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Biological relevance of decamethylcyclopentasiloxane (D5) induced rat uterine endometrial adenocarcinoma tumorigenesis: Mode of action and relevance to humans

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ABSTRACT

Decamethylcyclopentasiloxane (D5) is a cyclic siloxane used in the production and formulation of consumer products with potential exposure to manufacturing workers, consumer, and the general public. Following a combined 2-year inhalation chronic bioassay performed in Fischer 344 (F344) rats, an increase in uterine endometrial adenocarcinomas was noted at the highest concentration to which animals were exposed. No other neoplasms were detected. In this study, a dose of 160 ppm produced an incidence of 8% endometrial adenocarcinomas. Based on a number of experimental studies with D5, the current manuscript examines the biological relevance and possible modes of action for the uterine endometrial adenocarcinomas observed in the rat following chronic exposure to D5. Variable rates of spontaneous uterine endometrial adenocarcinomas have been reported for untreated F344 CrlBr rats. As such, we concluded that the slight increase in uterine endometrial adenocarcinomas observed in the D5 chronic bioassay might not be the result of D5 exposure but may be related to variability of the spontaneous tumor incidence in this strain of rat. However, if the uterine endometrial adenocarcinomas are related to D5-exposure, alteration in the estrous cycle in the aging F344 rat is the most likely mode of action. D5 is not genotoxic or estrogenic. The alteration in the estrous cycle is caused by a decrease in progesterone with an increase in the estrogen:progesterone ratio most likely induced by a decrease in prolactin concentration. Available data support that exposure to D5 influences prolactin concentration. Although the effects on prolactin concentrations in a number of experiments were not always consistent, the available data support the conclusion that D5 is acting via a dopamine receptor agonist-like mechanism to alter the pituitary control of the estrous cycle. In further support of this mode of action, studies in F344 aged animals showed that the effects of D5 on estrous cyclicity produced a response consistent with a dopamine-like effect and further suggest that D5 is accelerating the aging of the reproductive endocrine system in the F344 rat utilized in this study. This mode of action for uterine endometrial adenocarcinoma tumorigenesis is not relevant for humans.

1. Introduction

1.1. Background

Decamethylcyclopentasiloxane (D5) is a cyclic siloxane used in the production of industrial and consumer products with potential exposure to consumers, the general public and manufacturing workers. D5 is used as an intermediate in the production of polydimethylsiloxanes and has a number of secondary uses as a component in household care and personal care products. D5 is highly volatile. Based on these properties, consumers, the general population, and workers may be exposed to D5 by the dermal and inhalation exposure routes. Dermal absorption of D5 is very limited, and most of the D5 applied to skin rapidly evaporates (Jovanovic et al., 2008; Reddy et al., 2007). A number of the toxicity and pharmacokinetic and metabolism studies with D5 used inhalation

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as the route of exposure (Burns-Naas et al., 1998a,b; McKim et al., 1999; Siddiqui et al., 2007; Tobin et al., 2008; Reddy et al., 2008; Jean et al., 2015). The available toxicity and mechanistic studies on D5 are summarized (Dekant and Klaunig, 2015). In a two-year inhalation chronic bioassay in Fischer 344 (F344) rats (Jean et al., 2015), a small but statistically significant increase in the incidence of uterine endometrial adenocarcinoma in female rats at the highest dose level of D5 studied (160 ppm) was seen. No other toxicologically significant neoplastic or non-neoplastic findings were reported. Given the small increase in the incidence of uterine endometrial adenocarcinoma seen in the Jean et al. (2015) study at the highest dose only, a more in-depth evaluation of the statistical analysis was conducted (Young and Morfeld, 2015) to address the question of whether the observed uterine endometrial adenocarcinoma increase could be spurious. This analysis indicated that changing the results in one or two animals would impact the conclusions regarding statistical significance. However, the statistical analysis supported the conclusion that D5 at the highest dose examined resulted in an increased incidence of uterine endometrial adenocarcinoma in F344 rats.

The purpose of this manuscript is to evaluate the biological relevance of the uterine endometrial adenocarcinoma in the rat and to evaluate the potential human relevance of this finding.

1.2. Carcinogenicity of D5

D5 was tested for carcinogenicity in a two-year chronic toxicity/carcinogenicity study by inhalation (Jean et al., 2015). Inhalation exposure was selected because of the rapid loss to evaporation of D5 applied to the skin, because inhalation is the second most prevalent exposure route for humans, and because of the similarity in kinetic behavior between this route and dermal exposure, the most prevalent route for humans. Male and female F344 rats were exposed to 0, 10, 40, and 160 ppm D5 (0, 163, 653, and 2610 mg/m^3) for a period of up to two years. A concentration of 160 ppm D5 in air is the highest vapor concentration of D5 that can be reliably achieved because aerosols develop at higher vapor concentrations.

The carcinogenicity study was composed of a primary group of rats exposed for two years and three additional groups exposed to D5 for different durations. In the primary group, rats (60 animals per sex per group) were exposed for 6 h/day and 5 days/week to D5 vapor at 0, 10, 40, and 160 ppm. One group of additional rats (6 per sex for each of the four D5 concentrations) were exposed to D5 vapor for 6 months and then sacrificed with the major aim to determine D5 concentrations in tissues. A second group of rats, consisting of 10 animals per sex for each of the four D5 concentrations, was sacrificed after 12 months of exposure to D5, and a third group (20 animals/sex for each of the four D5 concentrations) was also exposed to D5 vapor for 12 months and then observed for an additional 12 months (recovery) with no exposure before sacrifice.

No toxicologically relevant effects were seen, except an increased incidence of hyaline inclusions in the nasal cavity in both males and females exposed to 160 ppm D5 for six months. Some statistically significant increases in liver weight were also observed, but these increases were neither D5 concentration- nor exposure-duration related, suggesting that adaptive responses likely due to the capacity of D5 to induce liver cytochromes P450 were in effect (Dekant and Klaunig, 2015).

After 2 years, hyaline inclusions in the nasal cavity were seen in both male and female rats at 160 ppm in the primary group and in the 12-month exposure/12-month recovery groups and in males at 40 ppm. The incidences of neoplastic changes in males and females were not statistically significantly different from control, with the exception of the incidence of uterine tumors. Incidences of uterine tumor and uterine tumor precursor lesions observed in the oncogenicity study with D5 by Jean et al. (2015) are shown in Table 1.

An increased incidence of uterine endometrial adenocarcinoma was seen only in the 160 ppm exposure group after two years of exposure. However, when comparing the untreated control group to the 160 ppm exposure treated group, the incidence of uterine endometrial adenocarcinoma was either significantly increased or of borderline significance depending on whether one or two sided statistical analysis was performed (Young and Morfeld, 2015; Jean et al., 2015). Neither the incidence of uterine endometrial adenoma nor the incidence of uterine endometrial adenomatous polyps increased; the observed incidence in uterine endometrial glandular hyperplasia, which is considered to be an obligatory precursor of uterine adenocarcinoma in rats, was also not increased at any exposure concentration. No significant increases in uterine endometrial adenocarcinoma or precursor incidences were observed in the satellite groups (Table 1).

In addition, in rats exposed to D5 vapor for two years, a significant negative trend for the incidence of fibroadenomas of the mammary gland (0 ppm, 8/60; 10 ppm, 7/60; 40 ppm, 7/60; 160 ppm, 2/60) was noted.

To understand the possible biological relevance of the uterine endometrial adenocarcinoma induction following D5 exposure and the potential impact of the tumor finding for human risk assessment, a number of factors were considered, including the incidence of spontaneously occurring uterine endometrial adenocarcinoma in F344 rats, the uterine tumor types occurring in F344 rats, events associated with reproductive aging in the female F344 rat, and the etiology and pathology of adenocarcinoma of the endometrium in women. These factors are described further below.

2. Overview of rodent uterine endometrial carcinogenesis

The rat uterus is divided into the uterine cervix and corpus. In rats, tumors of the uterus include both epithelial and non-epithelial neoplasms. The epithelial neoplasms include adenomas, adenocarcinomas, squamous cell papillomas and adenoacanthomas. Non-epithelial tumors including fibrosarcomas, endometrial stromal

Table 1
Uterine tumor incidence in rats exposed to D5 by inhalation.

Exposure concentration (ppm D5)	2 year exposure group		1 year exposure/Recovery group	
	Cystic endometrial hyperplasia[a]	Endometrial adenocarcinoma[a]	Cystic endometrial hyperplasia	Endometrial adenocarcinoma
0	9/60 (15%)[b]	0/60 (0%)[b]	0/20 (0%)	1/20 (5%)
10	Not examined	1/60 (2%)	Not examined	1/20 (5%)
40	Not examined	0/60	Not examined	0/20 (0%)
160	9/60 (15%)	5/60 (8%)	2/20 (10%)	2/20 (10%)

[a] Incidence of lesion seen.
[b] Percent incidence of lesion.

polyps, leiomyomas, and hemangiomas have also been described. Rat uterine adenomas can be polypoid or sessile while uterine endometrial adenocarcinomas are frequently polypoid and consist of irregular glandular structures lined with cuboidal or columnar cells. The nuclei of the uterine endometrial adenocarcinomas are usually hyperchromatic and have frequent mitotic figures. While these neoplasms occasionally metastasize to distant tissues they frequently invade the neighboring muscle and adjacent tissue in the uterus.

In most strains of rats, spontaneous uterine endometrial adenocarcinomas are seen with low incidence however Maekawa et al. (1983) have described a high incidence of uterine endometrial adenocarcinomas in aged Donryu rats. Atypical glandular hyperplasia of the endometrium is also frequently seen in the rat prior to the development of uterine adenocarcinomas and may represent a reversible preneoplastic change (Yoshida et al., 2012). While most studies assessing the progression of uterine changes preceding uterine endometrial adenocarcinoma formation have been reported in the Donryu rat, a similar pathologic progression (from focal glandular hyperplasia to adenomatous hyperplasia to adenocarcinoma) appears to also occur in the F344 rat (Tang et al., 1984).

2.1. Overview of the reproductive endocrinology of the aging female F344 rat

As female F344 rats age, normal estrous cycling ends and the rats enter a short period of constant estrus (Brown and Leininger, 1992) followed by repetitive pseudopregnancy and anestrus. Since the D5 chronic bioassay was performed in F344 rats, it is important to examine the events associated with reproductive aging in this strain of rat. Constant estrus is characterized by persistently elevated estrogen levels (Brown and Leininger, 1992; Lu et al., 1981, 1980b, 1977) and repetitive pseudopregnancy is characterized by sustained progesterone and prolactin levels. In anestrus, ovarian activity ceases. Remaining in constant estrus for any sustained period of time is relatively uncommon for F344 rats (Nagaoka et al., 1994). Pseudopregnancy occurs at a much higher incidence in the aging female F344 rat compared to other rat strains. In the aging female F344 rat, blood prolactin concentrations increase and dopaminergic inhibition of prolactin secretion via the tuberoinfundibular dopaminergic (TIDA) neurons decreases. Prolactin normally maintains corpus luteum function and stimulates the synthesis of progesterone. As a consequence of aging, female F344 rats enter a state of pseudopregnancy in which elevated progesterone concentrations are sustained. In pseudopregnancy, rather than regress, as occurs in young rats that are cycling normally, the corpora lutea persist and continue to secrete progesterone. A pseudopregnancy episode usually lasts about 2 weeks, although longer durations are possible. Animals that become pseudopregnant usually do so multiple times. As with pregnancy, pseudopregnancy is associated with high levels of progesterone and prolactin and low levels of estrogen, luteinizing hormone (LH), and follicle stimulating hormone (FSH) (Demarest et al., 1982; Huang et al., 1976; Huang et al., 1978; Lu et al., 1980a; Neumann, 1991; Peluso, 1992; Smith et al., 1975). Pseudopregnancy episodes and elevation in progesterone occur as early as 12 months of age (Nagaoka et al., 1994). Consequently, in the aging female F344 rat, a high level of progesterone relative to estrogen (e.g., a decreased estrogen/progesterone ratio) from repetitive pseudopregnancy is the predominant signal to the endometrium.

As female F344 rats enter reproductive senescence and the age-related changes in the reproductive cycle occur, there are also age-related changes in the uterus. These include endometrial hyperplasia, uterine stromal polyps, and endometrial adenomas (Leininger and Jokinen, 1990). There are two types of endometrial hyperplasia (Leininger and Jokinen, 1990). The first type, diffuse cystic endometrial hyperplasia, is thought to result from prolonged estrogen stimulation and is not believed to be preneoplastic. The second form of hyperplasia, focal glandular hyperplasia (adenomatous hyperplasia), however, may be a precursor to the development of adenocarcinomas. Uterine stromal polyps, the most common uterine tumor in rats, form in response to prolonged progesterone stimulation (Leininger and Jokinen, 1990).

The cause of spontaneous uterine endometrial adenocarcinoma in rats is unknown, but hormones, in particular a constant estrous state, and chronic infection have both been suggested to play an important role (Elsinghorst et al., 1984). Tang et al. (1984) have examined the effect of changing endocrine status on the uterine endometrium of the F344 rat in control aging rats, after neonatal androgenization and after estrogen implant in ovariectomized rats. These investigators showed that either induced or naturally present constant estrous status at 12 months of age led to focal glandular hyperplasia in the F344 rat that did not progress to more advanced adenomatous hyperplasia until 29 months of age. The authors suggested that development of a progesterone-insensitive state associated with the aged endometrium may be required for the progression to advanced adenomatous hyperplasia and uterine endometrial adenocarcinoma in the aged F344 rat.

2.2. Incidences of spontaneous uterine tumors in rats

Uterine endometrial adenomas and adenocarcinomas are usually of relatively low incidence in the F344 rat (Table 2) (Goodman et al., 1979; Haseman et al., 1998; Maekawa et al., 1983; Solleveld et al., 1984). Typically, the incidence of endometrial adenocarcinoma has been reported to be less than 1% at 24 months of age (Maekawa et al., 1983; Haseman et al., 1998). In F344 rats older than 24 months, the incidence of endometrial adenocarcinoma increases to about 8–12% after 30 months (Nyska et al., 1994). In the F344 rat, the most frequent spontaneous tumor is the endometrial stromal polyp followed in incidence by the endometrial adenocarcinoma and endometrial sarcomas. A recent review of the tumor incidence in control groups from the National Toxicology Program (NTP) chronic bioassays utilizing the F344 rat has revealed additional sources of information (Ando et al., 2008; CRL, 1990; Kuroiwa et al., 2013; Rao et al., 1990; Young and Morfeld, 2015) that challenge the conclusion of a rare occurrence of these tumors in F344 rats, particularly in certain sub-strains. The D5 chronic bioassay was originally compared to NTP historical controls that use the N sub-strain rather than the historical control data from Charles River, CrlBR sub-strain, the sub-strain used in the current bioassay.

Nyska and coworkers (Nyska et al., 1994) examined the incidence of spontaneous uterine endometrial adenocarcinoma in F344 rats in their laboratory and compared the uterine endometrial adenocarcinoma incidence to that reported in the NTP bioassay by Rao and coworkers (Rao et al., 1990). The F344 inbred rats (F-344 [CrlBR]) in the Nyska laboratory were obtained from Charles River Laboratories, UK. These rats were used in two studies, an earlier chronic 2-year study (LSRI-1) and a later chronic bioassay (LSRI-2). The incidences of uterine endometrial adenocarcinomas in the first study (LSRI-1) (13.3%) were similar to that reported for the NTP (11%) rats (Rao et al., 1990). However in a later study in the Nyska Laboratory (LSRI-2) performed approximately 3 years after the LSRI-1, the incidence of uterine endometrial adenocarcinomas were approximately double (24%) compared to the LSRI-1 and the NTP studies. No difference in adenomas was detected between the two Nyska studies and the NTP data set. In another study (Kuroiwa et al., 2013), the incidence of uterine endometrial adenocarcinoma in F344 rats in chronic bioassays (2-year duration) in the animal facility was reported for three periods of time. Kuroiwa et al. (2013)

Table 2
Incidences of spontaneous uterine tumors in various rat strains.

Study	Strain/Substrain	Years studies performed	Adenoma[a]	Adeno-carcinoma[a]	Reference
NTP studies	F344 N	Up to 2012	0.29%	0.29%	(NTP, 2013)
NTP (Rao)	F344 N	1971–1981	0.2%	11.0%	(Rao et al., 1990)
Nyska Study	F344 CrlBR	1989–1993			(Nyska et al., 1994)
	LSRI-1		0%	13.3%	
	LSRI-2		0%	24.0%	
	NTP		0.2%	11.0%	
Kuroiwa	F344 DuCrlCrlj				(Kuroiwa et al., 2013)
		1990–1999	ND	3.3%	
		2000–2004	ND	12.0%	
		2005–2009	ND	13.5%	
Charles River Laboratory	F344 CrlBR	1980–1990	ND	8.0%	(CRL, 1990)
Haseman	F344 N	1971–1997	0.4%	0.7%	(Haseman et al., 1998)
Dinse	F344 N	1995–2005		0.22%	(Dinse et al., 2010)

ND = no data presented.
[a] Indicates the percentage of tested animals with tumors.

reported the incidence of uterine endometrial adenocarcinomas in F344 rats as 3.3% in studies performed from 1990 to 1999, as 12.0% in rats in studies from 2000 to 2004, and as 13.5% in rats from 2005 to 2009 (Table 2). Data from Charles River (Charles River data, 1990 compilation) demonstrated that the incidence of uterine endometrial adenocarcinoma was greater (mean of 8%; 79/950 animals) in F344/CrlBr used in their facility as compared to the F344/N used by NTP (0.7% in feeding studies) (Haseman et al., 1998). A more recent analysis of the NTP database (Dinse et al., 2010) confirmed an incidence of uterine endometrial adenocarcinoma of 0.22% in F344/N rats used by NTP.

As noted in Table 2, the incidence of spontaneous uterine endometrial adenocarcinoma varies with different substrains of F344 rats and the incidences in sub-strains can change with time. The higher incidence cited in these additional references was notably greater than the 0.4–0.7% cited by NTP (Haseman et al., 1998) that was originally compared to the D5 chronic bioassay study. The variability the in uterine tumor incidence has important implications in understanding the relevance of the incidence of these uterine tumors in the D5 chronic bioassay. Given the relatively low incidence tumors (only in the high dose), the lack of a dose response in the tumor response and the lack of any tumors seen in the control group in the D5 chronic bioassay this new information on the substrain variability of spontaneous uterine tumors in the F344 rat, a re-evaluation of the statistical analysis of the bioassay was conducted (Young and Morfeld, 2015). For this analysis, additional historical controls were included in this evaluation Four sets of control groups specific to this sub-strain were formed, with varying heterogeneity. One control group included the concurrent controls for the D5 24-month exposure but also the control group for the group with 12 months of exposure followed by 12 months of recovery. In addition to the 12-month exposure/12-month recovery control group in the D5 study, historical control groups from three additional studies, each of 24-month duration, were considered: Octamethylcyclotetrasiloxane (D4) (Lee, 2004), hexamethyldisiloxane (HMDS) (Dotti et al., 2005), and polydimethylsiloxane (PDMS) (Mertens, 2003). Each of the studies had a 12-month exposure/12-month recovery and a 24-month exposure control group, both of which were considered as potential historical controls. All studies used the F344 rats of the same strain and from the same source. The effect of D5 was either statistically significant or showed a borderline significance when comparing all control incidences to the 160 ppm D5 24-month group. Analysis indicated that changing the results in one or two animals would impact the conclusions regarding statistical significance. The evidence, therefore, supports the conclusion that D5 at the highest dosage level results in an increased incidence of uterine endometrial adenocarcinomas. However, given the borderline nature of the statistics and the variability of the incidence of this spontaneous uterine tumor in historical controls in F344 rats, it is important to assess the biological relevance of this finding to further substantiate or counter this effect. It is important to note that the increased incidence of uterine endometrial adenocarcinomas seen with D5 at the highest dose studied cannot be completely ruled out as being due to variable rates of spontaneous tumors in the control F344 CrlBr rat substrain.

2.3. Comparison of the histology of the uterine endometrial adenocarcinoma seen in the D5 study to F344 controls

To better understand the histomorphology of the spontaneous uterine endometrial adenocarcinoma in aged control F344 rat and the relationship of these tumors to the uterine endometrial adenocarcinomas found in the D5 two-year bioassay, an independent pathology review of the uterine tumors was conducted of all F344 female control animals in the NTP historical database that had a diagnosis of either uterine endometrial adenocarcinoma or uterine endometrial adenoma and compared to the uterine tumors in the D5 study (Mann, 2003). The review included NTP control animals sacrificed at 24 months as well as control animals that were allowed to reach 30 months of age.

The D5-associated uterine endometrial adenocarcinomas were histologically indistinguishable from uterine endometrial adenocarcinomas found in control F344 rats from the NTP studies. There was a much lower incidence of non-neoplastic changes (cystic endometrial hyperplasia, epithelial hypertrophy) in the uteri of D5-exposed F344 rats with uterine endometrial adenocarcinomas that were present in control F344 rats in the NTP studies. This lack of preneoplastic changes is inconsistent with typical precursor changes seen in women given unopposed estrogen (Inoue, 2001) or chemically induced uterine endometrial adenocarcinoma in rodents (Maekawa et al., 1999; Verdeal et al., 1986).

The review of the historical NTP database was based on the reasoning that the similarity of the morphology of the spontaneous uterine endometrial adenocarcinomas and uterine endometrial adenocarcinomas seen in the D5 study might be presumptive evidence that D5 was causing an earlier onset of a tumor that increases spontaneously as the female F344 rat ages and in particular in this sub-strain of F344 rat. The histological characteristics of the uterine endometrial adenocarcinomas observed in female rats following exposure to D5 were indeed indistinguishable from the uterine endometrial adenocarcinomas present in the control animals in the NTP studies, providing evidence that D5 may simply cause a slightly earlier onset of a spontaneous tumor in female F344 rats.

2.4. Agents that induce uterine endometrial adenocarcinoma in the rat

Information on a possible mode-of-action for the induction of uterine endometrial adenocarcinoma can be derived from a comparatively large database on chemicals that were tested for carcinogenicity and induced uterine endometrial adenocarcinoma in the rat. The agents include genotoxic compounds such as nitrosamines and aromatic amines (Tanaka and Mori, 1983; Ogino et al., 1989), hormones and hormonally active chemicals such as 17β-estradiol (E2) (Yoshida et al., 2012) and tamoxifen (Carthew et al., 2000), chemicals such as indole-3-carbinol that induce cytochrome P450 1A enzymes resulting in a subsequent modulation of estrogen metabolism (Yoshida et al., 2012) and dopamine receptor agonists such as bromocriptine (Alison et al., 1994; Griffith, 1977). Chemicals with genotoxic properties presumably induce uterine endometrial adenocarcinoma by an interaction of electrophilic decomposition products/metabolites with DNA. A direct proliferative response on the endometrial tissue is the most likely basis for uterine endometrial adenocarcinoma induction by hormones or hormonally active chemicals or by chemicals that modulate estrogen metabolism favoring formation of estrogen metabolites with higher potency that strongly activate estrogen receptors. In contrast, dopamine agonists inhibit prolactin secretion from the pituitary in rats causing alteration of the estrogen/progesterone ratio causing estrogen dominance resulting in persistent endometrial stimulation ultimately causing endometrial tumors.

In summary, based on the analysis of mechanisms of uterine endometrial adenocarcinoma in rats, four basic mechanisms can be derived, including 1) genotoxicity, 2) direct estrogenicity, 3) interference of administered chemicals with hormone balance such as p450 1A inducers or active chemicals, and 4) dopamine agonism. The last three mechanisms all are consequences of a proliferative stimulus on the endometrium by changes in hormone levels and include direct hormonal activity and indirect modulation of estrogen concentrations by dopamine receptor agonists or agents that alter estrogen biotransformation.

In the examination of the mechanisms and the biology of uterine endometrial adenocarcinoma formation in the rat, the majority of the work performed has used the Donryu rat (Yoshida et al., 2002). The Donryu rat displays a high incidence of spontaneous endometrial adenocarcinomas that increases with aging. The tumors induced have morphological and biological similarities to those found in humans. Spontaneous uterine endometrial adenocarcinoma development in the Donryu strain has also been linked to an age-related imbalance of ovarian hormones, specifically an increased estrogen:progesterone ratio that occurs much earlier as compared to the F344 rat. The F344 rat on the other hand represents a strain that exhibits a slow progression of hyperplasia to adenocarcinoma (Tang et al., 1984) that is related to age-related imbalance of ovarian hormones that occurs late in life in the F344 rat leading to an increase in uterine endometrial adenocarcinoma typically only after 24 months of age.

3. Etiology and pathology of adenocarcinoma of the endometrium in women

3.1. Occurrence

The most common malignancy of the human uterine corpus is endometrioid adenocarcinoma of the endometrium. Endometrial malignancies are mostly adenocarcinomas, although sarcomas rarely are seen. Among adenocarcinomas, endometrioid histomorphology is the predominant subtype. Papillary serous carcinoma occurs in 3–10% of endometrial adenocarcinomas, and clear cell carcinoma occurs in 0.8–5.5% of endometrial adenocarcinomas, and both subtypes confer a worse prognosis than does endometrioid adenocarcinoma (Cirisano et al., 1999). Undifferentiated tumors and carcinosarcomas are rare and also are associated with a poor prognosis. Cancer of the uterine corpus increases in the years prior to menopause and peaks in the first decade after menopause.

3.2. The role of estrogen

Most endometrioid endometrial adenocarcinoma is associated with excessive estrogenic stimulation of the uterine endothelium. This view was reinforced historically by the observation that women given estrogen for menopausal therapy demonstrated an increase in hyperplastic and malignant lesions of the endometrium (Montgomery et al., 2004).

Among younger women who are diagnosed with endometrioid endometrial adenocarcinoma, chronic disorders of ovulation are common (Navaratnarajah et al., 2008). An Australian study of women younger than 50 with a diagnoses of endometrial adenocarcinoma found an increased likelihood that affected women reported a diagnosis of polycystic ovarian syndrome (PCOS) or symptoms of chronic oligo/anovulation (irregular menses, severe acne as an adult, hirsuitism), odds ratio 4.3, 95% confidence interval 1.8–10.2 in comparison to a control group without endometrial adenocarcinoma (Fearnley et al., 2010). A typical feature of PCOS is irregular or absent ovulation with prolonged exposure of the endometrium to the proliferative effects of ovarian estrogen without the counterbalancing effects of luteal progesterone.

Obesity is another risk factor for endometrial adenocarcinoma, presumably because adipose tissue aromatizes adrenal androgens to estrogens and produces more frequent or sustained exposure of the endometrium to estrogenic stimulation. In the Australian study on PCOS, adjustment for obesity attenuated the increase in risk, and obesity has been found to be an independent risk factor for endometrial adenocarcinoma (reviewed by Fader et al., 2009). Diabetic women are also at risk for developing endometrial adenocarcinoma due to the association of diabetes with obesity and to an independent decrease in sex-hormone binding globulin associated with hyperinsulinemia, which results from insulin resistance. A decrease in sex-hormone binding globulin results in a higher plasma concentration of free (unbound) estrogens.

3.3. Adenocarcinoma of the endometrium not associated with estrogen in women

Although most endometrioid adenocarcinoma of the endometrium is associated with excess estrogen stimulation of the endometrium, some endometrial adenocarcinoma, including serous, clear cell, undifferentiated, and carcinosarcoma types, arise independent of estrogenic stimulation (reviewed by Sherman, 2000). A distinction between estrogen-dependent “Type 1” adenocarcinoma and estrogen-independent “Type 2” adenocarcinoma was suggested in 1983. The Type 1 adenocarcinomas arise from a precursor lesion called atypical hyperplasia, are less aggressive and, in their earliest stage, may respond to progestin therapy (Randall and Kurman, 1997). Type 2 tumors, by contrast, do not arise from hyperplasia, do not respond to progestin, and arise by mechanisms not involving unopposed estrogen (Yang et al., 2013). Type 1 lesions are associated with microsatellite instability and with mutations in *K-RAS*, *β-catenin*, *PIK3CA*, and *PTEN*, while Type 2 lesions are associated with mutations in *P53* and with chromosome abnormalities (Arafa et al., 2010).

Approximately 1 in 5 women younger than 50 years of age with endometrial adenocarcinoma have a dominant mutation in a DNA mismatch repair gene (Walsh et al., 2008). This hereditary

condition is called Lynch syndrome, also known as hereditary nonpolyposis colon cancer, and predisposes affected individuals to cancer of the colon, stomach, ovary, and ureter as well as endometrium. Among women with Lynch syndrome, the lifetime risk of endometrial adenocarcinoma is up to 60% (Aarnio et al., 1999). Epigenetic silencing of DNA mismatch repair genes may be responsible for endometrial adenocarcinoma in some women without the Lynch syndrome; in these cases, microsatellite instability may be detected in endometrium prior to histological evidence of carcinoma (Faquin et al., 2000). Epigenetic mechanisms in endometrial adenocarcinoma are discussed further below.

3.4. Mediating factors

3.4.1. CYP 1A1

The naturally occurring estrogens E2 and estrone are oxidized to catechol estrogens by CYP 1A1 and CYP 1B1. The catechol estrogens are further oxidized by CYP 1A1 to semiquinones and quinones, which generate reactive oxygen species through redox cycling. The reactive metabolites can bind to nucleic acids, producing DNA damage through adduction (Akanni and Abul-Hajj, 1999).

In humans, it has been proposed that single nucleotide polymorphisms (SNPs) of CYP 1A1 that confer increased enzyme activity might increase the risk of endometrial adenocarcinoma through a genotoxic mechanism. In support of this proposal, case–control studies of women with endometrial hyperplasia and adenocarcinoma showed that the presence of a valine (for isoleucine) in codon 462 of exon 7 of CYP 1A1 was associated with these disorders in populations in Spain and Turkey (Esinler et al., 2006; Esteller et al., 1997). A threonine for asparagine in codon 461 of CYP 1A1 was also associated with endometrial adenocarcinoma in Spain (Esteller et al., 1997). However, the isoleucine-to-valine SNP at codon 462 of exon 7 was not shown to be associated with endometrial carcinoma in a Japanese population (Sugawara et al., 2003), and both CYP 1A1 SNPs were associated with a *decreased* risk of endometrial carcinoma in a US sample (Doherty et al., 2005).

3.4.2. Glutathione-S-transferase

Glutathione-*S*-transferases (GSTs) play a role in the detoxification of the reactive oxidative metabolites of catechol estrogens. In humans, SNPs that are associated with a decrease in GSTP1 activity have been reported to be associated with an increased risk of endometrial carcinoma (Chan et al., 2005). Other GST polymorphisms have not been associated with endometrial cancer risk (Doherty et al., 2005; Esteller et al., 1997).

3.4.3. Epigenetic changes

In human uterine tumors, hypermethylation of the promoter region of tumor-suppressor genes is common in type 1 but not type 2 adenocarcinoma of the endometrium (reviewed by Arafa et al., 2010). Epigenetic inactivation of *MLH1*, a mismatch repair gene, and of *PTEN* are early events in the estrogen-associated hyperproliferation leading to adenocarcinoma.

The finding that tumor-suppressor genes are methylated in atypical hyperplasia and in histologically normal endometrium located near foci of endometrioid adenocarcinoma suggests that epigenetic changes, perhaps estrogen-induced, are early steps in the carcinogenic process (Arafa et al., 2008).

3.5. Relatedness of human and rat endometrial adenocarcinoma

Female rats appear to develop endometrial adenocarcinoma by mechanisms similar to those operating in women (Table 3). As discussed in Section 2.4, above, the most work on spontaneous adenocarcinoma of the endometrium in rats comes from inbred strains including Donryu, DA/Han, and BDII/Han, which have high tumor incidences with age due to mutations in *K-ras* or tumor suppressor genes (reviewed by Vollmer, 2003). The uterine adenocarcinomas arising in these animals are morphologically similar to the endometrioid adenocarcinomas arising in women, occur on a background of endometrial hyperplasia, and are most likely estrogen-responsive until they become advanced and undifferentiated (Yoshida et al., 2012). The Donryu rat has been shown to have an increase in estrogen exposure with respect to progesterone exposure at 12 months of age compared to F344 rats, attributable to disruptions of cycling (Nagaoka et al., 1990). As a consequence, Donryu rats have a 61% incidence of endometrial hyperproliferative lesions (endometrial adenocarcinoma, hyperplasia, and adenoma) with advanced age. This phenomenon is analogous to the increased exposure to endogenous estrogens in women with disturbances of ovulation and with obesity.

4. Biological relevance for D5 uterine endometrial adenocarcinoma induction in rats

From the available literature, agents that induce uterine tumors in rodents appear to function through either DNA interaction by genotoxic compounds with uterine epithelial cells or through nongenotoxic effects, specifically direct activation of estrogen receptors or modulation of estrogen levels. In the case of the former, a number of genotoxic carcinogens including nitroso compounds (ENNG, MNNG, and MNU) have been reported to induce adenocarcinomas in the rat uterus Estrogen or compounds that mimic or modulate estrogen or induce constant estrus have also been shown to induce uterine endometrial adenocarcinomas in rats. In addition, oxidative stress and damage derived from estrogen metabolism and catechol estrogen formation have been proposed (Yoshida et al., 2012) as possible mechanisms for the induction of uterine endometrial adenocarcinomas in rats. Modulation of dopamine levels in the rat have also been linked to uterine cancer formation due to a modulation of estrogen (Alison et al., 1994).

Based on the available experimental data for D5 coupled with published studies on the potential mechanisms by which chemicals induce uterine endometrial adenocarcinoma in the rat as well as proposed mechanisms involved in the process of chemical carcinogenesis, we identified and evaluated several possible modes of action by which D5 might induce uterine endometrial adenocarcinoma in the F344 rat to assess the biological relevance of this borderline finding: 1) genotoxicity, 2) direct endocrine (estrogenicity, androgenicity and progestogenic) activity, 3) oxidative stress/damage/inflammation/cytotoxicity, and 4) alteration of pituitary control of the estrous cycle. Of these possible modes of action, our review of the data from D5 studies supports an alteration of pituitary control of the estrous cycle as the most probable to be considered further. Prior to presenting the support for these final conclusions, we will present a summary of the other modes of action considered and why they are unlikely.

4.1. Consideration of other possible modes of action of D5 induced rat uterine endometrial adenocarcinoma

4.1.1. Genotoxicity as a possible mode of action of D5 induced rat uterine endometrial adenocarcinoma

Multiple genetic toxicity assessments for D5 have been performed in bacteria, mammalian cells *in vitro* and in intact animals *in vivo* and none have provided evidence that D5 is a genotoxic chemical (Isquith et al., 1988; Litton, 1978; Dekant and Klaunig, 2015). It was concluded that the genotoxic mode of action does not apply to D5 induction of uterine tumors in F344 rats.

Table 3
Comparison of endometrial adenocarcinoma in rat and human.

Feature	Rat	Human	Comments
Endometrial hyperplasia as a precursor lesion	Han-Wistar rat adenocarcinoma starts as nodular hyperplasia (Deerberg et al., 1981). There are two types of rodent endometrial hyperplasia. The first type, diffuse cystic endometrial hyperplasia, is thought to result from prolonged estrogen stimulation and is not believed to be preneoplastic. Focal glandular hyperplasia (adenomatous hyperplasia), however, may be a precursor to neoplasia (Leininger and Jokinen, 1990)	Endometrial hyperplasia regarded as a precancerous stage of endometrial adenocarcinoma (reviewed in Elsinghorst et al., 1984). Human cystic endometrial hyperplasia is not precancerous, while more complex hyperplasia with cytologic atypia is premalignant.	Elsinghorst et al. (1984) mentions that it is not known if the nodular hyperplasia in rats corresponds to any human hyperplasia subtype
Relationship to parity	Virgin Han-Wistar rats used in aging studies demonstrated 39% incidence of endometrial adenocarcinoma (reviewed in Deerberg et al., 1981).	Nulliparous women have an increased risk of endometrial adenocarcinoma (Dossus et al., 2010; Karageorgi et al., 2010; Pfeiffer et al., 2009).	Schonfeld et al. (2013) report that nulliparity alone does not modify the risk of endometrial adenocarcinoma over that associated with hormone-related risk factors, which may result in subfertility.
Metastasis to lungs in advanced stage	In Han-Wistar, metastasis to lungs is commonplace (Deerberg et al., 1981).	Infrequent metastasis to lungs	
Types of adenocarcinoma	Appears to be estrogen-dependent; not clear that an estrogen-independent form has been observed in rats.	Type I (estrogen-dependent) and Type II (estrogen-independent) adenocarcinoma have been described (Bokhman, 1983)	
Role of prolactin	Prolactin is luteotropic in rats (Harleman et al., 2012), maintaining a condition of continuous progesterone in aged rats, which decreases risk of endometrial adenocarcinoma.	Prolactin may be a risk factor for endometrial cancers (Levina et al., 2009). However, prolactin is not luteotropic in humans (reviewed in Harleman et al., 2012).	
Role of obesity	Zucker fa/fa obese rats exposed to estrogen demonstrated significantly higher pro-proliferative genes cyclin A and c-Myc mRNA expression in the endometrium of obese rats compared to lean control rats. In addition, anti-proliferative genes RALDH2 and sFRP4 were not affected by estrogen in obese rats, but were more strongly induced in lean rats (Zhang et al., 2009).	Obesity is a major risk factor for endometrial cancer (reviewed by De Pergola and Silvestris, 2013)	No studies were located that showed more endometrial adenocarcinoma among obese compared to lean rats.
Relation between endometrial and breast cancer	Rats with spontaneous breast cancer may be less prone to endometrial adenocarcinoma, and vice versa (Harleman et al., 2012)	Increased exposure to estrogens is a risk factor for carcinoma at both sites.	
PHF5A/*PHF5A* expression	Changes in expression of *Phf5a*/*PHF5A* were found in rat endometrial adenocarcinomas (Falck and Klinga-Levan, 2013)	Changes in expression of *Phf5a*/*PHF5A* were found in human endometrial adenocarcinomas (Falck and Klinga-Levan, 2013)	The authors report that the pattern of changes was not consistent between rats and humans.
Molecular genetic features	BDII-Han rats have molecular features closer to higher-grade human type I tumors with significant down regulation of *Pten*, *Cdh1*, *p16*, *Erbb2*, and *Ctnnb1* (Samuelson et al., 2009)	Type I endometrial adenocarcinoma is characterized by frequent microsatellite instability and *PTEN*, *K-RAS*, and *CTNNB1* mutations; type II adenocarcinoma displays recurrent *TP53* mutation, *CDKN2A* (P16) inactivation, over-expression of *ERBB2*, and reduced *CDH1* expression (Samuelson et al., 2009)	This rat strain is specifically inbred to produce a model of human endometrial adenocarcinoma and may not be generalizable to other rat strains.
Insulin-like growth factor (IGF) signaling and susceptibility to endometrial hyperplasia	IGF-1 signaling is present in rat endometrium after treatment with estrogen (McCampbell et al., 2008)	Over-expression of IGF-1 is present in complex endometrial hyperplasia (McCampbell et al., 2008)	

4.1.2. Direct endocrine activity as a mode of action of D5-induced rat uterine endometrial adenocarcinoma

To understand the potential for a direct sustained response of D5 on endometrial cells, D5 was also assessed for endocrine activity and was found to not possess any significant estrogenic, anti-estrogenic, progestagenic, androgenic, or anti-androgenic activity (Quinn et al., 2007b).

Estrogenicity of D5 was assessed in a rat uterotrophic assay in both Sprague–Dawley and F344 rats after D5 inhalation (160 ppm, whole body, 16 h/day for 3 days). No effects of D5-exposure on the estrogenic endpoints measured were observed in either strain of rat. In addition, D5 (10 μM) did not show the capacity to compete with E2 for binding to the estrogen receptor (ER) *in vitro* and D5 (10 μM) did not result in a response in an *in vitro* reporter gene assay using MCF-7 cells transiently transfected with a plasmid for ER-α and the luciferase gene. D5 was also negative in a Hershberger assay assessing potential androgenicity *in vivo*, and D5 did not interact with progesterone receptors alpha and beta *in vivo*.

In a review (Dekant and Klaunig, 2015) to assess a possible role of membrane and nuclear ER, animals were exposed by inhalation to D5 (one exposure for 6 or 16 h). Possible D5-mediated effects on estrogen-dependent endpoints were compared to the response to ethinyl estradiol and 4-hydroxytamoxifen. Ethinyl estradiol, which activates both the membrane and nuclear ER, and 4-hydroxytamoxifen, which activates membrane ER but is a partial agonist/antagonist for nuclear ER, served as positive controls. Both positive controls resulted in the expected response, but D5 did not influence any of the estrogen-responsive endpoints, including uterine weight, estrous state, epithelial cell height (luminal and glandular), histopathology (uterus and mammary gland), and proliferation (BrdU incorporation) in luminal, glandular, stromal, and total cells of the uterus and mammary gland. Finally, the histopathology from the numerous rat exposure studies with D5 (Jean et al., 2015; Dekant and Klaunig, 2015) has also failed to support a direct endocrine effect for the observed uterine tumors. Based on the results noted above, it is concluded that a direct endocrine activity mode of action does not apply to D5 induction of uterine tumors in F344 rats.

4.1.3. Oxidative stress/damage as a possible mode of action of D5-induced rat uterine endometrial adenocarcinoma

Oxidative stress/damage has been implicated as a cause and/or promoting component in tumorigenesis (Klaunig, 2004; Klaunig et al., 2011). In the case of D5, four possible sources of oxidative damage can be considered in the etiology of uterine tumor induction. These include the induction of oxidative damage directly from D5, induction of oxidative damage due to the formation of catechol estrogens and redox cycling, and oxidative damage from inflammation or cytotoxicity. Oxidative stress can result in DNA damage through formation of oxidative adducts, inhibition of normal DNA repair, or the production of lipid peroxidation products that form DNA adducts. Reactive oxygen species can also modify gene expression, specifically those genes involved in apoptosis and cell proliferation.

Oxidative metabolism of estrogens to catechol estrogens has been suggested as a potential inducer of uterine carcinogenesis. Estrogens have been shown to undergo oxidative metabolism by cytochromes P450 1A1 and 1B1 to produce 2-hydroxyestradiol (2-OHE2) and 4-OHE2. 4-OHE2 has been suspected to have a role in carcinogenesis as a result of its oxidization to quinone derivatives (2, 3-OHE2-*o*-quinone and 3, 4-OHE2-*o*-quinone). These quinones have the potential to function at both the initiation and promotion stage of the cancer process. Ortho-quinones derived from catechol estrogens are electrophilic DNA reactive compounds. These quinones are also redox cycling agents that are able to generate reactive oxygen species (ROS).

In general toxicology studies changes in liver weight in rats exposed to high levels of D5 following both oral and inhalation exposure have been demonstrated. Additional work assessing the enzyme induction profile in rats treated with D5 demonstrated a pattern consistent with the classical response observed following phenobarbital treatment. As is the case for phenobarbital, D5 has been shown to induce liver enlargement, hepatocellular hypertrophy/hyperplasia, and hepatic cytochrome P450 induction (CYP2B1/2 primarily) in the rat (McKim et al., 1999; Jean et al., 2005). While a 1.8 fold increase in EROD activity was observed, no change in CYP 1A1/2 immunoreactive protein occurred and EROD activity returned to normal in 14-day post exposure rats.

To assess the impact of D5 on estrogen metabolism, serum was collected from aging F344 rats exposed to D5 for ~12 months. D5 exposure did not demonstrate a difference in the presence of estrogen-related analytes (including catechol estrogens) as compared to controls (Sloter, 2013; Fuhrman et al., 2014). With no experimental evidence supporting an oxidative or catechol estrogen mode of action, it is unlikely this mode of action is responsible for uterine adenocarcinomas seen following treatment with D5.

Oxidative stress can also be produced subsequent to acute and chronic inflammation. In the case of D5, there is no evidence for an inflammation mode of action in the subchronic and chronic inhalation studies with D5. Inflammatory changes were not observed in the uterus in the detailed histopathological examinations of a variety of studies (Jean et al., 2015; Dekant and Klaunig, 2015).

Chronic cytotoxicity with persistent regeneration or compensatory hyperplasia of a target tissue has been proposed as a mode of action for chemical carcinogens. However, there is no evidence from the subchronic and chronic inhalation studies with D5 for chronic cytotoxicity in the target tissue based on the histopathologic evaluation of uterine tissues in the oncogenicity study or other repeated dose studies with D5 (Dekant and Klaunig, 2015).

4.2. Alteration of pituitary control of estrus cycle as a mode of action of D5-induced rat uterine endometrial adenocarcinoma

4.2.1. Alteration of pituitary control following D5 exposure

This mode of action for the rat uterine tumor induction based on changes in the estrous cycle associated with D5 exposure is consistent with some of the experimental data available for D5. There is evidence that exposure to D5 influences prolactin concentration, although the effects on prolactin are not consistent from *in vivo* to *in vitro* studies (Dekant and Klaunig, 2015). Therefore, the precise mechanism-of-action for alteration of pituitary control of the estrus cycle by D5 is not known with certainty. 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. 1).

There are no data showing that D5 treatment alters LH in the rat in a similar manner as suggested by data for D4. D4 has been shown to interfere with reproduction in the rat (Meeks et al., 2007) through suppression of LH (Quinn et al., 2007a). Although D4 and D5 are structurally similar and have several metabolites in common, they differ in reproductive endpoints. D4 inhalation was associated with a decrease in implantations and live litter size (Quinn et al., 2007a), whereas D5 inhalation had no adverse effects on reproduction in a two-generation study using the same rat strain (Siddiqui et al., 2007). In addition, in a two year study with D4, uterine adenomas with an associated increase in cystic endometrial hyperplasia, which is consistent with elevated endogenous E2 from ovarian cysts (common with modulations of LH), were seen. Therefore, the available reproductive data for D5 and

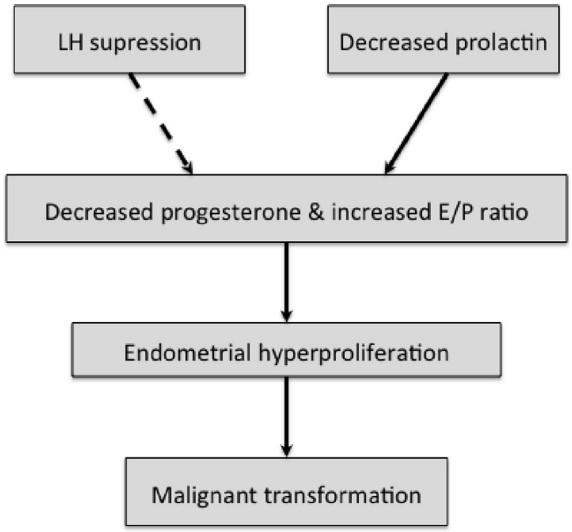

Fig. 1. Proposed alteration in estrous cycle mode of action for D5 induce rat uterine endometrial adenocarcinoma by D5. Alteration of pituitary control of the estrous cycle in the rat could occur either by alteration in LH or prolactin. The absence of evidence for D5-associated LH-mediated cycle disruption is represented by the broken line linking this theoretical mechanism to subsequent events. E/P = estrogen:progesterone.

differences in the pattern seen in the two year studies with D4 and D5 do not provide support for D5 alteration of the estrous cycle via modulation of LH.

Previously studies (Jean, 2005c,b; Jean et al., 2005) suggested disruption of prolactin/progesterone dominance through dopamine receptor agonism following exposure to D5. More recent work, including a chronic aged rodent study (Dekant and Klaunig, 2015; Sloter, 2013) supports the conclusion that D5 does not act directly as a dopamine agonist. However, the following information is suggestive of dopamine receptor agonist-like and/or partial agonist/antagonist activity of D5. Mode of action studies show that D5 decreases pituitary lactotroph release of prolactin *in vitro* and modulates circulating prolactin levels *in vivo*. Further studies *in vitro* confirm the effect but suggest it may be an effect on one or more downstream components of the dopamine signal transduction pathway. Also, repeated D5 exposure via inhalation did not significantly affect circulating prolactin levels after exposure for one and five days. However, the average prolactin levels of rats exposed to D5 for five days strongly trended higher four and eight hours post-exposure.

4.2.2. Alteration of estrus cycle following D5 exposure

Data from the aged-animal study show an alteration of the estrous cycle by D5. To put this observation into perspective, the regulation of the estrous cycle in the rat needs to be reviewed. Progesterone from the corpus luteum counters the proliferative effects of estrogen on the endometrium at least in part by down-regulating estrogen receptors (reviewed by Oehler et al., 2000). Endometrial proliferation reflects a balance of stimulatory effects of estrogen and inhibitory effects of progesterone, a relationship that can be described by the estrogen:progesterone ratio. The typical rat 4-day estrous cycle is characterized by an increase in estrogen on proestrus followed by the LH surge and ovulation. The corpus luteum produces a peak in progesterone on estrus, with maintenance of progesterone production through the rest of the cycle and into the next cycle. Corpus luteum function is maintained by prolactin in the rat, and elevated concentrations of prolactin are associated with persistence of corpora lutea from previous cycles, producing a progesterone-dominant pseudopregnancy state. Female rats experience a decreasing number of estrous cycles as they age and, as discussed above, have prolonged periods of diestrus that eventuate in persistent diestrus or pseudopregnancy. The more estrous cycles in an animal's life, the more proestrus days it will have experienced and the more estrogen will have stimulated the endometrium.

Support for alteration of the estrous cycle following exposure to D5 comes from the study of Sloter, 2013, in which F344 females were treated with D5 from 11 to 25 months of age with monitoring of estrous cycle stage by daily vaginal lavage. During the first four months of the exposure period, rats treated with D5 spent more time in an estrogenic state than did controls and were in an estrogenic state for more consecutive days than controls (Fig. 2). In the D5-exposed rats, the percent of days spent in an estrogenic state was increased by an average of 44% during the first 45-day interval and 78% during the second 45-day interval. The percentage was still increased during the third 45-day interval, even though the control percentage had increased markedly during this time. Frequency of estrogenic state was not different from controls subsequently, as both control and D5-exposed animals converted to a repeated pseudopregnant state characteristic of the reproductively senescent F344 rat. A plot (Fig. 3) of the cumulative number of days in an estrogenic state reveals two inflection points, one when the rats progress from 4- or 5-day cycles to being more frequently in an estrogenic state (approximately 14 months of age in controls) and the second when they progress to repetitive pseudopregnancy (approximately 18 months of age in controls).

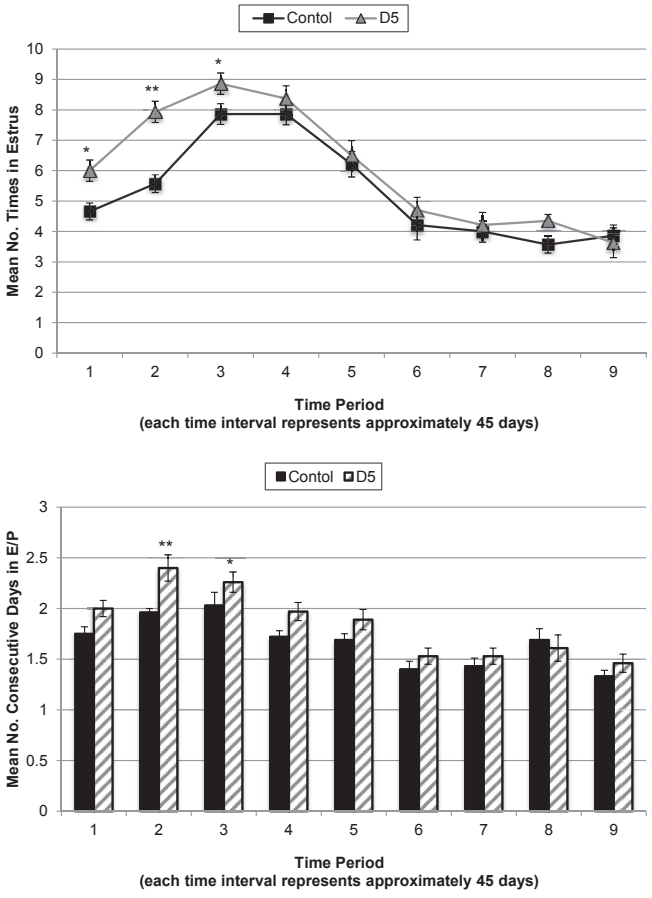

Fig. 2. Number of times in an estrogenic state (top panel) and number of consecutive days in an estrogenic state (lower panel). **Statistically different from control at $P < 0.05$, 0.01. From Sloter (2013).

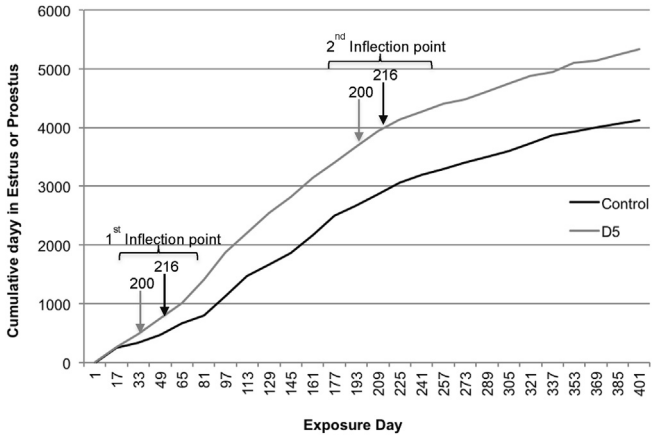

Fig. 3. Cumulative days in a high estrogen state associated with D5 and D4 treatment. From Sloter (2013).

Both inflection points were shifted to the left (i.e., occurred at earlier ages) in the D5-exposed group: 23 days earlier for the first inflection point, 16 days earlier for the second. This result suggests an advancement of aging in the D5-exposed reproductive tracts. The larger cumulative number of days of estrogen exposure would be expected to increase the risk of endometrial hyperproliferation. This study did not show effects on circulating prolactin levels, but because blood samples were taken only at 3–4-week intervals and were not normalized to estrous cycle phase, these data cannot provide much, if any, weight of evidence. Another set of aged F344 rats also were utilized (Dekant and Klaunig, 2015) to evaluate the effect of an exposure to D5 on cyclicity, prolactin, and estrogen:progesterone ratio following exposure at later age. Female F344 rat aged 22 months were exposed to D5 by inhalation (160 ppm, 6 h/day, 5 days/week) for three months. The rats were already in a state of persistent diestrus/pseudopregnancy when the study began. The effect of D5 was subtle with regard to cyclicity; however, there was an increase in the days in proestrus/estrus with the 160 ppm D5 exposure. For the D5 exposed animals, there was also a subtle effect (albeit using infrequent sampling) on serum progesterone concentrations, but no real change in prolactin. In addition, there was an increase in the combined incidence and severity of focal glandular endometrial hyperplasia (including the papillary subtype) in the D5 treated animals.

4.2.3. Endometrial hyperproliferation as a precursor lesion

As female rats age, the uterine estrogen and progesterone receptors show alterations. Uterine trauma in young F344 rats (8–10 months old) produces a decidual cell response comparable to a pseudopregnant state, but old rats (>20 months old) do not show this reaction as readily (Saiduddin and Zassenhaus, 1979). Administration of E2 to ovariectomized rats normally results in an increase in estrogen receptor; administration of progesterone results in a decrease in estrogen receptor. In aging rats, there is a smaller increase in estrogen receptor after administration of estrogen and a smaller decrease in estrogen receptor after administration of progesterone (Saiduddin and Zassenhaus, 1979). Young and old rats have the same amount of uterine progesterone receptor, suggesting that the attenuated response to progesterone in older rats may also be due to impairment of the function of the receptor or post-receptor machinery. The decrease in progesterone receptor function with age would be expected to result in less progesterone opposition to estrogen-associated endometrial proliferation, perhaps accounting for the susceptibility of aging F344 rats to endometrial hyperproliferative lesions (Nyska et al., 1994).

The association between estrogen exposure and endometrial hyperplasia has been demonstrated in aged Fischer rats *in vitro* and *in vivo* (Tang et al., 1982, 1984). The development of hyperplasia is prevented in the rat by the cyclic appearance of progesterone associated with pseudopregnancy until 29 months of age where hyperplasia and adenocarcinoma develop despite the presence of elevated progesterone, suggesting that the development of more advanced adenomatous hyperplasia and adenocarcinoma may be due to a decrease in sensitivity to progesterone. Adenocarcinoma has been for the most part only rarely observed spontaneously in control F344 rats before 24 months of age. Focal glandular hyperplasia is considered to be a precancerous lesion, in contrast to cystic hyperplasia, which is not. The distinction between focal glandular hyperplasia and true neoplasia often is difficult because of the gradual transition from hyperplasia into adenoma or carcinoma, which becomes the predominant lesion. Although there was no reported increase in focal glandular hyperplasia following D5 exposure for 24 months, at least one tumor in the 160 ppm D5 treated group had glandular hyperplasia associated with the tumor (Jean et al., 2015). Possible explanations for the lack of obvious associated precursor lesions include focal precursor lesions that were not detected as a consequence of sampling and sectioning or onset of low-grade adenocarcinomas months prior to the development of persistent diestrus/pseudopregnancy, a state during which precursor lesions may have regressed. The latter explanation is supported by the observation (Dekant and Klaunig, 2015) that D5 treatment was associated with an increase in days in estrus shortly after the initiation of treatment but not thereafter in aged F344 rats exposed to 160 ppm D5 starting at 11 or 22 months of age. Another possible explanation is that presence of focal glandular hyperplasia as a precursor lesion was lost as the tumors progressed to such an advanced stage that the tumor became the predominant lesion. This possibility is supported by the shorter term study when aged F344 rats exposed to D5 staring at 22 months showed an increase in the combined incidence and severity of focal glandular hyperplasia (including the papillary subtype) in the D5 treated animals. This finding suggests that the initial cycle disruption leads to the precursor lesion (focal glandular hyperplasia) that progress to an advanced tumor after continued exposure.

4.3. Discussion of proposed mode of action on relevance to humans

As indicated above, reproductive senescence of aging female F344 rats is characterized by a pseudopregnancy state that is caused by hypothalamic aging. Hypothalamic aging gives rise to the loss of dopamine regulation of pituitary prolactin release. As a consequence, prolactin levels are elevated resulting in persistent corpora lutea, elevated progesterone and low estrogen. This unique aspect of reproductive senescence in F344 rats can lead to subtle changes in these aging animals that are not possible in humans due to species differences in hormonal regulation. For example, dopamine agonists such as bromocriptine can inhibit prolactin secretion from the pituitary in rats causing luteolysis and new follicle development resulting in estrogen dominance (Alison et al., 1994). This estrogen dominance can lead to endometrial stimulation, which may lead to endometrial carcinoma. Bromocriptine, a well-known dopamine agonist, has been reported to produce uterine endometrial hyperplasia and adenocarcinomas in rats following chronic administration (NDA 17-962). This carcinogenic effect has not been demonstrated in any other species including humans (Burke et al., 1988). In addition, clinical studies with bromocriptine show no effect on LH, FSH, estrogen levels, progesterone levels, or endometrial histopathology in women.

In women and other primates, reproductive senescence is very different from that in rats and occurs relatively late in life (about 51

years of age in humans). In women, reproductive senescence (menopause) is the result of ovarian follicular depletion and is not at all related to aging of the hypothalamus. At the time of menopause, ovarian follicles are essentially depleted and estrogens and progesterone are severely reduced, but the capacity of the hypothalamus is normal. Also, prolactin decreases after menopause. Thus, in human females, the post-menopausal period is associated with elevated LH and FSH secretion and reduced secretion of estrogens, progesterone, and prolactin. Based on these differences and the lack of effects seen in the clinical studies, the tumorigenic effect of dopamine agonists in female rats should be considered a species-specific effect with no risk to human health (Burke et al., 1988).

The weight-of-evidence suggests that if even if D5 is responsible for a slight increase in uterine adenocarcinomas in the rat, this result is a rat-specific (and possibly sub-strain-specific) and has no relevance to the induction of human uterine adenocarcinomas.

5. Conclusions

Variable rates of spontaneous uterine endometrial adenocarcinomas have been reported for untreated F344 CrlBr rats. As such, we concluded that the slight increase in uterine endometrial adenocarcinomas observed in the D5 chronic bioassay might not be the result of D5 exposure but may be related to variability of the spontaneous tumor incidence in this strain of rat. However, if the uterine endometrial adenocarcinomas are related to D5-exposure, a plausible mode of action exists in the rat that involves alteration in the estrous cycle in the aging F344 rat. The alteration consists of a decrease in progesterone with an increase in the estrogen:progesterone ratio caused most likely by a decrease in prolactin concentration. Suppression of prolactin has been shown in some but not all studies with D5. It is possible that the *in vivo* studies that failed to show suppression of prolactin were not sensitive to alterations that were restricted to a subgroup of susceptible animals or the lack of consistency may be due to the technical difficulty of measuring serum hormones that by their nature are highly cyclical.

A decrease in progesterone and an increase in the estrogen:progesterone ratio is supported by data in 11–25-month-old rats exposed to D5. The increase in estrogen exposure was documented during the first four months of the exposure period and led to an increase in the cumulative days of estrogen exposure over the exposure period when compared to the control animals.

Taken as a whole, the mode of action data of D5 indicates that it is acting possibly via a dopamine receptor agonist-like mechanism to alter the pituitary control of the estrous cycle. Like dopamine receptor agonists, pharmacology studies show that D5 decreases pituitary lactotroph release of prolactin *in vitro* and decreases circulating prolactin levels *in vivo* in specific animal models designed to optimize the release of prolactin, an effect that can be competed for by a dopamine receptor agonist. Further studies *in vitro* confirmed the effect but suggest it may be an effect on one or more downstream components of the dopamine signal transduction pathway. Studies in aged animals show that the effects of D5 on estrous cyclicity are consistent with a dopamine-like effect and further suggest that D5 might be accelerating the aging of the reproductive endocrine axis in this strain of rat. These results are consistent with a mode of action for uterine endometrial adenocarcinoma tumorigenesis that is not relevant for humans.

Conflicts of interest

Dr. Klaunig, Dr. Dekant and Dr. Scialli report personal fees from the American Chemistry Council. Dr. Plotzke's employer, Dow Corning Corporation, manufactures and sells silicone based chemical products.

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