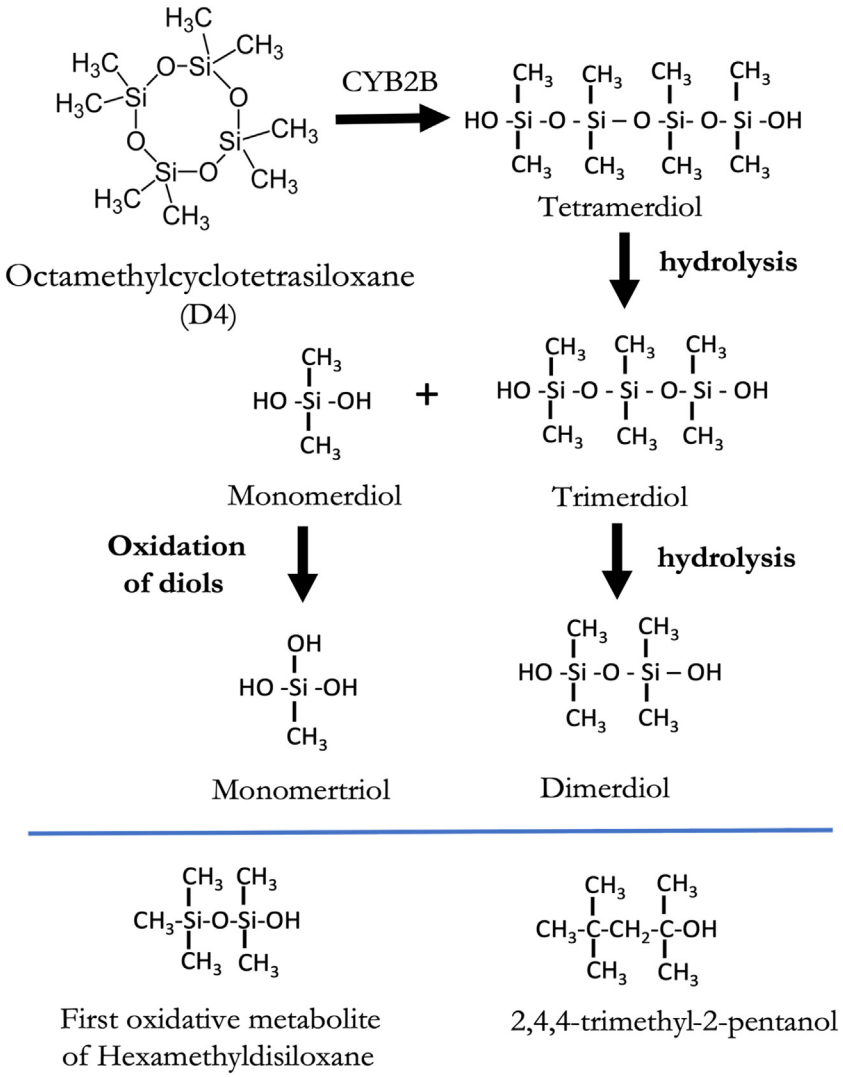

Fig. 1. Structures of D4, several silanol metabolites, and 2,4,4-trimethyl-2-pentanol, a hydrocarbon alcohol associated with male rat nephropathy. The metabolism of D4 produces a variety of silanols that have been characterized in urine. Oxidative metabolism, catalyzed by cytochrome P450 enzymes, first produces an O-methoxy intermediate (not shown) which then hydrolyzes to give a silanol with hydroxy groups on both the alpha- and omega-silicon. This first product still has four dimethyl silane groups and is termed tetramer diol. The first metabolite undergoes further hydrolysis to trimer, dimer and eventually to monomer diols. There is also some oxidation of silanol methyl groups to produce triols. With linear siloxanes, such as hexamethyl disiloxane (HMDS) oxidation produces silanol metabolites with a single hydroxyl group (bottom left) whose structure is similar to the prototypical hydrocarbon, 2,4,4-trimethyl-2-pentanol, that causes male rat nephropathy than are the silanol metabolites of D4.

increasing days of exposure. Consistent with its low blood/air partition coefficient (Table 1), blood D4 concentrations reached near maximum levels within minutes during 6-hr inhalation exposures and free D4 in plasma was eliminated rapidly with cessation of exposure. The elimination half-life of free blood D4 exposures was in the order of minutes. However, the more detailed time course of D4 over many hours after cessation of exposure indicated that blood contained both free D4 (available for exhalation and transport into tissues) and a much lower concentration of a less-available form believed to be D4 packaged into blood lipids and lipoprotein particles (Andersen et al., 2001; Campbell et al., 2017). This inhalation PBPK model has incorporated PK data from dermal and oral dose routes (Sarangapani et al., 2003; McMullin et al., 2016; Campbell et al., 2017) and from PK results from human volunteers following both inhalation and dermal exposures (Reddy et al., 2003, 2007; Reddy et al., 2008).

In 6-hr, multi-day vapor inhalation exposures, D4 caused induction of liver cytochrome P450 enzymes like those induced by phenobarbital (McKim et al., 2001a; Meeks et al., 2022). Increases in these enzymes also increased D4 metabolism, leading to kinetic non-linearities in plasma and tissue concentrations at exposures above a few hundred ppm (Jean et al., 2017). In a 14-day repeat PK vapor inhalation study, the blood levels on day 14 were similar to those seen after a single 6-hr exposure and fat concentrations were increased only 4 or 5-fold. PBPK modeling showed that the increased D4 concentration in fat over the 14 days of exposure was simply due to a slower equilibration of D4 into fat, i.e., fat concentrations increase until the fat/blood partition coefficient is reached with a rate constant for filling of fat tissues determined by both the fat-blood partition coefficient and diffusion-limitation on uptake from plasma to tissues (Andersen et al., 2001). Importantly, D4 due to rapid clearance by both liver metabolism and exhalation does not bioaccumulate during multiday inhalation exposures (Andersen et al., 2008); over more prolonged exposures of several weeks, concentrations in fat simply approach an equilibrium condition based on fat/blood partitioning.

PK studies were also conducted with 10 human volunteers exposed by vapor inhalation to 10 ppm D4 and blood D4 and urinary metabolites were measured at various times after a 1 -h exposure (Utell et al., 1998). The PK behavior in humans for both

inhalation (Reddy et al., 2003) and dermal exposures (Reddy et al., 2007) was similar to that seen in rats and was well described with the PBPK model that had been developed for rats. In addition, the human PK data sets showed that the terminal half-lives in blood for D4 and total radioactivity differed by a factor of about 5, reflecting more rapid clearance for parent compound and somewhat less rapid clearance of metabolites (Reddy et al., 2003). Similar patterns in elimination of D4 and metabolites were noted in rats (Andersen et al., 2001).

2.2. Oral and dermal pharmacokinetics

Uptake, distribution, and elimination of D4 after oral or dermal exposures have also been studied. In some of the first PK studies, high doses of D4 were administered by oral gavage to rats either as undiluted D4, mixed with food oil vehicles or in simethicone (a long chain polydimethylsiloxane - PDMS). The time courses after oral dosing in these vehicles were more complex than after inhalation or dermal dosing and appeared to reflect slower uptake from the gut and absorption of lipid-complexed D4 rather than free forms of D4 (Sarangapani et al., 2003). To better understand D4 uptake from the gut without the complicating factor of food oil vehicles, rats have also been dosed with either 30 or 300 mg ^{14}C-D4/kg using a rodent liquid diet (Domoradzki et al., 2017a). These results were also modeled assuming the systemic uptake was in a lipid-bound form arising from uptake into the liver and passing into the venous blood rather than uptake as free D4 (Campbell et al., 2017). As noted with inhalation exposures at higher concentrations, the uptake from liquid diet showed evidence of dose-dependent kinetics across the 10-fold increase in dose.

While the MOA analyses here are all based on biological responses following inhalation exposures, dermal PK studies may be important for assessing some tissue effects of siloxanes such as the deposition and uptake of aerosol or vapor D4 in epithelial tissues throughout the respiratory tract. Following application to human skin preparations *in vitro*, 88 % of the applied dose evaporated from the surface and most of the initially absorbed dose diffused back to the surface and then evaporated into the air over the next 24 h rather than being available for systemic absorption (Reddy et al., 2007). Overall, less than 1 % of the applied dose was absorbed into the systemic circulation and the dermally applied D4 that is absorbed into the blood showed blood kinetics that were like the kinetic behavior of inhaled D4, indicating uptake of free D4 into the bloodstream.

2.3. Metabolism – rats and humans

Metabolism of D4 involves initial oxidation of a methyl group (Fig. 1) with hydrolysis of the resulting hydroxymethyl or O-methyl-D4 to silanols of varying chain lengths (Varaprath et al., 1999, 2003). Evaluating the detailed time course of elimination of silanols in urine from human volunteers indicated some methyl-group oxidation of silanols (Reddy et al., 2003) that produced shorter chain length triols. Overall, pathways of hydrolysis and urinary excretion are more important for controlling the blood time course and tissue exposures of silanol metabolites than is methyl group oxidation of the silanols. Based on estimated elimination rate constants for total metabolites in rats (Andersen et al., 2001) or specific silanols in humans (Reddy et al., 2003), the half-lives of these silanols were of the order of several days in both rats and humans.

3. Toxicity

Multiple responses (Table 2) have been reported in a diverse array of comprehensive animal studies conducted with D4. In this section, each of the endpoints considered for assessing MOA and human relevance is described along with information on the inhalation exposure concentrations and study duration at which responses were observed. In some studies, resolution of tissue response was evaluated in protocols where groups of animals were held after cessation of the exposures. Most adverse effects in these animal studies were seen in inhalation studies. Some of the early inhalation studies used nebulizers to generate atmospheres and later studies relied on vaporization of D4 into chamber inlet air stream at a maximum concentration of 700 ppm generated on a day-to-day basis. Both methods, but especially the use of the nebulizer, are expected to produce a mixture of vapor and aerosol depending on the exposure concentration (Krieger et al., 2021; Pauluhn, 2021).

3.1. Pulmonary irritation

In a 28-day nose-only inhalation exposure with rats, atmospheres were generated at 0, 226, 417, 700 and 1154 ppm for 6 h/day and 5 days/week using a Hospitak nebulizer (Dow Corning, 1995; Franzen et al., 2017). Pulmonary responses included alveolar inflammation and goblet cell proliferation. A subsequent three-month study using whole body inhalation exposure with rats (Burns-Naas et al., 2002), also using the Hospitak nebulizer, with concentrations of 0, 25, 90, 412 and 989 ppm, reported alveolar macrophage accumulation and leukocyte infiltration in the highest exposure groups (412 and 989 ppm). A peer review pathology reexamination of these tissues (Dow Corning, 2000) concluded that the pulmonary responses were attributable to formation of aerosol by the exposure system and focal effects associated with droplet deposition in the respiratory tract coupled with stress from nose-only restraint. All other inhalation studies were conducted with vaporization of D4 into the airstream rather than using a nebulizer.

Table 2
Effects Considered in Evaluations of MOAs and related Measures of Dose.

Endpoint	Key References	Relevant Mechanistic Studies/Considerations
Irritation in the Upper and Lower Respiratory Tract with high dose hemorrhage in the alveolar region	(Burns-Naas et al., 2002; Jean and Plotzke, 2017)	(Pauluhn, 2018; Pauluhn, 2021
Leukocytosis & Altered thymus and adrenal weights in female rats	(Jean and Plotzke, 2017; Dow Corning, 1995; Burns-Naas et al., 2002)	(Pauluhn, 2018; Pauluhn, 2018)
Hepatic Hypertrophy	(Dow Corning, 1995; Burns-Naas et al., 2002; Jean and Plotzke, 2017)	(McKim, 1998; McKim et al., 2001a; Sarangapani et al., 2002)
Pigment Accumulation and Bile Duct Proliferation	(Dow Corning, 2001)	(HarlanLaboratories Ltd., 2010)
Nephropathy	(Jean and Plotzke, 2017)	(Cassidy et al., 2001; Meyers et al., 2013)
Ovarian and uterine responses	(Burns-Naas et al., 2002; Jean and Plotzke, 2017)	(McKim et al., 2001b; He et al., 2003; Lee et al., 2015; Quinn et al., 2007b; Jean et al., 2017; Dow Corning, 2012)
Delayed ovulation; reduced implantation sites; reduced litter size	(Meeks et al., 2007)	(Quinn et al., 2007a; Baker, 2010; Knobil, 1974; Norman et al., 1973b; Toyoda and Chang, 1969)

The chronic toxicity and oncogenicity study of D4 in Fisher 344 (F344) rats using inhalation exposures was conducted at 0, 10, 30, 150 and 700 ppm with 6/h/day whole-body vapor inhalation exposures for up to 104 weeks and provided the most detailed information about pulmonary and nasal cavity responses (Jean et al., 2017). The nasal cavity showed responses typical of a mild irritant with increased incidence of goblet cell hyperplasia in males and females at 12 and 24 months of exposure to 700 ppm D4. Squamous epithelium hyperplasia was only elevated at 700 ppm. Eosinophilic globules were increased in females at 150 and 700 ppm at 24 months. The increase in the incidence of nasal cavity squamous epithelium hyperplasia was evident in both males and females exposed to 700 ppm D4 for 12 months. These tissue effects were much less evident 12-months after cessation of the 1-year exposures. While there were statistically significant trends for eosinophilic globules at 24 months, the recovery group and the groups exposed for the full 24 months had similar incidences of these globules. These eosinophilic globules are commonly observed in aging F344 rats with mean incidences of 22 % in males, ranging from 8 to 62 %, and 38 % in females, ranging from 12 to 84 % (Nagano et al., 1997).

Other effects on the lungs in females involved a slight, but statistically significant increased incidence of hemorrhage after 24 months of exposure to 150 and 700 ppm D4. In addition, there was a slight, but statistically significant increase in the incidence of alveolar sub-pleural chronic inflammation in the 700 ppm females at 24 months (8/60) with a significant trend test across the exposure concentrations. This finding was not present in the recovery groups. Males showed a non-significant increase in hemorrhage incidence at 24 months, but not at 12 months or in the recovery group, with a positive trend test for the response. Overall, the lungs showed consistent evidence of irritant activities in the upper and lower regions of the respiratory tract at 700 ppm that resolved after cessation of exposure in the groups exposed for 12 months and held another year without exposure. The incidences, even at 700 ppm, were low; the two largest responses were seen for lung hemorrhage at 24 months in males (8/59) and subpleural chronic inflammation in females (8/60). Neither was there a consistent dose response, even in the groups showing a positive trend for specific effects in the deep lung.

3.2. Stress-related effects, leukocytosis, and dose response

Lymphocytic leukocytosis occurred in both sexes in the chronic two-year toxicity study (Jean and Plotzke, 2017) with a consistent and statistically significant increase in white blood cell count attributable to an elevation in the number of leukocytes for males and females exposed to 700 ppm at 3, 6, and 12 months. The high variability and lack of a clear-cut dose-response made it difficult to determine the lowest effect exposure concentration. Importantly, leukocytosis occurred in concert with signs of respiratory tract irritation at the highest exposures. Other responses were also observed that were consistent with irritation and stress responses. In the 3-month nose- only study using the nebulizer-based D4-generation method, there were decreases in thymus weight in female rats at both the 488 and 989 ppm and increases in adrenal weights only at 989 ppm (Burns-Naas et al., 2002). The pathology peer review panel concluded that the nose-only restraint procedures and presence of aerosol likely contributed to these stress-related responses (Dow Corning, 2000).

3.3. Hepatic hypertrophy

This response was first reported in 14-day repeated oral dose study conducted in SD rats by gavage of a D4 emulsion in a 0.5 % weight-to-volume mixture of methocel A4M in distilled water (Dow Corning, 1990; Franzen et al., 2017) at doses of 0, 25, 100, 400 or 1600 mg D4 /kg bw/day. There were statistically significant decreases in body weight in male and female rats at 1600 mg/kg bw/day and statistically significant increases in liver/body weight ratios in both male and female rats and a statistically significant increase in absolute liver weight in females following administration of 400 or 1600 mg/kg bw/day.

Liver weight increases were also evident at 6, 12, and 24-months exposure for both sexes in the 104-week inhalation bioassay study with F344 rats (Jean et al., 2017). For males, the liver weight increases were associated with exposure to $\geq$ 30 ppm D4 at 6 months, $\geq$150 ppm at 12 months, and 700 ppm D4 at 24-months. Female liver weight increases were also statistically significant at all three time points (6, 12, and 24 months) at 700 ppm and at 12 and 24 months with exposure to 150 ppm D4. Despite increases in liver weight, no increases in neoplastic responses were noted in liver. Similar liver responses had also been noted in rats in the 90-day nose-only-exposures (Burns-Naas et al., 2002).

Several other liver responses were reported in a two-generation inhalation reproductive toxicity and developmental neurotoxicity study (Dow Corning, 2001) conducted with exposure groups at 0, 70, 300, 500 and 700 ppm. These rats were exposed for 70 consecutive days for 6 h/day. A golden-brown pigment accumulated in the periportal zones of the liver, and its incidence and severity were increased in both males and females at 300, 500 and 700 ppm. The observed incidence was 0/30, 2/30, 7/29 and 16/29 for males in these 4 exposure groups and 0/30, 2/30, 4/29 and 9/20 for the females. Increased incidence of BDH was also observed in the 700 ppm male rats (18/29 versus 3/30) and in the 500 and 700 ppm females (2/29 and 6/29 versus 0/30). Only the increase in BDH incidence at 700 ppm in males as statistically significant. Neither pigment accumulation nor BDH were reported as findings in the two-year study with F344 rats.

3.4. Kidney

Chronic inhalation exposure of rats to D4 for 24 months increased kidney weights relative to controls (Jean and Plotzke, 2017) and nephropathy was a common finding in both males (80 %–100 %) and females (28 %–92 %) in all groups including controls. No treatment-related differences in incidence or severity were evident at 12 months or in recovery groups. There was a statistically significant increase in incidence of chronic nephropathy at 24 months for females at $\geq$ 30 ppm. Although not statistically significant, the severity scores were generally increasing with increasing exposure concentration for both males and females at 24 months. Kidney weights were increased at both 6- and 12-months exposures at 700 ppm for females and at lower exposures, 30 ppm and above, in males. Despite the observation of nephropathy there was no increase in kidney tumors.

3.5. Female reproductive effects

A comprehensive reproductive toxicity study was completed with SD rats at concentrations of 0, 70, 300, 500 or 700 ppm 6 h per day for at least 70 consecutive days prior to mating and continuing through weaning of the pups on postnatal day 21 (Siddiqui et al., 2007). The responses included prolonged estrous cycles, decreased mating and fertility indices in the F1 generation, significant reductions in the mean number of pups born and mean live litter size in the 500 and 700 ppm groups for both the F0 and F1 generations, and reduced implantation sites at 700 ppm for both F0 and F1 generations. No adverse effects were observed at any exposure level on anogenital distance, vaginal patency and preputial separation or for male functional reproductive parameters, including spermatogenic endpoints, microscopic evaluation

of male reproductive tissue, or mating success with the unexposed females. Reproductive toxicity was due to effects in the females.

3.6. Female reproductive tissue responses

In the highest concentration group of a 3-month nose-only nebulizer-generated exposure, the components of the female reproductive tract were the primary targets for D4 (Burns-Naas et al., 2002) with an increase in the numbers of rats in the diestrus portion of the reproductive cycle, reduced ovary weights, ovarian hypoactivity and vaginal mucification. In the two-year study (Jean and Plotzke, 2017), significant increases in uterine weights were reported in females exposed to 700 ppm (24 months) and a small increase in the incidence of endometrial epithelial hyperplasia following exposure to 700 ppm for 24 months. Benign uterine endometrial adenomas were present in four of sixty animals exposed to 700 ppm D4 for 24 months, and, although not statistically significant compared to controls, the incidence profile trend for this response across the dose groups was statistically significant. In examining the estrus cycling and reproductive senescence in aged female rats (Jean et al., 2017), reproductively senescent female F344 rats exposed to D4 had reduced incidence of pseudopregnancy and increased periods of proestrus – the portion of the estrus cycle associated with elevated blood estradiol (E2).

This set of mammalian responses – pulmonary irritation/leukocytosis, liver hypertrophy/pigment accumulation and bile duct hyperplasia, mild nephropathy with small increases in kidney weights, vaginal mucification, ovarian hypoactivity, reduced female fertility with alterations in estrus cycling and endometrial hyperplasia and benign uterine adenomas - served as the key endpoints for this hypothesis-based assessment of likely MOAs and human relevance of these MOAs.

4. Mechanistic studies/consideration of measures of dose

4.1. Respiratory effects

Although the maximum expected vapor concentration of D4 at 25 °C is about 1300 ppm (Table 1), the methods of generating atmospheres for inhalation exposure will produce some level of aerosol at much lower exposure levels. The older studies (Dow Corning, 1995; Burns-Naas et al., 2002) used nebulizers to bring aerosols into an airstream that was then diluted with chamber input air in which most of the aerosol was expected to become vapors. In the more recent studies, test atmospheres were generated by vaporization of D4 into the chamber inlet air stream. The vapor generator consisted of a fiberglass wick in contact with a heated cylinder located within the chamber's inlet compartment. Review of these studies have suggested that both these methods produce aerosols (Pauluhn, 2021). Based on the chamber monitoring method used in the inhalation studies, it was not possible to quantitatively measure aerosol contributions across exposure concentration. Nonetheless, the highest exposure generated with the nebulizer, 898 ppm (Burns-Naas et al., 2002), was already close to the saturation concentration. More recent studies with direct measurement of aerosol in studies with the fiberglass wick and heated cylinder showed increasing amounts of aerosol at atmospheric concentrations above 150 ppm (Krieger et al., 2021) and theoretical analyses indicate that the aerosols would form dependent on exposure concentration relative to the maximum vapor concentration saturation achievable at particular temperature and methods of atmospheric generation (Pauluhn, 2021).

Aerosol droplets larger than about 3 μm and those of about 0.02 μm preferentially deposit in the head/nose in the upper airway (Heyder, 2004). The larger particles deposit primarily by sedimentation and the smaller by diffusion to surfaces. In this way, aerosol uptake to epithelial surfaces can occur in both the head/nasal regions of the airways and the deep lungs. Deposition of aerosol along the pulmonary epithelium leads to high local exposure to epithelial surfaces, affecting respiratory and olfactory epithelium and other structures along the airways. Aerosol deposition of D4 with local irritant effects might also cause more systemic responses. The tissue irritation would lead to cytokine release into the bloodstream and deposition at sites in olfactory epithelium or the vomeronasal organ (VMO) could affect odorant or pheromone signaling associated with olfactory cues for reproduction (Asaba et al., 2014).

Many of the responses in the nasal region are consistent with aerosol deposition. The responses reported in the nasal epithelium include macrophage accumulation, interstitial inflammation, and leukocyte infiltration (Burns-Naas et al., 2002). Females showed responses for the first two of these responses at 122 ppm and above and the males only at 488 ppm and higher. Leukocyte infiltration was only seen in the 898 ppm group. In the chronic two-year study (Jean and Plotzke, 2017), respiratory epithelial goblet cell hyperplasia and squamous epithelium hyperplasia were elevated at the highest exposure (700 ppm). Eosinophilic globules were increased in females at all concentrations above 30 ppm at 24 months. Aerosol deposition in the nose would also be expected to produce nociceptor responses (Pauluhn, 2018). Deposition of D4 aerosol in the deep lung would spread onto the epithelial surface, disrupting surfactant turnover, and bringing macrophages to scavenge the disrupted surfactant layers. The observation of increased macrophages and local alveolar hemorrhage is consistent with aerosol exposure in the deeper lung at the high exposure concentrations.

Aerosol deposition along the airways has similarities with dermal applications of D4. As noted in the PK section, following dermal application about 10 % of applied D4 penetrated the skin. Of this initially absorbed amount, the majority diffused back to the epidermal surface in the 24 h post-application and volatilized to the atmosphere (Reddy et al., 2007). Mixed aerosol-vapor exposures along the respiratory tract would lead to locally high epithelial concentrations compared to vapor-only exposures. For the respiratory tract, the greater preponderance of lesions at 122 ppm and 488 ppm and higher in studies using a nebulizer for atmosphere generation and the responses predominately at 700 ppm in the two-year study with vaporization into the airstream are supportive of a conclusion that there were combined aerosol-vapor exposures with deposition of aerosols. The respiratory tract changes seen at the lower exposure concentrations, eosinophilic globules (Jean and Plotzke, 2017), are a commonly observed response in aged F344 rats and the significant trend test for responses in the alveolar region are driven by small increases in incidence at 700 ppm and inconsistent changes at the lower exposure concentrations.

With the expectation of a more complex dose response in the deep lung associated with aerosol deposition at the highest exposure concentration, 700 ppm, in the 2-year exposures in F344 rats, there would be value is looking at alternative dose-response models that focus on the lower exposures – 0, 10, 30 and 150 ppm. For the three endpoints showing small increases in responses in the deep lung, i.e., lung hemorrhage in males and lung hemorrhage and subpleural chronic inflammation in females, the incidences at 0, 10, 30 and 150 ppm were 3/60, 3/59, 5/60 and 5/60 for hemorrhage in the males; 0/59, 1/60, 0/59 and 2/60 for hemorrhage; in females and 0/59, 2/60, 3/59 and 2/60 for subpleural chronic inflammation. These dose response trends could be evaluated separately (without the 700 ppm group) and sensitivity of the trends examined by running multiple dose response relationships where incidences in a single group was

changed by ± 1 responding animal, either an increase of 1 or of decrease of 1 responding animal, to assess the sensitivity of these trends to small changes in incidence.

4.2. Hepatic responses

While no mechanistic studies have queried the brown pigment or bile duct proliferation, mechanistic studies of enzyme induction and liver hypertrophy (McKim et al., 2001a) provided a detailed inhalation dose-and-time profile for liver responses to D4 that was subsequently more quantitatively examined by PBPK modeling (Sarangapani et al., 2002). The full dose response for this PBPK evaluation, including groups exposed to 0, 1, 7, 30, 70, 150, 300, 500, 700 and 900 ppm for 6 h/day for 5 consecutive days, was from an earlier study (McKim, 1998). The half-saturation air phase concentration for increased CYP2B induction was about 70 ppm. Plasma, liver, and fat tissue concentrations at this exposure concentration were 0.49 ± 0.06, 8.1 ± 0.31 and 38.65 ± 1.85 μg/mL, with an estimated liver tissue/free D4 affinity constant for induction of 0.67 μM. The dose response for increased liver weight differed from that of CYP2B induction. Liver weight was still increasing at the higher exposure levels and the predicted dose response had a Km of 3.36 μM, representing an inhaled concentration of about 800 ppm (Sarangapani et al., 2002). The dose response for BDH and pigment accumulation in the SD rats (Dow Corning, 2001) is more like the dose response of liver enlargement than that of CYP induction.

CYP2B oxidizes D4 and its induction increases the metabolic clearance of D4 several fold, leading to non-linearities in the relationship of exposure level to plasma conncentations (Sarangapani et al., 2002; Jean et al., 2017). Phenobarbital (PB) was used as a positive control compound and produced liver enlargement and enzyme induction like that caused by D4. However, unlike D4, PB is known to cause liver cancer in chronic exposures (Elcombe et al., 2014). A key difference in the tissue PK behavior expected for D4 and PB is persistence of elevated blood levels throughout treatment with PB compared to elevation for only a restricted 6-hr exposure period with D4. The half-life of PB in rats is of the order of 20 h (Loscher, 2007) and active concentrations once achieved will persist throughout the entire day for the drinking water dosing protocol. From the liver results, the responses to D4 and PB indicate that these two compounds may both have effects on constitutive androstane receptor (CAR) signaling. The diminished time of concentrations sufficient for pathway activation – 6 h for D4 versus the entire 24 h for PB – likely explains why D4 causes PB-like induction of liver enzymes and hepatocyte proliferation without an increase in hepatocellular cancer on longer term exposures. Nonetheless, the evidence is very strong that liver, enlargement, and proliferation caused by D4 has a MOA similar to PB, i.e., through activation of CAR-receptor signaling (Elcombe et al., 2014).

To confirm involvement of CAR, mechanistic studies were conducted with HepG2 cells transiently transfected with plasmids coding for either rat or human CAR) and a luciferase reporter plasmid containing a 1.8 kb human CYP2B6 promoter (Dow Corning, 2005a). D4 dissolved in ethanol increased reporter gene expression for both human and rat CAR, showing a larger response with human (11-fold) than with rat CAR (about 5-fold) as compared to a control. Doses tested were 0, 1, 5, 15, 31, 62.5, 125, 250, 500 and 1000 μM D4 with a 24 h treatment time. Maximum induction of CAR was seen at a nominal dose of 62.5 μM for both rat and human CAR. In an *in vitro* study with an human pregnane X receptor (PXR) gene assay, reporter gene expression was increased 6.4-fold at a nominal D4 concentration of 125 μM (Dow Corning, 2005b). While these nominal concentrations are much higher than the half-saturation liver D4 concentration for inducing Cyp2B expression *in vivo* (0.67 u M), the measured concentrations *in vivo* and nominal assay concentrations *in vitro* are not easily compared due to the high lipophilicity and volatility of D4, factors that complicate the *in vitro* studies. Nonetheless, these mechanistic studies showed that D4 would be expected to activate both CAR and PXR-signaling in the liver when concentrations in the liver were sufficiently high.

4.3. Kidney responses

While specific mechanistic studies were not pursued with D4 to examine the rodent nephropathy response, kidney pathology has also been reported after inhalation exposures to two linear siloxanes, hexamethyldisiloxane (HMDS) and octamethyltrisiloxane (L3). With HMDS (Cassidy et al., 2001), exposures at 0, 50, 194, 593, 1509 and 5012 ppm for 6/hour/day, 5days/week for 13 weeks in male and female F344 rats caused dose-related increases in various markers of renal effects – hyaline casts, tubular regeneration, tubular mineralization, papillary mineralization and hyaline droplets – with increased incidence starting at the 593 ppm exposures and increasing severity at higher doses. With L3, male-specific protein droplet nephropathy was also noted in male SD rats exposed to 800, 1600 or 3200 ppm L3 for 6 h/day in an exposure design with 29 days exposure (Meyers et al., 2013). No dose-response information was provided in describing these effects. While the structures of linear and cyclic siloxanes differ substantially, the metabolites, i.e., silanols of varying chain lengths, show more commonality among the three substances (Fig. 1). Metabolism of the linear siloxanes begins with methyl group oxidation followed by hydrolysis to monomeric and dimeric silanols as found with D4. One difference, though, is that metabolites of the linear siloxanes include trimethylsilanols (Varaprath et al., 2003).

Two mechanistic studies evaluated alpha-2u globulin responses following HMDS exposure of Fisher 344 rats. Six rats were exposed via nose-only vapor inhalation at 5000 ppm for 6 h/day for 6 days. The morning after the last exposure rats were necropsied and kidneys taken for immunochemistry with a mouse monoclonal alpha-2u globulin antibody. The area and density of staining was increased by HMDS exposure with a change in appearance from a fine stippled appearance to larger droplets with needle-like or rhomboid shapes. Some tubules showed sloughing of necrotic cells into the lumen (Dow Corning, 2007). In another study, male F344 rats were dosed with ^{14}C-HMDS by oral gavage in corn oil at 0, 10, 100 and 1000 mg/kg/day. Hydrophobic Interaction Chromatography (HIC) isolated alpha-2u globulin from kidney cytosol, and radioactivity (either HMDS or metabolites) co-eluted with the protein. D-limonene oxide, a chemical with high affinity for alpha-2u globulin displaced or reversed binding by HMDS-derived radioactivity. No binding was found in kidney cytosol from female rats (Dow Corning, 2003).

4.4. Reproductive Toxicity in the female rat

Studies were pursued to determine the windows of exposure that caused reproductive toxicity. Female SD rats were exposed to D4 at concentrations of 0, 70, 300, 500 or 700 ppm, 6 h a day seven days a week during selected phases of the reproductive cycle: an overall phase, ovarian phase, fertilization phase, and implantation phase (Meeks et al., 2007). The overall phase included exposure from 28 days prior to mating, through mating and until gestation day 19; the ovarian phase was from 31 days prior to mating until three days prior to the start of mating; the fertilization phase included the three days prior to mating until gestation day three; and the implantation phase study was from gestation day two through gestation day five. In the overall phase, a significant decrease was reported in the number of corpora lutea in female

rats exposed to concentrations above 300 ppm D4, in the number of uterine implantation sites and in number of fetuses in rats exposed to 500 or 700 ppm. No significant treatment-related changes were reported in the ovarian or implantation phases. In the fertilization phase, uterine implantation and post-implantation losses were increased in females exposed to 700 ppm and there was a significant decrease in the number of corpora lutea at the same concentration. In another portion of this study, female SD rats were exposed during pre-mating and post-mating phases to a concentration of 0 or 700 ppm D4 via whole body inhalation for six hours per day at various times around ovulation and implantation. In only one group, those exposed on the day prior to mating, was there a decreased pregnancy rate. Clearly, the effects of D4 on rat reproduction are exquisitely dependent on the timing of exposure during the estrus cycle.

Detailed studies were also completed in female SD exposed to rats at 700 or 900 ppm D4 to assess the blood concentrations of various hormones around the time of ovulation and to examine histopathology of the ovary in female rats after exposure to 700 or 900 ppm D4 on diestrus days 1 and 2 and on proestrus (Quinn et al., 2007a). The rats were staged by vaginal lavage and histology. Two groups of rats were used. The first group using cannulated rats received a shorter exposure on proestrus (2.5 h) with blood collection at 10 a.m.. The second group of cannulated rats had 3 days of 6 h/day exposures and serial blood collection starting from 2 pm through 10 a.m. the first day of estrus. In examining the full cohort of rats at each exposure level, the average LH values at various times on the day of proestrus were reduced in the 700 and 900 ppm groups. Evaluation of oviducts on the morning of estrus showed that some animals from each group including controls did not ovulate. When grouped by ovulation status, there were no differences between treated and untreated animals in terms of the LH surge. However, there were differences in the proportion of rats that ovulated: 79 % in controls versus 42 and 31 % for the groups exposed at 700 and 900 ppm, respectively. Females that failed to ovulate had no LH surge. In the cannulated rats, there was reduced prolactin at the 2:00 pm sampling and increase in progesterone and smaller increases in estrogen and estrone at the 10:00 am

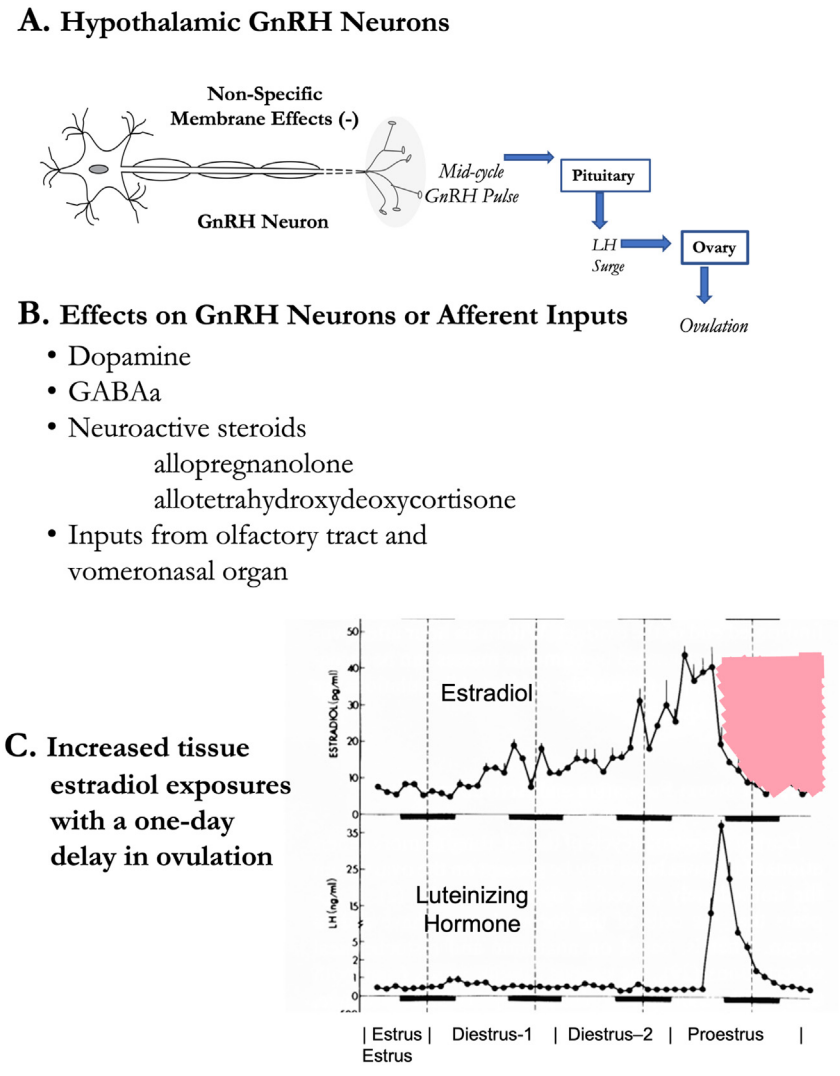

Fig. 2. Modes of Action of D4 in modulating the mid-cycle GnRH pulse with subsequent prolongation of tissue estrogen exposures. A. Rat GnRH neurons are primed for a GnRH surge by exposures to E2 from the growing follicle through estrus, diestrus and proestrus. The GnRH surge drives LH release, and the LH surge drives ovulation. **B.** The sensitivity of the GnRH neurons is affected by a variety of inputs. GABAa provides inhibitory control on neuronal functions and is affected by neuroactive steroids, phenobarbital, and inhalation anesthetics. Neuronal tracts from the nose (the olfactory epithelium and vomeronasal organ) affect female receptivity and readiness for mating (Asaba et al., 2014). **C.** If the GnRH surge is delayed by a single day, tissue E2-exposure approximately double (shaded region in lower right). Data on hormone levels (Smith et al., 1975) were digitized and the figure is from a PhD thesis (Toporikova, 2007).

sampling on the day of proestrus. These observations clarified the timing of exposures that led to impaired reproductive effects in female rats, showing that the exposures on proestrus affected the mid-cycle LH surge required to initiate a LH surge and ovulation.

4.5. Estrogenic modes of action

Multiple mechanistic studies have examined the potential for D4 to act via estrogenic/antiestrogenic modes of action in rodents. Each of these have included uterotrophic assays using various protocols – (i) oral dosing of immature SD and F344 rats using D4 in a sesame seed oil vehicle for 4 consecutive days (McKim et al., 2001b), (ii) subcutaneous administration in immature rats (Lee et al., 2015), (iii) oral administration in corn oil in ovariectomized mice for three consecutive days (He et al., 2003), (iv) inhalation exposure of both SD and F344 ovariectomized female rats at 700 ppm for 6 h/day for 3 consecutive days (Dow Corning, 2004); (v) inhalation exposures to 700 ppm for 16 h/day for three consecutive days in SD and F344 (Quinn et al., 2007b); and, (vi) inhalation exposures of ovariectomized F344 rats for both standard 6-hr and extended 16-hr exposures (Dow Corning, 2012).

Using the full dose response of orally administered D4 (0, 10, 50, 100, 250, 500 and 1000 mg/kg/day – and EE – 1, 3, 10 and 30 μg/kg/day), both the level of activity and the extent of response were estimated. The activity on a delivered dose basis was about 6 orders of magnitude greater for EE than for D4 and the maximum response in terms of uterine weight increase for EE (350 %) was more than double that of D4 (160 %) (McKim et al., 2001a). In mice (He et al., 2003), the potency of EE for increasing uterine weight was much greater than that of D4 and subcutaneous administration in immature rats did not produce changes in uterine weight (Lee et al., 2015).

Since most of the toxicology and mechanistic studies of D4 that bear on risk assessment have been by the inhalation route, the estrogenic responses following inhalation exposure allow a better comparison to in-life toxicity results. In a study using both SD and F344 rats with 6-hr exposures at 700 ppm (Dow Corning, 2004) changes in uterus weight in SD rats did not reach statistical significance, while there were significant increases in both wet and blotted weight with the F344 rats. In the F344 rats, control uterus weights were 0.053 ± 0.013 g and for the ethinyl estradiol (3.0 μg/kg/day) and 700 ppm D4 groups, the values were 0.280 ± 0.013 g and 0.106 ± 0. 012 g, respectively. Another protocol used 16 -h exposures at 700 ppm (Quinn et al., 2007b). The average uterus wet weight in the SD rats following inhalation exposures were 0.064 g (control), 0.186 g (D4) and 0.414 g (3 μg EE/kg/day). The net increases with rats exposed to D4 using the 16 h inhalation protocol were 33.5 % (SD rats) and 32.5 % (F344) of the response seen with EE. Simultaneous treatment with both EE and D4 (Quinn et al., 2007b) showed a small reduction compared to EE alone.

The six-hour exposures had a much-reduced uterotrophic response to 700 ppm D4 compared to the EE-control examined. The differential response to 6- and 16 -h exposures at 700 ppm were also examined more closely in ovariectomized F344 rats (Dow Corning, 2012). Parameters evaluated in the study included wet and blotted uterine weight, cell heights for both lumen and glandular regions, cell proliferation by BrdU-labelling for luminal, glandular and stromal portions of the uterus and mammary tissue cell proliferation. Ethinyl-estradiol (EE), an agonist for both nuclear estrogen receptor (ESR1; ERα) and for the G-protein coupled

Table 3
Modes of Action, Key Events, Dose Metrics, and Risk Assessment Relevance for diverse endpoints seen in toxicology testing with D4.

Endpoints	Mode of Action and Key Events	Associated Dose Metrics	Expected Dose Response	Human Risk Assessment Relevance
Responses in Pulmonary Airways and Alveolar region	Equilibration of lipophilic vapors with epithelial tissues and deposition of aerosols onto, retention in and irritation of epithelial tissues	Tissue concentration of D4 and persistence of elevated D4 in regions with aerosol deposition	Non-linear with sharp break in dose response for inhalation of vapor atmospheres alone versus those with aerosols	Not relevant endpoint for risk assessments for low concentration vapor only exposures, i.e., below several hundred ppm
Liver hypertrophy with increased cell proliferation	Activation of CAR-signaling by D4, increasing patterns of gene expression of genes for metabolizing enzymes (Cyp2B1) and cellular pathways controlling cell proliferation in the rat	Time above a minimally active concentration of a CAR- receptor complex or for inhibiting EGFR signaling	Non-linear, where the appearance of hypertrophy and hepatocellular carcinoma are limited by short duration of action of inhaled D4, and some level of CAR activation required to produce transcriptional responses	Not a human relevant endpoint. The key responses – liver enlargement and cell proliferation – do not occur in humans exposed to CAR-activators
Liver pigmentation and bile duct hyperplasia in SD Rats	Uncertain	Uncertain and the preferred dose metric would be exposure concentration	Low-dose non-linear with a threshold since no evidence of tumor formation in liver in chronic studies	Use to establish a NOEL or BMD for assessing risks of D4
Mild nephropathy and kidney responses	Chronic progressive nephropathy (CPN) and some binding of silanol metabolites with alpha-2u globulin to impair protein processing and leading to kidney toxicity	CPN and some persistence of silanol- alpha-2u globulin binding requiring some minimally active concentration of the complex to affect protein processing	Expectation of non-linear behavior where some minimal level of impaired processing of protein is required to cause kidney responses	Not human relevant since the endpoint is specific to the rat, especially the male rat.
Reproductive responses- reduced litter size, decreased implantation sites and corpora lutea	Hypothalamic or nasal epithelial D4 affecting afferent GABA pathways innervating GnRH neurons to block/delay GnRH surge	D4 accumulating in lipid membranes or intramembrane binding to GABA receptors to inhibit GnRH neuron function.	Low-dose non-linearity since it would be necessary to activate GABAergic suppression to some minimal degree to block LH surge or achieve tissue D4 sufficient for non-specific effects	Not human relevant due to differences in GnRH input required to regulate the LH surge in rodents and humans
Uterine, ovarian, and vaginal responses and altered patterns of cycling in senescent female rats	D4 alterations of GnRH surge & LH surge delays ovulation leading to longer periods of tissue exposures to endogenous estrogen	Increased E2 levels and activation of estrogen receptor signaling due to prolonged proestrus	Low-dose non-linearity due to need for sufficient concentrations of D4 to affect GnRH neuronal function and block mid-cycle LH surge	Not human relevant due to differences in GnRH control of estrus cycle LH surge in rats and women

estrogen receptor (GPER1), and 4-hydroxytamoxifen (4-OHT), an agonist of GPER1 and partial agonist/antagonist of ESR1, were used as positive control compounds. G-protein coupled receptors (GPCRs) are transmembrane proteins that transmit signals from the extracellular environment, most often via intracellular phosphorylation cascades. With respect to uterine weight, the increase with the 16-hr exposure was 70 % as large as seen with EE. The increase for the 6-hr exposures was only 30 % of the EE control. The increase in uterine wet weight for D4 for the 6-hr exposures was more like the increase with 4OHT. With BrdU labelling (% labelled cells), the 16-hr D4 group was similar (55.5 ± 2.9) to the EE group (63.4 ± 2.8) while the increase with the 6-hr exposures were more similar for D4 (35.2 ± 8.5) and 4OHT (33.9 ± 6.1). Control values for BrdU labelling were (4.3 ± 3.7). Perhaps, the most important conclusion from this study was that the 6-hr D4 exposure group produced responses much more like those with 4OHT, a GPER1 agonist, than those with E2. Importantly, the 6-hr exposure duration was used in the toxicology studies and in the examination of LH surge and ovulation. Other tests of receptor binding and measurements of estrogen biomarker gene expression showed that D4 exhibits only weak binding with ESR1 (He et al., 2003; Quinn et al., 2007b).

4.5.1. Quantitative Contributions of D4 to estrogenicity in a mature female rat

Although most of these mechanistic studies of uterine proliferation with immature or ovariectomized female rats showed weak biological responses to D4, these study designs do not take into account the more complex time courses of tissue E2-exposures in intact female rats that experience 4-to-5-day estrus cycles with appreciable circulating estrogens throughout the cycle. For instance, E2 concentrations during the estrus cycle (Fig. 2C) range from 5 to 40 pg/mL (Smith et al., 1975). Even in diestrus there are significant concentrations of E2 with maximal E2 concentrations on the morning of estrus of about 0.15 nM. Plasma levels of D4 with 700 ppm exposures were 8.03 ± 0.95 μg/mL, about 27 μM. In the inhalation studies with ovariectomized rats the relative potency of E2 is nearly 200,000 times greater than D4, and the overall range of E2 throughout the estrus cycle is less than 10-fold between diestrus and proestrus. Thus, the contribution of D4 to estrogenic responses in the intact, mature female rat, unlike juvenile females or ovariectomized adult females, would be insignificant compared to the cyclic tissue exposures to E2 itself in the sexually mature female rat (Fig. 2C) where there are appreciable E2-concentrations throughout the estrus cycle. Rather than expecting the excess estrogenic activity seen in the reproductive tract tissues to arise from D4, it is the prolongation of proestrus from a delay in ovulation that increases total tissue E2 exposures.

5. Explicit consideration of MOAs, Key Events and Tissue Dose Measures

Integrating information on PK, tissue distribution, toxicity, mechanistic studies, and comparisons to other compounds provides the opportunity to develop hypotheses for specific MOAs in each of the key tissues and make decisions about endpoints that could be used in risk assessments with D4 based on evaluation of their relevance for human exposures to D4 vapors. This section integrates the various data streams to propose/support MOAs and, where possible, compares various possible MOAs to suggest those that are more likely than others based on current information. These MOAs then used to assess the dose measures for D4, or silanol metabolites likely associated with these responses. Table 3 outlines, dose measures and proposed MOAs for each of these endpoints.

5.1. Upper and lower pulmonary region toxicity

Exposure to atmospheres of D4 containing both vapors and aerosols lead to equilibration with vapors and deposition of variable amounts of D4 aerosol along the airways. The site of deposition depends on particle size with both larger and smaller aerosol droplets preferentially depositing in the head and nasal cavity (Heyder, 2004). The aerosol dose will be greater at the higher exposures consistent with observations of the markers of toxicity. In addition, the kinetic behavior on skin indicates that, after aerosol droplet deposition in the respiratory tract and D4 uptake into the epithelium, the local concentrations of D4 are expected to remain elevated for several hours after exposure with some portion moving back into the airstream after cessation of the exposure period. The high exposures created using nebulizers would produce a greater percentage of aerosol production with nociceptor-related changes (Pauluhn, 2018) involving increased thymus and adrenal weights (Burns-Naas et al., 2002), direct tissue irritation/hemorrhage in the deeper lung and concomitant increases in corticosterone release. In the deeper lung, deposited D4 or high concentrations of D4 would be expected to disrupt surface tension properties based on its spreading characteristics. The effects in the deeper lung areas seen with longer-term exposures are consistent with alterations of surfactant homeostasis and localized irritation (Pauluhn, 2021). For the respiratory tract, the dose metric would be epithelial tissue concentrations of D4 and amounts of deposited aerosol that would lead to epithelial uptake of deposited D4 with short-term, i.e., several hours, local retention as indicated in the PK studies of dermal uptake. Increasing tissue concentrations would activate adaptive stress responses, leukocytosis, altered surfactant processing with attendant macrophage scavenging of surfactant, and increases in corticosteroids in blood. The key event in the lung is uptake of vapors and aerosol into the epithelium at high exposure concentrations causing tissue irritation (Table 3; Supplemental Fig. 1- Panel 1).

As an alternative hypothesis, metabolism of D4 in metabolically competent cells in the respiratory and olfactory epithelium in the nose and in club cells along the pulmonary epithelium might convert D4 to locally high concentrations of reactive metabolites and cause tissue toxicity. Fitting of a PBPK model to closed chamber gas-uptake time course curves with D4 in male Fisher 344 rats required including 5 % of total whole-body metabolism in the respiratory epithelium (Dobrev et al., 2008). Some compounds metabolized to reactive compounds at sites in the pulmonary or olfactory epithelium cause epithelial toxicity – including styrene (Cruzan et al., 1997, 2002), naphthalene (Campbell et al., 2014) and vinyl acetate (Bogdanffy et al., 2001). With these compounds, oxidation (styrene and naphthalene to ring epoxides) or enzymatic hydrolysis (vinyl acetate to acetaldehyde and acetic acid) in the epithelial tissues produce metabolites that cause toxicity. This alternative hypothesis is very unlikely for D4 since there is no evidence that the linear silanols produced by metabolism of D4 are reactive and there were no signs of reactivity-based hepatotoxicity in the liver where there is much higher enzymatic activity for D4 metabolism.

5.2. Kidney

Chronic progressive nephropathy (CPN) is common in many strains of laboratory rats, and its incidence and severity is increased by exposures to various chemicals (Hard et al., 2009). In addition, inhalation studies with unleaded gasoline and various short-chained, branched hydrocarbons cause a male-specfic rat nephropathy (Swenberg, 1993). The compounds causing this nephropathy interact with a male rat-specific protein, alpha-2u

globulin leading to protein accumulation, nephropathy and small increases in kidney cancer. Among compounds causing alpha-2u globulin accumulation are various low molecular weight, branched chain hydrocarbons, exemplified by 2,2,4-trimethyl pentane. After exposures, a metabolite, 2,4,4-trimethyl-2-pentanol is found bound to alpha-2u globulin (Borghoff et al., 1991). Although silicon atoms are significantly larger than carbon, the parent pentane and active metabolite have structural similarities with HMDS and its monohydroxylated metabolite (Fig. 1) and HMDS or metabolites co-eluted with alpha-2u globulin in HMDS-exposed male rats (Dow Corning, 2003). The key event in the kidney with HMDS would arise from silanol metabolites similar to those produced by HMDS interacting with alpha-2u globulin. The dose measure for this endpoint would be related to both achieved concentrations of silanol metabolites formed and their affinity for alpha-2u globulin. The kidney effects with D4, however, are most likely from a combination of CPN (affecting both male and female rats) with some small contribution of male-specific nephorpathy increasing the severity of the response in males.

While is not evident that there are any other compelling alternative hypotheses, it would be possible to assess binding of D4 or its metabolite silanols to male rat alpha-2u globulin *in vivo*, as done with HMDS (Dow Corning, 2003), or estimating binding with appropriate QSAR methods that would estimate quantitative differences in binding to alpha-2u globulin compared to hydrocarbons (Barratt, 1994). However, in the light of the lack of relevance of this endpoint for human risk assessment, there would be no urgency to conduct further investigations of a silanol-based MOA for kidney effects.

5.3. Liver

CAR signaling pathway activation in liver by D4 increases the clearance of D4 from the body – a form of auto-induction of metabolism. Similar metabolic processes are evident with barbiturate induction of CAR pathway activation. The MOA for PB induction of CYP2B enzymes appears to be related to effects on PB on phosphorylation and activation of the constitutive androstane receptor -CAR (Mutoh et al., 2013). Activation of CAR by PB exposures leads to a transcriptionally active form of the CAR receptor that upregulates a suite of genes involved in xenobiotic metabolism and in cell cycle progression and hepatocyte proliferation in the rat.

PB does not bind directly to CAR. Instead, the mechanism of induction appears to be PB altering epidermal growth factor receptor (EGFR) signaling at the cell surface to affect downstream CAR phosphorylation (Mutoh et al., 2013). EGFR is a transmembrane receptor and interacts with various activators or inhibitors at the cell membrane interface (Supplemental Fig. 1-Panel 2). Several possible docking sites were identified for PB on both the active and inactive form of EGFR. However, it is not known if D4 and PB work in a similar manner to activate CAR, i.e., through effects on EGFR. Nonetheless, the most plausible MOA for liver enlargement and hepatocyte proliferation would be that D4, as does PB, activates CAR-driven transcriptional programs whether through binding to EGFR directly or through actions on some other portion of the CAR-response pathway in liver. While the liver enlargement and hepatocyte proliferation associated with activation of CAR pathways are not relevant for human risk assessment (Elcombe et al., 2014), other responses, notably pigment accumulation and bile duct hyperplasia seen at the highest concentration (700 ppm) in the reproductive toxicity study, have not been not associated with CAR activation and have to be assessed differently (Dow Corning, 2001; Siddiqui et al., 2007). The dose response for these effects follows that for increased liver weight rather than that of CYP-induction. The relevance of these responses for humans is unknown at present.

5.4. Female rat reproductive effects

The reproductive effects of D4 in the rat have been clearly associated with modulation of the mid-cycle LH surge required to drive ovulation (Meeks et al., 2007; Quinn et al., 2007a). In the rat, a mid-cycle GnRH surge leads to the ovulation-producing LH surge on the morning of proestrus. In humans, LH release from the pituitary occurs in response to patterns of GnRH release from the hypothalamus at different times during the estrus cycle and the activity of GnRH neurons is controlled by a diverse array of inputs (Fig. 2B). Several hypotheses for the modulation of the GnRH/LH surges in female rats were considered.

5.4.1. D4 is not a dopamine agonist

One proposal to explain the reduced or delayed LH surge was D4 serving as a dopaminergic agonist to alter prolactin release (Dekant et al., 2017). Prolactin is required for normal ovulation in rats, but it does not appear to play a role in human ovulation. D4 might act as a dopamine agonist and reduce prolactin release. However, a series of pharmacological studies provided no strong evidence for D4 actions with these receptors (Baker, 2010). In addition, pergolide mesylate (PM), a dopamine receptor agonist, was also included in studies of altered cycling in the aging female rat (Jean et al., 2017). Effects similar to those caused by D4 were seen with PM; however, there were differences in hormone levels of progesterone, estradiol, prolactin and corticosterone, between D4- and PM-treated rats. Altered dopamine signaling did not appear to be the cause of the modulation of the LH surge and ovulation in D4 exposed rats.

5.4.2. Non-specific, anesthetic-like membrane effects of D4

Another possible effect would be non-specific consequences of high lipid phase concentrations of D4 affecting neurotransmission (Antkowiak, 2001; Lynch, 2008), a mechanism of action for volatile anesthetics originally outlined separately by Overton (Overton, 1895) and Meyer (Meyer, 1899) over a hundred years ago. While models for anesthetic potency have been developed for anesthetic gases, they have not been applied to volatile compounds with oil/air or octanol/air partition coefficients as large as that of D4 (Table 1). With conventional volatile anesthetics, a simple relationship permits estimation of the minimal anesthetic concentration (MAC) based on a single parameter, the oil/gas partition coefficient (Sosis, 2017). The fitted relationship is [(MAC) = (octanol/gas partition coefficient)**0.9542)/108.67. The estimated MAC for D4 with this equation would be 0.0101 % by volume or 101 ppm. The likely reason for this low estimate of the MAC (compared to observations in the animal exposures) is the very high lipophilicity of D4 compared to the other volatile anesthetics used in developing the relationship between MAC and log oil/air (Antkowiak, 2001) and the slower filling of membranes and brain tissues expected with intermittent 6 h/day exposures with a highly lipophilic volatile. Nonetheless, this estimation of a MAC for D4 emphasizes that non-specific membrane effects are not quantitatively unreasonable given the high lipophilicity of D4 and that this non-specific anesthetic substance-like activity could affect the activity of GnRH neurons themselves or of afferent inputs to these neurons (Fig. 2A and B).

Effects of D4 on membrane fluidity have been examined in liposomes and rat pituitary and human umbilical vein endothelial cells – HUVEC - in unpublished reports (IONTOX, 2018, 2019). Liposomes were prepared and labelled with 1,6-diphenyl-1,3,5-hexatriene (DPH). Sodium dodecyl sulfate was a positive control and the effect of D4 on membrane fluidity assessed following treatment with increasing concentrations of D4 (0.3, 1, 3 10 and 30 %). The mixture of neat D4 and the aqueous test solution was mixed, vortexed and then allowed to sit. After the separation of D4

from the aqueous phase, fluorescence depolarization showed a dose response across all concentrations. The Fluorescence Polarization (FP) values decreased from about 160 units at 0 % D4 to about 40 units at 30 % D4. The decrease in polarization indicates an increase in membrane fluidity, i.e., moving to a more disordered state, and the treatment causing approximately a 50 % maximal loss was between 1 and 3 % D4.

Functional *in vitro* studies of trans-membrane proteins after treatment with D4 were also pursued with HUVEC cells. Treatment of these cells with vascular endothelial growth factor (VEGF) leads to ERK1/2 phosphorylation. Cells were treated with increasing concentrations of VEGF and either D4 alone or D4 in combination with sunitinib maleate, a VEGF receptor inhibitor. D4 increased ERK1/2 phosphorylation compared to vehicle control at the highest treatment level and this increase was not affected by sunitinib. It appears that D4 increases fluidity, i.e., a non-specific change in membrane packing, and these non-specific changes lead to enhanced ERK1/2 phosphorylation. Although it is difficult to accurately determine the aqueous phase exposure to D4 in these experiments, qualitatively these results indicate that D4 at aqueous concentrations achievable in these mixed suspension experiments alters membrane integrity and membrane-associated biological functions. The results with HUVEC cells do not indicate that D4 caused specific alteration of ERK function but simply that D4 has an ability to change membrane fluidity and associated behaviors of membrane proteins/lipoproteins of various kinds via non-specific mechanisms.

5.4.3. Alterations in GABA signaling

Binding of D4 with cellular proteins or partitioning of D4 into membrane components would also affect GnRH neuronal function. Both PB and progesterone block the mid-cycle LH surge and ovulation in rodents (Everett and Sawyer, 1950; Toyoda and Chang, 1969; Arimura and Schally, 1970; Tyler and Gorski, 1980). While these observations were noted some 50–60 years ago, the neural basis of these responses has only become clarified with expanding knowledge of the control of the activity of neurotransmitter pathway signaling in the hypothalamus. Gamma-aminobutyric acid (GABA) is the dominant inhibitory neurotransmitter in the hypothalamus, where 50 % of all synaptic contacts in the rat hypothalamus are estimated to be GABAergic (Decavel and Van den Pol, 1990). The GABA receptor is a transmembrane chloride-ion channel protein (Supplemental Fig. 1- Panel 3). Several neurosteroids affect GABA neuronal activity, by enhancing inhibitory input through activation of chloride ion signaling. Two of the better studied neurosteroids are allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC), formed respectively from progesterone and corticosteroids (Paul and Purdy, 1992). In the two-year study of the aging female rat, there was a consistent increase in corticosterone (Jean et al., 2017) that could form THOC either in the periphery or in tissues within the brain. An increased stress response with increased corticosteroid levels from pulmonary tract irritation associated with exposure to mixed aerosol/vapors at the higher exposure concentrations would increase THOC in brain and could increase inhibitory tone with GABA neurons and affect GnRH release.

Allopregnanolone (3-α-hydroxy-5-α-prenan-20-one) binds GABA-receptors with high affinity, augmenting GABA-activated chloride channels, in a manner similar but not identical to PB (Paul and Purdy, 1992) and suppresses GnRH release (Calogero et al., 1998). Binding sites for neurosteroids have been identified by using photolabeling analogs of the progesterone derived neurosteroids with a human α1β3 GABAa receptor and docking studies to predict amino acid residues involved in neurosteroid binding (Chen et al., 2019). Similarly, PB binding sites in a α1β2γ2 GABAa receptor were solved by electrophysiology and molecular dynamics simulations to identify binding sites between α-β and α-γ interfaces (Kim et al., 2020). Studies with several general anesthetics, isoflurane, halothane, and chloroform, coupled with site specific mutagenesis defined a binding pocket for these anesthetics in an amphipathic cavity on the alpha-subunit with a molecular volume of ~250 to 370 $Å^3$ (Jenkins et al., 2001). The molecular volume of D4 is close to this range, approximately 390 $Å^3$ (Personal communication, G. Kozerski, Dow Chemical). An overlapping target for PB and D4 affecting GABA receptors in the hypothalamus would also be consistent with their common activity in increasing CAR receptor pathway-mediated transcriptional programs in the liver.

5.4.4. Non-specific effects on GnRH neuron activity arising from olfactory/VMO afferents in rodents

While the pituitary and hypothalamus concentrations of D4 were similar to concentrations in other tissues at the same exposure levels (Plotzke et al., 2000; Andersen et al., 2001; Krieger et al., 2021), afferent inputs to the hypothalamus arising from the olfactory epithelium (OE) or the vomeronasal organ (VMO) in rodents might experience higher concentrations through aerosol deposition and direct vapor equilibration in the nasal epithelium coupled with movement of deposited D4 into neuronal tract tissues immediately proximal to nasal deposition sites. Nasal delivery of various particles and stabilized lipid carriers have been used to deliver compounds to the brain and bypass the blood/brain barrier (Crowe et al., 2018). This uptake route is composed of two pathways. Endocytosis brings the particles into the olfactory epithelium followed by axonal transport to the synaptic clefts in the olfactory bulb for exocytosis. Olfactory neurons then distribute compounds to other brain regions. Nasal delivery bypasses the systemic circulation and can deliver drugs that would be rapidly degraded during circulation or those whose uptake into brain would be limited by the blood-brain-barrier. Easily biodegradable test materials were first conjugated with a carrier or incorporated into stable particles before intranasal exposure. However, it is unlikely that deposited lipophilic D4 aerosols would maintain structural stability after deposition on the epithelium to allow endocytosis. Whether or not the pathway is significant for delivery of vapor or deposited liquid droplet aerosols of D4 to the hypothalamus, uptake into the nasal epithelium and local diffusion into the olfactory bulb structures could produce exposures to neuronal pathway that communicate to the hypothalamus, which in turn could also affect GnRH afferents.

5.4.5. Non-specific effects on GnRH neuron activity arising from nociceptor stress response in rodents

Deposition of high concentrations of D4 vapor or aerosol droplets in the nasal cavity of rodents could produce nociceptor responses (Pauluhn, 2018). The high exposure concentrations in the rat inhalation studies produced a greater percentage aerosol and nocioreceptor-related responses (Pauluhn, 2018) involving secondary changes in thymus and adrenal weights (Burns-Naas et al., 2002) and signs of tissue irritation/hemorrhage in the deeper lung. Deposited D4 aerosol in the deep lung would spread onto the epithelial surface, disrupting surfactant turnover and recruiting macrophages to scavenge the disrupted surfactant layers. The observation of increased macrophages and local hemorrhage is consistent with aerosol exposure in the deeper lung. These types of changes in the pulmonary tract can lead to concomitant increases in corticosterone release as a stress response. In the two-year study of female rat reproductive senescence, there was a consistent increase in corticosterone (Jean et al., 2017) that could potentially form THOC, a corticosteroid metabolite, either in the periphery or in tissues within the brain. An increased stress response with increased corticosteroid levels from pulmonary tract irritation associated with exposure to mixed aerosol/vapors at the higher

exposure concentrations would increase THOC in brain, and further increase the inhibitory tone of GABA neurons.

Overall, the MOA for the reproductive toxicity in female rats with the strongest support is that a combination of non-specific membrane fluidity changes and enhanced GABAa-related disruption of GnRH neurons either in the hypothalamus or in afferent pathways affecting hypothalamic GnRH neurons modulate the mid-cycle GnRH surge required for mid-cycle LH release and ovulation in the rat (Supplemental Fig. 1; Panel 3).

5.5. Female rat reproductive tissue responses

Specific tissue responses (vaginal/uterine/ovarian tissue) observed during toxicity evaluations with high levels of mixed aerosol/vapor showed signs of estrogenic stimulation that included uterine hyperplasia, vaginal mucification and minimal evidence for benign uterine adenomas. These responses contrasted with a lack of estrogenic stimulation on other tissues that are normally adversely affected by excess estrogenic activity, including adverse effects on prostate, testes, and spermatogenesis in males (Siddiqui et al., 2007). The increased estrogenic signaling in the mature, cycling female rat exposed to 700 ppm D4 is associated with the delay in ovulation, producing extended periods of proestrus (Fig. 2C). Any potential activation of estrogen receptors by D4 would be swamped by normal exposures to E2 during estrus cycling in the intact, mature female rat (Borgert et al., 2018) where the potency of E2 is 5 or 6 orders of magnitude greater than that of D4 (McKim et al., 2001b). The estrogenic MOA of D4 in ovariectomized female rats indicates action as a weak agonist, primarily affecting signaling through GPER1 that would not contribute significantly to estrogenicity in a mature, cycling female rat.

6. Relevance of rat MOAs for human risk assessment

Table 3 includes a column assessing human relevance of the MOAs of toxicity in rats. Both the mild degree of nephropathy and liver enlargement with increased cell proliferation are rodent-specific responses and of no relevance for human risk assessment either qualitatively or quantitatively (Hard et al., 2009; Elcombe et al., 2014). However, neither the pigment accumulation nor bile duct hyperplasia, responses that were also reported for the linear siloxane L3 (HarlanLaboratories Ltd., 2010) are considered part of the PB-like responses to CYP2B-inducer. These responses were observed at the highest exposure concentrations with D4 and were statistically significant at 700 ppm. The relevance of these endpoints is unknown at the present time. NOEL or BMDs for D4 could be derived for these responses for more formal risk assessments.

Of the remaining endpoints (Table 3), the pulmonary responses at the exposure concentrations above 300 ppm arise from combined aerosol/vapor exposures and are not relevant for low inhaled concentrations where only vapor would be present in inhaled air. The increased eosinophilic globules in females at exposures of 30 ppm and above at 24 months had a clear dose response. However, these globules are commonly observed with highly variable incidence in old F344 rats and have a higher incidence in females than males. This slight increase in a common geriatric endpoint in these rats would not be an appropriate endpoint for risk assessment.

The evidence for impaired female rat reproductive function and specific reproductive tissue responses very strongly supports MOAs related to some combination of non-specific membrane fluidity responses and GABAa inhibition of GnRH neuronal function (i.e., increased inhibitory tone of the GABAa neurons) as the cause of delaying the LH surge and ovulation, with the subsequent extended time in estrus. The dose-response for effects on ovulation in the rat would be non-linear with a threshold since the measure of dose for a nonspecific membrane disruption process, a response secondary to a stressor or a receptor mediated process, would require some minimal level of dose maintained over a sufficient duration to have any measurable effect on enhanced GABA inhibition of the GnRH neurons. However, a more significant issue for extrapolation of relevance of this endpoint to humans arises from differences between rats and humans in relation to interspecies difference in control of GnRH and the LH surge. The rodent MOA here is not relevant for humans.

Fertile female rats have 4- to 5-day estrus cycles and ovulation is governed by the large mid-cycle GnRH surge driving the ovulation-inducing LH pulse on the day of proestrus. Extending the time in estrus by a single day very significantly adds to the net estrogen exposure during a cycle (Fig. 2C). The human menstrual cycle is more prolonged and LH release affecting ovulation differs from that in the rat. The human female LH surge occurs in the absence of a midcycle GnRH discharge, and is generated by facilitatory activity between pulsatile GnRH input to the pituitary and activating actions of ovarian estradiol on the pituitary (Plant, 2012). In contrast to the rodent (Toyoda and Chang, 1969; Norman et al., 1973b, a; Majewska et al., 1986), barbiturate treatment does not block the LH surge in rhesus monkeys (Knobil, 1974; Plant, 2012) and this rodent response, altering the mid-cycle GnRH surge with postponed ovulation and extended proestrus, would not be considered relevant for assessing risks in women.

6.1. Tissue MOAs of D4

This hypothesis-driven MOA analysis indicates that there may be non-specific, membrane fluidity effects arising from partitioning of the lipophilic D4 into membranes, together with some degree of specificity for D4 action with two proteins - GABAa and EGFR-that are integrated into cell membranes. Supplemental Fig. 1 illustrates these MOAs by showing uptake of D4 into cell membranes for all endpoints other than the kidney. With current technologies for modeling small molecule-protein binding, it would be feasible to evaluate possible binding sites for D4 on GABA and EGFR computationally and compare results with those of PB (for GABAa and EGFR). However, as discussed in relation to binding of silanol metabolites of D4 with alpha-2u globulin, there is no pressing need for further evaluation of binding to these proteins due to the lack of relevance of the altered mid-cycle GnRH response for human risk assessment.

The responses of D4 in the uterotrophic assays in ovariectomized rats suggested that D4 was more similar in action to 4OHT than EE and was likely interacting through GPER1 (Supplemental Fig. 1; Panel 4). As with GABAa and EGFR, various computational tools – molecular modeling studies, molecular dynamics and docking algorithms – have also been used to examine GPER1 binding sites for E2 and for agonists and antagonists (Mendez-Luna et al., 2015) and there is continued progress in refining understanding of binding sites within GPCRs (Chan et al., 2019). D4, with the silicon atoms surrounded by methyl groups, would likely have higher potential for binding with more hydrophobic regions of binding pockets or for insertion within the lipid bilayers. Binding and functional studies pf D4 binding with GABAa receptors could also be useful in more accurately determining affinities and binding sites on the receptor and differentiating non-specific membrane and GABAa-related MOAs. However, since the-reproductive tissue/reproductive responses are not relevant for human risk assessment due to differences in regulation GnRH release from the hypothalamus, control of the LH surge and the estrogenicity related to prolonged estrus rather than D4 (Table 3; Fig. 2), further experiments or computational modeling to evaluate

binding of D4 with GPER1 would have little value for D4 risk assessment. The estrogenicity in rodents arises from actions of more prolonged E2-exposures (Table 3) rather than any effect of D4.

7. Conclusions

Integration of evidence from toxicology, metabolism, and pharmacokinetics indicates that the MOAs for most of the relevant toxicological responses to D4 in mammalian species are either species-specific for rodent (liver hypertrophy, nephropathy, and the PB-like delay of the mid-cycle LH surge) or high dose pulmonary responses from inhaled aerosols that are not expected to contribute substantially at lower exposures. The reproductive tissue responses in the rat, including uterine hyperplasia, vaginal mucification and minimal evidence for uterine benign adenomas are secondary to delaying the LH surge on the day of proestrus, an effect enhanced by the presence of inhaled aerosols.

The mid-cycle, ovulation-producing GnRH surge is a process specific to the rodent; ovulation in human females occurs through pulsatile GnRH release and priming of the anterior pituitary by estrogen to prepare the anterior pituitary for an LH surge that drives ovulation. Importantly, none of these responses should be considered relevant for human risk assessments and, in view of differences in the way GnRH regulates LH release and ovulation between rats and humans, the reproductive effects should not drive regulatory labelling of D4. Neither should D4 be regarded as an estrogenic endocrine disruptor since the responses (reproductive effects and reproductive and uterine/vaginal morphology) following exposure to D4 in adult intact animals are due to prolonged endogenous E2 from delaying the rat-specific mid-cycle LH release. Since the reproductive tissue, uterine adenoma and effects on reproduction are due to a rodent-specific MOA, D4 should not bear carcinogenic, mutagenic or reproductive toxicity (CMR) labelling nor be considered an endocrine disruptor. A NOAEL or BMD could be established for increased liver pigmentation and bile duct hyperplasia and serve as a point of departure for developing a risk assessment. Importantly though, at this point the MOA of this endpoint with its differential sensitivity in SD and F344 rats, is unknown as its relevance for humans.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Melvin Andersen reports financial support and administrative support were provided by American Chemistry Council Silicones Environmental Health and Safety Center. Melvin Andersen reports a relationship with American Chemistry Council Silicones Environmental Health and Safety Center that includes: consulting or advisory.

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Appendix A. Supplementary data

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