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Biological relevance of effects following chronic administration of octamethylcyclotetrasiloxane (D4) in Fischer 344 rats

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HIGHLIGHTS

- Inhalation of octamethylcyclotetrasiloxane (D4) induces uterine adenomas in rats.
- Biological relevance of this effect for human risk characterization is assessed.
- Alterations in the estrous cycle in the aging F344 rat is the most likely mode of action.
- Cycle alterations likely induced indirectly via a dopamine-like mechanism.

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ABSTRACT

Octamethylcyclotetrasiloxane (D4) is a cyclic siloxane primarily used as a monomer or intermediate in the production of silicone polymers resulting in potential exposure of workers, and potential low level inhalation or dermal exposure for consumers and the general public. Following a two-year inhalation toxicity study with D4 in rats, increases in uterine endometrial cystic hyperplasia and adenomas were observed at the highest concentration of D4 administered (700 ppm). No other neoplasms were increased with D4 treatment. In addition, chronic inhalation exposure of rats to D4 induced changes in relative liver and kidney weights, and produced a chronic nephropathy.

This manuscript examines the biological relevance and possible modes of action for the effects observed in the F344 rat following chronic inhalation exposure to D4. D4 is not genotoxic and appears to exert its effects through a nongenotoxic mode of action. An alteration in the estrous cycle in the aging F344 rat was the most likely mode of action for the observed uterine effects following chronic inhalation exposure. Data support the conclusion that D4 acts indirectly via a dopamine-like mechanism leading to alteration of the pituitary control of the estrous cycle in aging F344 rats with a decrease in progesterone and an increase in the estrogen/progesterone ratio most likely induced by a decrease in prolactin concentration. D4 also inhibited the pre-ovulatory LH surge causing a delay in ovulation, persistent follicles and thus a prolonged exposure to elevated estrogen in the adult Sprague Dawely rat. A lengthening of the estrous cycle in the F344 rat with an increase in endogenous estrogen was also induced by D4 inhalation. Although the mode of action responsible for induction of uterine adenomas in the female F344 rat has not been clearly confirmed, the subtlety of effects on the effects of D4 on cyclicity may prevent further assessment and definition of the mode of action. The occurrence of uterine endometrial adenoma in the rat is not relevant for human risk characterization because (1) there are differences in ovulatory cycle regulation in rats compared to humans, (2) cystic hyperplasia without atypia in women is not a cancer precursor, and (3) there is no endometrial lesion in women that is directly analogous to endometrial adenoma in the rat. The effects of D4 on liver are due to a phenobarbital-like mechanism that results in induction of cytochrome P450 and other enzymes of xenobiotic

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biotransformation. The liver effects are adaptive and not adverse. Kidney findings included chonic progressive nephropathy, a rat lesion that has no counterpart in the human and that should not be used in human risk assessment.

1. Introduction

Octamethylcyclotetrasiloxane (D4) is a cyclic siloxane primarily used as a monomer or intermediate in the production of silicone polymers. Potential human exposure includes workers, consumers, and the general public. D4 is highly lipopilic (log K_{ow} of 6.5) with very low water solubility (50 ppb) and a boiling point of 175 °C and is highly volatile. Inhalation of D4 vapor and dermal contact with D4-containing formulations are the major expected routes of human exposures (SCCS, 2010). However, dermal absorption of D4 is very limited and most of the D4 applied to skin rapidly evaporates due to the high volatility (DCC, 2000d, 2000c; Jovanovic et al., 2008). Because D4 does not have a potential for bioaccumulation (Andersen et al., 2008), dietary exposures of humans are considered of little relevance.

Toxicity studies on D4 have been performed addressing the acute, subchronic, and chronic effects of D4 in rodents (Franzen et al., 2017). Most of these studies used inhalation as the route of exposure due to the high volatility of D4, the very limited dermal absorption (DCC, 1998a, 2000c), and the unique toxicokinetics of D4 after oral administration (Sarangapani et al., 2003; DCC, 2006a; Andersen et al., 2008). Inhalation studies following established study protocols have been performed to address most endpoints relevant to risk characterization (Burns-Naas et al., 2002; Batelle, 2004; Meeks et al., 2007; Siddiqui et al., 2007). In addition, mechanistic studies have been performed with D4 both *in vivo* and *in vitro* to provide information on the underlying mechanisms of the effects observed in the hazard assessment studies and the relevance of the D4 effects observed in rodents for human risk characterization.

This manuscript summarizes the results of the D4 experimental studies with regard to the understanding of those mechanisms by which D4 elicited effects including the increases in liver weight, nephropathy, and the incidence of proliferative uterine endometrial lesions after chronic exposure in F344 rats.

2. Absorption, distribution, metabolism, excretion of D4

The toxicokinetics of D4 are well characterized, and studies investigating both single and repeated inhalation, dermal application, and intravenous (i.v) administration have been performed in experimental animals and, for some routes of administration, in human subjects. Toxicokinetics following single oral exposures also have been assessed in humans and rats (Utell et al., 1995, 1998; DCC, 1996b, 1997b, 1997c, 2000b, 2001c, 2006a; Kala et al., 1998; Varaprath et al., 1999; Plotzke et al., 2000; Dobrev et al., 2008). The biotransformation of D4 was investigated in experimental animals after inhalation and intravenous administration. Initial oxidation of D4 occurs by cytochromes P450 to give heptamethylcyclotetrasiloxanol (DCC, 1997a, 1997b, 2006b) (Fig. 1); this cyclic siloxanol is then further hydrolyzed to dimethylsilanediol ($Me_2Si(OH)_2$), methylsilanetriol ($MeSi(OH)_3$), and a number of other minor metabolites (DCC, 2001c). These polar metabolites are excreted in urine (Fig. 1).

In human subjects exposed to a D4 concentration of 10 ppm in air, absorption of D4 from the lung after inhalation is limited and exhalation of absorbed parent D4 is rapid. In addition, D4 not exhaled is cleared by biotransformation to polar metabolites and excretion in urine (DCC, 1996b, 1996c, 2000b, 2000a, 2002a; Utell et al., 1998; Plotzke et al., 2000; Reddy et al., 2003). Systemically

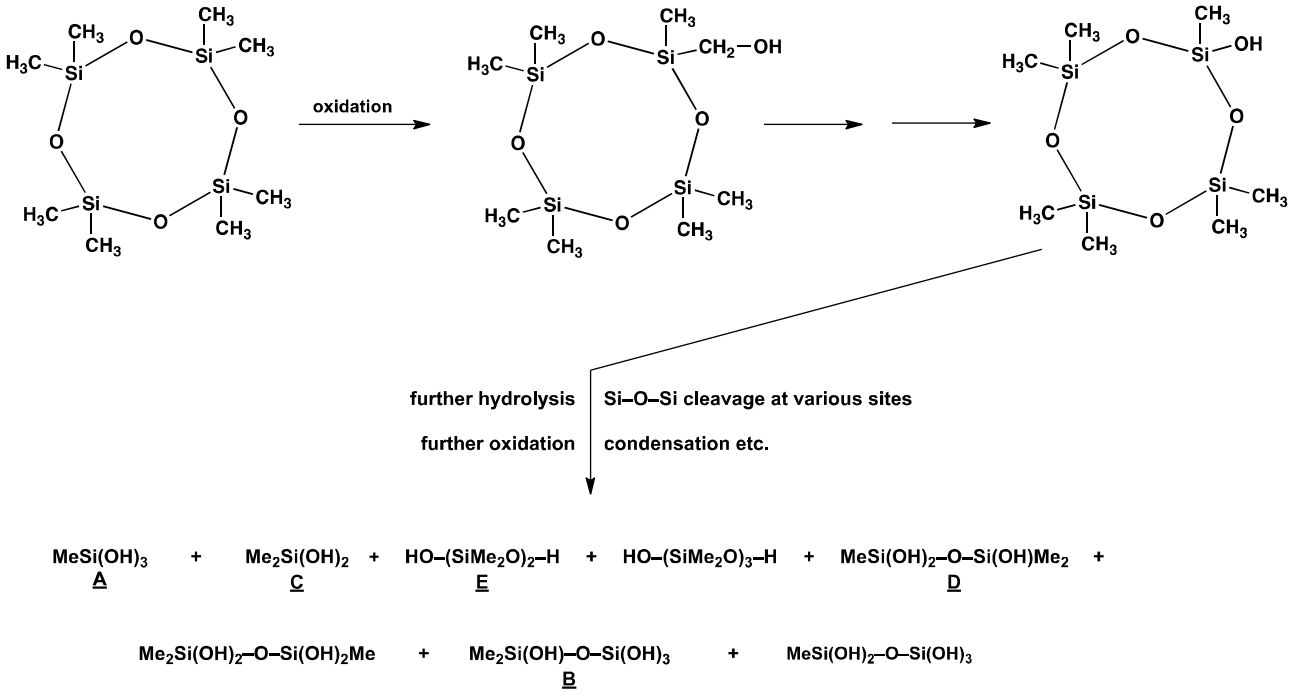

Fig. 1. Biotransformation of D4 in rodents.

available D4 is widely distributed to tissues, and maximum tissue concentrations of D4 in most tissues except fat were reached within three hours after the end of the inhalation period; high D4 concentrations in fat were sustained for 48 h. The D4 concentrations in liver and plasma after repeated inhalation exposures were similar to those seen after a single inhalation exposure. As a consequence, despite the high octanol/water partitioning, D4 has no tendency for bioaccumulation due to rapid elimination. After oral administration, the extent of absorption of D4 depends on carrier and dose, and most of the absorbed D4 is also rapidly eliminated in urine, feces, and in expired air (SEHSC, 1995). Dermally applied D4 is rapidly absorbed into the outer layers of the skin, but systemic absorption of D4 from the skin is very limited (approximately 0.5%) due to the rapid evaporation of D4 applied to the skin (DCC, 1998a, 2000c, 2000d; Zareba et al., 2002; Reddy et al., 2007; Jovanovic et al., 2008).

Physiologically based pharmacokinetic (PBPK) models show that the lipophilicity and the high volatility of D4 have major influences on D4 kinetics (Andersen et al., 2001, 2002, 2008; Reddy et al., 2003, 2007; Sarangapani et al., 2003). The high lipophilicity of D4 results in high lipid partitioning and in retention of D4 in blood lipids, but systemically available D4 is rapidly cleared by exhalation. Inhalation and dermal administration result in a similar pharmacokinetic profile, presumably due to diffusion of D4 molecules through biological membranes while the distribution and kinetics of D4 after oral dosing differs significantly from the predictions of the PBPK model suggesting that D4 is transferred from the gastrointestinal tract to the blood as microemulsions of D4 that are distributed as such.

3. D4 toxicity

The toxicity of D4 has been reviewed in detail in a companion manuscript (Franzen et al., 2017). D4 has low acute toxicity and is not an eye or skin irritant or a skin sensitizer (SCCS, 2010). Repeated dose toxicity studies with D4 have been performed using oral, dermal, and inhalation exposures (Bayer, 1988; DCC, 1988c, 1988b, 1988a, 1989a, 1989b, 1990, 1992, 1995a, 2001b; GSPA, 1991; DCC, 1995b; Burns-Naas et al., 2002). In all studies, D4 inhalation induced changes in liver weight and, inconsistently, changes in

Table 1
Summary of other Key Toxicity Studies on Octamethylcyclotetrasiloxane (D4) using inhalation as route of exposure.

Animal model	Study design and dosing	NOAEC/ LOEC	Observations	Reference
Sprague Dawley rats	90-day inhalation toxicity study with whole body exposure to 0, 5, 10, and 300 ppm D4 for 6 h/day, 5 days/week; groups of 10 males and 10 females/group including recovery groups	NOAEC of 300 ppm	Increased liver weight in male and female animals at 300 ppm. Liver weight changes reversible	(IRDC, 1991)
Sprague Dawley rats	90-day inhalation toxicity study with whole body exposure to 0, 50, 300, and 700 ppm D4 for 6 h/day, 7 days/week; groups of 10 males and 10 females/group including recovery group of 10 male and 10 female rats exposed to 0 and 700 ppm observed for 28 days after termination of exposure	NOEC of >700 ppm	Statistically significant increase in relative liver weight in males at all D4 exposure concentrations and in mid and high dose females, reversible in males; significant decrease in ovary weight in high dose females	(DCC, 1989b)
Fisher F344 rats	90-day inhalation study with nose-only exposure to targeted concentrations of 0, 35, 122, 488, and 898 ppm D4 for 6 h/day, 5 days/week; groups of 20 male and 20 female rats including additional recovery group of 10 male and 10 female rats exposed to 0 and 898 ppm observed for 28 days after termination of exposure	LOAEC of 35 ppm for lung effects	Changes in hematology and biomarkers of liver damage at >122 ppm, increases in liver weight at >488 ppm in males and >122 in females, increases in goblet cell proliferation in the nasal cavity at 898 ppm in both males and females, increased incidence of alveolar macrophage foci and chronic interstitial inflammation in the lungs in all D4-exposed groups, increased incidence of ovarian atrophy and vaginal mucification at 898 ppm	(Burns-Naas et al., 2002)
Fisher F344 rat	2-year inhalation study with whole body exposure to 0, 10, 30, 150, or 700 ppm D4, 6 h/day, 5 days/week, main group with exposure for two years consisted of 60 animals/sex/D4 concentration Groups of 10 animals/sex/D4 concentration exposed for one year with immediate sacrifice Group of 20 animals/sex/D4 concentration exposed for one year with sacrifice one year after termination of exposure Group of 6 animals/sex/D4 sacrificed after 6 month, used mainly for analysis of tissue levels	NOAEC of 150 ppm	Increased incidences of endometrial adenoma (4 out of 60) and a 50% incidence rate of uterine endometrial hyperplasia with a mean severity rate of 2.5 in the 700 ppm exposure group after 2 year inhalation exposure, increased chronic nephropathy and kidney weights at 700 ppm, increased liver weights without histopathological changes at 700 ppm after 2 year inhalation Increased liver and kidney weight at 700 ppm exposure groups after 1 year inhalation exposure to D4 Increases in mean/body/liver weight ratio without histopathological correlate at 700 ppm for one year with 12 month recovery Increased liver weights, significant at 700 ppm, after 6 months	(Batelle, 2004)
Sprague-Dawley rats	Developmental toxicity study (30 pregnant rats/group) following OECD 414 applying 0, 100, 300, and 700 ppm D4 by whole body inhalation	NOAEC of 700 ppm	No effects on fetal development	(IRDC, 1993b)
New Zealland White Rabbits	Developmental toxicity study (30 pregnant dams/group) following OECD 414 applying 0, 100, 300, and 500 ppm D4 by whole body inhalation	NOAEC of 500 pppm	No effects on fetal development	(IRDC, 1993a)
Sprague-Dawley rats	One-generation reproductive toxicity study by whole body inhalation to 0, 70, and 700 ppm D4 for 6 h/day for a min. of 28 days prior to mating and through the day of sacrifice (22 male and female animals/group). Exposures of females suspended from GD 21 to lactational day 4, offspring examined after sacrifice on PND 28	NOAEC of 70 ppm	Decreased number of implanation sites, reduced mean live litter size at 700 ppm	(DCC, 1997f)
Sprague-Dawley rats	2-generation reproductive toxicity study by whole body inhalation to 0, 70, 300, 500, and 700 ppm of D4 for 6 h/day for at least 70 consecutive days prior to mating through weaning of pups on postnantal day 21 performed in accordance with US EPA OPPTS Health Effects Test Guideline 870.3800	NOAEC of 300 ppm	Reduced mating and fertility index in F1 at 700 ppm Reduction in mean live litter size and mean number of pups in F0 and F1 at 500 and 700 ppm Increase estrous cycle length in F1 females at 700 ppm Reduction in corpora luea and reduced numbers of pregnacies at 700 ppm No adverse effects on male reproductive endpoints	(Siddiqui et al., 2007)

liver biomarkers. The liver changes were partially reversible during the recovery periods. In addition, in F344 rats, an increased incidence of nasal mucosa goblet cell proliferation (slight to minimal degree) and chronic interstitial inflammation of the lungs were seen in high-dose animals consistent with inhalation exposure to a slight irritant (Table 1).

3.1. Carcinogenicity and chronic toxicity of D4

D4 was tested for carcinogenicity in a two-year chronic toxicity/carcinogenicity study by inhalation using nominal concentrations up to 700 ppm, the highest achievable vapor concentration that could be maintained consistently for long term repeated exposures (Batelle, 2004; Jean et al., 2016b). Male and female F344 rats (60 rats/sex/dose) were exposed to 0, 10, 30, 150, or 700 ppm D4 vapor for 6 h/day, 5 days/week for up to 104 weeks in whole-body inhalation chambers. Three additional treatment groups included a 6-month treatment time, a 12-month treatment time, and a 12-month treatment and 12 month recovery period.

In the animals exposed to D4 for less then two years, some exposure-related changes in liver biomarkers and changes in liver weight, sometimes in combination with centrilobular hypertrophy of hepatocytes, were seen at the six- and 12-month sacrifices. Some increases in absolute and/or relative kidney weight were also observed. In addition, as observed in one of the 90-day inhalation studies, there were increased incidences of goblet cell hyperplasia in the nasal mucosa. After 24 months of inhalation exposure to D4, absolute and/or relative liver and kidney weights were increased in both sexes in the 700-ppm group, and a substantial increase in absolute and relative uterine weight was observed. Survival of rats in all treatment groups (except the 700-ppm male group) was not significantly different from untreated controls (Batelle, 2004; Jean et al., 2016a). Histopathologic analysis indicated liver, kidney, lung, and uterus as the target organs for chronic inhalation of D4 (Tables 2 and 3). In the uterus of D4 exposed rats, there was an increase in the incidence and mean severity of endometrial cystic hyperplasia. Although the incidence of endometrial adenoma in the 700 ppm D4 group was low (6.7%) and not statistically different from the control (0% incidence) on pairwise comparison, the incidence profile across the treatment groups was statistically significant for trend. Jean et al., 2016a, concluded that the increase in uterine weight, the increased incidence of endometrial hyperplasia and adenoma, and low historical incidence of uterine adenoma in F344 rats (0.3%, Haseman et al., 1998) identified the uterus as a target organ. In the liver, centrilobular hypertrophy was observed in males in the highest exposure group, and an increase in the severity of chronic progressive nephropathy occurred in both sexes in the 700-ppm group (Table 2). There was a lower incidence of nasal lesions in the 24-month exposure group compared to animals exposed to D4 for only 12 months.

3.2. Reproductive and developmental toxicity

The reproductive and developmental toxicity of D4 was evaluated in a large number of studies after inhalation exposures at concentrations up to 700 ppm (Table 1) (IRDC, 1993b, 1993a; DCC, 1996c, 1996d, 1997d, 1997e, 1997f, 1998b, 1999b, 2001a; Meeks et al., 2007; Siddiqui et al., 2007). No developmental effects of D4 were observed in the developmental toxicity studies, and reproductive effects induced by D4 inhalation were not observed in male animals in any of the studies. In females, reproductive effects included decreases in the number of corpora lutea, number of uterine implantation sites, number of pups born, mean litter size, increased estrous cycle length, and persistent follicles in ovaries. Most of these effects were restricted to the 700 ppm exposure groups. The timing and duration of exposure of female rats to D4 required to induce female-specific reproductive toxicity is limited to a very narrow time-window immediately prior to ovulation since a single 6-h exposure to D4 on the day prior to mating resulted in a statistically significant reduction in fertility (Meeks et al., 2007).

4. Mechanistic studies with D4

4.1. Genotoxicity of D4

The genotoxicity of D4 was evaluated in bacteria, cultured mammalian cells, and in rats after D4 inhalation (Isquith et al., 1988a, 1988b; Vergnes et al., 2000). D4 did not show a genotoxic response when assessed for gene mutations in bacteria and chromosome aberrations in mammalian cells and was also negative when assessed *in vivo* using the induction of chromosomal aberrations in bone marrow. In addition, an extended dominant lethal assay performed in rats after oral administration of up to 1000 mg/kg bw for 8 weeks was negative. In summary, genotoxicity tests with D4 were consistently negative demonstrating that D4 does not have genotoxic properties.

4.2. Hepatomegaly and enzyme induction

D4 caused reversible hepatomegaly that almost never was associated with histopathologic changes in the liver in rats after inhalation exposures (DCC, 1996a; McKim et al., 2001a; Burns-Naas et al., 2002). Hepatic enzyme induction was identified as the potential mechanism responsible for the liver weight increases (DCC, 1999a; Zhang et al., 2000). D4 inhalation caused large

Table 2
Non-neoplastic changes in Fischer 344 rats after chronic vapor inhalation exposure to octamethylcyclotetrasiloxane (D4) for 24 months.

Target Organ	Gender		0 ppm	10 ppm	30 ppm	150 ppm	700 ppm
Liver	Male						
		Basophilic focus	25/60	27/60	15/60	15/60	20/60
		Hypertrophy	0/60	0/60	0/60	0/60	5/60*
	Female						
		Basophilic focus	45/59	42/59	45/60	44/60	26/60*
		Hypertrophy	0/59	0/59	0/60	0/60	0/60
Lung	Male	Hemorrhage	3/60	3/59	5/60	5/60	8/59
		Subpleural Chronic Inflammation	2/60	1/59	2/60	3/60	3/59
	Female	Hemorrhage	0/59	1/60	0/59	2/60	4/60*
		Subpleural Chronic Inflammation	0/60	2/60	3/59	2/60	8/60*
Kidney	Male	Chronic Nephropathy	56/58	58/59	57/59	57/60	60/60
	Female	Chronic Nephropathy	17/59	25/59	33/59*	46/60*	55/60*

* Statistically different from control ($p < 0.05$).

Table 3
Effect of 24 months inhalation exposure of octamethylcyclotetrasiloxane (D4) on uterine histopathology in Fischer 344 rats. A statistically significant trend for endometrial adenoma was observed.

Uterine pathology	0 ppm	10 ppm	30 ppm	150 ppm	700 ppm
Endometrial Epithelial Hyperplasia	11/59 (19%)	8/59 (14%)	5/59 (8%)	13/60 (22%)	30/60* (50%)
Endometrial adenoma	0/59 (0%)	0/59 (0%)	0/59 (0%)	0/60 (0%)	4/60 (7%)
Stromal polyp	11/59 (19%)	17/59 (29%)	14/59 (24%)	17/60 (28%)	15/60 (25%)

* Statistically different for trend ($p < 0.05$).

increases (>20 fold) in cytochrome P450B1/2 activity and protein concentrations (DCC, 2002b) suggesting that D4 may act as a "phenobarbital-like" inducer. The pattern of liver changes induced by D4 was similar to the pattern of liver changes induced by phenobarbital, and D4 activated the constitutive androstane receptor (CAR) in reporter gene assays (DCC, 2005a). Therefore, the liver effects seen in rats exposed to D4 appear to occur through CAR-mediated processes similar to those seen with other CAR activators such as phenobarbital (Elcombe et al., 2014).

4.3. Direct endocrine activity

A series of experiments were conducted to examine the ability of D4 to interact with endocrine pathways (McKim et al., 2001a; He et al., 2003; Jean et al., 2005; Quinn et al., 2007a). The potential estrogenicity of D4 was assessed in uterotrophic assays *in vivo* and *in vitro* in both an estrogen responsive reporter cell line and by estrogen-receptor binding studies (McKim et al., 2001b; He et al., 2003; Quinn et al., 2007b). D4 has very weak estrogenic and antiestrogenic activity and a low affinity for estrogen receptor-α, five to six orders of magnitude below that of the positive control ethinylestradiol. D4 did not show androgenic activity in the Herschberger assay with male F344 rats through whole body D4 inhalation (Quinn et al., 2007b). In *in vitro* ligand binding assays including assessment of receptor binding to calf uterine progesterone receptor and to recombinant human progesterone receptor (alpha and beta forms), there was no indication of binding of D4 to the progesterone receptor (Jean et al., 2005). Assessment of D4 in a cell-based reporter gene assay showed no activation of recombinant human progesterone receptor-β (Jean et al., 2005).

Although D4 has weak estrogenic/antiestrogenic activity (He et al., 2003; Quinn et al., 2007b; McKim et al., 2001b), there were no reported indications of estrogenic or anti-estrogenic effects in male rats, in estrogen-sensitive tissues in females, or in hormone-related developmental landmarks, including anogenital distance, in rat pups in a two-generation reproductive developmental study with D4. It is unlikely that the very weak activity of D4 in estrogenic assays is responsible for the increase in the endometrial proliferative lesions seen in the 2-year chronic bioassay.

4.4. Dopamine agonisim

A dopamine-related mode-of-action was considered as an explanation for the observed effects of D4 on the uterus in rats after inhalation exposure to 700 ppm D4 for two years. Dopamine agonists inhibit prolactin secretion from the pituitary in rats, causing estrogen dominance resulting in persistent endometrial stimulation that ultimately induces proliferative endometrial lesions (Alison et al., 1990).

Studies investigating the role of dopamine agonism were performed in two animal model systems (reserpine-pretreated female rats and aging female F344 rats) and *in vitro* (DCC, 2005b, 2009a, 2010a,b; Jean et al., 2005). Reserpine administration to rats depletes brain dopamine, blocks the dopamine inhibition of prolactin secretion into blood, and induces a pronounced increase in circulating prolactin, providing a model to investigate the potential for a chemical to interact with the dopamine D2-receptor. In the aging F344 rat, altered hypothalamic control of dopamine causes elevated prolactin secretion and gives rise to increased prolactin concentrations in blood. Administration of dopamine receptor agonists also reduces prolactin in this system.

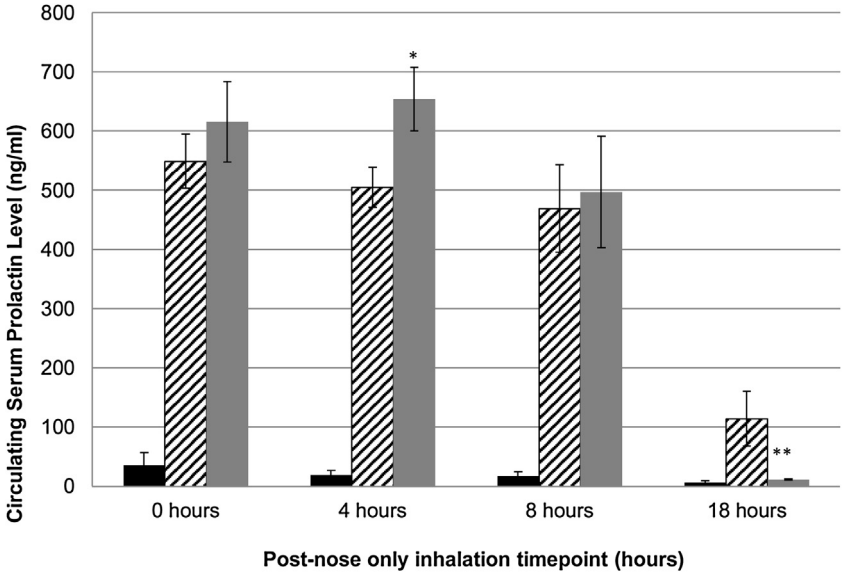

Fig. 2. Mean ± SEM serum prolactin concentration in dopamine-depleted rats over 18 h after a 6-h inhalation exposure to D4 (700 ppm). **$P < 0.02$ compared to reserpine-treated control, n = 4 (outlier removed); $P < 0.02$ compared to reserpine-treated control, n = 6 (outlier removed). From (DCC, 2010b).
■, reference control; ▨, reserpine treated, ▩, D4 exposed.

Support for the dopamine agonist mode of action was obtained after D4 inhalation in reserpine-treated rats (DCC, 2005c). Reserpine administration to rats caused a six-fold increase in prolactin concentration. D4-inhalation (nose-only, 700 ppm for six hours) reduced this reserpine-induced increase in prolactin concentration by 85% in samples taken at the end of the inhalation exposure (Fig. 2) (DCC, 2010b). Administration of the dopamine receptor antagonist sulpiride prior to treatment blocked the D4 effect on serum prolactin providing support for the conclusion that D4 has dopamine agonist-like effects on the pituitary in rats.

A series of *in vitro* studies determined the ability of D4 to stimulate prolactin release from specific cells and evaluated D4 affinity for dopamine receptors (DCC, 2009b). While D4 completely blocked maitotoxin-induced prolactin secretion in MNQ-cells, a direct interaction of D4 with dopamine receptors was not established (DCC, 2009b; Baker, 2010; DCC, 2011). Therefore, D4 is unlikely to interact directly with dopamine receptors.

4.5. Effect of D4 inhalation on the LH surge

Experiments were performed to assess the effect of D4 exposures on the LH surge (Quinn et al., 2007a). D4 was shown to inhibit the pre-ovulatory LH surge causing a delay in ovulation, persistent follicles, and a prolonged exposure to elevated estrogen in the adult Sprague-Dawley (SD) rat (Quinn et al., 2007a, 2007b). This study was conducted to assess the affect of D4 on the pre-ovulatory LH surge, to assess the ability of D4 to block or delay ovulation, and to evaluate the effects of exposure to D4 on other hormones related to normal reproductive function. Whole body vapor inhalation exposure of rats to D4 at 700 ppm or 900 ppm resulted in an increased number of rats with suppressed pre-ovulatory LH surge compared to controls, whereas the number of the rats that failed to ovulate appeared to be within the normal range (25–30%) (Aschheim, 1983; Lu, 1983; Cooper and Goldman, 1999). It is important to note that a concentration of 900 ppm D4 is the highest possible vapor concentration that can be reliably generated in a short term exposure and a concentration of 700 ppm D4 was the highest vapor concentration reliably generated in long term reproductive studies. Evaluation of individual animal plasma LH data indicated that failure of the LH surge at 6 p.m. on the day of proestrus was accompanied by blocked or reduced ovulation. Persistent mature follicles in D4-exposed animals continued to secrete estradiol leading to higher estradiol (E2) concentrations on the morning of estrus as a result of an attenuated LH surge and blocked ovulation. The D4 treated ovulators had slightly higher E2 concentrations on the morning of estrus compared to the controls. These findings might have been due to retention of large follicles in both ovulating and non-ovulating treated animals.

An increased number of estrogenic days demonstrated by vaginal cytology in the early exposure phase could have been related to LH surge suppression as this finding was also seen in D4 treated animals during the LH surge suppression study. Hormone evaluation in shorter term studies that evaluated estradiol levels just following suppression of LH surge in cycling animals demonstrated an increase in circulating estradiol as compared to control animals that had ovulated. This increase, even if intermittent, would increase the lifetime exposure of estrogen-sensitive tissues including uterus and vagina. As discussed below, cystic endometrial hyperplasia (as seen in this study and the chronic bioassay) results from prolonged estrogen stimulation and is not believed to be preneoplastic in the absence of atypia (Leininger and Jokinen, 1990).

A study in F344 rats attempted to evaluate effects of D4 inhalation exposure on LH, prolactin, FSH, and estradiol concentrations. This study was confounded by cycle disruption in control and D4-exposed animals after a 4-day exposure regimen, preventing interpretation of potential compound-associated effects. The cycle disruption was attributed to stress associated with the inhalation procedure, perhaps related to environmental noise.

4.6. Effect of D4 inhalation on estrous cycles

Exposure of cycling adult female F344 rats to D4 by whole-body inhalation at 700 ppm for 35 days resulted in estrous cycle prolongation to 5.7 days compared to 5.0 days in control animals. The increased cycle length was attributable to an increase in time in diestrus. By the end of the treatment period, 17 of 20 D4-treated animals and all 20 control animals were cycling normally. D4 treatment was associated with an increase in large follicles in animals sacrificed in estrus. The large follicles might have represented unovulated follicles that continued to secrete E2, and there was a statistically significant increase in serum E2 concentration on the morning of estrus in D4-treated animals (30.5 pg/mL as compared to 26.6 pg/mL in controls).

F344 females treated with D4 from 11 to 25 months of age with monitoring of estrous cycle stage by daily vaginal lavage showed an increased time in an estrogenic state compared to controls, and females were in an estrogenic state for more consecutive days than controls (Fig. 3) (WIL, 2013; Jean et al., 2016b). The larger cumulative number of days of endogenous estrogen exposure is expected to increase the risk of endometrial hyperproliferation

Data on circulating prolactin levels were collected, but because blood samples were taken only at three to four week intervals and were not normalized to estrous cycle phase, these data are not informative. Histomorphology of the uterine and vaginal tissue at 24 months was consistent with the cyclicity data suggesting an increase in endogenous estrogenic influence.

5. The biological relevance for human risk characterization of D4-induced effects in animals following chronic exposure

5.1. Non-cancer endpoints

Longterm exposure of rats to D4 by inhalation induced non-neoplastic effects on the liver, the kidney, and the respiratory tract (Tables 1 and 2). In addition, chronic inhalation of D4 induced an increased incidence of endometrial hyperplasia and endometrial adenoma in rats (Table 3). Our evaluation of these endpoints used available data on the mode-of-action and the relevance of the observations from chronic exposure in the human hazard assessment for D4 (Jean et al., 2016a) and other key toxicity studies on D4 using inhalation as the route of exposure.

5.2. D4-induced hepatomegaly

Repeated administration to rats of D4 by inhalation and oral administration caused increases in liver weights; however, histopathological indications of hepatocellular damage were not present. Liver effects of D4 (Tables 1 and 2) observed in several of the repeated dose toxicity studies (increase in weight, hypertrophy) and dedicated experiments (induction of cytochromes P450) are consistent with those observed with phenobarbital, suggesting that D4 acts as an enzyme inducer in the same way as phenobarbital (Zhang et al., 2000). Phenobarbital-induced changes in liver weight are not considered adverse and phenobarbital-induced liver tumors in rats after long-term administration are not considered relevant to human risk characterization (Elcombe et al., 2014). Based on the absence of liver tumors and liver changes at the final sacrifice of the two-year inhalation study with D4, D4 is considered as a weaker enzyme inducer than phenobarbital. The absence of pathologic and carcinogenic liver effects after long-term

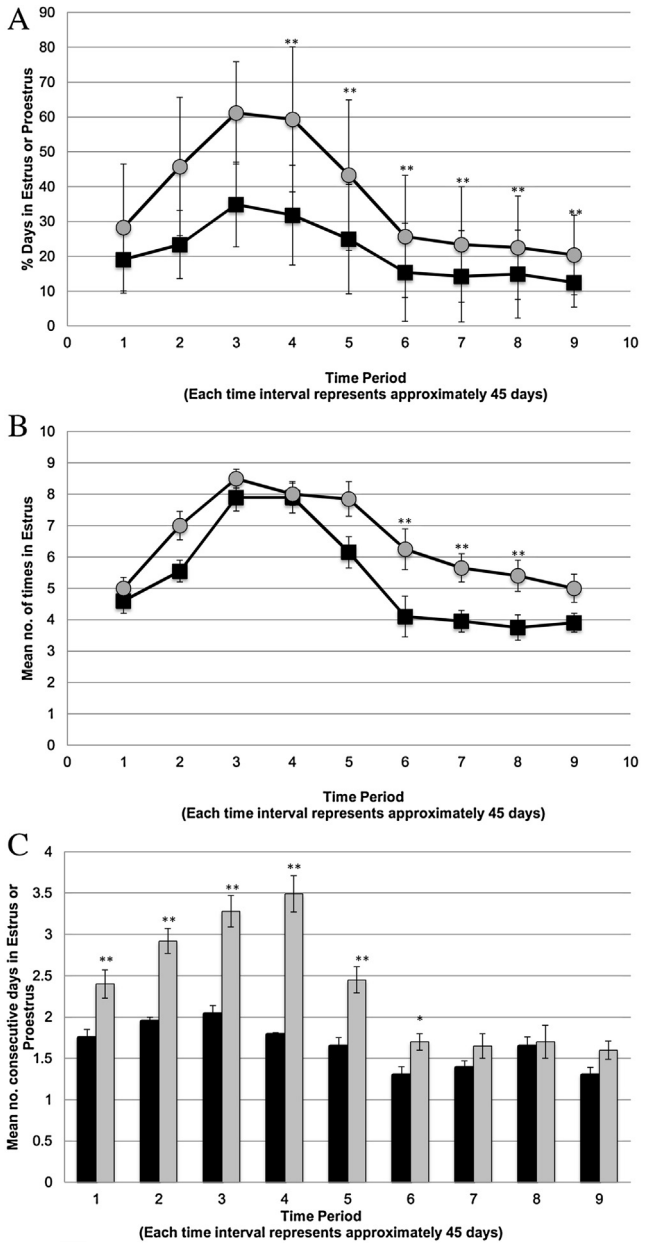

Fig. 3. Number of times in an estrogenic state (A), number of consecutive days in an estrogenic state (B), and length of estrogenic state (C) in aged Fisher F344 rats exposed to 700 ppm of D4 (whole body exposure, 6 h/day, 5 days/week). Exposure was started at an age of 47/48 weeks and was continued for 58 weeks. *, ** Statistically different from control at $P < 0.05$, 0.01. From (WIL, 2013). ■, control; ▒, ◯, D4 exposed.

inhalation exposure to D4 further supports the conclusion that the liver weight changes represent an adaptive response.

In conclusion, there is sufficient evidence to conclude that the liver effects induced by D4 are consistent with a CAR-mediated mode of action similar to that seen with phenobabrbital (Elcombe et al., 2014). The liver changes induced by D4, therefore, are not considered adverse changes but represent adaptive reversibile changes and should not be used in human risk characterization (Andrew, 2005; FDA, 2009; EMEA, 2010; Hall et al., 2012).

5.3. Chronic nephropathy

Inhalation exposure of rats to D4 at 700 ppm increased the absolute and/or relative kidney weight and resulted in a significant increase in chronic nephropathy in both sexes of rats exposed for two years (Table 2). Chronic progressive nephropathy (CPN) is a spontaneous degenerative disease in the commonly used strains of laboratory rats, and its incidence and severity is frequently exacerbated by chronic administration of chemicals (Hard et al., 2009). While the underlying initial events of CPN in rats are not well defined, the available evidence indicates that CPN is a distinctive disease entity in rats that has no human counterpart based on clinical manifestation, disease progression, and influencing factors. Therefore, the kidney effects observed after chronic inhalation of D4 at the highest concentration of 700 ppm likely have no relevance for human risk characterization (Hard et al., 2009, 2013).

5.4. Uterine endometrial epithelial hyperplasia and endometrial adenoma

Information on possible modes of action for the induction of endometrial hyperplasia and endometrial adenoma can be derived from a comparatively large database on chemicals that were tested for carcinogenicity and the established/presumed modes of action for these chemicals, especially for the occurrence of proliferative uterine lesions (CPDB, 2011; Klaunig et al., 2016). Most recently, Klaunig et al. (2016) identified and evaluated several possible modes of action by which another cyclic siloxane, decamethylcyclopentasiloxane (D5), might induce an endometrial proliferative lesion (endometrial adenocarcinoma) in the F344 rat. These modes of action included (1) genotoxicity, (2) direct endocrine (estrogenic, androgenic, and progestogenic) activity, (3) oxidative stress/damage/inflammation/cytotoxicity, and (4) alteration of pituitary control of the estrous cycle (Klaunig et al., 2016).

Chemicals with genotoxic properties presumably induce uterine tumors by an interaction of electrophilic decomposition products/metabolites with DNA. However, a direct proliferative response of the endometrial tissue is the most likely basis for uterine tumor induction by hormonally active chemicals of sufficient potency (Klaunig et al., 2016). The other proposed modes of action all include changes in the exposure of the uterine endometrium to endogenous estrogen. For example, dopamine agonists inhibit prolactin secretion from the pituitary in rats causing estrogen dominance resulting in persistent endometrial stimulation ultimately causing endometrial tumors (Alison et al., 1990; Alison et al., 1994). High dose levels of tetrabromobisphenol A inhibit estrogen sulfation resulting in increased exposure to endogenous estogens, and induction of cytochrome P450 1A results in a modulation of estrogen metabolism (Gosavi et al., 2013).

5.4.1. Genotoxicity

As reviewed above in Section 3.1, multiple genetic toxicity assessments for D4 in bacteria, mammalian cells *in vitro*, and in intact animals *in vivo* have failed to provide evidence that D4 is genotoxic. Support for a genotoxic mode of action in the induction of uterine tumors by D4 is absent, and a direct genotoxic mode of action is unlikely.

5.4.2. Direct endocrine activity

D4 possesses only very weak estrogenic and antiestrogenic activity in rats and has a low affinity for estrogen receptor-α (He et al., 2003). D4 had no estrogenic/antiestrogenic activity on pubertal timing in male or female rats in a two-generation study (Siddiqui et al., 2007). D4 does not have progestagenic, androgenic, or anti-androgenic activity (Quinn et al., 2007b). A direct hormonal effect of D4 on endometrial cells is unlikely as a mode of action for D4-associated endometrial hyperplasia and adenoma in the aging F344 rat.

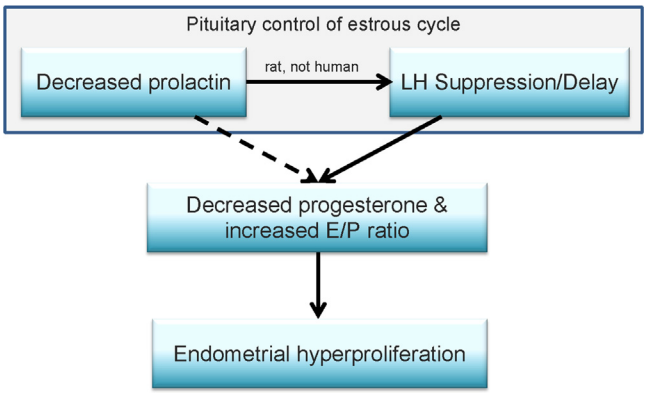

Fig. 4. Proposed alteration in estrous cycle mode of action for D4 induced rat uterine proliferative lesions by D4. Alteration of pituitary control of the estrous cycle in the rat occurs by alteration of prolactin, which might exert its effects through a delay in or suppression of the LH surge. The dashed arrow indicates uncertainty about whether a D4-mediated decrease in prolactin has a direct effect on corpus luteum progesterone production. E/P = estrogen: progesterone.

5.4.3. Induction of cytochrome P450 1A enzymes by D4

The profile of enzymes induced by D4 in rat liver has been characterized. D4 exposure increased cytochrome P450 2B1/2 protein concentrations and activity as indicated by increased metabolic rates for specific marker substrates (DCC, 2002b). Only small changes were observed in cytochrome P450 1A activity and protein concentration indicating that D4 exposure does not induce this enzyme. There are no data to support the conclusion that uterine effects of D4 are indirectly induced by modulation of cytochrome P450 1A activity (Zhang et al., 2000).

5.4.4. Alteration of pituitary control of the estrous cycle

As indicated in Klaunig et al., 2016; estrogen dominance in the rat could occur through a decrease in LH leading to anovulation and prolonged secretion of endogenous E2 from the ovary or alteration of prolactin leading to modulation of progesterone release from the ovary (Fig. 4). This mode of action is based on changes in the estrous cycle associated with D4 exposure (Quinn et al., 2007a). The inhibition of the pre-ovulatory LH surge in rats causes a delay in ovulation, persistent follicles, and prolonged exposure of the endometrium to endogenous estrogen. There are two types of endometrial hyperplasia (Leininger and Jokinen, 1990). The first type, diffuse cystic endometrial hyperplasia as seen following D4 exposure, is thought to result from prolonged estrogen stimulation and is not believed to be preneoplastic. Because the uterus of rats seems to be highly sensitive to changes in endogenous estrogen exposure, this mode of action may contribute to the induction of uterine endometrial hyperplasia and thus play a role in the development of the benign uterine tumors. In rats, dopamine agonists inhibit prolactin secretion from the pituitary resulting in an increase in the estrogen:progesterone ratio, which results in persistent estrogen stimulation of the endometrium. Administration of dopamine agonists to rats, but not to other species, produces uterine cystic endometrial hyperplasia and uterine tumors (Burke et al., 1988; Alison et al., 1994). A change in estrogen:progesterone ratio induced by D4 through interaction with the dopamine system thus may contribute to the development of endometrial hyperplasia and benign endometrial tumors. Studies performed to characterize the interaction of D4 with this pathway indicated that D4 is neither a potent nor a direct agonist at the dopamine receptor; however, the results suggested an indirect interaction with the dopamine pathway distal to the receptor.

6. Human relevance of D4 associated endometrial proliferative lesions

6.1. Endometrial neoplasms in women

The most common human endometrial neoplasms are hyperproliferative lesions associated with unopposed or inadequately opposed estrogen. During the normal month-long menstrual cycle, E2 is produced by the granulosa cells of the dominant ovarian follicle. E2 increases endometrial thickness and gland activity. Ovulation at midcycle is followed by conversion of the granulosa cells to lutein cells in the corpus luteum and a switch from production of predominantly E2 to production of progesterone. Progesterone decreases estrogen receptors in the endometrium and decreases endometrial proliferation. If fertilization of the ovum does not occur, the corpus luteum involutes, and the endometrium is sloughed in the menstrual flow, taking the endometrium down to the relatively inactive basal level, permitting the cycle to restart.

In the absence of ovulation, continued estrogen stimulation of the endometrium can result in hyperplasia, a condition in which the endometrial gland and stroma cells over-proliferate. The hyperplastic glands can appear as piled-up layers of cells with out-pocketing, bridging of glands by hyperplastic epithelium, and crowding. Endometrial hyperplasia is not malignant, but cellular atypia within the hyperplastic glands is considered a premalignant change that can progress to adenocarcinoma.

An association between unopposed estrogen and hyperplastic disorders of the endometrium, including endometrial adenocarcinoma, was suspected in humans based on the experience of menopausal women who were given unopposed estrogens (reviewed by Montgomery et al., 2004). Younger women who develop endometrial adenocarcinoma often have ovulatory disturbances such as polycystic ovarian syndrome (Navaratnarajah et al., 2008; Fearnley et al., 2010). Obese women are at higher risk of proliferative disorders of the endometrium, including endometrial hyperplasia and adenocarcinoma, because adipose tissue aromatizes adrenal androgens to estrogens resulting in greater exposure of the endometrium (reviewed by Fader et al. (2009)).

In chronically treated F344 rats, D4 produced an increase in uterine weight, cystic endometrial hyperplasia, and adenomas in the 700-ppm dose group after 24 months. Cystic hyperplasia is analogous to a proliferative change of the endometrium in women with disruption of the menstrual cycle. Cystic hyperplasia, more commonly called simple hyperplasia, is characterized by enlargement of the endometrial glands with occasionally out-pocketing of glands without the heaping up of layers of gland cells or crowding of stroma that characterizes complex hyperplasia. In simple hyperplasia, there is no atypia of the gland epithelium. Based on the low levels of the proliferation marker Ki-67, it has been suggested that many of these simple lesions are not proliferative at all (Ambros, 2000). Cystic hyperplasia without atypia in women is not a cancer precursor.

A role for estrous cycle disruption in the genesis of cystic endometrial hyperplasia in the rat D4 chronic toxicity study is consistent with D4 effects in this study as well as other studies. Inhalation exposure to D4 resulted in prolongation of estrous cycles in a rat reproductive study (Siddiqui et al., 2007) and an inhibition of the luteinizing hormone surge (Siddiqui et al., 2007) leading to elevated concentrations of E2 on the morning of estrus. These alterations would be expected to lead to an increased endogenous E2 signal to the uterus over time. Second, any dopamine agonist-like activity following inhalation exposure to D4 that would inhibit prolactin secretion from the pituitary would also result in persistent endogenous estrogen stimulation of the endometrium. Diffuse cystic endometrial hyperplasia as seen

following D4 exposure is more likely to result from prolonged endogenous estrogen stimulation (Leininger and Jokinen, 1990).

Endometrial adenomas occurred in four out of 60 female rats in the 700-ppm dose group in the chronic toxicity study and were not observed in any other treatment group or the control. Endometrial adenoma is an unusual lesion in rats that has been associated with focal proliferation of the surface rather than glandular epithelium (Dixon et al., 2014). These lesions can form polypoid masses that project into the uterine lumen. Adenomas do not invade adjacent tissues and have no malignant potential. There is no endometrial lesion in women that is directly analogous to the endometrial adenoma in the rat, although the rat lesion has some histological similarity to the human endometrial polyp. Rodent adenomas have little if any stromal proliferation, whereas polyps in women have well developed stroma. Carcinomas can develop in human endometrial polyps whereas rat endometrial adenomas are not premalignant.

6.2. Cycle control in women

Control of the female reproductive cycle is different in rats and primates (reviewed by Plant, 2012). Endometrial proliferation occurs in both cases under the influence of estrogen, chiefly E2, produced by developing follicles. In female rats, there is a circadian neural signal that results in generation of a gonadotropin-releasing hormone (GnRH) surge leading to release of luteinizing hormone (LH) surge when concentrations of E2 are adequate. In female primates, there is no midcycle surge of gonadotropin-releasing hormone preceding the LH surge; rather, there is intermittent release of GnRH from a hypothalamic pulse generator throughout the menstrual cycle. The response of pituitary LH release to the intermittent GnRH pulse can be directly regulated by E2.

Corpus luteum function in rodents but not in primates relies on adequate secretion by the pituitary gland of prolactin (reviewed by Bachelot and Binart, 2007). Although hyperprolactinemia can be associated with antagonism of ovulation in women (Al-Suleiman et al., 1979) normally prolactin is not involved in menstrual cyclicity. To the extent that D4 interferes with prolactin production through a dopaminergic mechanism, it would be expected to interfere with estrous cyclicity in rats but not menstrual cyclicity in women.

7. Synthesis regarding endometrial proliferative findings

It is likely that cycle disruption occurred over time in F344 females exposed to D4 due to either an inhibition by D4 of pituitary prolactin production (Fig. 4) and/or through modulation of the LH surge leading to an increased endogenous E2 signal to the uterus. Neither mechanism would be relevant to human risk due to differences between rat and human in pituitary control of the female reproductive cycle (Plant, 2012; Klaunig et al., 2016).

Although the specific mode of action responsible for induction of uterine adenomas in the female F344 rat has not been confirmed, the subtlety of the effects following exposure to D4 may prevent further assessment and definition of a precise mode of action. However, the available data provide important insight into the potential human relevance of the uterine tumors in rats. Relevant findings include:

- D4 has not been shown to be mutagenic or genotoxic in multiple *in vitro* and *in vivo* experimental models designed to evaluate this potential;
- No tumors were associated with chronic D4 exposure of male F344 rats and no organs other than the uterus developed treatment-related proliferative lesions in female F344 rats following chronic D4 exposure;
- Uterine cystic endometrial hyperplasia and adenoma in the female F344 rat arose during the 2nd year of exposure, a period of marked changes in physiology and onset of a reproductive senescence that is unique to the F344 rat, distinctly different from human, and often associated with increased endogenous E2 from ovarian cysts;
- The affinity of D4 for the estrogen and progesterone receptors is low to non-existent. It is unlikely that the demonstrated weak estrogenicity of D4 was involved in the uterine effects that developed in the aging F344 rat in response to D4 exposure either in the chronic bioassay or in animals treated from 11 to 25 months of age, because there were no other indications of a weak estrogenic response in either males or females in the chronic bioassay or a two-generation study (Batelle, 2004; Siddiqui et al., 2007; Jean et al., 2016a);
- Although D4 is not a direct dopamine agonist, there were slight alterations in the dopamine activation pathway and modulation of prolactin concentrations following exposure to D4 that are suggestive of some interference with this pathway.
- D4 exposure inhibits ovulation and can prolong exposure of the endometrium to endogenous estrogen in the rat. In addition, in aged rats (Jean et al., 2016b), D4 exposure produced a higher percentage of days for which the vaginal lavages exhibited a more estrogenic character. The higher percentage of days in proestrus/estrus in the D4 group appeared to be the result of prolonged estrogenic phases during the first half of the study (consistent with the LH surge study) followed by increased cycling (i.e., greater numbers of times in proestrus/estrus) during the second half of the study. If alteration of the LH surge with subsequent prolonged exposure of the uterine endometrium to endogenous estrogen is responsible for cystic endometrial hyperplasia and adenomas, it is unlikely this effect would occur in humans due to the marked differences in reproductive function, brain regulation of LH secretion, and the mechanism of reproductive aging and the hormonal environment of reproductive senescence in the rat versus human;
- Cystic hyperplasia without atypia in female humans is not a cancer precursor, and there is no endometrial lesion in women that is directly analogous to endometrial adenoma in the rat.
- The uterine effects were only seen following exposure to the highest exposure concentration of D4 (700 ppm). Sarangapani et al., 2003 developed a pharmacodynamic extension to a physiologically based pharmacokinetic (PBPK) model to characterize dose-response behaviors of cytochrome P450 induction following inhalation exposure to D4. This evaluation showed that at exposures greater than ~300 ppm there was an apparent saturation of liver enzymes with subsequent decreasing liver metabolism suggesting that the high doses of D4 used in the toxicity studies may have exceeded the rats' physiological capacity to handle the chemical. The effects seen above this exposure concentration are of questionable toxicological relevance when compared to actual human exposures.

In summary, the available information suggests that the induction of benign proliferative endometrial lesions in the rat after chronic D4 inhalation has no relevance for human risk characterization. Due to the absence of genotoxicity of D4 and absence of any appreciable direct hormonal activity of D4, the induction of cystic endometrial hyperplasia and the significant trend for an increased incidence of uterine endometrial adenoma observed across D4 dose levels in the two-year inhalation study are likely due to interferences of D4 with rat estrous cycle control that are only seen at doses that exceed the metabolic capacity of animals and not relevant to women.

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