OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

Proposition 65

Nitrapyrin

A chemical listed “as causing cancer” by the authoritative bodies mechanism and under review by the Carcinogen Identification Committee

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Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
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The Office of Environmental Health Hazard Assessment's (OEHHA) Reproductive and Cancer Hazard Assessment Branch was responsible for the preparation of this document.

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PREFACE

Proposition 65[1] requires the publication of a list of chemicals "known to the state" to cause cancer or reproductive toxicity. The Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency maintains this list in its role as the lead agency for implementation of Proposition 65[2].

The US Environmental Protection Agency (US EPA) is a designated "authoritative body" whose formal identification of carcinogens serves as the basis for listing chemicals under Proposition 65[3]. OEHHA listed nitrapyrin as a carcinogen under Proposition 65 on October 5, 2005, based upon its classification by US EPA (US EPA, 2000)[4] as "likely to be carcinogenic in humans." In 2012, US EPA revised the classification of nitrapyrin to "Suggestive Evidence of Carcinogenic Potential" (US EPA, 2012a)[5].

When a chemical listed in this manner is no longer formally identified by the authoritative body as causing cancer and no other administrative basis for listing applies, then the chemical is referred to the Carcinogen Identification Committee (CIC)[6], the state's qualified experts for carcinogenicity determinations under Proposition 65[7]. The CIC then determines whether the chemical has been "clearly shown through scientifically valid testing according to generally accepted principles to cause cancer." If the CIC makes such a determination, the chemical remains on the Proposition 65 list. Otherwise, the chemical is removed from the list.

On November 4, 2015, the CIC is scheduled to deliberate on the carcinogenicity of nitrapyrin. OEHHA developed this document with information on the evidence of carcinogenicity of nitrapyrin to assist the CIC in its deliberations on whether or not nitrapyrin should remain on the Proposition 65 list. The original reports and papers discussed in the document will also be provided to the CIC as part of the hazard identification materials. Comments on this hazard identification document received

[1] The Safe Drinking Water and Toxic Enforcement Act of 1986, codified at Health and Safety Code 25249.5 *et seq*., commonly referred to as Proposition 65.
[2] Health and Safety Code section 25249.12, Title 27, Cal. Code of Regs., section 25102(o)
[3] Title 27, Cal. Code of Regs., section 25306
[4] US Environmental Protection Agency (US EPA, 2000). Cancer Assessment Document. Evaluation of the Carcinogenic Potential of Nitrapyrin (Second Review). Cancer Assessment Review Committee. Health Effects Division. Office of Pesticide Programs. May 5, 2000.
[5] US Environmental Protection Agency (US EPA, 2012a). Cancer Assessment Document. Evaluation of the Carcinogenic Potential/Mode of Action for Mouse Liver Tumors. Nitrapyrin. PC Code 06923. Cancer Assessment Review Committee. Health Effects Division. Office of Pesticide Programs. March 1, 2012.
[6] Title 27, Cal. Code of Regs., section 25306(j)
[7] Title 27, Cal. Code of Regs., section 25305(a)(1)

during the public comment period also form part of the hazard identification materials, and are provided to the CIC members prior to their formal deliberations.

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1. EXECUTIVE SUMMARY

Nitrapyrin is a pesticide that acts as a bactericide, a nitrification inhibitor, and a plant growth regulator (Powell and Prosser, 1986; Tice and Carson, 1999; US EPA, 2005c). In California, nitrapyrin is registered for use on corn, wheat, cotton, sorghum, strawberries, and sudangrass (CDPR, 2013b). There are no registered residential uses in California or the US (US EPA, 2005c). The potential for dietary exposure is considered very low. Agricultural workers may be exposed through loading, mixing, or applying nitrapyrin, or re-entering treated sites.

Nitrapyrin was listed as known to the State of California to cause cancer under Proposition 65 on October 5, 2005 through the authoritative bodies listing mechanism. The Proposition 65 listing was based on the US Environmental Protection Agency (US EPA) classification of nitrapyrin as "Likely to be carcinogenic in humans" (US EPA, 2000). In 2012, US EPA reclassified nitrapyrin as "Suggestive Evidence of Carcinogenic Potential" (US EPA, 2012a).

When a chemical is no longer formally identified by US EPA as causing cancer and no other administrative basis for listing applies, then the chemical is referred to the Carcinogen Identification Committee (CIC)[8], the state's qualified experts for carcinogenicity determination under Proposition 65[9]. The Office of Environmental Health Hazard Assessment (OEHHA) has prepared this document to provide the CIC with information relevant to the assessment of the evidence of carcinogenicity of nitrapyrin.

Human Carcinogenicity

No epidemiology studies were identified that investigated the risk of cancer associated with exposure to nitrapyrin in humans.

Animal Carcinogenicity

Evidence for the carcinogenicity of nitrapyrin comes from long-term carcinogenicity studies (two in rats and four in mice) exposed via feed (Szabo *et al.*, 1989; Quast *et al.*, 1990; Stebbins and Cosse, 1997). Tumors were observed at a number of sites.

[8] Title 27, Cal Code of Regs., section 25306(j)
[9] Title 27, Cal. Code of Regs., section 25305(a)(1)

Kidney tumors

- In the male F344 rat study by Szabo *et al.* (1989), the combined incidence of rare renal tubule cell adenoma and adenocarcinoma was significantly increased in the high-dose group. Statistically significant dose-response trends were observed in the incidence of renal tubule cell adenoma, adenocarcinoma, and combined adenoma and adenocarcinoma.

Liver tumors

- In the male B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidences of hepatocellular adenoma and combined hepatocellular adenoma and carcinoma were significantly increased in the 250 mg/kg/day dose group, with significant dose-response trends.
- In the female B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidences of hepatocellular adenoma and combined hepatocellular adenoma and carcinoma were significantly increased in the 125 and 250 mg/kg/day dose groups, with significant dose-response trends.

Forestomach tumors

- In the male B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidences of forestomach squamous cell papilloma and combined squamous cell papilloma and carcinoma were significantly increased in the 125 and 250 mg/kg/day dose groups, with significant dose-response trends observed for papilloma, carcinoma, and combined papilloma and carcinoma.
- In the female B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidences of forestomach squamous cell papilloma and combined squamous cell papilloma and carcinoma were significantly increased in the 125 and 250 mg/kg/day dose groups, with significant dose-response trends.

Epididymal tumors

- In the male B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidence of rare epididymal histiocytic sarcoma was significantly increased in the 250 mg/kg/day dose group, with a significant dose-response trend.

Harderian gland tumors

- In the female B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidence of Harderian gland adenoma was significantly increased in the 125 and 250 mg/kg/day dose groups, with a significant dose-response trend.

Mechanisms and Other Relevant Data

Nitrapyrin is rapidly and extensively absorbed following oral administration in rats, mice, chickens, goats, and dogs. Dermal application of nitrapyrin also leads to a substantial uptake in rats, the only species in which a dermal exposure study was conducted. Upon entering the circulatory system, nitrapyrin is rapidly distributed throughout the body. Metabolism studies in rats, mice and dogs indicate that nitrapyrin is metabolized to 6-chloropicolinic acid (6-CPA), which is then conjugated with glycine to form *N*-(6-chloropicolinoyl)glycine (6-CPG) (or taurine in mice) and excreted. No metabolism studies in human tissue or cells were identified.

Nitrapyrin has been assessed for a limited number of genotoxicity endpoints. In the *Salmonella* reverse mutation assay, nitrapyrin induced concentration-related and reproducible increases in frameshift and base pair substitution mutations in multiple tester strains in two studies (Zeiger *et al.*, 1988; Mecchi, 2007), but not in a third (Kennelly, 1985). Nitrapyrin did not induce reverse mutations in *E. coli* strain WP2*uvr*A, hypoxanthine-guanine-phosphoribosyl transferase mutations in mammalian Chinese hamster ovary cells, micronuclei in mouse bone marrow *in vivo*, or unscheduled DNA synthesis *in vitro* in rat hepatocytes or *in vivo* in mouse liver.

Nitrapyrin activates the constitutive androstane receptor (CAR) and studies suggest activation of the aryl hydrocarbon receptor, pregnane X receptor, and peroxisome proliferator-activated receptor alpha. CAR activation has been proposed as a nongenotoxic mode of action (MOA) for liver tumors in mice. Some of the key events in the proposed CAR MOA were observed with nitrapyrin, including increased liver *Cyp*2b10 expression, hypertrophy, absolute and relative liver weight, and basophilic foci. However, total liver cytochrome P450 protein content was not increased, and some of the key events in the proposed CAR MOA were not observed following nitrapyrin exposure. Specifically, nitrapyrin did not increase pentoxyresorufin O-dealkylase activity and, as noted by US EPA, the liver cell proliferation response pattern was not typical of other CAR activators (US EPA, 2012a). In addition, CAR knockout mice exhibited many similar liver changes as wild-type mice when treated with nitrapyrin (increased liver *Cyp1a1*, *Cyp3a11*, and *Cyp4a10* gene expression, hypertrophy, and liver weight) (Murphy *et al.*, 2014b). Thus, it appears that many of nitrapyrin's effects in mouse liver are independent of CAR activation.

α_{2u}-Globulin accumulation, mineralization of the loops of Henle, hyaline droplet accumulation, and increases in dilated tubules with proteinaceous casts were observed in the kidneys of male rats (Szabo *et al.*, 1989). α_{2u}-Globulin accumulation was proposed as a possible mechanism for renal tubule cell tumor development in male rats,

but several International Agency for Research on Cancer (IARC) criteria for determining whether an agent causes kidney tumors through an α_{2u}-globulin-associated response in male rats (IARC, 1999) were not met, such as “lack of genotoxic activity” and “male rat specificity for nephropathy and renal tumorigenicity”. Specifically, evidence of genotoxicity was observed in *Salmonella* in multiple strains in two studies, and effects of nitrapyrin on the kidney were observed in female rats (increases in dilated tubules with proteinaceous casts at 12 months in treated females, increased absolute and relative kidney weights at 12 and 24 months, and increased blood urea nitrogen (BUN) at 24 months, Szabo *et al.,* 1989). With regard to three of the other IARC criteria, data were not available to evaluate whether the criteria had been met. Given the genotoxicity findings in *Salmonella* and the effects of nitrapyrin on female rat kidney, it is likely that nitrapyrin acts through additional carcinogenic mechanisms.

The biological activity of nitrapyrin was compared to 11 structurally related pyridine compounds. Of these 11 comparison compounds, one compound, pyridine, is listed under Proposition 65 as causing cancer, and the only other comparison chemical reported to induce tumors in animals is 3-(chloromethyl)pyridine. Pyridine and/or 3-(chloromethyl)pyridine induced tumors at sites in common with nitrapyrin, *e.g.*, mouse liver, rat kidney, mouse forestomach, and rat testes (although at a different site within the testes), but comparisons across the larger set of structurally similar chemicals are limited by the lack of carcinogenicity testing data for several of these compounds. Several of the structurally-related compounds are genotoxic, and induce mutations in *Salmonella* and/or mutations or chromosomal aberrations in the mouse lymphoma cell assay (2-chloropyridine, 3-chloropyridine, 2,3-dichloropyridine, 2-(chloromethyl)pyridine, 3-(chloromethyl)pyridine, and 2-chloro-5-(trichloromethyl)pyridine).

2. INTRODUCTION

This document summarizes the evidence of carcinogenicity for nitrapyrin, a pesticide that acts as a bactericide, a nitrification inhibitor, and a plant growth regulator (Powell and Prosser, 1986; Tice and Carson, 1999; US EPA, 2005c).

Nitrapyrin was listed as known to the state of California to cause cancer under Proposition 65 on October 5, 2005 through the authoritative bodies listing mechanism. The Proposition 65 listing was based on the US Environmental Protection Agency (US EPA) classification of nitrapyrin as "Likely to be carcinogenic in humans" (US EPA, 2000). In 2012, US EPA reclassified nitrapyrin as "Suggestive Evidence of Carcinogenic Potential" (US EPA, 2012a).

Given that nitrapyrin is no longer formally identified by US EPA as causing cancer, the Office of Environmental Health Hazard Assessment (OEHHA) has prepared this document to provide the Carcinogen Identification Committee (CIC) with information relevant to the assessment of the evidence of carcinogenicity of nitrapyrin.

2.1 Background on US EPA Carcinogenicity Reviews of Nitrapyrin

Between 1992 and 2011, US EPA conducted four evaluations of the carcinogenicity of nitrapyrin (US EPA, 1992; 2000; 2005a; 2012a). These evaluations are briefly summarized below.

The carcinogenicity of nitrapyrin was first reviewed by US EPA in 1992 (Attachment 1). This review focused on unpublished two-year feeding studies on nitrapyrin conducted in male and female F344 rats (Szabo *et al.*, 1989) and male and female B6C3F_1 mice (Quast *et al.*, 1990), and a published study on the mutagenicity of nitrapyrin in *Salmonella* (Zeiger *et al.*, 1988). Additional information considered in this review by US EPA (1992) included unpublished studies in *Salmonella* by Kennelly (1985), a Chinese hamster ovary (CHO) cell gene mutation assay by Linscombe and Gollapudi (1986), an *in vivo* mouse bone marrow micronucleus (MN) assay by Kirkland (1985), an *in vitro* rat hepatocyte unscheduled DNA synthesis (UDS) assay by Mendrala and Schumann (1982), a metabolism study, and structure-activity correlations. US EPA classified nitrapyrin in Group D – not classifiable as to human carcinogenicity due to inadequate evidence. The US EPA classification was based on a statistically significant increase in renal tubule adenomas and adenocarcinomas in male rats. US EPA further elaborated that "since there appeared to be potential for kidney, and possibly liver, effects in mice, and there is apparent genotoxic potential for nitrapyrin, an acceptable mouse study is required for complete assessment and whether the assessment will totally focus on the

α_{2u}-globulin mechanism or not" (US EPA, 1992). US EPA determined that the dosing in the mouse study was inadequate and requested an additional study at higher doses.

The carcinogenicity of nitrapyrin was next reviewed by US EPA in 2000 (Attachment 2). This review included all of the information considered in the previous review, as well as additional unpublished two-year feeding studies conducted at higher doses in male and female $B6C3F_1$ mice (Stebbins and Cosse, 1997), as well as unpublished two-week feeding studies on hepatocyte proliferation and apoptosis in male and female mice (US EPA, 2000). In reviewing the new two-year mouse studies, US EPA concluded "there was an increase (both pair-wise and trend) in liver and [fore]stomach tumors in $B6C3F_1$ male and female mice, epididymal sarcomas in male mice, and Harderian gland tumors in female mice". US EPA re-classified nitrapyrin as "Likely to be carcinogenic to humans". The decision was based on the observation of tumors at multiple sites in both sexes of mice, the available mutagenicity data supporting a mutagenic mode of action (mutagenicity "in *Salmonella typhimurium* strains TA97, TA98, and TA100 in the presence of S9 activation"), and the structural relationship between nitrapyrin and the chlorinated pyridines that are mutagenic and carcinogenic in mice and rats (US EPA, 2000).

In response to a request from the registrant, Dow Chemical Company, US EPA conducted a third review on the carcinogenicity of nitrapyrin in 2005 (Attachment 3). This review included all of the information considered in the previous reviews, as well as additional information submitted by the registrant, in the form of a presentation and unpublished report (Hardisty, 2004) from a "histopathology peer review and scientific advisory group" convened by the registrant. In this third review of the carcinogenicity of nitrapyrin, US EPA maintained the classification of nitrapyrin as "Likely to be Carcinogenic to Humans". The US EPA classification was based on the following: "(i) The treatment-related mouse liver tumors occurred in both sexes and, although benign, showed a robust increase in incidence compared to the control, (ii) There was insufficient data to accept the cytotoxic mechanism for the mode of action for induction of liver tumors, (iii) There were rare, malignant tumors in the mouse epididymis at the high dose, and (iv) Although there was insufficient data to classify nitrapyrin as a mutagenic carcinogen there remains a concern based on a weak response in the *Salmonella* assay and structural activity relationships".

The most recent US EPA review of the carcinogenicity of nitrapyrin was in 2012 (Attachment 4). In this review US EPA evaluated additional information submitted by the registrant, including an unpublished overview of the mouse carcinogenicity studies (Eisenbrandt *et al.*, 2010b), an unpublished reevaluation of the genotoxicity of nitrapyrin (Zeiger, 2010), a new unpublished *in vivo* mouse UDS assay (Pant and Celestin, 2009),

new unpublished mutagenicity assays in *S. typhimurium* and *E. coli* (Mecchi, 2007), and an unpublished review of mouse epididymal tumors by a Pathology Working Group (PWG) sponsored by the registrant (Hardisty, 2010). Additionally, US EPA reviewed four unpublished reports submitted by the registrant related to nitrapyrin and activation of the constitutive androstane receptor (CAR) in the mouse liver (Daly, 1995; Eisenbrandt *et al.*, 2010a; LeBaron, 2010; LeBaron *et al.*, 2010). In its 2012 review, US EPA reclassified nitrapyrin as "Suggestive Evidence of Carcinogenic Potential". The US EPA decision was based on the following: "(i) There was no evidence of carcinogenicity in male or female rats, (ii) The undifferentiated epididymal sarcomas in male mice have been reclassified as histiocytic sarcomas and shown to be not treatment-related, (iii) Liver tumors, which were seen in both sexes of mice, were attributable mainly to adenomas (i.e., no progression to malignancy) and the tumors occurred late in the course of treatment (i.e., no reduction in survival), (iv) Data are not sufficient to support the proposed MOA [mode of action] for liver tumors, and (v) There was no evidence for a mutagenic mode of action for the liver tumors". US EPA also states, "the evidence from the nitrapyrin animal data is suggestive of carcinogenicity, which raises a concern for carcinogenic effects, but is judged not sufficient for quantification of cancer risk in humans" (US EPA, 2012a).

2.2 Identity of Nitrapyrin

Nitrapyrin (2-chloro-6-trichloromethyl pyridine) is a colorless or white crystalline solid with a mildly sweet odor (NIOSH, 2015). Figure 1 shows the chemical structure of nitrapyrin. Table 1 lists some physical and chemical properties of nitrapyrin.

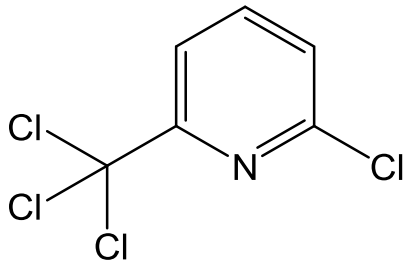


Figure 1. Chemical structure of nitrapyrin

Table 1. Some physical and chemical properties of nitrapyrin (US EPA, 2005c; NIOSH, 2015)

Common Name	Nitrapyrin
IUPAC Name	2-Chloro-6-(trichloromethyl)pyridine
Chemical Class	Pyridine
CAS Registry Number	1929-82-4
Molecular Formula	$C_6H_3Cl_4N$
Molecular Weight	230.9
Density	1.55 g/mL
Melting Point	62-63ºC
Boiling Point	128-129ºC
Flash Point	100°C
Water Solubility	92 ppm (at 25ºC)
Vapor Pressure	2.8×10^{-5} mmHg (at 23ºC)
Henry's Law Constant	2.03×10^{-5} atm-m^3/mole (at 25ºC)
Log P (Octanol-water coefficient)	3.41
Synonyms/Trade Names	Nitrapyrine, α,α,α,6-Tetrachloro-2-picoline, N-Serve® nitrogen stabilizer, N-Serve TG®, N-Serve 24E®, N-Serve 24®, Stay-N 2000®, Dowco-163, Instinct®II

2.3 Occurrence, Use and Exposure

2.3.1 Use and Occurrence

Nitrapyrin is a pesticide that functions as a bactericide and nitrification inhibitor, and as a plant growth regulator (Powell and Prosser, 1986; Tice and Carson, 1999; US EPA, 2005c). It is applied along with urea and nitrogen fertilizer to delay the loss of nitrogen from soil. Nitrapyrin delays nitrification by selectively inhibiting *Nitrosomonas* bacteria, which oxidizes ammonium to nitrite (Tice and Carson, 1999).

In 1998, the US EPA's Office of Pollution Prevention and Toxics High Production Volume Chemicals list recorded an annual production volume of 1.6 to 2.9 million pounds of nitrapyrin (Tice and Carson, 1999). Nitrapyrin is no longer included on the US EPA's list of high production volume chemicals (US EPA, 2012b). OEHHA was unable to identify current production volume information for nitrapyrin.

In California, nitrapyrin is registered for use on corn, wheat, cotton, sorghum, strawberries, and sudangrass (CDPR, 2013b). There are no registered residential uses in California or the US (US EPA, 2005c). Nitrapyrin is applied directly to the soil as a broadcast treatment, band treatment, top dressing treatment, side-dress, or injected into

the soil (US EPA, 2005c). It can be applied before, during, or after planting and/or post-harvest.

Active registered formulations in the US containing nitrapyrin include: N-Serve 24, N-Serve TG, Instinct, N-Serve® XT, and GF-2937 from Dow AgroSciences LLC, and Nitrapyrin Technical and N-Lock Nitrogen Stabilizer from Makhteshim Agan of North America, Inc. (NPIRS, 2013). Three registered formulations of products containing nitrapyrin are active in California: N-Serve 24, N-Serve 24 CA, and N-Serve TG (CDPR, 2013b). From 1999 to 2012, an annual average of 70.3 pounds of nitrapyrin was applied in California (CDPR, 2014a).

Nitrapyrin is removed from the soil through volatilization into the ambient air and degradation through hydrolysis and photolysis in water (Redemann *et al.*, 1964). Nitrapyrin is monitored for in California water (CDPR, 2013c), but has not been detected in groundwater or surface water (CDPR, 2014b).

2.3.2 Exposure

Nitrapyrin can be absorbed by plants and livestock (Redemann *et al.*, 1965; Mullison and Norris, 1976; Iwata *et al.*, 1981; Kallio and Sandholm, 1982; Bjerke, 1987; Bjerke and Martin, 1987; Bjerke *et al.*, 1990; US EPA, 2004); however, concentrations measured in plants and livestock are generally very low (Jensen, 1971; Mullison and Norris, 1976; US EPA, 2004). Nitrapyrin has never been detected in foods tested by pesticide residue monitoring programs in California (CDPR, 2013a) or by the US Food and Drug Administration (FDA) (US FDA, 2011). Therefore, the potential for dietary exposure is considered very low.

Agricultural workers may be exposed through loading, mixing, or applying nitrapyrin, or re-entering treated sites. The National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health from 1981 to 1983 estimated that 399 workers were exposed to nitrapyrin (RTECS, 2002). Nitrapyrin was detected at a concentration of 18.0 ng/m^3 air in a commercial pesticide storage building (Yeboah and Kilgore, 1984). Nitrapyrin exposures of commercial loaders and farmer applicators were measured by Honeycutt *et al.* (1993). Average respiratory exposure was 0.0010 to 0.031 mg/kg body weight/day and average dermal exposure was 0.0037 to 0.094 mg/kg body weight/day. Both respiratory and dermal exposure to farmers in closed-cab tractors was lower than exposure to commercial loaders (Honeycutt *et al.*, 1993).

3. DATA ON CARCINOGENICITY

3.1 Carcinogenicity Studies in Humans

No epidemiological studies on the effects of human exposure to nitrapyrin were identified in a recent literature search conducted by OEHHA.

3.2 Carcinogenicity Studies in Animals

The carcinogenicity of nitrapyrin has been studied in rats and mice. Six animal carcinogenicity studies, consisting of two unpublished dietary studies in Fischer 344 (F344) rats (Szabo *et al.*, 1989), and four unpublished dietary studies in $B6C3F_1$ mice (Quast *et al.*, 1990; Stebbins and Cosse, 1997) were submitted to US EPA. In its first evaluation of the carcinogenicity of nitrapyrin, US EPA (1992) considered the four carcinogenicity studies available at that time (Szabo *et al.*, 1989; Quast *et al.*, 1990). In subsequent evaluations conducted in 2000, 2005, and 2012, US EPA considered all six studies (Szabo *et al.*, 1989; Quast *et al.*, 1990; Stebbins and Cosse, 1997).

3.2.1 Studies in Rats

3.2.1.1 Two-year feeding studies in F344 rats (Szabo et al., 1989) (see also US EPA, 1992)

Szabo *et al.* (1989) conducted two long-term carcinogenicity studies of nitrapyrin: one in male rats and one in female rats. Nitrapyrin (CAS number 1929-82-4, purity 93.3%) was administered in the diet to groups of 50 male and 50 female F344 rats at doses of 0, 5, 20, or 60 mg/kg body weight per day for two years. An additional 10 rats per dose group were included in each study for interim sacrifice at 12 months. All animals were four weeks old at the start of the studies.

Males

In the two-year study in males, there was a dose-dependent increase in mortality, with a statistically significant difference in survival between the high-dose males and controls. Forty-six percent of the high-dose males died prior to terminal sacrifice, with most deaths occurring during the last five to six months of treatment, compared to 24 percent of the controls dying prior to terminal sacrifice. The authors of the study ascribed the higher mortality in high-dose males to an increased severity of chronic progressive glomerulonephropathy (CPN) that occurred during the second year of treatment (1 CPN/12 deaths in controls; 10 CPN/23 deaths in high-dose males).

Mean body weights of males were statistically significantly reduced from day 483 to termination at the high dose (2.2 percent lower than controls at day 483) and from day 595 to termination at the mid dose (3.2 percent lower than controls at day 595). At study termination, mean body weights of the high- and mid-dose groups were 14.8 and 6.7 percent lower than controls, respectively. There was no appreciable difference in food consumption among the treatment groups.

Tumor incidences observed at the end of the two-year study in male rats are given in Table 2. Renal tubule cell (tubular) adenomas and adenocarcinomas were observed only in the high-dose group, and significant dose-response trends in the incidences of renal tubule cell adenomas, adenocarcinomas, and combined adenomas and adenocarcinomas were observed. The incidence of combined renal tubule cell adenomas and adenocarcinomas was significantly increased in the high-dose group as compared to controls, and exceeded laboratory historical control data from studies conducted between 1983 and 1991 (0/150) (US EPA, 1992). These data and data from other laboratories on the incidence of renal tubule cell tumors in untreated male F344 rats indicate that renal tubule cell adenomas (1/1794; 0.05%) and adenocarcinomas (2/1794; 0.11%) are rare (Goodman *et al.*, 1979).

No renal tumors were observed in male rats in the interim sacrifice at 12 months.

Table 2. Incidence[1] of treatment-related kidney tumors in male F344 rats administered nitrapyrin in feed for two years (Szabo *et al*., 1989)

Tumor Site and Type		Dose (mg/kg/day)				Trend test p-value[2]
		0	5	20	60[3]	
Kidney	Renal tubule adenoma (r)	0/39	0/40	0/39	3/32	<0.01
	Renal tubule adenocarcinoma (r)	0/38	0/40	0/38	3/28	<0.01
	Combined (r)	0/39	0/40	0/539	6/32**	<0.001

[1]Number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor.
[2]p-values from exact trend test conducted by OEHHA
[3]Fisher pairwise comparison with controls
** p < 0.01. (r) denotes a rare tumor type. See text for details.

Non-neoplastic findings

Both absolute and relative liver weights were significantly increased in the high-dose males at 12 and 24 months. At 24 months, absolute and relative liver weights were increased by 41 and 67 percent, respectively, compared to the controls. Absolute and relative kidney and adrenal weights were also increased at 24 months in the high-dose

males. Absolute and relative kidney weights were increased by 17 and 38 percent, respectively, relative to the controls. Absolute and relative adrenal weights were increased by 69 and 90 percent, respectively, relative to the controls. Increases in the absolute and relative weights of the adrenals at 24 months were probably a physiologic response to renal failure resulting from chronic progressive glomerulonephropathy, according to the study authors.

Changes in clinical chemistry parameters and histopathology observed in high-dose males (a 40 percent increase in serum cholesterol and slight to moderate diffuse hepatic centrilobular hypertrophy and vacuolation) were consistent with fatty change in the liver. Additionally, serum glutamate-pyruvate transaminase (SGPT), now called alanine transaminase (ALT), was increased by 23 percent in high-dose males compared to controls, indicating non-specific liver injury.

At the 12-month interim sacrifice, increased incidences of mineralization of the loops of Henle and hyaline droplet accumulation in the P2 section of proximal tubules were observed in the high-dose males, as well as increases in dilated tubules with proteinaceous casts among the low- and mid-dose males. An immunoperoxidase assay was conducted to identify the presence of α_{2u}-globulin, a male-rat-specific urinary protein, in hyaline droplets in the kidneys from control and high-dose males. All the high-dose males showed clear and positive staining for α_{2u}-globulin in the P2 section of the proximal tubule, whereas slight and scattered staining was observed in control males. At 24 months, there was a significant increase in incidence of severe chronic progressive glomerulonephropathy in the high-dose males (33/50) compared to the controls (2/50).

Females

In the two-year study in female rats, no treatment-related differences were observed in mortality. In the high-dose females, mean body weights were significantly reduced from day 49 (2.3 percent lower than controls) to termination (9.9 percent lower than controls). There was no appreciable difference in food consumption among the treatment groups. No treatment-related tumors were observed.

One kidney tubule cell adenoma was observed in the control group, and none were observed in the treated groups at two years, or in any of the animals examined at the 12 month interim sacrifice.

Non-neoplastic findings

Both absolute and relative liver weights were significantly increased in high-dose females at 12 and 24 months. At 24 months, absolute and relative liver weights of the high-dose females were increased by 46 and 64 percent, respectively, relative to controls, and absolute and relative kidney weights were increased by 10 and 24 percent, respectively, relative to controls. At 12 months, only relative kidney weights of the high-dose females were increased (by 10 percent).

At the 12-month interim sacrifice, an increase in dilated tubules with proteinaceous casts was observed in the kidneys for the mid- and high-dose females (1/10 and 6/10 rats, respectively) as compared to 0/10 controls. At 24 months, slight and moderate chronic progressive glomerulonephropathy was observed in female rats, but was not statistically significant compared to controls. At 24 months, slight hepatic centrilobular hypertrophy and vacuolation were observed in the high dose group.

3.2.2 Studies in Mice

3.2.2.1 Two-year feeding studies in B6C3F$_1$ mice (Quast et al., 1990) (See also US EPA, 1992)

Quast *et al.* (1990) conducted two long-term carcinogenicity studies of nitrapyrin: one in male mice and one in female mice. Nitrapyrin (technical grade N-SERVE TG, 93.3 percent purity) was administered in the diet to groups of 50 male and 50 female B6C3F$_1$ mice at doses of 0, 5, 25, or 75 mg/kg body weight/day for two years. Mice were sacrificed at 105 weeks. An additional 10 mice per dose group were included in each study for interim sacrifice at 12 months.

Males

In the two-year study in male mice, there were no differences in survival, body weight or food consumption between the various dose groups and the controls. US EPA concluded that since the highest test dose produced minimal toxicity, the dose was not sufficient to assess the carcinogenicity of nitrapyrin, and requested that an additional carcinogenicity study of nitrapyrin be performed in male mice (US EPA, 1992).

One renal cortical (tubule cell) adenoma was observed in each of the 25 and 75 mg/kg/day dose groups, with none observed in controls. Spontaneous renal cortical tubule cell adenomas are rare in B6C3F$_1$ mice (2/1351; 0.1 percent) (Haseman *et al.*, 1998). No treatment-related increases in tumors were observed at other sites, and no kidney tumors were observed at the 12-month interim sacrifice.

Incidence data for liver and epididymal tumors are reported here for informational purposes, since increased tumor incidences at these sites were reported in the male mouse study of Stebbins and Cosse (1997). In the Quast *et al.* (1990) study, the incidence of liver hepatocellular adenomas was 20/49, 12/48, 18/50, and 20/48 in the 0, 5, 25, and 75 mg/kg/day dose groups, respectively, and the incidence of liver hepatocellular carcinomas was 9/47, 11/47, 6/45, and 7/48 in the 0, 5, 25, and 75 mg/kg/day dose groups, respectively. One hepatocellular adenoma was observed in each of the dosed groups (5, 25, and 75 mg/kg/day) at the 12-month interim sacrifice. No hepatocellular tumors were observed in the control group at interim sacrifice.

Quast *et al.* (1990) observed three tumors of the epididymis in the control group and one in the 75 mg/kg/day dose group. Two of the epididymal tumors in the control group and the tumor in the 75 mg/kg/day dose group were originally described as Leydig cell tumors by the study authors (Quast *et al.*, 1990). The third tumor in the control group was classified as a histiocytic sarcoma. A Dow Chemical Company-sponsored PWG (Pathology Working Group) review in 2010 reclassified all the epididymal tumors as histiocytic sarcomas (Hardisty, 2010). Histiocytic sarcomas of the epididymis are considered rare in mice (Creasy *et al.*, 2012). No epididymal tumors were observed at the 12-month interim sacrifice.

Non-neoplastic findings
Kidney absolute and relative weights at 24 months were statistically significantly increased (5.5 percent and 7.0 percent, respectively) in the high-dose males compared to controls. At the 12-month interim sacrifice, serum ALT activity was increased in the high-dose males by 37 percent (relative to the controls), which was likely associated with mild liver pathology. At the 12- and 24-month sacrifices, livers of the high-dose males exhibited altered cytoplasmic homogeneity with centrilobular distribution.

Females

In the two-year study in female mice, there were no differences in survival, body weight or food consumption between the various dose groups and the controls. US EPA concluded that since the highest test dose produced minimal toxicity, the dose was not sufficient to assess the carcinogenicity of nitrapyrin, and requested that an additional carcinogenicity study of nitrapyrin be performed in female mice (US EPA, 1992).

A single incidence of renal pelvis transitional cell carcinoma was observed in a female administered 25 mg/kg/day. Renal pelvis transitional cell carcinomas are rare in $B6C3F_1$ mice (0/1349) (Haseman *et al.*, 1998). No renal tumors were observed at the 12-month interim sacrifice.

Incidence data for liver tumors and Harderian gland tumors are reported here for informational purposes, since increased tumor incidences at these sites were reported in the female mouse study of Stebbins and Cosse (1997). In the Quast *et al.* (1990) study, the incidence of liver hepatocellular adenomas was 10/47, 6/47, 7/47, and 14/50 in the 0, 5, 25, and 75 mg/kg/day dose groups, respectively, and the incidence of liver hepatocellular carcinomas was 1/45, 1/46, 1/47, and 1/46 in the 0, 5, 25, and 75 mg/kg/day dose groups, respectively. One hepatocellular adenoma was observed in the 25 mg/kg/day dose group at the 12-month interim sacrifice.

The incidence of Harderian gland adenomas was 5/50, 1/15[10], 3/10[11], and 0/50 in the 0, 5, 25, and 75 mg/kg/day dose groups, respectively. One Harderian gland adenoma was observed in the control group at the interim sacrifice[12].

Non-neoplastic findings
Liver absolute and relative weights at 24 months were statistically significantly increased (7.8 percent and 9.1 percent, respectively) in the high-dose females as compared to controls.

3.2.2.2 Two-year feeding studies in B6C3F$_1$ mice (Stebbins and Cosse, 1997) (See also US EPA, 2000; 2005a; 2012a)

Stebbins and Cosse (1997) conducted two long-term carcinogenicity studies of nitrapyrin: one in male mice and one in female mice. Nitrapyrin (N-SERVE Nitrogen stabilizer; 95.4% purity) was administered in the diet to groups of 50 male and 50 female B6C3F$_1$ mice/dose at doses of 0, 125, or 250 mg/kg/day for two years. An additional 10 mice per dose group were included in each study for interim sacrifice at 12 months.

Males

In the two-year study in male mice, mortality in the control, 125, and 250 mg/kg/day dose groups was 20, 4, and 34 percent, respectively, with the majority of deaths in each group occurring after 92 weeks (Stebbins and Cosse, 1997, p. 24 and p. 60). Mortality

[10] Histopathologic examination of the Harderian gland was performed on only 15 animals in the 5 mg/kg/day dose group.
[11] Histopathologic examination of the Harderian gland was performed on only 10 animals in the 25 mg/kg/day dose group.
[12] At the 12-month interim sacrifice histopathologic examination of the Harderian gland was performed on the control and 75 mg/kg/day animals, but not on any animals in the 5 mg/kg/day dose group and on only one animal in the 25 mg/kg/day dose group.

in the 125 mg/kg/day dose group was statistically significantly lower than that of controls (2/50 vs. 10/50, respectively). Mortality in the 250 mg/kg/day dose group was increased (17/50) as compared to controls, but the increase did not reach statistical significance at the $p < 0.05$ level. Mean body weights in the 250 mg/kg/day dose group were statistically significantly reduced as compared to controls, starting on day 19 (3.1 percent lower than controls) and continuing through to study termination (8.8 percent lower than controls). No significant difference in food consumption was observed among the treatment groups. US EPA concluded that the dosing was adequate and not excessive to assess the carcinogenic potential of nitrapyrin in this study in male mice (US EPA, 2000).

Tumor incidences observed at the end of the two-year study in male mice are summarized in Table 3. There were statistically significant increases in hepatocellular adenomas and combined hepatocellular adenomas and carcinomas in the 250 mg/kg/day dose group compared to the controls, with positive dose-response trends. At the 12-month interim sacrifice, the incidence of hepatocellular adenomas was 2/10, 0/10, and 3/10 in the 0, 125, and 250 mg/kg/day dose groups, respectively. No hepatocellular carcinomas were observed at interim sacrifice.

As shown in Table 3, the incidences of forestomach squamous cell papillomas and combined squamous cell papillomas and carcinomas were statistically significantly increased at both doses, with positive dose-response trends observed for papillomas, carcinomas, and papillomas and carcinomas combined. Forestomach carcinomas are considered rare in male $B6C3F_1$ mice (background incidence of 2/1355; 0.1 percent) (Haseman *et al.*, 1998). No forestomach tumors were observed at interim sacrifice.

In addition, tumors of the epididymis were elevated in both dose groups, with a significant dose-response trend and a statistically significant increase in the 250 mg/kg/day dose by pairwise comparison with controls. The original study authors classified these tumors as malignant undifferentiated epididymal sarcomas (Stebbins and Cosse, 1997). A Dow Chemical Company-sponsored PWG review in 2010 reclassified these tumors as histiocytic sarcomas (Hardisty, 2010) (see Section 3.3.3 Animal Tumor Pathology for details). Histiocytic sarcomas of the epididymis are considered rare in mice (Creasy *et al.*, 2012). No epididymal tumors were observed at interim sacrifice.

Table 3. Incidence[1] of treatment-related lesions in male B6C3F$_1$ mice administered nitrapyrin in feed for two years (Stebbins and Cosse, 1997)

Tumor Site and Type		Dose (mg/kg/day)[2]			Trend test p-value[3]
		0	125	250	
Liver	Hepatocellular adenoma	12/49	19/50	45/48***	<0.0001
	Hepatocellular carcinoma	7/49	3/50	12/49	NS
	Combined	17/49	20/50	46/49***	<0.0001
Forestomach	Squamous cell papilloma	1/43	9/49*	12/36***	0.0002
	Squamous cell carcinoma (r)	0/43	0/49	3/38	0.0236
	Combined	1/43	9/49*	15/38***	<0.0001
Epididymis	Histiocytic sarcoma (r)	0/40	2/48	4/33*	0.0177

NS = not significant
[1]Number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor.
[2]Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls
[3]Exact trend test conducted by OEHHA
* $p < 0.05$; *** $p < 0.001$. (r) denotes a rare tumor. See text for details.

Non-neoplastic findings

Both absolute and relative liver weights were significantly increased in the 125 and 250 mg/kg/day dose males at 12 and 24 months. At 24 months, absolute and relative liver weights in the 125 mg/kg/day dose group were increased by 29 and 24 percent, respectively, compared to the controls. Absolute and relative liver weights of the 250 mg/kg/day dose group were increased by 82 and 101 percent, respectively, compared to the controls. Absolute and relative kidney weights were also increased in the 125 mg/kg/day dose group at 24 months (10 and 7 percent greater than the controls, respectively).

There were no significant differences in hematological parameters. Histopathological examination revealed hepatocellular necrosis associated with compensatory cell proliferation in both the 125 and 250 mg/kg/day dose males. At the end of the two-year study, the incidence of single cell hepatocellular necrosis was increased in the treated males (96 percent in both doses vs. 10 percent in the controls). Individual necrotic hepatocytes were randomly located in centrilobular or midzonal portions of the liver lobules (Stebbins and Cosse, 1997). Liver inflammation was examined with a proliferating cell nuclear antigen staining assay and electron microscopy. Subacute to chronic liver inflammation was observed in 88 to 90 percent of the treated males compared to 10 percent in the controls at 24 months. Centrilobular multi focal pigment,

cytoplasmic inclusions, bile duct hyperplasia, hepatocellular foci of altered cells, and hepatocellular vacuolation were also observed in the high-dose males (US EPA, 2000).

Females

In the two-year study in female mice there were no differences in survival, body weight, or food consumption between the various dose groups and the controls. US EPA concluded that the dosing was adequate and not excessive to assess the carcinogenic potential of nitrapyrin in this study in female mice (US EPA, 2000).

Tumor incidences observed at the end of the two-year study in female mice are summarized in Table 4. Statistically significant increases were observed in hepatocellular adenomas and combined hepatocellular adenomas and carcinomas at both dose levels with positive dose-response trends. No hepatocellular tumors were observed at interim sacrifice.

The incidences of forestomach squamous cell papillomas and combined squamous cell papillomas and carcinomas were statistically significantly increased at both dose levels, with positive dose-response trends. Three forestomach squamous cell carcinomas were observed in the 250 mg/kg/day dose group, with none in controls. Forestomach squamous cell carcinomas are considered rare in female B6C3F_1 mice (background incidence of 0/1353) (Haseman *et al.*, 1998), and US EPA (2000) noted the biological significance of these carcinomas. No forestomach tumors were observed at interim sacrifice.

A significant increase in Harderian gland adenomas was noted in both doses, with a positive dose-response trend. The incidences of these tumors in both treated groups were above the concurrent control incidence and more than four-fold above the laboratory mean historical control incidence of 3.5 percent (US EPA, 2000). This mean laboratory historical control incidence is similar to that reported by National Toxicology Program (NTP) for female B6C3F_1 mice: 45/1353 (3.3 percent) (Haseman *et al.*, 1998). No Harderian gland tumors were observed at interim sacrifice.

Table 4. Incidence[1] of treatment-related lesions in female B6C3F$_1$ mice administered nitrapyrin in feed for two years (Stebbins and Cosse, 1997)

Tumor Site and Type		Dose (mg/kg/day)[2]			Trend test p-value[3]
		0	125	250	
Liver	Hepatocellular adenoma	6/47	27/48***	32/48***	<0.0001
	Hepatocellular carcinoma	0/47	1/48	2/48	NS
	Combined	6/47	28/48***	33/48***	<0.0001
Forestomach	Squamous cell papilloma	1/47	8/48*	21/48***	<0.0001
	Squamous cell carcinoma (r)	0/47	0/48	2/48	NS
	Combined	1/47	8/48*	22/48***	<0.0001
Harderian gland	Adenoma	1/47	8/48*	9/48**	0.0107

NS = not significant

[1]Number of tumor-bearing animals per number of animals alive at week 54. First liver tumor observed at week 75; first forestomach tumor observed at week 81; first Harderian gland tumor observed at week 81.

[2]Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls

[3]Exact trend test conducted by OEHHA

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (r) denotes a rare tumor. See text for details.

Non-neoplastic findings

Statistically significantly increased absolute and relative liver weights were present at 12 and 24 months in both doses groups. At 24 months, absolute and relative liver weights of the 125 mg/kg/day dose group were increased by 24 and 25 percent, respectively, compared to controls. Absolute and relative liver weights of the 250 mg/kg/day dose group were increased by 59 and 64 percent, respectively, compared to controls.

No significant differences in hematological parameters were observed. Histopathological examination revealed very slight to moderate hepatocellular necrosis associated with compensatory cell proliferation in both the 125 and 250 mg/kg/day dose groups. At the end of the two-year study, the total incidence of single cell hepatocellular necrosis was increased in the treated females (22 and 38 percent in the 125 and 250 mg/kg/day dose groups, respectively, vs. 6 percent in controls). The majority of the necrosis was very slight in severity. Hepatocellular foci of altered cells and hepatocellular vacuolation were seen in the 250 mg/kg/day dose group.

3.2.2.3 US EPA 2005 and 2012 reviews of the epididymal tumors observed in the male mouse studies of Quast et al. (1990) and Stebbins and Cosse (1997)

In 2005, US EPA (2005a) considered the information submitted by the registrant regarding epididymal tumors in the male mouse studies of Quast *et al.* (1990) and Stebbins and Cosse (1997). This included revised classification of epididymal tumors observed in the Quast *et al.* (1990) study and originally described as Leydig cell tumors to that of "undifferentiated sarcoma", according to an unpublished report by a "histopathology peer review and scientific advisory group" convened by the registrant (Hardisty, 2004). The registrant proposed to US EPA that the data for undifferentiated sarcoma of the epididymis be combined from the two male mouse studies by Quast *et al.* (1990) and Stebbins and Cosse (1997), as shown in Table 5 (and reported and discussed in US EPA, 2005a). The registrant concluded that the epididymal tumors seen in the 125 and 250 mg/kg/day dose groups were "a spurious finding as the result of biological variation unrelated to treatment with nitrapyrin" (as reported in US EPA, 2005a).

Table 5. Incidence[1] of undifferentiated sarcomas of the epididymis in male B6C3F$_1$ mice (Quast et al., 1990; Stebbins and Cosse, 1997, as reported in US EPA, 2005a)

Dose (mg/kg/day)	0 (control)	**0 (control)**	5	25	75	**125**	**250**
Undifferentiated sarcoma (%)	2/50 4%	**0/50 0%**	0/50 0%	0/50 0%	1/50 2%	**2/50 4%**	**4/50 8%**

[1]Incidences of the low dose study (Quast *et al.*, 1990) are unbolded and incidences of the high dose (Stebbins and Cosse, 1997) study are **bolded**.

US EPA (2005a) did not find that the epididymal tumors in the high dose group were spontaneous and a result of natural variation, and instead concluded that the "epididymal tumors are biologically significant and cannot be dismissed from the weight-of-evidence for carcinogenicity evaluation since they are malignant and a rare tumor in mice".

In 2012, US EPA considered additional information submitted by the registrant regarding epididymal tumors in the male mouse studies of Quast *et al.* (1990) and Stebbins and Cosse (1997). This included revised classification of the epididymal tumors from "undifferentiated sarcoma" to "histiocytic sarcoma" by a PWG convened by the registrant. In addition, tissues from all animals with a previously reported neoplasm of the epididymis were selected for immunohistochemical staining, and slides previously

stained with hematoxylin and eosin were reexamined. US EPA (2012a) summarized the additional data submitted by the registrant as shown in Table 6 below.

Table 6. Incidence of epididymal histiocytic sarcomas in male $B6C3F_1$ mice (Quast et al., 1990; Stebbins and Cosse, 1997, as reported in US EPA, 2012a)

	Quast *et al*. (1990)				Stebbins and Cosse (1997)		
Dose mg/kg/day	**0**	**5**	**25**	**75**	**0**	**125**	**250**
Number of animals examined	50	50	50	50	50	50	50
Number of animals with epididymis examined	50	8	10	50	50	50	50
Epididymis – histiocytic sarcomas	3	0	0	1	0	2	4

In evaluating this data, US EPA (2012a) states:

> "if one just looked at the Stebbins and [Cosse] study, it might appear that epididymal histiocytic sarcoma was related to Nitrapyrin treatment (none in the controls, two in the 125 mg/kg/day group and four in the 250 mg/kg/day group); however, the Quast data showed three in controls, none in either of the 5 and 25 mg/kg/day mice (in these two groups not all of the epididymides were evaluated microscopically) and only one in the 75 mg/kg/day (high dose) group. With the data from the two studies combined, it is clear that the occurrence of epididymal histiocytic sarcoma is incidental.... not related to the chemical."

Regarding comparison of the increased incidences observed in the Stebbins and Cosse (1997) study with historical controls, US EPA (2012a) noted that the range of historical control data for histiocytic sarcoma "hematopoietic, all sites" reported by NTP is "0.5% with a range of 0 – 4%.", and concluded:

> "Accordingly, the incidence seen at 125 and 250 mg/kg (2 and 4%, respectively) is within the NTP historical control range."

However, the correct percentages are 4 (2/50) and 8 (4/50) percent, respectively (Table 6), which are outside the NTP historical control range of 0 to 4 percent for histiocytic sarcoma "hematopoietic, all sites."

Discussion of the epididymal tumors observed in the male mouse studies

The US EPA Guidelines for Carcinogen Risk Assessment (US EPA, 2005b) provide the following guidance regarding historical control data:

> "The most relevant historical data come from the same laboratory and the same supplier and are gathered within 2 or 3 years one way or the other of the study under review; other data should be used only with extreme caution" (US EPA, 2005b).

In light of US EPA guidance on the appropriate timespan (+/- 2-3 years) for determining proper laboratory historical control data, it is apparent that caution should be used when comparing the control tumor incidences from the male mouse study of Quast *et al*. (1990, start date April 1987) with that from the male mouse study of Stebbins and Cosse (1997, start date August 1994), as the two studies were conducted seven years apart. Given this seven year difference, it is also apparent that each study should be compared to its own 'timespan appropriate' laboratory historical control data.

In the absence of laboratory historical control data, US EPA (2012a) looked to data from studies conducted by the NTP. Comparison of the epididymal histiocytic sarcoma incidence data from the Stebbins and Cosse (1997) $B6C3F_1$ mouse study performed in the Dow laboratory to the NTP's historical control data for histiocytic sarcoma occurring at all sites in $B6C3F_1$ mice is problematic, however. For example, the NTP historical control data are for histiocytic sarcomas at all sites, not just at the epididymis. Additionally, the Stebbins and Cosse (1997) study was not performed in the same laboratory as the NTP studies.

In fact, histiocytic sarcomas of the epididymis are considered rare in mice (Creasy *et al.*, 2012).

As reported above in Table 3, statistical analyses of the epididymal histiocytic sarcoma data from Stebbins and Cosse (1997) show a significant increase in the 250 mg/kg/day dose group ($p < 0.05$) (compared to the concurrent controls) with a positive and significant dose-response trend (exact trend test *p* value = 0.0177), indicative of a treatment-related effect.

3.2.2.4 US EPA 2005 review of the Harderian gland tumors observed in the female mouse studies of Quast et al. (1990) and Stebbins and Cosse (1997)

The registrant, Dow Chemical Company, proposed to US EPA that the data for Harderian gland tumors from the two female mouse studies by Quast *et al.* (1990) and Stebbins and Cosse (1997) be combined, as shown in Table 7 (and reported and discussed in US EPA, 2005a). The registrant's position was that if the control tumor incidence data from the two studies (Quast *et al.*, 1990; Stebbins and Cosse, 1997) are combined, the control incidence of Harderian gland adenomas in female mice becomes 6/100.

Due to the differences in control incidences between the two studies, the registrant concluded that the concurrent control group in the Stebbins and Cosse (1997) study has a "lower than expected incidence" for this tumor type (as reported in US EPA, 2005a). The registrant considered that the significant increase in incidence of Harderian gland tumors observed in nitrapyrin-treated female mice in the Stebbins and Cosse (1997) study is not treatment-related, but "it is simply a statistical anomaly resulting from the lower than expected incidence in the concurrent control group" (as reported in US EPA, 2005a).

Table 7. Incidence[1] of Harderian gland tumors in two female B6C3F$_1$ mice studies (Quast et al., 1990; Stebbins and Cosse, 1997, as reported in US EPA, 2005a)

Dose mg/kg/day	Quast *et al.* (1990)				Stebbins and Cosse (1997)		
	0	5	25	75	0	125	250
Harderian gland adenoma	5/50 (10%)	1/15[2]	3/10[3]	0/50 (0%)	1/50 (2%)	8/50 (16%)	9/50 (18%)

[1]Tumor incidence data as reported in US EPA (2005a) for the Quast *et al*. (1990) study are the number of tumor-bearing animals per number of animals examined; and for the Stebbins and Cosse (1997) study are the number of tumor-bearing animals per total number of animals in the dose group.
[2] Histopathologic examination of the Harderian gland was performed on only 15 animals in the 5 mg/kg/day dose group.
[3] Histopathologic examination of the Harderian gland was performed on only 10 animals in the 25 mg/kg/day dose group.

Using the data presented in Table 7, US EPA (2005a) noted significant differences in pairwise comparisons with concurrent controls for the 125 and 250 mg/kg/day dose groups, and a significant increasing trend. US EPA also noted that the tumor incidence in both the 125 and 250 mg/kg/day dose groups exceeded the historical control ranges for Harderian gland tumors in B6C3F$_1$ mice reported in the study archives provided by the registrant (4 to 14 percent), the NTP study archives (0 to 10 percent), and the 1989 Charles River Company study archives (0 to 4.3 percent) (US EPA, 2005a).

US EPA (2005a), in reaching its conclusion regarding the Harderian gland tumors, stated: 1) the incidence of Harderian gland tumors in the second study was "slightly outside the historical control range", 2) there was "a lack of a clear dose-response between 125 (16%) and 250 (18%) mg/kg/day", and 3) "the concurrent control for the second study is considered low relative to the first". US EPA (2005a) concluded that "the Harderian gland tumors were not considered to be treatment-related".

Discussion of the Harderian gland tumors observed in the female mouse studies

In light of the US EPA guidance noted earlier (US EPA, 2005b) on the appropriate timespan (+/- 2-3 years) for determining proper laboratory historical control data, it is apparent that caution should be used when comparing the control tumor incidences from the female mouse study of Quast *et al*. (1990, start date April 1987) with that from the female mouse study of Stebbins and Cosse (1997, start date August 1994), as the two studies were conducted seven years apart. Given this seven year difference, it is also apparent that each study should be compared to its own 'timespan appropriate' laboratory historical control data.

The US EPA Guidelines for Carcinogen Risk Assessment (US EPA, 2005b) provide the following guidance regarding interpretation of tumor incidence data:

> "Generally speaking, statistically significant increases in tumors should not be discounted simply because incidence rates in the treated groups are within the range of historical controls or because incidence rates in the concurrent controls are somewhat lower than average. Random assignment of animals to groups and proper statistical procedures provide assurance that statistically significant results are unlikely to be due to chance alone. However, caution should be used in interpreting results that are barely statistically significant or in which incidence rates in concurrent controls are unusually low in comparison with historical controls" (US EPA, 2005b).

As reported above in Table 4, statistical analyses of the Harderian gland adenoma data from Stebbins and Cosse (1997) show significant increases in both the 125 ($p < 0.05$) and 250 mg/kg/day dose groups ($p < 0.01$) (compared to the concurrent controls) with a positive and significant dose-response trend (exact trend test *p* value = 0.0107), indicative of a treatment-related effect.

3.3 Other Relevant Data

3.3.1 Pharmacokinetics and Metabolism

The pharmacokinetics of nitrapyrin has been studied *in vivo* in rats, mice, goats, and chickens in several unpublished studies, and in rats and dogs in two published studies. These are: two unpublished oral gavage studies in rats (Timchalk *et al.*, 1987; Domoradzki and Brzak, 1998), one published report on dietary administration to two rats (Redemann and Clark, 1967), one unpublished oral gavage study in mice (Domoradzki and Brzak, 1998), one unpublished dermal study in rats (Domoradzki and Gibson, 1997), one unpublished feeding study in chickens (Stafford, 1987b, as summarized in US EPA, 2004), another unpublished feeding study in goats (Stafford, 1987a, as summarized in US EPA, 2004), and one published report on dietary administration in dogs (Redemann *et al.*, 1966). These pharmacokinetic studies are discussed below.

3.3.1.1 Absorption

Oral Studies

Timchalk *et al.* (1987) presents a metabolism study on the oral administration of nitrapyrin to male and female Fischer 344 rats. ^{14}C-Nitrapyrin was administered by oral gavage to three groups of five male and five female F344 rats per group: a) a single dose of 1 mg/kg body weight, b) a single dose of 60 mg/kg body weight, or c) 14 daily doses of non-labeled nitrapyrin at 1 mg/kg/day, followed by a single dose of radiolabeled ^{14}C-nitrapyrin at 1 mg/kg on day 15. Non-labeled nitrapyrin (chemical purity of 93.3%) was spiked with ^{14}C-nitrapyrin (radiochemical purity of ≥99%). Radioactivity levels in the plasma, urine, and feces were monitored for 72 hours following administration of the ^{14}C-nitrapyrin dose. After the single oral dose of 1 or 60 mg/kg, plasma ^{14}C levels peaked at two hours post-treatment. Absorption half-lives ($t_{1/2}$) were 1.22 and 3.19 hours for the single treatments of 1 and 60 mg/kg, respectively, calculated from the plasma ^{14}C levels by the study authors. Although a slower absorption rate was noted in the 60 mg/kg group than the 1 mg/kg group, the total amounts of ^{14}C-nitrapyrin absorbed over a 72-hour period for both single-dose groups were proportional to the administered doses. Most of the radioactivity was excreted within the first 24 hours via the urine in all three treatment groups (77 to 83 percent), indicating that the chemical was rapidly absorbed through the gastrointestinal tract. A small amount of the radioactivity passed through the gastrointestinal tract unabsorbed (8 to 11 percent was found in the feces 24 hours post-treatment). There were no significant differences in the pharmacokinetics of nitrapyrin between the male and female rats or the single and multiple doses. Overall, these kinetic data demonstrate

that orally ingested nitrapyrin is rapidly and extensively absorbed via the gastrointestinal tract in rats (Timchalk *et al.*, 1987).

Following oral gavage administration, Domoradzki and Brzak (1998) examined the metabolism of nitrapyrin in male $B6C3F_1$ mice. Groups of 10 mice were given a single oral gavage dose of 25 or 250 mg/kg radiolabeled ^{14}C-nitrapyrin. Non-labeled nitrapyrin (chemical purity of ≥99%) was spiked with ^{14}C-nitrapyrin (radiochemical purity of 99.9%). Twenty-four hours post-treatment, 72 and 69 percent of the ^{14}C was recovered in the urine in the 25 and 250 mg/kg dose groups, respectively. While most of the radioactivity was absorbed rapidly from the gastrointestinal tract, some passed through unabsorbed (20 and 13 percent was recovered in the feces within 24 hours in the low- and high-dose groups, respectively).

Overall, ^{14}C-nitrapyrin was rapidly absorbed through the gastrointestinal tract upon oral exposure in both mice (Domoradzki and Brzak, 1998) and rats (Timchalk *et al.*, 1987).

Dermal study

Domoradzki and Gibson (1997) examined dermal absorption of ^{14}C-nitrapyrin in male F344 rats. Non-labeled nitrapyrin (chemical purity of 99.9%) was spiked with ^{14}C-nitrapyrin (radiochemical purity of 99%). Approximately 24 hours prior to dosing, the hair on the back of each rat was clipped. Dermal occlusive devices were constructed to hold test material on the skin without leaking and were fitted with barrier frames to prevent access to the dermal application site by the rat. Animals were housed individually. In the first group of four rats, a single dermal dose of 1 mg ^{14}C-nitrapyrin/cm^2 skin surface area was applied topically on a 10 cm^2 skin area. The dosed area was occluded, then the rats were sacrificed 24 hours post-dosing and the dose site was washed with an aqueous detergent solution. A second group of four male rats was given the same dose, but the dermal dose site was washed at 24 hours and then re-occluded until the animals were sacrificed at 72 hours post-dosing to determine the amount of ^{14}C-nitrapyrin absorbed post-washing. In the first group, 25 percent of the dose was absorbed (based on the total radioactivity found in the urine, feces, blood, kidneys, liver, and carcass). In the second group, 17 percent of the administered dose was excreted in the urine and feces in the first 24 hours. In this second group, absorption continued during the next 48 hours; 35 percent of the administered dose was recovered (based on the total radioactivity found in the urine, feces, blood, kidneys, liver, and carcass) 72 hours post-dosing. Substantial absorption of ^{14}C-nitrapyrin occurs following dermal exposure, with about 25 percent occurring in the first 24 hours and additional absorption occurring following washing of the skin (Domoradzki and Gibson, 1997).

3.3.1.2 Distribution

Following oral administration to F344 rats in the study by Timchalk *et al.* (1987), plasma ^{14}C levels peaked at two hours post-treatment and then gradually decreased within a 12 hour interval. A slower plasma clearance rate was observed in the 60 mg/kg single-dose group than in the 1 mg/kg single-dose group. Distribution of nitrapyrin was examined by measuring ^{14}C levels in tissue samples collected at 2, 10, 24, and 72 hours post-dosing from the liver, kidneys, plasma, and adipose tissue of the 60 mg/kg dose group. Radioactivity levels in the kidneys and fat peaked at a later time (10 hours post-treatment), followed by sharp decreases to 1 µg/g and non-detectable, respectively, at 72 hours post-treatment. At 72 hours post-treatment, irrespective of the doses, residual radioactivity in the body was minimal (ranging from 0.5 to 0.9 percent of the administered dose), with higher levels in the liver (0.48 to 0.84 percent of the administered dose), followed by negligible amounts in the kidneys, lungs, red blood cells, and plasma, and non-detectable in the fat. Nitrapyrin did not accumulate in any tissues. The study authors concluded that there are no significant differences in the overall disposition of nitrapyrin between sexes or among the three treatment protocols examined (two single dose and one multiple dose regimens) (Timchalk *et al.*, 1987).

After male F344 rats were dermally exposed to ^{14}C-nitrapyrin for 24 hours in the Domoradzki and Gibson (1997) study, the average total percentage of radioactivity present in the tissues was 4.5 percent of the applied dose (18 percent of the absorbed dose), with the majority in the carcass (3.6 percent of the applied dose) and trace levels in the liver, kidneys, and blood (0.62, 0.17, and 0.14 percent, respectively, of the applied dose). The second group of animals was exposed for 24 hours, followed by a skin wash at that time, then re-occluded and sacrificed at 72 hours post-dosing. The average total percentage of radioactivity in the tissues in this group was 0.81 percent of the applied dose (two percent of the absorbed dose), with the majority in the carcass (0.48 percent of the applied dose) and trace amounts in the liver, kidneys, and blood. Similar to oral administration, ^{14}C-nitrapyrin did not accumulate in the body following dermal exposure (Domoradzki and Gibson, 1997).

Following oral administration of radiolabeled nitrapyrin to mice in the Domoradzki and Brzak (1998) study, tissue burden at 72 hours accounted for less than one percent of the total administered radioactivity in both dose groups. In both groups, trace levels of radioactivity were found in the liver, skin, carcass, kidneys, and blood. These kinetic data suggest that, following rapid absorption through the gastrointestinal tract, nitrapyrin does not accumulate in the body in mice (Domoradzki and Brzak, 1998).

According to an unpublished feeding study in chickens (Stafford, 1987a, as summarized in US EPA, 2004), trace amounts of radioactivity were present in various tissues approximately six days following a single orally administered dose of ^{14}C-nitrapyrin. The highest level was detected in the liver, followed by the kidney, fat, and egg yolk. In another unpublished feeding study in goats (Stafford, 1987a, as summarized in US EPA, 2004), oral administration of ^{14}C-nitrapyrin resulted in trace amounts of radioactivity present in the kidney and liver at approximately three days post-dosing (Stafford, 1987a, as summarized in US EPA, 2004). These results demonstrate that oral intake of nitrapyrin does not result in significant accumulation in the body in chickens or goats.

3.3.1.3 Metabolism

Figure 2 illustrates the proposed metabolic pathway of nitrapyrin, based on studies in animals. The primary metabolites are 6-chloropicolinic acid (6-CPA) and its glycine conjugate, *N*-(6-chloropicolinoyl)glycine (6-CPG). Nitrapyrin undergoes oxidative dehalogenation at the trimethyl side group and hydrolysis to 6-CPA, which is conjugated with either glycine or taurine. The conjugation steps involve activation of 6-CPA with acetyl CoA, producing an acyl-CoA thioester that reacts with the amino group of an amino acid to form the conjugated metabolites (Domoradzki and Brzak, 1998).

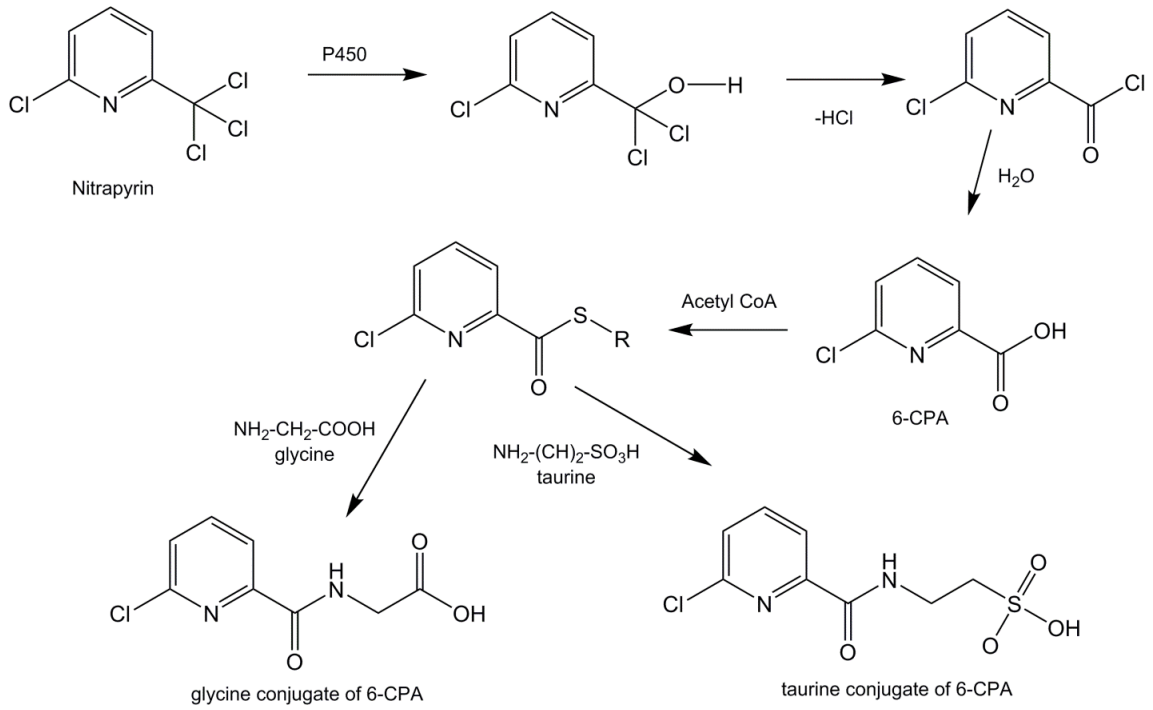


Figure 2. Proposed metabolic pathway of nitrapyrin in rodents (adapted from Domoradzki and Brzak, 1998).

Timchalk *et al.* (1987) observed that, following oral gavage in F344 rats, nitrapyrin was metabolized to 6-CPA and subsequently converted to its glycine conjugate, 6-CPG. Irrespective of the dose, 6-CPA and 6-CPG (in a ratio of approximately 40:60) were the only metabolites detected in the urine collected within the first 24 hours post-treatment. No nitrapyrin was found in the urine (Timchalk *et al.*, 1987). In another study, Domoradzki and Brzak (1998) identified nitrapyrin, 6-CPA, and 6-CPG in the urine collected over 24 hours post-treatment, from two male F344 rats exposed to a single oral gavage dose of 60 mg/kg nitrapyrin. The detection of unmetabolized nitrapyrin in the urine can be indicative of saturated nitrapyrin oxidation at high dose in the rats (Domoradzki and Brzak, 1998).

In the mouse study conducted by Domoradzki and Brzak (1998), following oral gavage of ^{14}C-nitrapyrin, four compounds were detected in the urine: nitrapyrin, 6-CPA, 6-CPG, and a taurine conjugate of 6-CPA. In the samples collected through 72 hours post-treatment, nitrapyrin (one percent of the administered dose) was identified in the samples from the 250 mg/kg group; no nitrapyrin was detected in samples from the 25 mg/kg group. The glycine conjugate of 6-CPA was the primary compound detected, comprising approximately 70 percent of the administered radioactivity in both treatment groups. Other minor urinary metabolites detected were 6-CPA (4 and 10 percent of the administered radioactivity in the 25 and 250 mg/kg dose groups, respectively) and the taurine conjugate of 6-CPA (approximately one percent of the administered radioactivity in both groups) (Domoradzki and Brzak, 1998). The study authors compared metabolites found in mice to those found in rats in the Timchalk *et al.* (1987) study, and concluded, “it appears that in the 0-24 hour interval that the mouse is able to convert a greater percentage of the dose found in the urine to the glycine conjugate of 6-CPA than is seen in the rat.” Additionally, there is no evidence that rats metabolize nitrapyrin to the taurine conjugate of 6-CPA (Domoradzki and Brzak, 1998).

Early studies conducted in dogs and rats fed radiolabeled ^{14}C-nitrapyrin in the diet also found 6-CPA and 6-CPG as the primary urinary metabolites. A female beagle dog was fed dog food containing 40 ppm nitrapyrin for three weeks before the beginning of a week of radiolabeled nitrapyrin feeding (tagged feeding). During the tagged feeding, the dog was fed two 120-gram portions containing 40 ppm ^{14}C-nitrapyrin eight hours apart. The authors found that most of the radiolabeled metabolite excreted in the urine was 6-CPG (Redemann *et al.*, 1966). In another study, two male albino rats were fed grains containing 100 ppm ^{14}C-labeled nitrapyrin. After a single day’s feeding, the two metabolites detected in the urine were 6-CPA (the major product) and 6-CPG (the minor product). Based on these two studies and kinetic studies completed in the same laboratory, the study authors hypothesized that 6-CPA is an intermediate in the formation of 6-CPG from nitrapyrin (Redemann and Clark, 1967).

US EPA (2004) reviewed additional metabolism studies of orally administered nitrapyrin conducted in chickens and goats, and concluded that the predominance of either 6-CPA or 6-CPG varies in different livestock tissues. Details on the timing of administration and collection methods were not specified by the US EPA report. In both chickens and goats, several unknown compounds are detected. According to US EPA, the chemical characterization in the metabolism studies suggested that the unknowns are conjugates of 6-CPA, but the studies do not specify which conjugates. In chickens, nitrapyrin, 6-CPA, 6-CPG, 2-chloro-6-(dichloromethyl)pyridine (DCM), and unknown compounds were detected in liver, fat, kidney, and egg yolk. In the liver, two unknown compounds were the predominant radioactive residues collected, along with minor amounts of 6-CPA. In chicken fat, the predominant compound present was nitrapyrin, with trace amounts of DCM. In chicken kidney, the predominant residue detected was an unknown, along with trace amounts of 6-CPA, nitrapyrin, 6-CPG, and DCM. In egg yolk, the primary compound detected was nitrapyrin, with trace amounts of 6-CPA, 6-CPG, DCM, and several unknowns (Stafford, 1987b, as summarized in US EPA, 2004).

As reported by US EPA (2004), nitrapyrin, 6-CPA, 6-CPG, DCM, and unknown compounds were detected in goat liver, kidney, and milk. In goat liver, the predominant compounds detected were unknowns, followed by 6-CPA, 6-CPG, DCM, and nitrapyrin. In goat kidney, the predominant compounds detected were unknowns, followed by 6-CPG, 6-CPA, and trace amounts of DCM and nitrapyrin. In goat milk, 6-CPG was the primary residue detected, with trace amounts of 6-CPA, DCM, and several unknowns (Stafford, 1987a, as summarized in US EPA, 2004).

The metabolism studies in mice, rats, and dogs indicate that nitrapyrin is metabolized to 6-CPA, which is then conjugated with glycine (or taurine in mice) and excreted. Although the US EPA (2004) report did not specify the methods used to identify metabolites or provide information on the identity of the unknowns, it appears that chickens and goats form additional metabolites besides 6-CPA and 6-CPG. These animals, therefore, may have slightly different metabolic profiles from mice and rats.

3.3.1.4 Excretion

Excretion of nitrapyrin was primarily via the urine in all mammalian species studied, following either oral or dermal administration.

Following oral administration of nitrapyrin to F344 rats in the study by Timchalk *et al.* (1987), similar excretion patterns were noted in all the dose groups. The total radioactivity recovery from the urine, feces, tissues, and cage wash was 95 to 99

percent of the administered dose, with the majority of the radioactivity recovered in the urine (80 to 85 percent), and 11 to 14 percent recovered in the feces over 72 hours post-treatment. Most of the radioactivity was excreted within the first 24 hours (a total of 85 to 94 percent of the administered dose), and ranged from 77 to 83 percent and 8 to 11 percent in the urine and feces, respectively. Thus, a small amount of ^{14}C-nitrapyrin likely passed through the gastrointestinal tract and was excreted in the feces without being absorbed. Very small amounts of ^{14}C were recovered in the feces after 24 hours (1.7 to 3.2 percent), suggesting that enterohepatic recirculation was minimal (Timchalk *et al.*, 1987).

In mice given a single oral gavage dose of nitrapyrin, most of the ^{14}C was eliminated within 24 hours post-treatment (Domoradzki and Brzak, 1998). Within 24 hours, 72 and 69 percent of the radioactivity was recovered in the urine in the 25 and 250 mg/kg dose groups, respectively. After 72 hours, total urinary excretion accounted for 76 and 82 percent of recovered radioactivity in the 25 and 250 mg/kg dose groups, respectively. The half-life for urinary elimination was about three hours for both doses. While most of the radioactivity was absorbed rapidly from the gastrointestinal tract, some passed through unabsorbed (20 and 13 percent was recovered in the feces within 24 hours in the low- and high-dose groups, respectively). Less than 4 percent of the radioactivity was recovered in the feces after 24 hours; therefore, significant enterohepatic recirculation is unlikely.

In dogs, at least 80 percent of the orally administered dose of nitrapyrin was found in the urine (Redemann *et al.*, 1966). In chickens, 88 percent of the nitrapyrin and its metabolites was found in the excreta, 0.1 percent in the egg yolk, and 0.004 percent in the egg white (Stafford, 1987b, as summarized in US EPA, 2004). A study in lactating goats found that 18 percent of the administered nitrapyrin (or its metabolites) appeared in the urine, 5 percent in the feces, and 0.06 percent in the milk (Stafford, 1987a, as summarized in US EPA, 2004).

Following dermal absorption in rats, the majority of the absorbed dose was excreted via the urine. At 24 hours following a single dermal dose of 1 mg ^{14}C-nitrapyrin/cm^2 skin surface area, in male F344 rats, 25 percent of the applied dose was absorbed. The amounts of radioactivity recovered in the urine and feces were 19 and 0.9 percent of the applied dose (78 and 4 percent of the absorbed dose), respectively. In a second group of male F344 rats, at 72 hours post-dosing, 35 percent of the applied dose was absorbed, with 31 percent of the applied dose being excreted via the urine and three percent via the feces (88 and 9 percent of the absorbed dose, respectively) (Domoradzki and Gibson, 1997).

Summary

Overall, these pharmacokinetic studies demonstrate that nitrapyrin is rapidly and extensively absorbed following oral administration in rats, mice, chickens, goats, and dogs. Dermal application of nitrapyrin also leads to a substantial uptake in rats. Upon entering the circulatory system, nitrapyrin is rapidly distributed throughout the body. Although both rats and mice metabolize nitrapyrin to 6-CPA and subsequently to 6-CPG, taurine conjugation of 6-CPA only occurs in mice. In addition to the glycine conjugate typically detected in rats and mice, other unidentified conjugated metabolites are formed in goats, chickens, and dogs. The metabolism of nitrapyrin may be saturable at high doses in rats. Nitrapyrin and its metabolites are unlikely to undergo enterohepatic recirculation in mice and rats, and do not accumulate to any significant extent in any tissues. Finally, the metabolites are rapidly eliminated primarily via urinary excretion in all species (with a bladder) studied. Chickens eliminate these metabolites in the excreta.

3.3.2 Genotoxicity

Nitrapyrin has been tested in a limited number of genotoxicity assays. Nitrapyrin was tested for the ability to induce gene mutations in *S. typhimurium* (three studies)*, E. coli* (one study), and CHO cells (one study); bone marrow MN formation *in vivo* (one study); and UDS (unscheduled DNA synthesis) in hepatocytes (one *in vitro* study and one *in vivo* study). One study in *S. typhimurium* is published in the peer-reviewed scientific literature (Zeiger *et al.*, 1988); the remaining studies are unpublished. The findings from the genotoxicity studies are summarized in Table 8 and described in greater detail below.

Table 8. Summary of *in vitro* and *in vivo* genotoxicity assays of nitrapyrin

Test system	System/ cell type	Dose/ concentration	Results[1]		Reference
			+ S-9	– S-9	
Gene mutation assays					
S. typhimurium reverse mutation	TA97	Up to 666 µg/plate	+	?[2]	Zeiger *et al.* (1988)
	TA98		+	-	
	TA100	Up to 333 µg/plate	+	-	
	TA1535		-	-	
S. typhimurium reverse mutation	TA97	Up to 500 µg/plate	-	-	Kennelly (1985), as reported by US EPA, 1992 [p. 18]; US EPA, 2005a [p. 3]; US EPA, 2012a [p. 13-14]; CDPR, 1997 [p. 8-9]; Yano *et al.*, 2008 [p. 54]
	TA98		-	-	
	TA100		-	-	
	TA1535		-	-	
S. typhimurium reverse mutation	TA98	Up to 1000 µg/plate (+S9) or 500 µg/plate (-S9)	+/-[3]	+/-[3, 4]	Mecchi (2007)[7]
	TA100		+/-[5]	+/-[5]	
	TA1535		+/-[6]	-	
	TA1537		-	-	
E. coli reverse mutation	WP2*uvr*A	Up to 500 µg/plate	-	-	Mecchi (2007)[7]
Mammalian cell forward mutation	CHO/HGPRT[8]	Up to 200 µg/ml	-	-	Linscombe and Gollapudi (1986), as reported by US EPA, 1992 [p. 18]; US EPA, 2005a [p. 3]; CDPR, 1997 [p. 9]; Yano *et al.*, 2008 [p. 54]
Micronucleus assays					
Mouse micronucleus formation	*In vivo* male and female mouse bone marrow	800 mg/kg sacrificed at 24, 48, and 72 hours	-		Kirkland (1985), as reported by US EPA, 1992 [p. 18]; US EPA, 2005a [p. 3]; CDPR, 1997 [p. 9]; Yano *et al.*, 2008 [p. 54]
Unscheduled DNA synthesis (UDS) assays					
Rat hepatocyte UDS	*In vitro* male rat hepatocytes	Up to 23 µg/ml	NT	-	Mendrala and Schumann (1982), as reported by US EPA, 1992 [p. 18]; US EPA, 2005a [p. 3]; CDPR, 1997 [p. 9]; Yano *et al.*, 2008 [p. 54]
Mouse liver UDS	Hepatocyte cultures harvested from livers of mice treated *in vivo*	Single oral dose of 0, 125, or 250 mg/kg, sacrificed 2-4 or 12-16 hrs to harvest hepatocytes	-		Pant and Celestin (2009), as reported by US EPA, 2012a [p. 13-14]

[1] Results as reported by study authors, unless otherwise specified.
[2] ?, Questionable mutagenic response
[3] Dose-related increases were observed without rat S-9 activation in the initial study and with or without S-9 activation in the follow-up study (as discussed by OEHHA)
[4] Weak positive in TA98, (as concluded by US EPA, 2012a)
[5] Dose-related increases were observed with S-9 in the initial study and with and without S-9 activation in the follow-up study (as discussed by OEHHA)
[6] Dose-related increases were observed with S-9 activation in the initial and follow-up studies (as discussed by OEHHA)
[7] Unpublished study released by US EPA, also reported by Yano *et al.* (2008) [p. 54] and US EPA (2012a) [p. 13-14])
[8] Chinese hamster ovary cells/Hypoxanthine-Guanine Phosphoribosyl-Transferase forward mutation assay
NT: Not tested

3.3.2.1 Gene mutation studies

Studies in *Salmonella typhimurium*

Zeiger et al. (1988)
The mutagenicity of 300 chemicals, including nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] (99% purity), was assessed in several *S. typhimurium* strains in a series of experiments where the investigators were blinded as to the identity of the test chemicals (Zeiger *et al.*, 1988). Specifically, nitrapyrin was tested in the bacterial reverse mutation assay in *S. typhimurium* strains TA97, TA98, TA100, and TA1535 using the preincubation method. The assays were run in both the absence of exogenous metabolic activation and in the presence of liver S-9 from Aroclor 1254-induced male Sprague-Dawley rats and Syrian hamsters (Zeiger *et al.*, 1988). In the preincubation method, the *Salmonella* culture is incubated with the test chemical in either the presence or absence of S-9 at 37º C for 20 minutes before plating.

Zeiger *et al.* (1988) described their data evaluation methods as follows:

> "Evaluations were made at both the individual trial and overall chemical levels. Individual trials were judged mutagenic (+), weakly mutagenic (+W), questionable (?), or nonmutagenic (-), depending on the magnitude of the increase of his^+ revertants, and the shape of the dose-response. A trial was considered questionable (?) if the dose-response was judged insufficiently high to support a call of "+W," if only a single dose was elevated over the control, or if the increase seen was not dose-related. The distinctions between a questionable mutagenic response and a nonmutagenic or weak mutagenic response are highly subjective. It was not necessary for a response to reach twofold over background for a chemical to be judged mutagenic."

> "A chemical was judged mutagenic (+) or weakly mutagenic (+W) if it produced a reproducible dose-related response over the solvent control in replicate trials. A chemical was judged questionable (?) if the results of individual trials were not reproducible, if increases in his^+ revertants did not meet the criteria for a "+W" response, or if only single doses produced increases in his^+ revertants in repeat trials. Chemicals were judged nonmutagenic (-) if they did not meet the criteria for a mutagenic or questionable response."

These methods are consistent with the data evaluation methods described in the *Handbook of Carcinogenic Potency and Genotoxicity Databases* (Gold and Zeiger, 1997):

> "A chemical was judged mutagenic (+) or weakly mutagenic (+w) if it produced a reproducible, dose-related increase in mutant colonies under any test condition. A chemical was judged questionable (?) if the results of individual trials were not reproducible, if increases in mutants did not meet the criteria for a "+w" response, or if only single doses produced increases in mutants in repeat trials. Chemicals were judged nonmutagenic (-) if they did not meet the criteria for a mutagenic or questionable response. A chemical was designated nonmutagenic only after it had been tested in at least 4 strains (i.e., TA98, TA100, TA1535, TA97, and/or TA1537), without activation and with rat and hamster S-9. A positive test result in one strain with one type of metabolic activation was sufficient to identify a chemical as a mutagen."

Nitrapyrin was reported by Zeiger *et al.* (1988) to be mutagenic in the *Salmonella* reverse mutation assay, inducing mutations in strains TA97, TA98, and TA100, but not in TA1535. Strains TA97 and TA98 detect frameshift mutations, while TA100 and TA1535 detect base pair substitutions. The data from the studies in TA97, TA98, and TA100 are briefly summarized below.

As shown in Table 9, nitrapyrin induced a dose-related increase in mean revertants per plate in *S. typhimurium* strain TA97 in the presence of metabolic activation with either 10% hamster liver S-9 or rat liver S-9. The authors concluded that nitrapyrin induced a mutagenic response in strain TA97 with hamster liver S-9 metabolic activation, a weak mutagenic response with rat liver S-9 activation, and a questionable response in the absence of S-9 metabolic activation (Zeiger *et al.*, 1988).

Table 9. Mutagenic response of nitrapyrin in *S. typhimurium* strain TA97 (Zeiger *et al*., 1988)

Dose (µg/plate)	Mean revertants per plate[1, 2]		
	No S-9	10% Aroclor 1254-induced hamster liver S-9	10% Aroclor 1254-induced rat liver S-9
	(?)[3]	(+)[3]	(+w)[3]
0 (solvent control[4])	177	195	181
10	187	220	186
33	248 (1.4)	248	186
100	186 (1.1)	262 (1.3)	203 (1.1)
333	t[5]	332 (1.7)	237 (1.3)
666		360 (1.9)	255 (1.4)
Positive control[6]	1405	965	647

[1]Results presented as mean his$^+$ revertants per plate; fold increase in parentheses
[2]A blank cell in a given column indicates that testing was not performed at that dose.
[3]Authors' data evaluation: ?, questionable response; +, mutagenic response; +w, weakly mutagenic response
[4]Dimethyl sulfoxide (DMSO)
[5]t: Complete clearing of background lawn (colonies not counted), indicative of cytotoxicity
[6]Positive control was 9-aminoacridine in assays without metabolic activation and 2-aminoanthracene in assays with metabolic activation.

As shown in Table 10, nitrapyrin induced a positive response in *S. typhimurium* strain TA98 in the presence of metabolic activation with either hamster or rat liver S-9. Dose-related increases in mean revertants per plate were observed in the presence of either 5%, 10%, or 30% hamster liver S-9, with more marked responses observed with increasing concentrations of hamster liver S-9. A clear dose-related increase in mean revertants per plate was also observed in the presence of 30% rat liver S-9. The authors concluded that nitrapyrin induced a mutagenic response in strain TA98 with 10% and 30% hamster liver S-9 and with 30% rat liver S-9, a weak mutagenic response with 5% hamster liver S-9, and a questionable response with 10% rat liver S-9 (Zeiger *et al.*, 1988).

Table 10. Mutagenic response of nitrapyrin in *S. typhimurium* strain TA98 (Zeiger *et al*., 1988)

Dose (µg/plate)	Mean revertants per plate[1,2]						
	No S-9	Aroclor 1254-induced hamster liver S-9			Aroclor 1254-induced rat liver S-9		
		5%	10%	30%	5%	10%	30%
	(-)[3]	(+w)[3]	(+)[3]	(+)[3]	(-)[3]	(?)[3]	(+)[3]
0 (solvent control[4])	17	28	25	21	27	23	17
10	13			22			
33	18	27	26	20	18	26	23
100	25s[5]	43 (1.5)	40 (1.6)	44 (2.0)	29	30 (1.3)	27 (1.6)
166		43 (1.5)	34 (1.4)		38 (1.4)	32 (1.4)	
333		42 (1.5)	46 (1.8)	70 (3.3)	46 (1.7)	30 (1.3)	47 (2.8)
666		54s (1.9)	51 (2)	73 (3.5)	25s	54 (2.3)	45 (2.7)
Positive control[6]	550			376			134

[1]Results presented as mean his[+] revertants per plate; fold increase in parentheses

[2] A blank cell in a given column indicates that testing was not performed at that dose.

[3]Authors' data evaluation: ?, questionable response; +, mutagenic response; +w, weakly mutagenic response; -, nonmutagenic response

[4]DMSO

[5]s: slight clearing of background lawn, indicative of some cytotoxicity

[6]Positive control was 9-aminoacridine in assays without metabolic activation and 2-aminoanthracene in assays with metabolic activation.

As shown in Table 11, nitrapyrin induced a positive response in *S. typhimurium* strain TA100 in the presence of metabolic activation with either hamster or rat liver S-9. Dose-related increases in mean revertants per plate were observed in the presence of 5%, 10% or 30% hamster liver S-9, with more marked responses observed with increasing concentrations of hamster liver S-9. Dose-related increases in mean revertants per plate were also observed in the presence of 10% and 30% rat liver S-9. The authors concluded that nitrapyrin induced a mutagenic response in strain TA100 with 10% and 30% hamster or rat liver S-9, a weak mutagenic response with 5% hamster liver S-9, and a questionable response with 5% rat liver S-9 (Zeiger *et al.*, 1988).

Table 11. Mutagenic response of nitrapyrin in *S. typhimurium* strain TA100 (Zeiger *et al*., 1988)

Dose (µg/plate)	Mean revertants per plate[1,2]						
	No S-9	Aroclor 1254-induced hamster liver S-9			Aroclor 1254-induced rat liver S-9		
		5%	10%	30%	5%	10%	30%
	(-)[3]	(+w)[3]	(+)[3]	(+)[3]	(?)[3]	(+)[3]	(+)[3]
0 (solvent control[4])	89	85	86	100	92	97	114
10	84			117 (1.2)			118
33	94 (1.1)	99	115 (1.3)	116 (1.2)	101 (1.1)	84	124 (1.1)
100	52s[5]	126 (1.5)	120 (1.4)	152 (1.5)	103 (1.1)	103	128 (1.1)
166		148 (1.7)	138 (1.6)		133 (1.5)	121 (1.2)	
333		153 (1.8)	152 (1.8)	208 (2.1)	148 (1.6)	159 (1.6)	173 (1.5)
666		119s (1.4)	171 (2.0)	245 (2.5)	84s	176s (1.8)	205 (1.8)
Positive control[6]	215			696			267

[1]Results presented as mean his$^+$ revertants per plate; fold increase in parentheses
[2]A blank cell in a given column indicates that testing was not performed at that dose.
[3]Authors' data evaluation: ?, questionable response; +, mutagenic response; +w, weakly mutagenic response; -, nonmutagenic response
[4]DMSO
[5]s: slight clearing of background lawn, indicative of some cytotoxicity
[6]Positive control was sodium azide in assays without metabolic activation and 2-aminoanthracene in assays with metabolic activation.

Kennelly (1985), as reported by US EPA (1992; 2005a; 2012a); CDPR (1997); Yano et al. (2008)

In an unpublished study using a plate incorporation protocol in *S. typhimurium* strains TA97, TA98, TA100, and TA1535 with or without rat liver S-9 activation, nitrapyrin (unspecified purity) at doses up to 500 µg/plate did not produce any positive mutagenic responses (Kennelly, 1985, as reported by US EPA, 1992; 2005a; 2012a; CDPR, 1997; Yano *et al.*, 2008). The doses of nitrapyrin were 0, 0.8, 4, 20, 100, and 500 µg/plate. The original study data were unavailable. According to the discussion of this study in

the US EPA (2005a) report, this plate incorporation study did not include a preincubation component and was tested with rat liver S-9 activation, but not with hamster liver S-9 activation (US EPA, 2005a).

Mortelmans and Zeiger (2000) indicate that, with few exceptions, the *S. typhimurium* reverse mutation assay preincubation protocol is more sensitive than the plate incorporation protocol. This is because the preincubation step provides greater opportunity for short-lived mutagenic metabolites to react with the bacterial DNA. Additionally, the use of hamster liver S-9 fraction for metabolic activation may be more sensitive than rat liver S-9 for some compounds (Prival and Mitchell, 1981; Haworth *et al.*, 1983; Kamber *et al.*, 2009).

Mecchi (2007)

Mecchi (2007) used the same preincubation protocol as the Zeiger *et al.* (1988) study to test nitrapyrin (unspecified purity) in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 with or without 10% rat S-9 activation (Tables 12 and 13) (unpublished study submitted to the US EPA and summarized in US EPA, 2012a). An initial study and a follow-up study with slight changes in dose (referred to as the confirmatory study by the author) were done. As shown in Tables 12 and 13, dose-related increases in the number of revertants per plate were observed in strains TA98, TA100, and TA1535 in both studies, but the higher doses tested often displayed a decrease in mean response, followed by signs of cytotoxicity. The dose-response curve for Ames (*Salmonella*) test data is often not monotonic, and commonly displays a decrease in mean response at high doses due to cytotoxicity of the tested chemical (Margolin, 1985). In strain TA98, a dose-related increase was observed without rat S-9 activation in the initial study and with or without rat S-9 activation in the follow-up study (Tables 12 and 13). In both the initial and follow-up studies, a greater than two-fold increase in revertants per plate was observed at a dose of 50 µg/plate without activation and in the follow-up study a two-fold or greater increase occurred at doses of 100 and 150 µg/plate with S-9 activation. Although not as strong a positive response, increases were observed in strain TA100 with S-9 in the initial study and with and without activation in the follow-up study and in strain TA1535 with S-9 activation in both the initial and follow-up studies. In strain TA1537 increases in mean revertants per plate were observed in the initial study only at one dose level without S-9, and without an apparent dose-response in the initial and follow-up studies with S-9, and in the follow-up study without S-9.

Mecchi (2007) employed the following criteria for determining whether a mutagenic response was observed in the four tester strains: at least a two-fold (for strain TA100) or three-fold (for strains TA98, TA1535, and TA1537) concentration-related and reproducible increase as compared to controls in mean revertants per plate. The author

concluded that, in the initial study, no positive increases in the mean number of revertants per plate were observed with any of the tester strains in either the presence or absence of S-9 activation. The author noted that an increase was observed with TA100 in the presence of S-9, but the increase did not meet the two-fold criteria needed for a positive evaluation. With regard to the follow-up study, Mecchi (2007) concluded that no positive increases were observed in any of the strains in the presence or absence of S-9 activation. The author noted that an increase in the mean number of revertants per plate was observed with TA100 in the presence of S-9, but, again, did not meet the two-fold requirement for a positive evaluation. In addition, an increase was also observed with TA98 in the presence and absence of S-9 activation, but it did not meet the three-fold criteria for a positive evaluation. Mecchi (2007) concluded that nitrapyrin did not cause a positive increase in mutations with any of the tester strains either in the presence or absence of S-9 activation.

Table 12. Mutagenic response of nitrapyrin in *S. typhimurium* in initial study (Mecchi, 2007)

Dose (µg/ plate)	Mean revertants per plate[1,2]							
	TA98		TA100		TA1535		TA1537	
	No S-9[3]	10% rat liver S-9	No S-9	10% rat liver S-9	No S-9	10% rat liver S-9	No S-9	10% rat liver S-9
0 (solvent control[4])	13	15	100	107	10	8	6	6
1			81		11		5	
5	21 (1.6)		102		10		6	
10	19 (1.5)	16	111 (1.1)	110	15 (1.5)	7	5	8 (1.3)
25	25 (1.9)	14	116 (1.2)	106	10	12 (1.5)	6	11 (1.8)
50	32 (2.5)	24 (1.6)	103	161 (1.5)	10	14 (1.8)	17 (2.8)	8 (1.3)
75	25 (1.9)							
100	3[5]	26 (1.7)	0[5]	181 (1.7)	4[5]	13 (1.6)	3[5]	12 (2.0)
200	0[5]	7[5]		15[5]		6[5]		7[5]
500		0[5]		0[5]		0[5]		0[5]
1000		0[5]		0[5]		0[5]		0[5]
Positive control	286[6]	274[7]	855[8]	1028[9]	781[8]	122[9]	1390[10]	160[9]

[1]Results presented as mean his$^+$ revertants per plate; fold increase in parentheses
[2]A blank cell in a given column indicates that testing was not performed at that dose.
[3]The mean vehicle control value in the initial mutagenicity study was not within the acceptable range for strain TA98, so the test was repeated in TA98 without S-9 activation. Results shown are from the repeat study.
[4]DMSO
[5]Reduced background lawn, indicative of cytotoxicity
[6]Positive control was 2-nitrofluorene
[7]Positive control was benzo[*a*]pyrene
[8]Positive control was sodium azide
[9]Positive control was 2-aminoanthracene
[10]Positive control was ICR-191 (acridine mutagen)

Table 13. Mutagenic response of nitrapyrin in *S. typhimurium* in follow-up study (Mecchi, 2007)

Dose (µg/ plate)	Mean revertants per plate[1,2]							
	TA98		TA100		TA1535		TA1537	
	No S-9	10% rat liver S-9	No S-9	10% rat liver S-9	No S-9	10% rat liver S-9	No S-9	10% rat liver S-9
0 (solvent control[3])	14	24	90	114	8	10	6	9
5	21		109 (1.2)		11 (1.4)		8	
10	21	18	116 (1.3)	109	14 (1.8)	11	11 (1.8)	8
25	25 (1.8)	19	132 (1.5)	132 (1.2)	9 (1.1)	9	6	6
50	37 (2.6)	27 (1.1)	141 (1.6)	143 (1.3)	11 (1.4)	14 (1.4)	9 (1.5)	9
100	0[4]	48 (2.0)	24[4]	217 (1.9)	7[4]	15 (1.5)	5[4]	16 (1.8)
150		61 (2.5)		184 (1.6)		13 (1.3)		9
200	0[4]	43 (1.8)	1[4]	133 (1.2)[4]	0[5]	8[4]	0[4]	13 (1.4)
500		0[4]		0[5]		0[4]		0[5]
Positive control	225[6]	285[7]	1024[8]	940[9]	588[8]	84[9]	1078[10]	119[9]

[1]Results presented as mean his$^+$ revertants per plate; fold increase in parentheses
[2]A blank cell in a given column indicates that testing was not performed at that dose.
[3]DMSO
[4]Reduced background lawn, indicative of cytotoxicity
[5]Absent background lawn, indicative of cytotoxicity
[6]Positive control was 2-nitrofluorene
[7]Positive control was benzo[*a*]pyrene
[8]Positive control was sodium azide
[9]Positive control was 2-aminoanthracene
[10]Positive control was ICR-191 (acridine mutagen)

Studies in *Escherichia coli*

Mecchi (2007)

Mecchi (2007) also tested nitrapyrin in a reverse gene mutation assay in *E. coli* (WP2*uvr*A) using the preincubation protocol in two studies. WP2*uvr*A detects base pair substitutions. Concentrations tested were 25, 50, 100, 200, 500, and 1000 µg/plate in the presence of S-9 mix and 10, 25, 50, 100, 200, and 500 µg/plate in the absence of S-9 mix in the initial study, and 10, 25, 50, 100, 200, and 500 µg/plate in the presence of S-9 mix and 5, 10, 25, 50, 100, 200, and 500 µg/plate in the absence of S-9 mix in the follow-up study. 2-Aminoanthracene was used as the positive control with S-9 mix and 4-nitroquinoline-N-oxide without S-9 mix. Nitrapyrin did not induce mutations in *E. coli* (WP2*uvr*A) at doses up to 1000 µg/plate.

Studies in mammalian cells

Linscombe and Gollapudi (1986), as reported by US EPA (1992; 2005a); CDPR (1997); Yano et al. (2008)
In an unpublished study, a hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) forward mutation assay was conducted in mammalian CHO cells with nitrapyrin (purity: 93.4%) at doses up to 200 µg/plate with and without rat liver activation. Concentrations tested were 0, 20, 40, 60, 80, 100 µg/ml without S-9 (five plates per concentration, three trials) and 0, 120, 140, 160, 180, 200 µg/ml with S-9 (one trial). There was no increase in mutation frequency (unpublished study submitted to US EPA, Linscombe and Gollapudi, 1986, as reported by US EPA, 1992; 2005a; CDPR, 1997; Yano *et al.*, 2008).

Micronucleus assay

Kirkland (1985), as reported by US EPA (1992; 2005a); CDPR (1997); Yano et al. (2008)
In an unpublished study, nitrapyrin did not induce chromosomal aberrations (CA) in a mouse micronucleus assay *in vivo*. Male and female mice (5/sex/group) were administered a single oral dose of 800 mg/kg nitrapyrin (purity: 90.64%) and sacrificed after 24, 48, or 72 hours. There were no increases in MN in the harvested bone marrow cells (unpublished study submitted to US EPA, Kirkland, 1985, as reported by US EPA, 1992; 2005a; CDPR, 1997; Yano *et al.*, 2008).

Unscheduled DNA synthesis (UDS) studies

Mendrala and Schumann (1982), as reported by US EPA (1992; 2005a); CDPR (1997); Yano et al. (2008)
In an unpublished study, nitrapyrin did not induce UDS in cultures of rat hepatocytes exposed *in vitro* to nitrapyrin (purity: 92%) at doses up to 23 µg/ml (unpublished study submitted to the US EPA, Mendrala and Schumann, 1982, as reported by US EPA, 1992; 2005a; CDPR, 1997; Yano *et al.*, 2008).

Pant and Celestin (2009), as reported by US EPA (2012a)
In an unpublished *in vivo* UDS assay, six male $B6C3F_1$ mice per dose were administered a single oral dose of 125 or 250 mg/kg body weight nitrapyrin (purity: 98.6%). Two additional groups of six mice each received a single oral dose of 10 ml/kg corn oil or 10 mg/kg dimethylnitrosamine as the negative and positive controls, respectively. Animals were sacrificed two to four hours or 12 to 16 hours after dosing, and the livers were removed for hepatocyte culture. No increases in UDS were

observed in the cell cultures (unpublished study submitted to US EPA, Pant and Celestin, 2009, as reported by US EPA, 2012a).

US EPA review of genotoxicity data

US EPA reviewed the available genotoxicity data for nitrapyrin in 1992, 2000, 2005, and 2012. In 1992, US EPA reviewed the *Salmonella* reverse mutation studies published by Zeiger *et al.* (1988) as well as the unpublished studies in *Salmonella* by Kennelly (1985), the CHO cell gene mutation assay by Linscombe and Gollapudi (1986), the *in vivo* mouse bone marrow MN assay by Kirkland (1985), and the *in vitro* rat hepatocyte UDS assay by Mendrala and Schumann (1982). In that review, US EPA concluded "there is apparent genotoxic potential for nitrapyrin" (US EPA, 1992). In 2000, US EPA reviewed the same genotoxicity studies, and concluded "although the mechanistic data are inadequate, the available mutagenicity data are supportive of a mutagenic mode of action" (US EPA, 2000). In 2005, the same genotoxicity data were reviewed and US EPA stated "the genotoxic potential [of nitrapyrin] cannot be discounted" (US EPA, 2005a).

In 2012, US EPA included the following additional information on genotoxicity in its review: the unpublished mutagenicity studies conducted in *Salmonella* and *E. coli* by Mecchi (2007), the unpublished UDS study conducted in mice exposed to nitrapyrin *in vivo* by Pant and Celestin (2009), and an unpublished report by Zeiger (2010). The 2012 review states: "in accordance with the U.S.EPA's *Framework for Determining a Mutagenic Mode of Action and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (US EPA, 2007)[13], the [US EPA] concluded that in absence of a mutagenic effect in at least two *in vivo* mutagenicity studies, there is no concern for a mutagenic mode of action" (US EPA, 2012a)[14].

Discussion and summary

The change in the US EPA's assessment of the genotoxic potential of nitrapyrin (US EPA, 2012a) was in large part based on a recent unpublished report authored by Zeiger (2010) and submitted to the Agency by the registrant. Zeiger (2010) states:

[13] This 2007 US EPA document is a draft that was released for external review and has never been finalized.

[14] The US EPA's 2012 review of nitrapyrin misquotes the language from the draft 2007 Framework document, which actually states, "Generally, for a chemical to be considered to have a mutagenic MOA [mode of action] for carcinogenicity, the data will include positive responses from one or more *in vivo* studies that are generally supported by *in vitro* gene mutation or cytogenetic assays" (US EPA, 2007).

> "The different conclusions by the authors in the tests with S9 resulted from the criteria used to determine a positive response. Both Kennelly (1985) and Mecchi (2007) used the "two-fold" rule for determining a positive response… In contrast, Zeiger *et al*. (1988) required only a reproducible, concentration-related response, with no requirement for a two- or three-fold increase over the solvent control background value. An examination of the test results shows that the fold increases obtained by Mecchi (2007) when 10% rat liver S9 was used tended to be equivalent to, or higher than, the responses obtained by Zeiger *et al*. (1988); the differences in judgment of positive or negative, therefore, were based solely on the criteria for a positive response."

However, long-standing data evaluation methods for the *Salmonella* reverse mutation assay published by Zeiger *et al.* (1988) and the *Handbook of Carcinogenic Potency and Genotoxicity Databases* (Gold and Zeiger, 1997) state that a two-fold increase over solvent control is not necessary for a compound to be judged mutagenic. Moreover, as explained by Mortelmans and Zeiger (2000) and Kim and Margolin (1999), the "two-fold" or "three-fold" approach has several weaknesses, such as a lack of validity as a decision rule for the Ames assay and concerns that it may be too conservative.

The 2012 US EPA assessment also noted with regard to the *Salmonella* reverse mutation studies by Mecchi (2007) that "the fold increases, which were within the established ranges of spontaneous mutant colonies for this strain (15-75 spontaneous revertants/plate) and the acceptable range of the reporting laboratory (8-60 revertants/plate) resulted from the extremely low spontaneous reversion frequency of strain TA98 (13-24 revertants/plate)" (US EPA, 2012a). However, since there is some variation between laboratories in the number of spontaneous revertant colonies, Mortelmans and Zeiger (2000) indicate that mutation frequencies observed in a given laboratory should be compared to that laboratory's historical control values and not to values from other laboratories.

Guidance on the testing and interpretation of bacterial reverse mutation tests was developed by US EPA and the Organisation for Economic Co-operation and Development (OECD) in the late 1990s (OECD, 1997; US EPA, 1998). This guidance states:

> "There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of the results should be considered first. Statistical methods may be

used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response" (OECD, 1997; US EPA, 1998).

Additional guidance for the interpretation of *in vitro* and *in vivo* genotoxicity studies has been developed by an Expert Working Group of the International Association of Environmental Mutagen Societies, published as Thybaud *et al.* (2007):

> "In some cases a clear and reproducible positive *in vitro* result is seen, yet the other assays in the initial battery, including any required *in vivo* test, are negative. The *in vitro* [positive] result is not automatically overruled by the negative *in vivo* result, and some follow-up testing or investigation is generally necessary to determine the relevance of the *in vitro* positive result….It might be assumed that the concern about the positive *in vitro* result lessens as the number and types of negative *in vivo* assay results increase. However, this assumption may not be valid since the *in vivo* assays may have different sensitivities and/or evaluate different genotoxic endpoints. It is important that relevant endpoints are examined in the most relevant tissues *in vivo*" (Thybaud *et al.*, 2007).

This is consistent with guidance from the International Programme on Chemical Safety (IPCS) Harmonized Scheme for Mutagenicity Testing, published as Eastmond *et al.* (2009), which states:

> "…multiple negative results may not be sufficient to remove concern for mutagenicity raised by a clear positive result in a single mutagenicity assay" (Eastmond *et al.*, 2009).

In summary, nitrapyrin has been assessed for a limited number of genotoxicity endpoints. Nitrapyrin did not induce reverse mutations in *E. coli* strain WP2*uvr*A in an unpublished study (Mecchi, 2007), HGPRT mutations in mammalian CHO cells (Linscombe and Gollapudi, 1986, as reported by US EPA, 1992; 2005a; CDPR, 1997; Yano *et al.*, 2008), MN in mouse bone marrow *in vivo* (Kirkland, 1985, as reported by US EPA, 1992; 2005a; CDPR, 1997; Yano *et al.*, 2008), or UDS *in vitro* in rat hepatocytes (Mendrala and Schumann, 1982, as reported by US EPA, 1992; 2005a; CDPR, 1997; Yano *et al.*, 2008) or *in vivo* in mouse liver (Pant and Celestin, 2009, as reported by US EPA, 2012a). In the *Salmonella* reverse mutation assay, nitrapyrin did induce concentration-related and reproducible increases in mutations in two studies (Zeiger *et al.*, 1988; Mecchi, 2007), but not in a third (Kennelly, 1985, as reported by US EPA, 1992; 2005a; 2012a; CDPR, 1997; Yano *et al.*, 2008). Specifically, a concentration-related and reproducible increase was observed in *S. typhimurium* strains

TA97, TA98, and TA100 in the presence of metabolic activation, with a greater response generally observed in the presence of hamster liver S-9, as compared with rat liver S-9 in the studies by Zeiger *et al.* (1988), and in strains TA98 and TA100 with or without rat liver S-9, and in TA1535 with S-9 in the studies by Mecchi (2007).

3.3.3 Animal Tumor Pathology

Rats

Renal tubule cell adenomas and adenocarcinomas were increased in male F344 rats treated with nitrapyrin (Szabo *et al.*, 1989). Both tumors are rare in male F344 rats, based on the reported spontaneous incidence of these tumors in untreated male rats of this strain. Reported incidences of renal tubule cell adenomas in untreated male F344 rats are 10/1352 (0.7%) (Haseman *et al.*, 1998), 5/2320 (0.2%) (Solleveld *et al.*, 1984), and 1/1794 (0.05%) (Goodman *et al.*, 1979), and the reported incidence of renal tubule cell adenocarcinomas in untreated male F344 rats is 2/1794 (0.11%) (Goodman *et al.*, 1979). Histological alterations in the kidneys noted by Szabo *et al.* (1989) included protein droplet accumulation in the epithelial cells of the proximal convoluted tubules at 12 months, increases in the severity of chronic progressive glomerulonephropathy, and the presence of primary renal tumors at 24 months.

Mice

Increases in the incidences of tumors of the liver, forestomach, and epididymis were observed in male B6C3F_1 mice treated with nitrapyrin, and in the liver, forestomach and Harderian gland of treated female B6C3F_1 mice.

Liver tumors

Liver adenomas and carcinomas were increased in both sexes of B6C3F_1 mice treated with nitrapyrin in the studies by Stebbins and Cosse (1997). In the unpublished report from the histopathology peer review and scientific advisory group convened by the registrant, Dow Chemical Company, (Hardisty, 2004), liver changes were described as “a progression of change from adaptation (hepatocellular) to hepatocellular toxicity (necrosis) to regenerative hyperplasic and eventually hepatocellular neoplasia”.

Hepatic adenomas and carcinomas arise from the same cell type, and adenomas can progress to carcinomas (Harada *et al.*, 1999). Hepatocellular carcinomas often have irregular borders, demonstrate necrosis and hemorrhage, and are not easily

distinguished from surrounding tissue. Abnormal growth patterns and cellular atypia are indicative of these tumors (Harada *et al.*, 1999).

Forestomach tumors

Forestomach squamous cell papillomas and carcinomas were increased in male and female B6C3F_1 mice treated with nitrapyrin in the studies of Stebbins and Cosse (1997). In these studies, treatment-related focal or multifocal hyperplasia of the nonglandular mucosa of the stomach was observed. These foci of hyperplasia were frequently accompanied by treatment-related focal or multifocal hyperkeratosis. In reviewing the pathological changes in the nonglandular stomach mucosa, the histopathology peer review and scientific advisory group convened by Dow Chemical Company stated, "Discrete foci of mucosal hyperplasia, papilloma, and squamous cell carcinoma of the mucosa of the forestomach probably represent a continuum in the development of treatment-related hyperplasia" (Hardisty, 2004). Forestomach carcinomas are rare in untreated B6C3F_1 mice, with incidences of 2/1355 (1.3%) in males and 0/1353 in females in historical controls from NTP studies conducted from 1990 to 1997 (Haseman *et al.*, 1998). Squamous cell carcinomas may arise from benign preexisting tumors or directly from altered mucosal epithelium (IARC, 2003).

Epididymal tumors

Epididymal tumors were increased in male B6C3F_1 mice treated with nitrapyrin in the study by Stebbins and Cosse (1997), and were identified by the study authors as undifferentiated epididymal sarcomas. In the Quast *et al.* (1990) study, three epididymal tumors were observed in the control group, and one was observed in the 75 mg/kg/day dose group. Quast *et al.* (1990) classified three of the epididymal tumors as Leydig cell tumors (two in the controls and one in the high dose) and one as a histiocytic sarcoma (control). In the unpublished report from the histopathology peer review and scientific advisory group convened by the registrant (Hardisty, 2004), the epididymal tumors seen in the Quast *et al.* (1990) study were reclassified to undifferentiated epididymal sarcomas. The report noted that the tumors originally described as Leydig cell tumors and those described as undifferentiated sarcomas have the same histology. US EPA's consulting pathologist concurred, superseding the original diagnosis. In 2005, US EPA reclassified all the epididymal tumors as undifferentiated sarcomas (US EPA, 2005a). Hardisty (2004) and the registrant, Dow Chemical Company, considered these tumors "a spurious finding as the result of biological variation unrelated to treatment of nitrapyrin." Historical control data were not provided. However, US EPA (2005a) determined that undifferentiated sarcoma is rare and malignant and thus biologically significant.

Following the US EPA's 2005 review, the registrant sponsored a PWG consisting of five expert veterinary pathologists which reevaluated the epididymal tumors (unpublished report, Hardisty, 2010). The PWG reexamined the slides from tissue sections of the epididymis and/or liver from all animals with a previous diagnosis of undifferentiated sarcoma or histiocytic sarcoma in the epididymis or liver in the studies of Quast *et al.* (1990) and Stebbins and Cosse (1997). Slides were stained with hematoxylin and eosin or stained immunohistochemically with primary antibodies against Mac-2 and F4/80 histiocyte/macrophage markers. Expression of Mac-2 and F4/80 is a distinguishing feature used to differentiate histiocytic sarcomas from Leydig cell adenomas (Creasy *et al.*, 2012). The PWG determined that the epididymal tumors in the Quast *et al.* (1990) and Stebbins and Cosse (1997) studies stained positive in immunohistochemical assays using the antibodies against the two histiocyte/macrophage markers (Hardisty, 2010) and, therefore, reclassified the epididymal tumors as histiocytic sarcomas. The PWG further described the histiocytic sarcomas as "infiltrative proliferations of round to oval cells in the interstitial regions of the epididymis surrounding the epididymal ducts." The neoplasms are reported to be "well circumscribed and appeared to be of low-grade malignancy with little evidence of aggressive local growth, no evidence of metastasis, and mitotic figures are uncommon" (Hardisty, 2010).

Histiocytic sarcomas can also be referred to as reticulum cell sarcoma type A, histiocytic lymphoma, and histiogenic tumor. Histiocytic sarcoma is rare and has a poor prognosis in humans (Vos *et al.*, 2005). Histiocytic sarcoma of the epididymis is a rare spontaneous tumor that arises from undifferentiated mesenchymal or histiocytic cells in mice, and may invade epididymal tissues and metastasize to other organs, mainly the peritoneum and liver (Creasy *et al.*, 2012). Historical control incidence data for epididymal histiocytic sarcomas are not available for $B6C3F_1$ mice. US EPA (2012a) noted that NTP has data on the incidence of histiocytic sarcomas 'hematopoietic, all sites' in untreated $B6C3F_1$ mice (mean 0.5%, with a range of 0-4%), but that no data were available from NTP on the spontaneous incidence of histiocytic sarcoma in the epididymis.

US EPA's consulting pathologist agreed with the reclassification of the epididymal tumors to histiocytic sarcomas, and stated that, "With the data from the two studies combined, it is clear that the occurrence of epididymal histiocytic sarcoma is incidental…not related to the chemical." (US EPA, 2012a). (See Section 3.2.2.3 for more discussion of these data.)

Harderian gland tumors

Harderian gland adenomas were increased in female B6C3F$_1$ mice treated with nitrapyrin in the study by Stebbins and Cosse (1997). The Harderian gland is an accessory lacrimal gland in the inner corner of the eye that excretes fluid to facilitate the movement of the third eyelid in some vertebrates (Huff *et al.*, 1989). Although the Harderian gland is not present in humans, these tumors are considered applicable in the assessment of carcinogenicity (Huff, 1992). Cells in these tumors are cuboidal to tall columnar with abundant, foamy, pale cytoplasm. The mitotic rate of these cells is low; tumors are not typically encapsulated; and they may occur in a number of different patterns, including cystic, papillary, and acinar (Botts *et al.*, 1999). All three of these pleomorphic types may progress to adenocarcinomas (Sheldon *et al.*, 1983). The mean historical control incidence of Harderian gland adenomas was 3.5% in the laboratory that conducted the study (US EPA, 2000). This is similar to the control incidence of Harderian gland adenomas in NTP carcinogenicity studies (45/1353; 3.3%) (Haseman *et al.*, 1998).

3.3.4 Structure Activity Considerations

Nitrapyrin (2-chloro-6-(trichloromethyl)pyridine) is composed of a six-membered heterocyclic aromatic ring containing a nitrogen atom (a pyridine ring), with chlorine and trichloromethyl substitutions ortho to the pyridyl nitrogen (also known as the ring nitrogen). Eleven structurally-similar chemicals are chosen for the structure activity comparison with nitrapyrin, based on the following criteria: (1) the presence of a pyridine ring, (2) with none, one, or two aromatic chlorine substitution(s) and none or one aromatic chloromethyl substitution, and (3) the availability of genotoxicity data. The selected compounds are pyridine, 2-chloropyridine, 3-chloropyridine, 2,3-dichloropyridine, 2,5-dichloropyridine, 2,6-dichloropyridine, 3,5-dichloropyridine, 2-(chloromethyl)pyridine, 3-(chloromethyl)pyridine, 4-(chloromethyl)pyridine and 2-chloro-5-(trichloromethyl)-pyridine (see Table 14 below). These compounds (except 2,5-dichloropyridine, 2,6-dichloropyridine, 3,5-dichloropyridine, and 4-(chloromethyl)pyridine) were included in the 1992 US EPA structure-activity comparison discussion for nitrapyrin (US EPA, 1992). The 1992 US EPA discussion included two additional compounds (trichloromethyl benzene and 1,4-dichlorobenzene) which lack a pyridine ring, and thus are not included in the structure activity comparison presented here. Information on the genotoxicity and carcinogenicity of each of the eleven comparison chemicals is briefly described below and in Table 14.

Pyridine has been listed as a carcinogen under Proposition 65 and has been classified by the International Agency for Research on Cancer (IARC) as a Group 3 carcinogen (IARC, 2000). Pyridine induced aneuploidy in *Saccharomyces cerevisiae* when tested without metabolic activation. Mixed results were obtained from the sex-linked recessive lethal mutation assay in *Drosophila melanogaster.* Administration of pyridine by injection produced a positive result in one of three studies and administration by feeding produced an equivocal result in one of three studies. Pyridine did not induce mutations in *Salmonella typhimurium* with or without metabolic activation or in mouse lymphoma L5178Y cells, and did not induce sister chromatid exchanges or CA *in vitro* in CHO cells, with and without S-9 activation. The single reciprocal translocation test in male *Drosophila* was negative, and pyridine did not induce CA or MN in bone marrow cells of male mice *in vivo* (IARC, 2000; NTP, 2000). Pyridine did not induce UDS in hepatocytes of male mice treated *in vivo* (MacGregor *et al.*, 2000). NTP conducted two-year carcinogenesis studies in male and female mice and rats administered pyridine in drinking water (NTP, 2000). NTP concluded there was clear evidence of carcinogenic activity in male and female $B6C3F_1$ mice based on increased incidences of hepatocellular tumors, some evidence of carcinogenic activity in male F344 rats based on incidences of renal tubule tumors, equivocal evidence in female F344 rats based on increased incidences of mononuclear cell leukemia, and equivocal evidence in male Wistar rats based on increased incidence of interstitial (Leydig) cell adenoma of the testis (NTP, 2000).

2-Chloropyridine was genotoxic in *Salmonella typhimurium* (strains TA97, TA98, TA100, TA102) in the presence of S-9 metabolic activation (Claxton *et al.*, 1987). It induced MN, gene mutations, and CA *in vitro* in mouse lymphoma cells without metabolic activation, and gene mutations and CA with metabolic activation (Dearfield *et al.*, 1993). It did not induce CA in cultured V_3 cells (an African Green monkey kidney cell line) (Anuszewska and Koziorowska, 1995). It has not been tested for carcinogenicity.

3-Chloropyridine was not genotoxic in *Salmonella* (Claxton *et al.*, 1987), but induced MN, gene mutations, and CA *in vitro* in mouse lymphoma cells without metabolic activation (Dearfield *et al.*, 1993). It also induced CA in cultured V_3 cells (Anuszewska and Koziorowska, 1995). It has not been tested for carcinogenicity.

2,3-Dichloropyridine was genotoxic in *Salmonella* strain TA97 with S-9 metabolic activation (Claxton *et al.*, 1987). It has not been tested in any mammalian cells, in any *in vivo* models for genotoxicity, or for carcinogenicity.

2,5-Dichloropyridine, 2,6-dichloropyridine, and 3,5-dichloropyridine were not genotoxic in *Salmonella* (Claxton *et al.*, 1987). None of these compounds have been tested in any mammalian cells, in any *in vivo* models for genotoxicity, or for carcinogenicity.

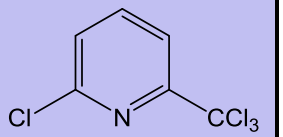
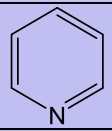
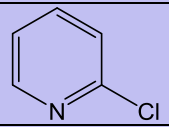
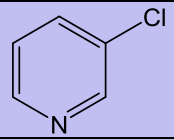
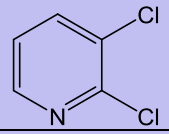
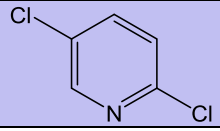
2-(Chloromethyl)pyridine was genotoxic in *Salmonella* strain TA100 with and without metabolic activation (Mortelmans *et al.*, 1986; Claxton *et al.*, 1987). Claxton *et al.* (1987) reported induction of CA and sister chromatid exchanges in CHO cells. In studies conducted by the National Cancer Institute (NCI), male and female F344 rats and $B6C3F_1$ mice were exposed to 2-(chloromethyl)pyridine via gavage for 99 weeks, followed by an observation period of six weeks for rats and five weeks for mice (NCI, 1979). Study authors noted that rats may have been able to tolerate a higher dose than the highest dose to which the rats were exposed. No treatment-related increases in tumors at any site were observed in female rats or male and female mice. A significant positive trend was observed in incidences of subcutaneous fibromas in male rats (but pairwise comparisons were not significant). NCI concluded that 2-(chloromethyl)pyridine was not carcinogenic to F344 rats or $B6C3F_1$ mice (NCI, 1979).

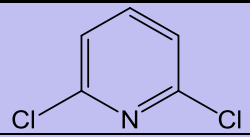
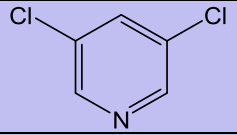
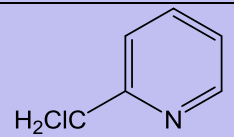
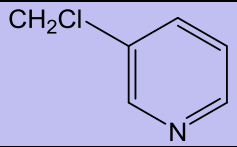
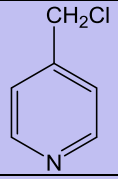
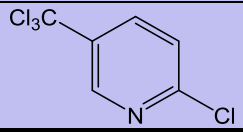
3-(Chloromethyl)pyridine was genotoxic in *Salmonella* strain TA100 with and without metabolic activation (Claxton *et al.*, 1987) and in the mouse lymphoma cell forward mutation assay *in vitro* both with and without metabolic activation (McGregor *et al.*, 1987). Claxton *et al.* (1987) reported induction of CA and increased sister chromatid exchanges (SCE) in CHO cells. In studies by NCI, male and female F344 rats and $B6C3F_1$ mice were exposed to 3-(chloromethyl)pyridine via gavage (NCI, 1978). Low-dose animals were dosed for 102 or 103 weeks, and high-dose animals were dosed for 81 or 83 weeks (discontinued due to early deaths). NCI concluded that 3-(chloromethyl)pyridine was carcinogenic in male rats and mice of both sexes, based on treatment-related forestomach tumors.

4-(Chloromethyl)pyridine was not genotoxic in *Salmonella* (Claxton *et al.*, 1987). It has not been tested in any mammalian cells, in any *in vivo* models, or for carcinogenicity.

2-Chloro-5-(trichloromethyl)-pyridine was genotoxic in *Salmonella* strain TA98 without S-9 metabolic activation, induced forward mutations *in vitro* in an L5178Y mouse lymphoma cell assay, and induced chromosomal damage in human lymphocytes *in vitro* both in the presence and absence of metabolic activation (US EPA, 1984). It has not been tested for carcinogenicity.

Table 14. Carcinogenicity and genotoxicity of nitrapyrin and structurally-related compounds

Name (CAS number)	Structure	Genotoxicity				Animal tumors observed							Cancer Classification	
		In vitro			*In vivo*	Liver	Fore-stomach		Kidney	Testis	Other		Proposition 65	IARC
		Salmonella	Mouse lymphoma	Other		Mouse	Rat	Mouse	Rat	Rat	Rat	Mouse		
Nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] (1929-82-4)		+	NT[1]	-[2]	-[3]	M, F	-	M, F	M	M[4]	-	F[5]	Currently under consideration	NE[6]
Pyridine (110-86-1)		-	-	±[7]	±[8]	M, F	-	-	M[9]	M[10]	F[11]	-	Listed	3[12]
2-chloropyridine (109-09-1)		+	+[13]	-[14]	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE
3-chloropyridine (626-60-8)		-	+[15]	+[16]	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE
2,3-dichloropyridine (2402-77-9)		+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE
2,5-dichloropyridine (16110-09-1)		-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE

Name (CAS number)	Structure	Genotoxicity				Animal tumors observed							Cancer Classification	
		In vitro			*In vivo*	Liver	Fore-stomach		Kidney	Testis	Other		Proposition 65	IARC
		Salmonella	Mouse lymphoma	Other		Mouse	Rat	Mouse	Rat	Rat	Rat	Mouse		
2,6-dichloropyridine (2402-78-0)		-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE
3,5-dichloropyridine (2457-47-8)		-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE
2-(chloromethyl) pyridine (4377-33-7 and 6959-47-3)		+	NT	+[17]	NT	-	-	-	-	-	M[18]	-	NE	NE
3-(chloromethyl) pyridine (3099-31-8)		+	+	+[17]	NT	-	M	M,F	-	-	-	-	NE[19]	NE
4-(chloromethyl) pyridine (1822-51-1)		-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE
2-chloro-5-(trichloromethyl)-pyridine (69045-78-9)		+	+	+[20]	NT	NT	NT	NT	NT	NT	NT	NT	NE	NE

[1]NT: Not tested
[2]Negative in *E. coli* reverse mutation assay, mammalian cell forward mutation assay in CHO/HGPRT cells, and rat hepatocyte UDS
[3]Negative for micronucleus formation in mouse bone marrow and mouse liver UDS
[4]Histiocytic sarcoma of the epididymis
[5]Harderian gland adenoma
[6]NE: Not evaluated
[7]Positive: induced aneuploidy in *Saccharomyces cerevisiae* (without metabolic activation); negative: sister chromatid exchanges and CA in CHO cells (with and without activation)
[8]Positive: sex-linked recessive lethal mutations in *Drosophila melanogaster* with injection in one study; negative: sex-linked recessive lethal mutation assay in *Drosophila* by feeding and injection in two studies, single reciprocal translocation test in male *Drosophila*, CA or MN formation in bone marrow cells of male mice, UDS in hepatocytes of male mice; equivocal: sex-linked recessive lethal mutation assay in *Drosophila* by feeding
[9]Renal tubule cell adenomas and combined renal tubule cell adenomas and carcinomas in high-dose F344 rats were significantly increased compared to controls.
[10]Interstitial (Leydig) cell adenoma of the testis in Wistar rats (NTP concluded this was equivocal evidence of carcinogenicity)
[11]Mononuclear cell leukemia incidences were significantly increased in mid- and high-dose F344 rats (NTP concluded this was equivocal evidence of carcinogenicity)
[12]IARC, 2000
[13]MN, gene mutations, and CA without metabolic activation; gene mutations and CA with metabolic activation
[14]Did not induce CA in cultured V_3 cells
[15]MN, gene mutations, and CA without metabolic activation
[16]CA in cultured V_3 cells
[17]CA and SCEs in CHO cells
[18]Subcutaneous fibroma (significant positive trend)
[19]No apparent exposure in California
[20]Induced chromosomal damage in human lymphocytes with and without metabolic activation

Some pyridine compounds, such as pyridine, 2-chloropyridine, and 3-chloropyridine, may undergo N-oxidation to generate reactive metabolites that may be involved in carcinogenicity (Cowan *et al.*, 1978; Gorrod and Damani, 1980; Namboodiri *et al.*, 1987). The pyridyl nitrogen serves as a primary nucleophilic center for this metabolic oxidation and forms highly polar N-oxide metabolites. The availability of the pyridyl nitrogen to undergo nucleophilic attack depends on the electronegativity, size, and positions of the substituents on the pyridine ring (Claxton *et al.*, 1987; Dearfield *et al.*, 1993; Anuszewska and Koziorowska, 1995).

The pyridyl nitrogen on nitrapyrin is closely surrounded by two electronegative and bulky substitutions, which potentially reduce nucleophilicity and accessibility of the nitrogen for metabolic oxidation. This may explain why N-oxide metabolites or N-oxide-related metabolites were not detected in the nitrapyrin metabolism studies in rats (Timchalk *et al.*, 1987), mice (Domoradzki and Brzak, 1998), dogs (Redemann and Clark, 1967), and other livestock (Stafford, 1987b, as summarized in US EPA, 2004) (See Section 3.3.1 Pharmacokinetics and Metabolism). Thus it appears unlikely that N-oxidation, common to some pyridine compounds, plays a role in the S-9 mediated mutagenic activity of nitrapyrin in the *Salmonella* reverse mutation assays reported by Zeiger *et al.* (1988) and Mecchi (2007) (See Section 3.3.2 Genotoxicity for details).

As summarized in Table 14, only one of the eleven comparison compounds [pyridine] is listed under Proposition 65[15], and only one of two additional comparison compound [3-(chloromethyl)pyridine] that have been tested has been reported to induce tumors in animals[16]. Pyridine and/or 3-(chloromethyl)pyridine induced tumors at sites in common with nitrapyrin, *e.g.*, mouse liver, rat kidney, mouse forestomach, and rat testes (although at a different site within the testes), but comparisons across the larger set of chemicals are limited by the lack of carcinogenicity testing data for most of these compounds. Perhaps the most interesting observation is that many of these structurally-related compounds are genotoxic. For example, six of the comparison compounds induce mutations in *Salmonella* and/or mutations or chromosomal aberrations in the mouse lymphoma cell assay; five induced genotoxicity in other test systems.

[15] Based on clear evidence of carcinogenicity in studies conducted in male and female mice by NTP (2000).
[16] NCI (1978) considered the compound to be carcinogenic based on studies in male rats and mice of both sexes.

3.3.5 High-throughput screening data

No studies were identified in the literature that specifically assessed nitrapyrin's ability to interact with molecular pathways and cellular processes related to carcinogenesis. The US EPA ToxCast database was searched to identify *in vitro* high-throughput screening (HTS) assays in which nitrapyrin was active (US EPA, 2014). Nitrapyrin was tested in 403 assays, and was active in seven (Table 15). These included five assays evaluating the upregulation of transcription factor activity (CEBPB, MTF1, POU2F1, NR1I2 [also known as pregnane X receptor, PXR], and VDR) and two assays evaluating the downregulation of chemokine gene expression (CXCL10 and CCL2).

Table 15. Positive high-throughput screening assay results from the ToxCast database (US EPA, 2014)

Assay name	Biological process target	Intended target family	Target gene	AC 50 (µM)
ATG_C_EBP_CIS_up	Regulation of transcription factor activity	DNA binding	CEBPB (CCAAT/Enhancer-Binding Protein, β)	42.2
ATG_MRE_CIS_up	Regulation of transcription factor activity	DNA binding	MTF1 (Metal-Regulatory Transcription Factor 1)	81.8
ATG_Oct_MLP_CIS_up	Regulation of transcription factor activity	DNA binding	POU2F1 (POU domain, Class 2, Transcription Factor 1)	28.7
ATG_PXRE_CIS_up	Regulation of transcription factor activity	Nuclear receptor	NR1I2 (Nuclear Receptor Subfamily 1, Group I, Member 2 [also called PXR])	46.4
ATG_VDRE_CIS_up	Regulation of transcription factor activity	Nuclear receptor	VDR (Vitamin D Receptor)	38.8
BSK_hDFCGF_IP10_down	Regulation of gene expression	Cytokine	CXCL10 (Chemokine, CXC Motif, Ligand 10)	13.8
BSK_LPS_MCP1_down	Regulation of gene expression	Cytokine	CCL2 (Chemokine, CC Motif, Ligand 2)	31.9

The Comparison Toxicogenomic Database (CTD, 2015) was used to ascertain if any of the seven target genes identified in the HTS testing of nitrapyrin are associated with cancer. Three of the target genes (CEBPB, PXR, and VDR) are linked to pathways that are associated with cancer (11-17% of the associations with diseases were specifically associated with cancer). The two chemokine-related target genes (CXCL10 and CCL2) were weakly associated with cancer pathways (less than 10 percent). The other two target genes (MTF1 and POU2F1) are not well-studied enough for associations to be reported in the Comparison Toxicogenomic Database.

Additional associations between these target genes and cancer pathways:

- The target gene CEBPB acts as a principal effector of the oncogene cyclin D1 activity in human cancer development (Lamb *et al.*, 2003). CEBPB, together with BCL2A1, has been found to be necessary to induce cell transformation or to sustain growth and survival of large cell lymphomas *in vitro* (Piva *et al.*, 2006). CEBPB was also able to upregulate P-cadherin promoter activity in breast cancer cells, and the expression of P-cadherin (an oncogene) and CEBPB were highly associated with human breast carcinomas (Albergaria *et al.*, 2010).
- PXR often dimerizes with CAR or retinoid X receptor (RXR) prior to binding to its DNA target sites, and plays a role in CAR activation.
- VDR plays a critical role in E-cadherin expression, which influences cell fate during colon cancer progression (Palmer *et al.*, 2004).
- CXCL10 is a chemokine that has angiostatic actions; suppression could contribute to an environment that is favorable to the growth of new blood vessels (Kleinstreuer *et al.*, 2013). CXCL10 was identified as a RAS (an oncogene) target gene and is overexpressed in the majority of colorectal cancers (Zhang *et al.*, 1997).

3.3.6 Mechanistic studies

Daly (1995) (unpublished study, as summarized in US EPA, 2012a) and LeBaron (2010) (unpublished study; also summarized in US EPA, 2012a)

A subchronic oral toxicity study was conducted in which nitrapyrin (purity: 90.0 and 92.05%) was administered in the diet to 10 male B6C3F_1 mice per group at dose levels of 0, 200, 300, 400, or 600 mg/kg/day and 10 female B6C3F_1 mice per group at dose levels of 0, 200, 400, 600, or 800 mg/kg/day for up to 95 or 96 days (Daly, 1995). The doses in this study are higher than those used in the two-year carcinogenicity study of Stebbins and Cosse (1997) (125 and 250 mg/kg/day). None of the animals in the 600 or 800 mg/kg/day dose groups survived to study termination. Mice administered 400 mg/kg/day exhibited decreased body weight gain, changes in hematological parameters, increased ALT, and other effects, suggesting that systemic and liver toxicity may have occurred at this dose level.

Treatment-related findings were observed in the livers of males and females. Significantly increased liver weights and centrilobular or panlobular hypertrophy occurred in all mice administered 200 to 400 mg/kg/day. Intracytoplasmic vacuoles and single cell necrosis of hepatocytes were observed in males administered 300 and 400 mg/kg/day and in females administered 400 mg/kg/day.

LeBaron (2010) conducted a retrospective evaluation on formalin-fixed liver tissue from Daly (1995). Archived liver tissue from each of the study animals (10 $B6C3F_1$ mice/sex/dose) was used for Ki-67 immunohistochemical staining analysis for cell proliferation. Statistically significant fold increases were observed in the midzonal and periportal regions in male mice in the 400 mg/kg/day dose group (as calculated by study authors with Dunnett's test, $\alpha \leq 0.05$). Increases in hepatocellular proliferation were observed in the female mice, but were not significantly increased compared to controls.

Yano and McFadden (1996) (unpublished study, as summarized in US EPA, 2012a)

Two-week studies were conducted in mice to measure hepatocyte proliferation and apoptosis (as part of the studies of Stebbins and Cosse, 1997). Five $B6C3F_1$ mice/sex/dose were administered 0, 200, or 400 mg/kg/day nitrapyrin in the diet for 2 weeks. Hepatocellular proliferation was analyzed via proliferating cell nuclear antigen (PCNA)-labeling. In male mice, statistically significantly increased cell proliferation was observed in the centrilobular region in the 200 mg/kg/day group and in the centrilobular and periportal regions in the 400 mg/kg/day dose group. US EPA (2012a) notes there was great variability in the data. An increase in the number of apoptotic cells in male mice was observed; however, this finding was not significant due to high standard deviations. No statistically significant changes in cell proliferation or apoptosis were observed in the livers of female mice.

LeBaron *et al.* (2010) (unpublished study; also summarized in US EPA, 2012a)

Nitrapyrin (purity: 98.6%) was administered in the diet for 7 or 14 days to 6 male $B6C3F_1$ mice per group (except the high-dose groups receiving treatment for 14 days and 14 days plus 21 day recovery, which had 9 mice) at dose levels of 0, 75, 250, and 400 mg/kg body weight/day. A recovery group was given the same doses of nitrapyrin for 14 days then switched to a control diet for 21 days of recovery.

Increased liver weights and hepatocyte hypertrophy were observed in the 250 and 400 mg/kg/day dose groups at 1 and 2 weeks. These changes were more pronounced in animals treated for longer times and at higher doses. No increases in liver weights were observed in the 75 mg/kg/day dose group or in the 21-day recovery group. Hepatocellular proliferation was analyzed via 5-bromo-2'-deoxyuridine (BrdU) incorporation. Slight, but significant, cell proliferation was observed in periportal hepatocytes in the 250 mg/kg/day dose group and in midzonal and periportal hepatocytes in the 400 mg/kg dose group after one and two weeks of treatment. No hepatocyte proliferation was observed in the 21-day recovery group.

Gene expression analysis of treated livers indicated increases in the *Cyp2b10* transcript levels in the 250 and 400 mg/kg dose groups in B6C3F$_1$ mice treated for 7 and 14 days, with only slight increases in the 21-day recovery group. Slight changes in gene expression were observed for *Cyp1a1* (2.03-fold increase in the 400 mg/kg/day dose group at 7 days), *Cyp3a11* (1.51-fold increase in the 400 mg/kg/day dose group at 7 days that decreased to 1.19-fold at 14 days and 1.23-fold in the 250 mg/kg/day group with a 21-day recovery) and *Cyp4a10* (6.75-fold increase in the 250 mg/kg/day dose group at 7 days). Pentoxyresorufin O-dealkylase (PROD) liver enzyme activity was also measured. No fold increases in PROD activity were observed for any of the treatment groups when compared to controls.

LeBaron *et al.* (2014) (unpublished study)

An *in vitro* study was conducted in rat liver microsomes to investigate mechanism-based (suicide) inhibition of cytochrome P450 enzyme activity. Phenobarbital (PB)-induced rat liver microsomes in the presence of pentoxyresorufin were incubated with either 0.1% DMSO (solvent control), 1- 500 µM phenobarbital (negative control for inhibition), 10-80 µM curcumin (positive control for inhibition), or 1- 500 µM nitrapyrin. Enzymatic activity was determined by rate of PROD conversion of pentoxyresorufin to resorufin as detected by a fluorometric plate reader. Nitrapyrin and curcumin inhibited the PROD activity in a dose-related manner, while PB had no effect on PROD activity. The authors concluded that nitrapyrin irreversibly inhibited *Cyp2b10*-mediated PROD activity of PB-induced microsomes.

Murphy *et al.* (2014a) (unpublished study reported in LaRocca *et al.*, 2015, an unpublished review)

A study was conducted to investigate differences in response to nitrapyrin in two mouse strains. Groups of six B6C3F$_1$ and six C57BL/6NTac male mice were administered nitrapyrin in the diet at 0 and 250 mg/kg/day for four or seven days. Increased liver weights and slight hepatocellular hypertrophy were observed in all treated groups after four and seven days of treatment. Increases in *Cyp2b10* mRNA transcript levels were observed in livers from both strains treated with nitrapyrin for 4 and 7 days. Slight increases were also observed in *Cyp1a1, Cyp3a11,* and *Cyp4a10* levels in both strains of mice. Hepatocellular proliferation was analyzed via immunohistochemical staining for BrdU-labeled nuclei. After four days of treatment, an increase in hepatocellular proliferation in the midzonal and periportal regions compared to controls was observed in both strains. After seven days of treatment, proliferation was increased in the

centrilobular, midzonal, and periportal regions in B6C3F$_1$ mice and in the periportal region in C57BL/6NTac mice.

Murphy *et al.* (2014b) (unpublished study)

A CAR knockout mouse study was conducted to evaluate hepatic response to nitrapyrin. Groups of six male wild-type C57BL/6NTac mice and six CAR knockout C57BL/6NTac mice were administered nitrapyrin (purity: 90.3%) in the diet at 0 or 250 mg/kg/day for 4 days. Mice were 12 weeks old at the start of nitrapyrin administration. Increased liver weights were observed in all treated mice (wild-type and CAR knockout) as compared to controls. Very slight hepatocellular hypertrophy was observed in all treated CAR knockout mice, and slight hypertrophy was observed in all treated wild-type. Increased *Cyp2b10* expression was observed in wild-type mice. Slight increases in expression levels of *Cyp1a1* (2.7-fold increase), *Cyp3a11* (2.2-fold increase), and *Cyp4a10* (1.5-fold increase) were also observed in the wild-type mice. In the CAR knockout mice, induction of *Cyp1a1* was observed. Slight increases in expression levels of *Cyp2b10* (2.2-fold increase), *Cyp3a11* (2.6-fold increase), and *Cyp4a10* (2.6-fold increase) were also observed. Murphy *et al.* (2014b) analyzed hepatocellular proliferation via BrdU incorporation and observed an increase in proliferation in the periportal region in wild-type mice treated with nitrapyrin. There were no increases in proliferation in CAR knockout mice.

Johnson and Kan (2015) (unpublished study)

An *in vitro* study was conducted in cultured mouse and human hepatocytes to evaluate cell proliferation. Primary CD-1 mouse hepatocytes and primary human hepatocytes were cultured and exposed to 0, 1, 3, 10, 30, or 100 µM nitrapyrin (purity: 90.3%) for 48 hours. Epidermal growth factor (25 ng/ml) was used as a positive control. Cell proliferation was measured by 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analog. Nitrapyrin exposure caused excessive cytotoxicity in cultured mouse hepatocytes at concentrations of 30 µM or greater. Nitrapyrin was not cytotoxic to cultured human hepatocytes up to the highest dose tested (100 µM). Nitrapyrin exposure significantly increased the proliferation of CD-1 mouse hepatocytes at a concentration of 10 µM. Nitrapyrin did not statistically significantly increase cell proliferation of human hepatocytes at any concentrations tested.

4. MECHANISMS

US EPA reviewed the carcinogenicity of nitrapyrin in 1992, 2000, 2005, and 2012. The mechanisms through which nitrapyrin induces tumors are not known. A number of mechanisms and hypotheses have been suggested, as described here. Also discussed are new data submitted by Dow Chemical Company related to its position that the MOA (mode of action) for liver tumors is through activation of the CAR (constitutive androstane receptor).

Genotoxicity

Nitrapyrin was tested for mutagenicity in bacterial cells. Positive activity was reported in *Salmonella* gene mutation studies employing a pre-incubation protocol with and without hamster or rat liver S-9 activation by Zeiger *et al.* (1988), but not in studies employing a plate incorporation protocol (Kennelly, 1985). A repeat set of studies by Mecchi (2007) using the more sensitive pre-incubation method in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 with and without 10% rat liver S-9 activation also indicate a weak positive response up to a dose of 100 µg/plate; higher doses may have caused toxicity to the test bacteria in this series of assays. Nitrapyrin did not induce reverse mutations in *E. coli* strain WP2*uvr*A, HGPRT mutations in mammalian CHO cells, MN in mouse bone marrow *in vivo*, or UDS *in vitro* in rat hepatocytes or *in vivo* in mouse liver. Positive genotoxicity results were observed with structurally-related chemicals. US EPA concluded that nitrapyrin has genotoxic potential in its 1992, 2000, and 2005 reviews. In 2012, US EPA concluded “there is no concern for a mutagenic mode of action” following the review of additional registrant-sponsored studies and an unpublished report by Zeiger (2010).

The genotoxicity studies are described in Section 3.3.2 Genotoxicity. It is to be noted that the concern about the positive genotoxicity results obtained in *Salmonella* by Zeiger *et al.* (1988) and Mecchi (2007) is not lessened by the other negative genotoxicity assays, as the negative assays used different and potentially less sensitive protocols or measured a different endpoint.

Nitrapyrin has not been tested for DNA binding, induction of oxidative DNA damage or DNA single strand breaks (*e.g.,* by single cell gel electrophoresis or in *S. typhimurium* strains sensitive to oxidative DNA damage such as TA102 and TA104), or in transgenic mouse models.

Since several structurally-related compounds are mutagenic and two are carcinogenic (mutagenic: 2-chloropyridine, 3-chloropyridine, 2,3-dichloropyridine,

2-(chloromethyl)pyridine, 3-(chloromethyl)pyridine, and 2-chloro-5-(trichloromethyl)pyridine; carcinogenic: pyridine is listed as a carcinogen under Proposition 65 and 3-(chloromethyl)pyridine induces tumors in animals, as discussed in Section 3.3.4 Structure Activity Considerations), and positive results were observed for nitrapyrin in *Salmonella*, the possible role of genotoxicity cannot be ignored. The contrary opinion of US EPA (2012a) appears to rely on a standard proposed in 2007 by the agency for positive identification of a mutagenic mode of action. This proposed standard is inappropriate for assessment of mutagenic or genotoxic potential in hazard identification. It was originally proposed in the context of deciding whether to use age-related sensitivity factors in quantitative cancer risk estimation. After undergoing external review, the proposal was never finalized.

α_{2u}-Globulin nephropathy and renal tubule tumors

As described in Section 3.2.1, nitrapyrin induced renal tubule adenomas and adenocarcinomas in male rats. During interim sacrifice at 12 months, increased incidences of mineralization of the loops of Henle and hyaline droplet accumulation in the P2 section of proximal tubules were observed in the high-dose males, as well as slight increases in dilated tubules with proteinaceous casts among the low- and mid-dose males. An immunoperoxidase assay was conducted to identify the presence of α_{2u}-globulin in hyaline droplets in kidneys from control and high-dose males. All high-dose males showed clear and positive staining of α_{2u}-globulin in the P2 section of the proximal tubule, whereas slight and scattered staining was observed in control males. Blood urea nitrogen (BUN) (an indicator of impaired kidney function) was increased in the high dose group at 6, 18, and 24 months. At 24 months, there was a significant increase in severe chronic progressive glomerulonephropathy in the high-dose males.

No renal tumors were observed in treated female rats. However, increases in dilated tubules with proteinaceous casts were observed in 1/10 mid-dose and 6/10 high-dose females at the 12-month interim sacrifice, compared to 0/10 in controls. And at 24 months, increased absolute and relative kidney weights and increased levels of BUN were observed in the high dose females (Szabo *et al.*, 1989).

Criteria for determining whether an agent causes kidney tumors through an α_{2u}-globulin-associated response in male rats has been developed by IARC (1999). These criteria are:

(1) Lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of *in vitro* and *in vivo* data
(2) Male rat specificity for nephropathy and renal tumorigenicity

(3) Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory
(4) Identification of the protein accumulating in tubule cells as α_{2u}-globulin
(5) Reversible binding of the chemical or metabolite to α_{2u}-globulin
(6) Induction of sustained increased cell proliferation in the renal cortex
(7) Similarities in dose-response relationship of the tumor outcome with the histopathological end-points (protein droplets, α_{2u}-globulin accumulation, cell proliferation)

With regard to IARC criterion 1, positive genotoxicity results were observed for nitrapyrin in *Salmonella* in multiple studies and test strains. Overall, considering the limited number of genotoxicity endpoints for which nitrapyrin has been assessed, these mutagenicity findings indicate that IARC criterion 1 is not met.

IARC criterion 2 is not met either, because the following effects of nitrapyrin treatment on female rat kidneys were observed: an increase in dilated tubules with proteinaceous casts at 12 months in treated females, increased absolute and relative kidney weights at 12 and 24 months, and increased BUN at 24 months (Szabo *et al.,* 1989). Effects of nitrapyrin on the kidney were also seen in mice. Rare renal tubule adenomas were observed in male $B6C3F_1$ mice (one each in the 25 and 75 mg/kg/day dose groups) in the study by Quast *et al*. (1990), and increases in absolute and relative kidney weights were observed at 24 months in the 250 mg/kg/day dose group in the study by Stebbins and Cosse (1997).

The following data gaps are noted with regard to evaluating the effects of nitrapyrin against the remaining criteria:

- Criterion 3 (histopathological changes in shorter-term studies), no data were provided to show protein droplet accumulation prior to 12 months of treatment
- Criterion 5, no data were provided to demonstrate reversible binding of nitrapyrin to α_{2u}-globulin
- Criterion 6, no data were provided to demonstrate sustained cell proliferation in the renal cortex.

A structurally similar compound, pyridine, was found to induce renal tubule tumors in male rats in a study by NTP (NTP, 2000). Some changes consistent with the α_{2u}-globulin response were observed in male rats treated with pyridine; however, NTP concluded that renal tubule tumors in male rats were not attributable to α_{2u}-globulin.

In summary, α_{2u}-globulin accumulation in the renal tubules is observed in nitrapyrin-exposed male rats, but several of the IARC criteria required for determining that an

α_{2u}-globulin-associated mechanism of carcinogenesis is operative are not met. This suggests that nitrapyrin may have additional mechanisms of action which contribute to the induction of renal tubule carcinomas.

Cytotoxicity

Liver tumors
Based on the liver changes found in nitrapyrin-treated mice in Stebbins and Cosse (1997) and Yano and McFadden (1996) (an unpublished 2-week study conducted as part of Stebbins and Cosse, 1997, summarized in US EPA, 2012a), the registrant, Dow Chemical Company, postulated that "nitrapyrin causes necrosis and regenerative hepatocyte proliferation and eventual neoplasia. Thus, the dose levels were excessive" (US EPA, 2005a).

The key events for liver tumor formation via a cytotoxic MOA are induction of cytotoxicity followed by regenerative cell proliferation and tumor formation (Cohen, 2010). Necessary data for the key events are evidence of hepatocellular necrosis, increased cell proliferation, and reversibility upon discontinuation of the chemical. Cytotoxicity can be identified either by histopathological determination of necrosis or by an increase in serum enzymes that are associated with liver toxicity. Hepatocellular toxicity also can occur due to an increase in apoptosis (Cohen, 2010).

Data on nitrapyrin and hepatocellular necrosis comes primarily from the two-year studies in mice by Stebbins and Cosse (1997). Data on nitrapyrin and hepatocellular proliferation comes from several studies (see Table 19), most of which were conducted in male mice. In the two-year male mice study by Stebbins and Cosse (1997), increased single cell necrosis, characterized as very slight hepatocellular necrosis, was observed in both dose groups. At 12 months these mice exhibited hepatocellular proliferation in the centrilobular zone, as measured by PCNA (Stebbins and Cosse, 1997). Yano and McFadden (1996) treated male mice for 14 days and observed statistically significantly increased cell proliferation in the centrilobular region in the 200 mg/kg/day group and in the centrilobular and periportal regions in the 400 mg/kg/day dose group (as measured by PCNA). In the two-year study in female mice by Stebbins and Cosse (1997) increased single cell necrosis was also observed in both dose groups, but no cell proliferation was observed. Similarly, no significant increase in hepatocellular proliferation was seen in female mice treated for 14 days by Yano and McFadden (1996).

US EPA reviewed the available data and "concluded that the highest dose [of 250 mg/kg/day] was adequate and not excessive to assess the carcinogenic potential of

nitrapyrin in both sexes" (US EPA, 2000). This position was maintained in the 2005 review. Additionally, US EPA (2005a) "determined that insufficient data were provided to support a cytotoxic mode of action for the induction of liver tumors. In particular, there were no temporal data showing time to onset of non-neoplastic conditions relative to the tumors and there was no reversibility data for the non-neoplastic lesions." More severe cytotoxicity may occur at higher doses, e.g., 400 mg/kg/day (Daly, 1995), but this is not indicative of a role for cytotoxicity in tumorigenicity.

Following the US EPA review in 2005, additional studies in mice were conducted that also exhibited hepatocellular proliferation. LeBaron (2010) conducted a retrospective evaluation on formalin-fixed liver tissue from Daly (1995). Male mice treated for 12 weeks with 400 mg/kg/day exhibited increased cell proliferation in the midzonal and periportal regions (as measured by Ki-67 staining), but no increase in hepatocellular proliferation was evident in female mice.

LeBaron *et al.* (2010), Murphy *et al.* (2014a), and Murphy *et al.* (2014b) completed additional studies in male, but not female, mice. LeBaron *et al.* (2010) observed slight, but significant, cell proliferation in periportal hepatocytes in the 250 mg/kg/day dose group and in midzonal and periportal hepatocytes in the 400 mg/kg dose group after one and two weeks of treatment. No hepatocyte proliferation was observed after 14 days of treatment and 21 days of recovery. Very slight necrosis was observed in mice treated for 7 days (1/6 and 3/6 in the 250 and 400 mg/kg/day dose groups, respectively) and 14 days (1/9 in the 400 mg/kg/day dose group). Murphy *et al.* (2014a) measured hepatocellular proliferation via BrdU staining in B6C3F_1 and C57BL/6NTac mice. After four days of treatment, an increase in hepatocellular proliferation in the midzonal and periportal regions was observed in both strains. After seven days of treatment, proliferation was increased in the centrilobular, midzonal, and periportal regions in B6C3F_1 mice and in the periportal region in C57BL/6NTac mice. Murphy *et al.* (2014b) observed an increase in proliferation in the periportal region in wild-type mice treated with 250 mg/kg/day nitrapyrin for 4 days (as measured by BrdU). Very slight necrosis was observed in 2/6 mice. There were no increases in hepatocellular proliferation in CAR knockout mice treated with nitrapyrin.

In 2012, US EPA reaffirmed its position that the doses used in the mouse studies of Stebbins and Cosse (1997) were not excessive. The US EPA's consulting pathologist explained:

> "Considering the non-neoplastic liver lesions, several of which are very common in aging mice, I find increase in the incidence rates of individual cell necrosis as well as some other lesions at 250 mg/kg/day at 'very slight' or 'slight' in severity.

> An increase in the size of the hepatocytes (either centrilobular or panlobular) was often graded as moderate, but there was no consistent dose relationship and this change is considered to be more of a physiological response that [sic] one of toxicity. There is nothing in this additional data to convince me that the two highest dose levels were excessive" (US EPA, 2012a).

US EPA concludes, "From this more recent MOA analysis, a case could be made for cytotoxicity; however, the necropsy data showed no clear indication of hyperplasia or necrosis" (US EPA, 2012a).

It appears that male and female mice have different short-term liver responses to nitrapyrin; however, both males and females exhibit a clear liver tumor response. Given that significant increases in hepatocellular proliferation and necrosis were not seen in female mice at doses that induced tumors, cytotoxicity is likely not involved in the mechanism of liver tumor development. Additionally, only slight increases in single cell necrosis were observed in male mouse liver and these observations do not support a cytotoxic MOA. A recent report from the registrant explains, "The absence of key events necessary to support cytotoxicity as the MoA (focal necrosis, increase serum enzymes) at tumorigenic dose levels substantiates that this is not a viable MoA for nitrapyrin-mediated liver tumors in mice" (LaRocca *et al.*, 2015).

Forestomach tumors

Dow Chemical Company suggested that the increased incidence of forestomach tumors was due to irritation of the forestomach (a storage area in rodents but not present in humans) by nitrapyrin, as evidenced by forestomach hyperplasia. US EPA (2012a) noted that nitrapyrin does not seem to be unusually more irritating than other chemicals that do not produce forestomach tumors. US EPA (2005) concluded that tumors in the forestomach of mice are treatment-related, but are not relevant for human risk assessment, based on the structure and physiological function of the stomach.

However, IARC does consider forestomach tumors in mice to be relevant for human risk assessment. IARC reviewed the issue of rodent forestomach tumors, and concluded the following:

> "While humans do not have a forestomach, they do have a comparable epithelial tissue in the oral cavity and the upper two-third of the esophagus. Thus, in principle, carcinogens targeting the forestomach squamous epithelium in rodents are relevant for humans. Also, the target tissue for carcinogens may differ between experimental animals and humans, and a forestomach carcinogen in rodents may target a different tissue in humans" (IARC, 2003).

IARC also noted that for chemicals that produce tumorigenic effects in other tissues, as well as the forestomach, these observations "indicate[ing] that there may be either general or multiple modes of action" (IARC, 2003).

Activation of constitutive androstane receptor (CAR)

Nitrapyrin was classified as likely to be carcinogenic in humans by US EPA in 2000 and 2005, based, in part, on the liver tumors observed in male and female mice. In 2010, the registrant, Dow Chemical Company, submitted data to US EPA from mechanistic studies to support its new position that the MOA for liver tumors is through activation of CAR. US EPA reviewed the submitted data in 2012. These studies and US EPA's position are described below, as well as a discussion of new mechanistic studies submitted to OEHHA by the registrant in 2015.

Background on CAR

CAR is a transcription factor that regulates the expression of many genes involved in the metabolism and transport of both xenobiotic and endogenous chemicals in humans and rodents. CAR is sequestered in the cytoplasm as a multiprotein complex and translocates to the nucleus upon chemical-mediated activation or deactivation through direct or indirect mechanisms. Once activated in humans, it binds to responsive elements upstream from the *CYP2B6* gene and upregulates the cytochrome P450 2B gene family that regulates hepatic drug metabolism. CAR is also known to control the inductive expression of several other CYP enzymes. Additionally, CAR modulates various hepatic functions involved in diverse physiological and pathophysiological processes, including energy metabolism, insulin signaling, cell proliferation, and tumor development (Yang and Wang, 2014).

Phenobarbital (PB) is a prototypical inducer of hepatic CYP2B enzymes in humans, rats and mice. PB has been studied in rodents as a possible model for understanding CAR activation and liver tumor development (Elcombe *et al.*, 2014). PB has been shown to promote liver tumors in rodents and is classified by IARC as a Group 2B carcinogen (possibly carcinogenic to humans) based on sufficient evidence in experimental animals and inadequate evidence in human (IARC, 2001). A study published after the IARC review examined cancer incidence among epileptic patients taking any of several antiepileptic drugs (the most commonly used drugs were PB, carbamazepine, and phenytoin) (Lamminpaa *et al.*, 2002). This study found excess risks of cancers of the brain and nervous system, larynx, liver, pancreas, colon, stomach, and lung among patients taking antiepileptic drugs. While a fraction of these tumors can be attributed to other causes, the authors concluded that "[t]he excess of some cancers might be

attributable to enzyme-inducing antiepileptic drugs." A recent experimental study shows that PB treatment induces liver tumors in mice expressing human CAR and PXR (pregnane X receptor) (Braeuning *et al.*, 2014).

Review of mechanistic data submitted by the Dow Chemical Company
Dow Chemical Company postulated that the MOA for nitrapyrin-induced mouse liver tumors is comparable to the proposed MOA for PB-induced mouse liver tumors (US EPA, 2012a; LaRocca *et al.*, 2015): mitogenesis through activation of CAR (Elcombe *et al.*, 2014). Several key events are required for the proposed CAR MOA. According to US EPA (2012a), the key events are:

(1) CAR activation
(2) Increased *Cyp*2b10 expression
(3) Increased pentoxyresorufin O-dealkylase (PROD) activity
(4) Increased hypertrophy
(5) Increased cell proliferation
(6) Increased liver weight
(7) Increased basophilic foci
(8) Liver tumors

Several studies of nitrapyrin have evaluated different parameters related to key events of the proposed CAR MOA. Each key event is briefly described below, followed by relevant data from the various mechanistic studies of nitrapyrin.

Key event #1: CAR activation
CAR activation is the molecular initiating event, which is demonstrated by induction of *CYP2b10*.

Key event #2: Increased Cyp2b10 expression
Three studies measured gene expression in mouse liver tissue. As shown in Table 16, nitrapyrin induces *Cyp2b10* gene expression, as well as *Cyp1a1*, *Cyp3a11*, and *Cyp4a10*, albeit at lower levels. PB induces *Cyp2b10*, as well as *Cyp1a1* and *Cyp3a11*, but not *Cyp4a10* (Table 16). *Cyp2b10* expression is an indicator of CAR activation in mice. *Cyp1a1* is associated with aryl hydrocarbon receptor (AhR) response, *Cyp3a11* is associated with PXR response, and *Cyp4a10* is associated with peroxisome proliferator-activated receptor alpha (PPARα).

In CAR knockout mice treated with nitrapyrin, a slight increase in *Cyp2b10* gene expression was observed (2.2 fold). Expression of *Cyp3a11* and *Cyp4a10* were similar in nitrapyrin-treated wild-type and CAR knockout mice, while expression of *Cyp1a1* in

the knockout mice was significantly increased, as compared to wild-type. The authors speculate that the increase in *Cyp1a1* (associated with AhR) is an adaptive (compensatory) response due to the absence of a functional CAR signaling pathway (Murphy *et al.*, 2014b).

Overall, these studies indicate that nitrapyrin activates multiple nuclear receptors, in addition to CAR.

Table 16. Summary of targeted gene expression in livers of male mice exposed to nitrapyrin, as reported in several mechanistic studies

Length of treatment	Dose (mg/kg/day)	Mouse strain	Gene[1]				Reference
			Cyp1a1	*Cyp2b10*	*Cyp3a11*	*Cyp4a10*	
4 days	250	$B6C3F_1$	2.2	370.7	1.6	2.5	Murphy *et al.* (2014a)
		Wild-type C57BL/6NTac	2.5	240.7	2.3	1.1	
			2.7	493.7	2.2	1.5	Murphy *et al.* (2014b)
		CAR knockout C57BL/6NTac	104.4	2.2	2.6	2.6	
7 days	75	$B6C3F_1$	1.11	4.05	0.86	1.32	LeBaron *et al.* (2010)
	250		1.96	351.02	1.38	6.75	
			2.1	562.5	1.6	4.3	Murphy *et al.* (2014a)
		Wild-type C57BL/6NTac	1.9	168.7	1.9	3.1	
	400	$B6C3F_1$	2.03	716.04	1.51	5.19	LeBaron *et al.* (2010)
	Positive control: 150 PB		2.06	807.43	6.93	1.13	
14 days	75		1.16	4.42	0.69	1.23	
	250		1.67	389.59	1.12	4.22	
	400		1.87	1092.32	1.19	2.91	
14 days (+21 day recovery)	75		1.02	1.60	1.08	1.21	
	250		0.92	2.91	1.23	1.57	
	400		1.04	2.70	0.88	1.33	

[1]Data are presented as relative fold-change compared to control

Key event #3: Increased PROD activity

PROD liver enzyme activity is used as a functional measure of the CYP2B10 enzyme, and increased PROD activity is characteristic of CAR activation (Lubet *et al.*, 1985). LeBaron *et al.* (2010) measured total cytochrome P450 content and PROD liver enzyme activity in male mice treated with nitrapyrin for 14 days and for 14 days plus 21 day recovery (Table 17). No substantial increases in total P450 protein content or PROD activity were observed for any of the treatment groups when compared to controls.

In reviewing these data, US EPA (2012a) stated “the absence of hepatic metabolic enzyme activity leaves a major uncertainty in the MOA analysis.”

Table 17. Total cytochrome P450 protein content and PROD liver enzyme activity in male B6C3F$_1$ mice treated with nitrapyrin (LeBaron *et al*., 2010)

Group	14-day exposure				14-day exposure and 21-day recovery			
Dose (mg/kg/day)	**0**	**75**	**250**	**400**	**0**	**75**	**250**	**400**
Total mean P450 protein content (nmol p450/mg protein)	0.33	0.31	0.42	0.43	0.32	0.32	0.47	0.49
Fold increase[1] of P450 protein content	1.0	<1	1.3	1.3	1.0	1.0	1.5	1.5
Fold increase[1] of PROD enzyme activity (pmol/mL)	1.0	1.0	1.1	1.0	1.0	1.0	1.1	1.1

[1]Relative fold-change compared to control activity

Key event #4: Increased hypertrophy

Liver hypertrophy is a key event in the proposed CAR MOA that usually occurs in the centrilobular region of the liver lobule (Whysner *et al.*, 1996; Holsapple *et al.*, 2006). Four studies measured hepatocellular hypertrophy in mice following nitrapyrin exposures up to twelve weeks (as shown in Table 18). Stebbins and Cosse (1997) also observed hepatocellular hypertrophy in male mice treated with nitrapyrin for one or two years. Centrilobular or panlobular hepatocyte hypertrophy occurred in 100 and 80 percent of male mice treated for one year and in 96 and 52 percent of male mice treated for two years in the 125 and 250 mg/kg/day dose groups, respectively. The study in female mice reported similar findings: hypertrophy was observed in 100 percent of female mice exposed for 1 year and in 92 and 88 percent of female mice exposed for two years in the 125 and 250 mg/kg/day dose groups, respectively (Stebbins and Cosse, 1997).

The newer mechanistic studies demonstrated similar findings (Table 18). All mice treated with ≥250 mg/kg/day nitrapyrin for 4 days, 7 days, 14 days, or 12 weeks demonstrated hypertrophy, with the exception of mice allowed a 21 day recovery period (none of which demonstrated hypertrophy) (Daly, 1995; LeBaron *et al.*, 2010; Murphy *et al.*, 2014a; Murphy *et al.*, 2014b). No differences in the liver hypertrophy responses were observed between wild-type and CAR knockout mice treated with nitrapyrin (i.e., 100% of the treated wild-type and CAR knockout mice exhibited very slight liver hypertrophy) (Murphy *et al.*, 2014b).

Table 18. Percentage of nitrapyrin-exposed male mice with increased centrilobular/midzonal liver hypertrophy, as reported in several mechanistic studies

Length of study	Mouse strain	Dose (mg/kg/day)	Percentage with liver hypertrophy[1]	Reference
4 days	$B6C3F_1$	250	100% (2)	Murphy *et al.* (2014a)
	Wild-type C57BL/6NTac	250	100% (1)	
		250	100% (2)	Murphy *et al.* (2014b)
	CAR knockout C57BL/6NTac	250	100% (1)	
7 days	$B6C3F_1$	75	0%	LeBaron *et al.* (2010)
		250	100% (1+2)	
		250	100% (2)	Murphy *et al.* (2014a)
		400	100% (2)	LeBaron *et al.* (2010)
14 days		75	0%	LeBaron *et al.* (2010)
		250	100% (1+2)	
		400	100% (3)	
14 days (+21 day recovery)		75	0%	
		250	0%	
		400	0%	
12 weeks		200	100% (2)	Daly (1995)
		300	100% (3)	
		400	100% (4)	
1 year		125	100% (2)	Stebbins and Cosse (1997)
		250	80% (3)	
2 years		125	96% (3)	
		250	52% (3)	

[1]Numbers in parentheses indicate the average severity grade for centrilobular/midzonal liver hypertrophy: 1 = very slight; 2 = slight; 3 = moderate; 4 = severe

Key event #5: Increased cell proliferation

Cell proliferation is a key event in the proposed CAR MOA. In PB-treated mice, increased cell proliferation in the liver is transient, and is generally observed after treatment for 7 days (possibly 14 or 28 days), but not usually after longer treatment (Elcombe *et al.*, 2014). Cell proliferation then returns to background levels (US EPA, 2012a). Seven studies investigated hepatocellular proliferation (Table 19).

In general, hepatocellular proliferation was observed in mice at the higher doses. Increased proliferation was observed in mice exposed to nitrapyrin for one year as well as mice exposed for periods of four days up to twelve weeks. US EPA (2012a) states "the weak response in the cell proliferation assay was not expected. For agents that induce CAR nuclear receptor activation, cell proliferation typically occurs within 1-4 days, which coincides with a burst of mitotic activity, and declines precipitously by day 7." The increase in cell proliferation is not expected to continue for the entire length of the study.

The newer mechanistic studies demonstrated similar findings (Table 19). After four days of treatment with 250 mg/kg/day, Murphy *et al.* (2014a) observed an increase in hepatocellular proliferation in the midzonal and periportal regions in both B6C3F_1 and C57BL/6NTac mice. After seven days of treatment with 250 mg/kg/day, proliferation was increased in the centrilobular, midzonal, and periportal regions in B6C3F_1 mice, but only in the periportal region in C57BL/6NTac mice. Murphy *et al.* (2014b) also observed an increase in proliferation in the periportal region in wild-type mice treated with 250 mg/kg/day nitrapyrin for 4 days, but not in CAR knockout mice.

Table 19. Hepatocellular proliferation in male mice exposed to nitrapyrin, as reported in several mechanistic studies

Length of treatment	Dose (mg/kg/day)	Mouse strain	Method used to assess proliferation	Liver lobular zones[1]				Reference
				CL[2]	MZ[2]	PP[2]	Total	
4 days	250	$B6C3F_1$	BrdU[3]	1.25	2.3*	5.9*	3.5*	Murphy *et al.* (2014a)[4]
		Wild-type C57BL/6NTac		-0.84	1.9*	4.8*	2.4*	
				0.8	1.4	2.4*	1.5*	Murphy *et al.* (2014b)[5]
		CAR knockout C57BL/6NTac		0.6	0.8	0.9	0.8	
7 days	75	$B6C3F_1$		-1.5	-1.9	-1.8	-1.8	LeBaron *et al.* (2010)[4]
	250			1.1	1.5	3.3*	2.0	
				1.8*	2.5*	6.2*	3.9*	Murphy *et al.* (2014a)[4]
		Wild-type C57BL/6NTac		1.12	1.46	3.79*	2.13	
	400	$B6C3F_1$		1.9	3.0*	8.0*	4.3*	LeBaron *et al.* (2010)[4]
14 days	75			1.0	-1.3	1.1	-1.1	
	200		PCNA[6]	16.7*		-0.8	3.3	Yano and McFadden (1996)
	250		BrdU[3]	2.1	1.7	3.8*	2.4*	LeBaron *et al.* (2010)[4]
	400			1.2	3.8*	11.2*	4.9*	
			PCNA[6]	105*		8.9*	23.4*	Yano and McFadden (1996)
14 days (+21 day recovery)	75		BrdU[3]	1.1	1.0	-1.1	1.0	LeBaron *et al.* (2010)[4]
	250			-1.0	1.2	1.2	1.1	
	400			-2.6	-3.0	-3.1*	-2.9	
12 weeks	200		Ki-67[7]	3	2.0	-0.3	2.0	LeBaron (2010)[4]
	400			5.5	6.0*	8.0*	8.0*	
1 year	125		PCNA[6]	4.8*		-0.25	2.5	Stebbins and Cosse (1997)[4]
	250			12.5*		9.5	11.2*	

[1] Data are presented as relative fold-change compared to control
[2] CL: centrilobular; MZ: midzonal; PP: periportal
[3] BrdU: 5-bromo-2'-deoxyuridine (a structural analog of thymidine)
[4] * $\alpha \leq 0.05$: pairwise comparison with controls by Dunnett's test (performed by study authors)
[5] * $\alpha \leq 0.05$: pairwise comparison with controls by t-test (performed by study authors)
[6] PCNA (a subunit of DNA polymerase)
[7] Ki-67 (a nuclear protein expressed only in cycling cells)

Key event #6: Increased liver weight

Increased liver weight, a key event in the proposed MOA that accompanies hepatocellular hypertrophy, was reported in five studies in male mice (Table 20). Overall, increases in relative liver weights generally mirrored the increased liver hypertrophy profile (US EPA, 2012a). Both liver hypertrophy and increased liver weights were observed in nitrapyrin-treated CAR knockout mice at levels similar to wild-type mice (Murphy *et al.*, 2014b). Therefore, it appears that these particular liver changes are not mediated solely via CAR.

Table 20. Percent increase in mean liver weights in male mice exposed to nitrapyrin compared to untreated controls, as reported in several mechanistic studies

Length of treatment	Dose (mg/kg/day)	Mouse strain	Percent increase in liver weight	Reference
4 days	250	$B6C3F_1$	27%	Murphy *et al.* (2014a)
		Wild-type C57BL/6NTac	28%	
			24%	Murphy *et al.* (2014b)
		CAR knockout C57BL/6NTac	23%	
7 days	75	$B6C3F_1$	3%	LeBaron *et al.* (2010)
	250		20%	Murphy *et al.* (2014a)
		C57BL/6 NTac	30%	
		$B6C3F_1$	19%	LeBaron *et al.* (2010)
	400		39%	
14 days	75		3%	
	250		24%	
	400		49%	
14 days (+21 day recovery)	75		<1%	
	250		1%	
	400		1%	
12 weeks	200		25%	Daly (1995)
	300		49%	
	400		70%	
1 year	125		24%	Stebbins and Cosse (1997)
	250		24%	
2 years	125		24%	
	250		101%	

Key event #7: Increased basophilic foci

Basophilic foci are preneoplastic liver lesions believed to eventually progress to adenomas and carcinomas (US EPA, 2012a). Stebbins and Cosse (1997) measured the incidence of eosinophilic and basophilic foci in male and female $B6C3F_1$ mice (Table 21). The increase in basophilic foci was significant at 250 mg/kg/day for both sexes, but the data do not show a correlation between the number of foci and tumor

incidence. Fewer nitrapyrin-treated mice had basophilic foci than had liver tumors in both the male and female studies. In the female mouse study, a statistically significant increase in liver tumors was observed at the low dose, but a corresponding increase in basophilic foci in that dose group is lacking.

Table 21. Incidence of hepatocellular foci and liver tumors in male and female B6C3F$_1$ mice treated with nitrapyrin for 2 years (Stebbins and Cosse, 1997, as reported in US EPA, 2012a)

Sex	Male			Female		
Dose (mg/kg/day)	**0**	**125**	**250**	**0**	**125**	**250**
Number of animals examined	50	50	50	50	50	50
Basophilic foci, focal/multifocal	0	1	8	1	1	12
Eosinophilic foci, focal/multifocal	5	5	12	2	2	23
Combined hepatocellular adenoma and carcinoma	17/49	20/50	46/49	6/47	28/48	33/48

Key event #8: Liver tumors

The final key event in the proposed CAR MOA is the development of liver adenomas or carcinomas. As shown in Table 21 and discussed in more detail in Section 3.2 (Carcinogenicity Studies in Animals), nitrapyrin induces liver tumors in male and female mice (Stebbins and Cosse, 1997).

US EPA review of the proposed CAR MOA for nitrapyrin

Overall, US EPA (2012a) concluded from their review of the MOA data that, "while there was some evidence of CAR activation, this finding was not supported by key data on P450 and specific enzyme induction (PROD). Similarly, the cell proliferation data did not show the typical profile…It was concluded, therefore, that the available data did not adequately support a CAR MOA." They did, however, add that "there is convincing evidence from the stop and recovery experiment that the effects were reversed when treatment ceased" (US EPA, 2012a).

US EPA (2012a) suggested further that study of "cell proliferation at earlier times in the dosing regimen to better define the time course of the proliferative response" be considered and that a CAR null mouse assay be considered for better characterization of the MOA.

Discussion of additional studies submitted by Dow Chemical Company

At the suggestion of US EPA (2012a), the registrant, Dow Chemical Company, conducted additional studies to support the proposed MOA. These include an *in vitro* study in rat liver microsomes to investigate possible suicide inhibition of cytochrome

P450 activity by nitrapyrin (LeBaron *et al.*, 2014), a CAR knockout mouse study (Murphy *et al.*, 2014b), and an *in vitro* study comparing cell proliferation in human and rat hepatocytes (Johnson and Kan, 2015).

When *Cyp2b10*-associated PROD activity was not detected in the *in vivo* study conducted by LeBaron *et al.* (2010), the registrant suggested this was due to mechanism-based (suicide) inhibition of the PROD enzyme *in vivo* by nitrapyrin administration. The registrant conducted an *in vitro* experiment with PB-induced liver microsomes (LeBaron *et al.,* 2014). In this system, addition of nitrapyrin or the positive control (curcumin) inhibited PROD activity in a dose-related manner, while addition of PB (as a negative control) had no effect. The authors proposed that nitrapyrin irreversibly inhibited Cyp2b10-mediated PROD activity of PB-induced microsomes.

A short-term four-day CAR knockout mouse study was conducted to investigate if CAR activation is necessary to induce hepatocellular proliferation (Murphy *et al.*, 2014b). The authors assert that, because CAR knockout mice exposed to nitrapyrin did not demonstrate hepatocellular proliferation or increased *Cyp2b10* gene expression, CAR activation is necessary for nitrapyrin-induced liver tumors. However, other liver changes were observed in both wild-type and CAR knockout mice, including induction of *Cyp1a1*, *Cyp3a11*, and *Cyp4a10*, centrilobular/midzonal liver hypertrophy, and increased liver weight. Therefore, it is apparent that nitrapyrin has other effects on the liver that are independent of CAR activation.

Johnson and Kan (2015) examined hepatocyte cell proliferation *in vitro* to compare human and mouse cell proliferation. Nitrapyrin exposure significantly increased the proliferation of CD-1 mouse hepatocytes at a concentration of 10 µM. Nitrapyrin had no effect on the proliferation of human hepatocytes at any concentrations tested. However, the human hepatocytes were primary cells isolated from two donors (a 61-year old Caucasian male and a 61-year old Caucasian female). In humans, CAR induces the *CYP2B6* gene, not the murine Cyp2b10 gene (Yang and Wang, 2014). CYP2B6 is a highly polymorphic gene, and large inter-individual differences exist in the expression of hepatic CYP2B6 induced by human CAR (Wang and Tompkins, 2008; Kobayashi *et al.*, 2015). Given the variability in expression of hepatic CYP2B6 induced by human CAR, it is not likely that these hepatocytes isolated from two human donors are representative of the human population.

Discussion of the proposed CAR MOA

As discussed by US EPA, the hypothesized CAR mode of action for PB involves several key events. Some of these key events were also observed in the studies of nitrapyrin, including *Cyp2b10* expression, liver hypertrophy, increased liver weight, and increased

basophilic foci. However, some of the key events were not observed with nitrapyrin treatment. Specifically, nitrapyrin did not increase PROD activity and did not produce the expected pattern in hepatocellular proliferation. Studies performed after the US EPA review in 2012 indicate that hepatocellular proliferation occurs following as early as 4 days of treatment with nitrapyrin. Yet the rapid decline in proliferation noted by US EPA as typical for chemicals that activate CAR was not observed in any of the nitrapyrin studies.

In additional studies conducted by Dow Chemical Company, CAR knockout mice exposed to nitrapyrin did not demonstrate hepatocellular proliferation. Both wild-type and CAR knockout mice administered nitrapyrin did, however, demonstrate centrilobular/midzonal liver hypertrophy and increased liver weights. *Cyp2b10* gene expression was much lower in CAR knockout mice than in wild-type mice. However, induction of *Cyp1a1*, *Cyp3a11*, and *Cyp4a10* in both wild-type and CAR knockout mice indicate that nitrapyrin interacts with other nuclear receptors, such as AhR, PXR, and PPARα. Thus, increased liver hypertrophy and liver weights and similar effects on expression of other *Cyp* genes in both wild-type mice and mice lacking the CAR receptor indicate that many of nitrapyrin's effects on the liver are independent of CAR.

Both nitrapyrin and PB activate CAR and induce liver tumors in mice; however, the nitrapyrin studies are limited in scope and provide limited information on the mechanisms through which nitrapyrin induces liver tumors in mice and no information on the induction of tumors at other sites. It is noteworthy that another group of chemicals hypothesized to act through a CAR mode of action, the conazoles, were shown to operate through very different molecular processes than PB (Nesnow *et al.*, 2009).

Relevance to humans

The registrant, Dow Chemical Company, argues that the key events for nitrapyrin-induced carcinogenic effects in the mouse liver are species-specific, and that the liver tumors observed are not relevant for human health risk assessment. The registrant proposed a PB-like CAR-mediated MOA for hepatocarcinogenesis, which supposedly would not be relevant to humans (Elcombe *et al.*, 2014).

However, in evaluating the carcinogenicity of PB, IARC concluded that PB is possibly carcinogenic to humans (Group 2B), based on sufficient evidence of carcinogenicity from studies in experimental animals, and inadequate evidence from studies in humans (IARC, 2001). An epidemiology study published since the IARC review by Lamminpaa *et al.* (2002) examined cancer incidence among epileptic patients taking any of several antiepileptic drugs, including PB. Patients were followed up for a mean of nearly 17

years. This study found statistically significant excess risks of cancers of the brain and nervous system, larynx, liver, pancreas, colon, stomach, and lung among patients taking antiepileptic drugs (Lamminpaa *et al.*, 2002). While this study was unable to assess cancer risk associated with PB specifically, the positive associations observed for antiepileptic drugs and cancers at multiple sites are of concern, and merit further investigation.

Recent studies in CAR/PXR humanized mice indicate that induction of mouse and human CAR/PXR lead to very similar responses. Luisier *et al.* (2014) examined early and late transcriptomic responses to sustained PB exposure (90 days) in liver tissue from double knockout CAR and PXR, double humanized CAR and PXR, and wild-type C57BL/6 mice. Transient induction of genes associated with DNA replication, cell cycle, and mitosis, and the proliferation-related nuclear antigen *Mki67* were observed in both humanized CAR/PXR mice and wild-type mice. These responses are consistent with hepatocyte proliferation. Peak expression occurred between 1 and 7 days of PB exposure. All of these responses were absent in the knockout mouse livers and were reversible in wild-type and humanized mice with a 4-week recovery period following exposure. These data suggest that the activation of both mouse and human CAR by PB leads to very similar hepatic xenobiotic and proliferative transcriptional responses in a C57BL/6 mouse genetic background (Luisier *et al.*, 2014).

In another study, male transgenic mice expressing human CAR and PXR were used to investigate possible differences between wild-type and humanized mice in their responses to PB (Braeuning *et al.*, 2014). In this tumor initiation/promotion study, a single initiating dose of N-nitrosodiethylamine was given, followed by PB treatment for 10 months. The authors state that the tumor response in PB-treated humanized mice was less pronounced regarding tumor volume fraction and tumor multiplicity, but that "phenobarbital-mediated tumor promotion clearly occurs in mouse liver expressing the human CAR and PXR receptors" (Braeuning *et al.*, 2014). Specifically, the liver tumor incidences observed in mice treated with the initiator alone were 7/15 adenomas in wild-type mice and 12/15 adenomas in humanized mice, and in mice treated with the initiator and with PB promotion the incidences were 14/14 liver adenomas in wild-type mice and 15/15 liver adenomas in humanized mice.

5. REVIEWS BY OTHER AGENCIES

Nitrapyrin has been reviewed and classified by US EPA as to its potential carcinogenicity in 1992, 2000, 2005, and 2012, as presented in some detail in preceding sections of this document. Currently, US EPA classifies nitrapyrin as "Suggestive

Evidence of Carcinogenic Potential" (US EPA, 2012a). Nitrapyrin has not been classified as to its potential carcinogenicity by NTP, FDA (US Food and Drug Administration), the National Institute for Occupational Safety and Health, or IARC.

6. SUMMARY AND CONCLUSIONS

6.1 Summary of Evidence

No epidemiology studies were identified that investigated the risk of cancer associated with exposure to nitrapyrin.

Evidence for the carcinogenicity of nitrapyrin comes from long-term carcinogenicity studies (two in rats and four in mice) exposed via feed. Tumors were observed at a number of sites.

Kidney tumors

- In the male F344 rat study by Szabo *et al.* (1989), the combined incidence of rare renal tubule cell adenoma and adenocarcinoma was significantly increased in the high-dose group. Statistically significant dose-response trends were observed in the incidence of renal tubule cell adenoma, adenocarcinoma, and combined adenoma and adenocarcinoma.

Liver tumors

- In the male B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidences of hepatocellular adenoma and combined hepatocellular adenoma and carcinoma were significantly increased in the 250 mg/kg/day dose group, with significant dose-response trends.
- In the female B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidences of hepatocellular adenoma and combined hepatocellular adenoma and carcinoma were significantly increased in the 125 and 250 mg/kg/day dose groups, with significant dose-response trends.

Forestomach tumors

- In the male B6C3F_1 mouse study by Stebbins and Cosse (1997), the incidences of forestomach squamous cell papilloma and combined squamous cell papilloma and carcinoma were significantly increased in the 125 and 250 mg/kg/day dose groups, with significant dose-response trends observed for papilloma, carcinoma, and combined papilloma and carcinoma.

- In the female B6C3F$_1$ mouse study by Stebbins and Cosse (1997), the incidences of forestomach squamous cell papilloma and combined squamous cell papilloma and carcinoma were significantly increased in the 125 and 250 mg/kg/day dose groups, with significant dose-response trends.

Epididymal tumors

- In the male B6C3F$_1$ mouse study by Stebbins and Cosse (1997), the incidence of rare epididymal histiocytic sarcoma was significantly increased, with a significant dose-response trend.

Harderian gland tumors

- In the female B6C3F$_1$ mouse study by Stebbins and Cosse (1997), the incidence of Harderian gland adenoma was significantly increased, with a significant dose-response trend.

Nitrapyrin has been assessed for a limited number of genotoxicity endpoints. Nitrapyrin did not induce reverse mutations in *E. coli* strain WP2*uvr*A, HGPRT mutations in mammalian CHO cells, MN in mouse bone marrow *in vivo*, or UDS *in vitro* in rat hepatocytes or *in vivo* in mouse liver. In the *Salmonella* reverse mutation assay, nitrapyrin induced concentration-related and reproducible increases in frameshift and base pair substitution mutations in multiple tester strains in two studies, but not in a third. Specifically, nitrapyrin induced concentration-dependent increases in *S. typhimurium* strains that detect frameshift mutations (TA97, TA98), and base pair substitution mutations (TA100) in the presence of metabolic activation, with a greater response generally observed in the presence of hamster liver S-9, as compared with rat liver S-9 in the studies by Zeiger *et al.* (1988), and in strains TA98 and TA100 with or without rat liver S-9, and in TA1535 (detects base pair substitutions) with S-9 in the studies by Mecchi (2007).

Metabolism studies in rats, mice and dogs indicate that nitrapyrin is metabolized to 6-CPA, which is then conjugated with glycine to form 6-CPG (or taurine in mice) and excreted. Additional nitrapyrin metabolites have been detected but not fully characterized in chickens and goats. No metabolism studies in human tissue or cells were identified.

Nitrapyrin activates CAR and studies suggest activation of AhR, PXR, and PPARα. CAR activation has been proposed as a MOA for the induction of liver tumors in mice. Some of the key events in the proposed CAR MOA were observed with nitrapyrin, including increased liver *Cyp*2b10 expression, hypertrophy, absolute and relative liver weight, and basophilic foci. However, total liver cytochrome P450 protein content was

not increased, and some of the key events in the proposed CAR MOA were not observed following nitrapyrin exposure. Specifically, nitrapyrin did not increase PROD activity and, as noted by US EPA, the liver cell proliferation response pattern was not typical of other CAR activators (US EPA, 2012a). In addition, CAR knockout mice exhibited many similar liver changes as wild-type mice when treated with nitrapyrin (increased liver *Cyp1a1*, *Cyp3a11*, *Cyp4a10* gene expression, hypertrophy, and weight) (Murphy *et al.*, 2014b). Thus, it appears that many of nitrapyrin's effects in mouse liver are independent of CAR.

α_{2u}-Globulin accumulation, mineralization of the loops of Henle, hyaline droplet accumulation, and increases in dilated tubules with proteinaceous casts were observed in the kidneys of male rats (Szabo *et al.*, 1989). α_{2u}-Globulin accumulation was proposed as a possible mechanism for renal tubule cell tumor development in male rats, but several IARC criteria for determining whether an agent causes kidney tumors through an α_{2u}-globulin-associated response in male rats (IARC, 1999) were not met, such as "lack of genotoxic activity" and "male rat specificity for nephropathy and renal tumorigenicity". Specifically, evidence of genotoxicity was observed in *Salmonella* in multiple strains in two studies, and effects of nitrapyrin on the kidney were observed in female rats (increases in dilated tubules with proteinaceous casts at 12 months in treated females, increased absolute and relative kidney weights at 12 and 24 months, and increased blood urea nitrogen (BUN) at 24 months, Szabo *et al.,* 1989). Additionally, kidney effects of nitrapyrin were observed in male mice: rare renal tubule adenomas (one each in the 25 and 75 mg/kg/day dose groups in the study by Quast *et al*., 1990), and increases in absolute and relative kidney weights at 24 months in the 250 mg/kg/day dose group in the study by Stebbins and Cosse (1997). With regard to three of the other IARC criteria, data were not available to evaluate whether the criteria had been met. Given the genotoxicity findings in *Salmonella* and the effects of nitrapyrin on female rat kidney, it is likely that nitrapyrin acts through additional carcinogenic mechanisms.

Nitrapyrin has been tested in 403 high-throughput screening assays in the US EPA ToxCast database, and was active in seven. Five of the active assays detect upregulation of transcription factor activity (CEBPB, MTF1, POU2F1, PXR, and VDR) and two detect downregulation of chemokine gene expression (CXCL10 and CCL2). Analysis using the CTD indicates that five of the seven target genes (CEBPB, PXR, VDR, CXCL10, and CCL2) have been associated with cancer pathways.

The biological activity of nitrapyrin was compared to 11 structurally related pyridine compounds: pyridine, 2-chloropyridine, 3-chloropyridine, 2,3-dichloropyridine, 2,5-dichloropyridine, 2,6-dichloropyridine, 3,5-dichloropyridine, 2-(chloromethyl)pyridine,

3-(chloromethyl)pyridine, 4-(chloromethyl)pyridine, and 2-chloro-5-(trichloromethyl)-pyridine. Of these 11 comparison compounds, just one compound, pyridine, is listed under Proposition 65 as causing cancer, and the only other comparison chemical reported to induce tumors in animals is 3-(chloromethyl)pyridine. Pyridine and/or 3-(chloromethyl)pyridine induced tumors at sites in common with nitrapyrin, *e.g.*, mouse liver, rat kidney, mouse forestomach, and rat testes (although at a different site within the testes), but comparisons across the larger set of chemicals are limited by the lack of carcinogenicity testing data for several of these compounds. Many of these structurally-related compounds are genotoxic, and induce mutations in *Salmonella* and/or mutations or chromosomal aberrations in the mouse lymphoma cell assay.

6.2 Conclusion

The evidence for the carcinogenicity of nitrapyrin comes from:

- Multiple studies in rats and mice
    - Renal tubule cell tumors, which are rare, in male F344 rats.
    - Liver tumors in male and female B6C3F_1 mice.
    - Forestomach tumors, including rare squamous cell carcinomas, in male and female B6C3F_1 mice.
    - Epididymal histiocytic sarcomas, which are rare, in male B6C3F_1 mice.
    - Harderian gland adenomas in female B6C3F_1 mice.
- Nitrapyrin was tested for a limited number of genotoxicity endpoints. It induced mutations in the *Salmonella* reverse mutation assay in multiple tester stains in two studies, but not in a third study.
- Nitrapyrin activated CAR and one unpublished study suggests activation of AhR, PXR, and PPARα.
- α_{2u}-Globulin accumulation was proposed as a possible mechanism for renal tubule cell tumor development in male rats, but several IARC criteria for determining whether an agent causes kidney tumors through an α_{2u}-globulin-associated response in male rats (IARC, 1999) were not met and it is likely that nitrapyrin acts through additional carcinogenic mechanisms.
- Some similarities in biologic activity between nitrapyrin and 11 structurally similar compounds, including pyridine, which is listed under Proposition 65 as being known to the state of California to cause cancer.

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APPENDIX A. Parameters for Literature Searches on the Carcinogenicity of Nitrapyrin

General searches of the literature on the carcinogenicity of nitrapyrin were conducted under contract by the University of California, Berkeley (Charleen Kubota, M.L.I.S.). The goal was to identify peer-reviewed open source and proprietary journal articles, print and digital books, reports and gray literature that potentially reported relevant toxicological and epidemiological information on the carcinogenicity of this chemical. The search sought to specifically identify all literature relevant to the assessment of evidence on cancer.

Databases

The literature search utilized the following search platforms/database vendors:

- PubMed (National Library of Medicine)
- EMIC (National Library of Medicine)
- SciFinder®: CAS (Chemical Abstracts Service)
- TOXNET (National Library of Medicine): Toxicology Literature Online (TOXLINE), Genetic Toxicology Data Bank (GENE-TOX)
- Web of Knowledge: BIOSIS Previews®, Web of Science® (Thomson-Reuters, Inc.)

Search Process

Relevant subject terms were entered into the PubMed Search Builder to execute a search.

The following is a typical chemical search strategy used to search PubMed:
(“*chemical name*” [MeSH] OR “CAS *registry number”*[RN]) AND ("bioassay"[MeSH] OR "carcinogenicity"[MeSH] OR "cancer"[MeSH] OR "tumor"[MeSH]) OR "neoplasm"[MeSH]) OR "genotoxicity"[MeSH]) OR "mutagenicity"[MeSH]) OR "metabolism"[MeSH]) OR "absorption"[MeSH]) OR "pharmacokinetics"[MeSH]) OR "structure activity relationship"[MeSH])

In PubMed, MeSH (Medical Subject Headings) terms at the top of hierarchical lists of subject headings are automatically “exploded” in a search to retrieve citations with more specific MeSH terms. For example, the heading “carcinogenicity” includes broad conditions that are related to cancer induction in animals and humans.

Additional databases listed above were then searched. The search strategies were tailored according to the search features unique to each database. Web of Science, for

example, was searched by entering chemical terms and refining the search by applying Web of Science categories Toxicology and/or Public, Environmental and Occupational Health. The search term used includes either the CAS registry number or the chemical name and its available synonyms. Sometimes other databases not listed here were searched as needed.

Additional focused searches were performed by OEHHA as needed. The search strategies are briefly described as follows:

Sections 3.1 – 3.3.3, Section 4

Focused searches were conducted for nitrapyrin. Relevant literature was also identified from citations in individual articles.

- Databases and other resources used: Google search engine, ChemSpider (Royal Society of Chemistry), MeSH (Medical Subject Headings) (National Library of Medicine), GENE-TOX, PubMed & PubChem BioAssay (National Library of Medicine), TOXLINE (National Library of Medicine).
- ChemSpider was searched to gather synonyms, CAS registry number, MeSH terms and Chemical Abstracts Service headings before searching bibliographic databases.
- PubMed search strategy: relevant subject terms were entered into the PubMed Search Builder to execute a PubMed search.
- The search strings applied to all databases listed above as: ("nitrapyrin" [MeSH] OR "2-chloro-6-(trichloromethyl)pyridine" OR “1929-82-4[RN]”) AND (“Neoplasms” [MeSH] OR “Cancer” [MeSH] OR “Mutation” [MeSH] AND “Toxicity” [MeSH] OR “Mechanism” [MeSH]).

Section 3.3.4:

- Structurally-related chemicals were first identified from US EPA (1992). Additional structurally-related chemicals were identified using the following applications: ChemoTyper (Molecular Networks GmbH and Altamira LLC, 2013), OECD QSAR Toolbox (OECD QSAR Toolbox v. 3.2, 2013), and VEGA (VEGA NIC v1.0.8, 2013).
- Databases and other resources used: Google search engine, ChemSpider (Royal Society of Chemistry), MeSH (Medical Subject Headings) (National Library of Medicine), PubMed & PubChem BioAssay (National Library of Medicine), TOXLINE (National Library of Medicine), iCSS Dashboard v0.5 (US EPA ToxCast Phase II data) and CTD (Comparative Toxicogenomics Database).
- The same search methods used in previous sections were used to identify data for structurally-similar chemicals.

Section 3.3.5

- The nitrapyrin ToxCast/Tox21 data and assay information were found through iCSS Dashboard v0.5 (US EPA ToxCast Phase II data, http://actor.epa.gov/dashboard/) and PubChem BioAssay (National Library of Medicine)
- The toxicogenomic data on nitrapyrin were found through CTD (Comparative Toxicogenomics Database, http://ctdbase.org/) and PubMed searches.
- 6 papers related to associations between the target genes and cancer pathways were identified and selected using standard expert knowledge-based review practices.

In summary, 328 references, including government reports, peer-reviewed journal articles, and books, were identified through these search strategies. Among these, 122 references were cited in this document.

Appendix A References

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