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THIFENSULFURON-METHYL: NEW DATA TO SUPPORT NO CLASSIFICATION FOR HUMAN HEALTH HAZARDS UNDER CLP

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TABLE OF CONTENTS

TABLES

FIGURES

APPENDICES

THIFENSULFURON-METHYL: NEW DATA TO SUPPORT NO CLASSIFICATION FOR HUMAN HEALTH HAZARDS UNDER CLP

1.0 SUMMARY

DuPont Crop Protection (DuPont) supports the proposal to not "classify for any human health hazard classes" as concluded in the CLH report for thifensulfuron-methyl (March 2016) prepared by the United Kingdom, the Rapporteur Member State.

The additional evaluations and new data presented in this document confirm that the requirements for carcinogenicity and developmental toxicity (based on observations in the rat developmental toxicity study) classifications under Regulation (EC) 1272/2008 have not been met. It also supports the conclusion that observed distribution of mammary tumours in the 2-year rat study are neither related to thifensulfuron-methyl administration nor are associated with an endocrine-mediated mode-of-action.

With respect to evaluations of mammary tumor incidence and potential for an endocrine-mediated mode-of-action, the new data, according to the OECD Conceptual Framework (OECD, 2012), demonstrate lack of a mode-of-action relevant to Sprague-Dawley rats and humans. Thifensulfuron-methyl was:

- Inactive in *in vitro* oestrogen receptor binding and transactivation assays in published screens for oestrogen agonist and antagonist activities; and in *in silico* assessments of interactions with oestrogen receptors;
    - *In vitro* oestrogen receptor binding and transactivation and *in silico* binding assessments also demonstrated inactivity with the triazine metabolite (IN-A4098)
- Inactive *in vivo* for oestrogenic activity (a uterotrophic assay in overiectomized Sprague-Dawley rats);
- Inactive *in vitro* in a dopamine binding assay, and absence of structural alerts for dopamine receptor interaction; and
- Inactive *in vivo* in the induction of prolactin secretion (via the dopamine pathway).

The CLH report concludes that mammary tumour incidences observed in the chronic rat study are not treatment-related, but are within the range of variability for Sprague-Dawley rats (a strain of rats highly susceptible to mammary gland tumourigenesis). These new data also demonstrate that the tumour incidences were not associated with the two, non-genotoxic, endocrine-mediated modes-of-action most relevant for this species (i.e., oestrogenic activity and dopamine receptor activation with subsequent increased prolactin concentration).

With respect to reproductive toxicity, new data are provided to demonstrate that there were no alterations observed in the foetal rat kidneys in a developmental toxicity

study in rats specifically designed to examine reproducibility of findings in the renal papilla:

- No foetal renal abnormalities were observed at 800 mg/kg/day (highest dosage tested in the previous rat developmental study). In the new study, dosing was performed throughout the gestation period (Gestation days (GD) 6-20), which was in contrast to the previous study in which the last day of dosing was gestation day 15 (major organogenesis period only). In addition, kidney evaluation procedures were performed according to current guidelines on GD 21 (instead of GD 20 in the former study). Results confirm that the original observations were the result of normal biological variation in kidney development.

These data support the interpretation that the apparently increased incidences of absent/small renal papilla observed in the original study are within the expected high spontaneous background rates observed for developing Sprague-Dawley rats. As such, requirements for developmental toxicity classification under Regulation (EC) 1272/2008 have not been met.

Further, reconstruction of systemic exposures in the carcinogenicity and developmental toxicity studies shows clear escalation of systemic exposure after oral dosing; there is no relevant saturation of exposure. Treatment-related findings would be expected to show a clear dose-response, which is not present for any finding of potential relevance to classification.

In conclusion, the additional data presented in this document confirm that the requirements for carcinogenicity and developmental toxicity classification of thifensulfuron-methyl under Regulation (EC) 1272/2008 have not been met. Additional evaluations also demonstrate the absence of endocrine-mediated activity most relevant to mammary tumour induction in Sprague-Dawley rats and in humans. Therefore, the interim provisions of Annex II, Point 3.6.5, paragraph 4 of Regulation (EC) No 1107/2009 defining substances with potential to have endocrine disrupting properties have not been met with thifensulfuron-methyl.

2.0 INTRODUCTION AND OBJECTIVES

Thifensulfuron-methyl has an existing harmonized classification under Directive No 1272/2008, and is not classified for human health hazards, in particular for carcinogenicity, mutagenicity, or toxicity for reproduction (CMR).

Thifensulfuron-methyl was first authorized for European use in 1993 and the initial Annex I listing was completed in 2001 (European Commission, 2001a). During the preceding ECCO peer reviews, it was concluded that no classifications for human health effects were warranted (European Commission, 2001b). Renewal dossiers were submitted to UK CRD (RMS) in 2012 under the AIR2 program.

During the recent AIR2 review and commenting processes for the thifensulfuron-methyl draft RAR, the carcinogenicity potential for this active substance was discussed. These discussions stemmed from observations in the 2-year study of mammary tumour incidences among female Sprague-Dawley rats that exceeded the

concurrent control. These same data had been previously reviewed and subsequently resulted in ECCO recommendations not to propose the classification of thifensulfuron-methyl for carcinogenicity (European Commission, 2001b and 2001c). Information relevant to interpretation of mammary tumour incidences among female Sprague-Dawley rats (e.g., laboratory historical control data and tumour latency analyses) are included and discussed in detail in the CLH report (UK CRD 2016). During the RAR review process, there were interpretation differences regarding whether the observed tumour incidences were test-substance related (UK CRD 2015a). The Expert Consultation meeting number 125 resulted in the majority of participants concluding that mammary tumours were test-substance related; however, the experts agreed that "no classification and labelling for carcinogenicity would be required for thifensulfuron-methyl" (UK CRD, 2015b; EFSA, 2015c). EFSA considered the lack of mechanistic data, according to the OECD Conceptual Framework (OECD, 2012) as a data gap with respect to the possible endocrine-mediated mode of action for mammary gland tumours observed in rats; and identified it as an issue that could not be finalized. The RMS did not agree.

To support the conclusion that thifensulfuron-methyl should not be classified for carcinogenicity, and that the apparent increase in mammary tumours is an artefact of the high spontaneous background incidence in the species and strain, this document summarizes new data generated according to the OECD Conceptual Framework (OECD, 2012) and provides:

- Summary of new *in silico* information from QSAR evaluation of thifensulfuron-methyl and metabolites for potential endocrine activity;
- Summary of new *in vitro* high throughput screening data available from the US EPA EDSP program for endocrine endpoints for thifensulfuron-methyl;
- Summary of new *in vitro* studies available for oestrogen receptor binding or dopamine receptor binding on thifensulfuron and the triazine metabolite IN-A4098;
- Summary of new *in vivo* data available regarding the potential for thifensulfuron to affect oestrogenic and prolactin mediated endpoints; and
- A weight-of-evidence assessment and interpretation of mammary tumour significance, taking into account the ECHA Guidance on Application of CLP Criteria, supporting non-classification of thifensulfuron-methyl for carcinogenicity.

To support the conclusion that thifensulfuron-methyl should not be classified for developmental toxicity, this document provides:

- A discussion of the multigeneration reproduction study in which microscopic evaluation of kidneys from F2b weanlings did not show evidence of any test substance-related effects on the kidney

- Dosimetry comparison between the multigeneration reproduction study and the developmental study based on data available from the metabolism studies that demonstrates absence of kidney effects in F2b offspring at maternal plasma concentrations exceeding the maternal plasma concentrations in the rat developmental study during time of peak renal papilla development
- A new, focused developmental study in rats to evalute the reproducibilty of foetal renal papillary observations reported at the highest dose (800 mg/kg/day) in the previous rat developmental study. In the study, dosing occurred throughout gestationt(GD 6-20), which was in contrast to the previous study in which the last day of dosing was GD 15 (major organogenesis period only). In addition, kidney evaluation procedures were performed on GD 21 according to current guidelines (instead of GD 20 in the former study). A weight of the evidence conclusion regarding kidney effects taking into account the ECHA Guidance on Application of CLP Criteria, supports no classification of thifensulfuron-methyl for developmental toxicity.

These additional evaluations and data clearly demonstrate that the criteria for classification for carcinogenicity and reproduction endpoints according to EU 1272/2008 have not been met.

3.0 ADDITIONAL INFORMATION REGARDING THE 2-YEAR RAT CHRONIC TOXICITY AND ONCOGENICITY STUDY (RAR ANNEX B.6A; MARCH 2015 UPDATE)

The 2-year rat chronic toxicity and oncogenicity study (DuPont 1986a) is presented in the CLH dossier (UK CRD 2016). DuPont agrees with the discussion of historical control data in the CLH report; differences in the distribution of mammary tumours showed no dose response, were not statistically significant and were within the historical control range.

Further evidence of the high spontaneous incidence of mammary tumours in Sprague-Dawley rats is the finding that 97% of the control animals in the Thifensulfuron-methyl cancer study had mammary gland hyperplasia, a precursor lesion of mammary tumours, and that incidences of 72%, 69% and 82% were seen at 25, 500 and 2500 ppm (CLH dossier, UK CRD 2016).

3.1 Metabolism kinetics (new data)

Although the incidences of mammary tumours appeared slightly higher in female rats at doses of 500 and 2500 ppm thifensulfuron-methyl, the incidence was not linearly related to dose (CLH dossier UK CRD 2016), which could be indicative of a saturation in absorption between the 500 and 2500 ppm dietary concentrations. While the high dose utilized in the ADME study (2000 mg/kg bw) (DuPont 1986b) suggests saturation of absorption relative to the lower dose (20 mg/kg bw) in that study, the dose selection is not directly applicable to the 2-year dietary study where the highest dose tested in female rats is 133 mg/kg bw. The use of gavage dosing instead of dietary administration, the contribution of the dosing vehicle, and the large

dose-spacing in the ADME study impair direct extrapolation. A recent evaluation of blood levels of radiolabeled thifensulfuron-methyl in the ADME study is provided (see Section 4.4; DuPont 2015f). In this evaluation, evidence for slight saturation over the 20 to 2000 mg/kg bw (or 100-fold) dosing range is presented. This shows there is very low potential for saturation in the chronic study over the 500 to 2500 ppm dose range (26 and 133 mg/kg bw, respectively, up to approximately 6-fold higher than the 20 mg/kg dose utilized for the ADME study). Therefore, the absence of clear dose-response in mammary tumour incidences cannot be explained on the basis of saturation of absorption.

3.2 Similarity to other triazine herbicides and triazinyl SUs (new data)

During the re-authorization process, comments were received comparing thifensulfuron-methyl to atrazine (a chloro triazine herbicide), known to cause an increase in mammary tumours (Cooper et al, 2007). DuPont rejects this comparison. There are important differences in chemical structure and biological activity between atrazine and thifensulfuron-methyl and its metabolites. The herbicidal mode of action for thifensulfuron-methyl (acetolactate synthase inhibitor) differs from that for atrazine (Photosystem II inhibitor). The triazine metabolite of thifensulfuron-methyl (IN-A4098) also differs in structure and is not herbicidally active. Furthermore, in studies with atrazine, tumour latency was demonstrated to be decreased (Eldridge et al, 1999), whereas, tumour latency was not decreased in the case of thifensulfuron-methyl (CLH dossier, UK CRD 2016). Atrazine caused a dose-dependent decrease in prolactin release (Cooper et al, 2007), whereas no effects on serum prolactin were observed in rats administered thifensulfuron-methyl (see Appendix 1.5 DuPont 2015b). These observations demonstrate that the apparent slight increase in mammary tumors in Sprague-Dawley rats administered thifensulfuron-methyl is not related to the atrazine mode of action.

During the evaluation period for the re-authorization process, EFSA suggested a possible link between structural similarities and mammary tumour incidences reported with thifensulfuron-methyl and tribenuron-methyl (UK CRD 2014). EFSA's comments suggested that the mammary tumour response in Sprague-Dawley rats represented a common pattern among triazinyl-sulfonylurea active substances. An increased mammary tumour incidence might be implied in Sprague-Dawley rats at exposures to tribenuron-methyl exceeding the MTD (EFSA, 2004). EFSA however concluded "the carcinogenic effect in female rats was not considered to be of sufficient evidence for classification of tribenuron-methyl as a carcinogen according to the EEC Evaluation Criteria (Directive 67/548/EEC) of dangerous substances and preparations" (EFSA, 2004).

As new data provided by DuPont, an evaluation of publically available toxicological reviews of 10 triazinyl-sulfonylurea active substances (including thifensulfuron-methyl and tribenuron-methyl) did not demonstrate a pattern of mammary tumour induction (Table 1). Chronic studies with most of these active substances utilized the Sprague-Dawley rat strain, which is noted for its high spontaneous mammary tumour incidence rate. With the exception of tribenuron-methyl, any mammary tumour incidences were confirmed to be within the background variability for this strain; and even tribenuron-methyl is not classified for carcinogenicity. Therefore, there has

been no common pattern of mammary tumour induction demonstrated for 10 triazinyl-sulfonylurea active substances. Additionally, the observation of mammary tumours only at tribenuron-methyl doses exceeding the MTD does not meet the criteria "structural similarity to a substance(s) for which there is good evidence for carcinogenicity" (ECHA, 2013).

Furthermore, the absence of correlation in mammary tumour incidences between metsulfuron-methyl, tribenuron-methyl, chlorsulfuron-methyl, and thifensulfuron-methyl, which all produce the common triazine metabolite IN-A4098, demonstrate that IN-A4098 is unlikely to be associated with the induction of mammary gland tumours.

Table 1
Summary of carcinogenic effects observed with triazinyl-sulfonylureas

Active Substance	Tumour Response[a]	Reference
Chlorsulfuron-methyl	Not carcinogenic[b]	ECHA, 2014 EFSA, 2008a
Ethametsulfuron-methyl	Not carcinogenic	EFSA, 2014
Iodosulfuron	Not carcinogenic	EU Commission, 2003
Metsulfuron-methyl	Not carcinogenic	EFSA, 2015a
Prosulfuron	Not carcinogenic	EU Commission, 2013a
Thifensulfuron-methyl	Not carcinogenic[c]	UK CRD, 2015b
Triasulfuron	Not carcinogenic	EU Commission, 2013b; EFSA, 2015b
Tribenuron-methyl	Not carcinogenic[d]	EFSA, 2004
Triflusulfuron-methyl	C3, R40 (proposed C2 under Regulation (EC)1272/2008)[e]	EFSA, 2008b
Tritosulfuron	Not carcinogenic[f]	Germany, 2002

[a] Test substance related tumours, outside of the historical control range.

[b] Slight increase in Leydig cell tumours did not result in classification.

[c] Existing classification is non-carcinogenic. No classification was proposed in the EFSA conclusion report (EFSA, 2015c).

[d] Increased rat mammary tumours at doses exceeding the MTD were considered not sufficient for classification.

[e] Classification based on increased Leydig Cell tumour incidence; aromatase inhibition considered the mode-of-action.

[f] Tritosulfuron containing < 0.02% of the process impurity AMTT was considered to be non-carcinogenic.

3.3 Evaluation of Endocrine Mode Of Action for mammary tumours (new data)

Substances may induce rodent mammary tumours by several non-genotoxic modes-of-action, but principally by oestrogenic activity; or action that results in increased prolactin (e.g., via interaction with dopamine receptors – dopamine antagonists) (Allison et al, 1994; Harvey, 2005; Simpkins et al, 2011). In order to evaluate the potential for thifensulfuron-methyl to interact with oestrogen or dopamine receptors, the following ***new*** evaluations and studies were undertaken:

- *In silico* evaluation of potential interactions with oestrogen receptors (see Section 3.3.1.1; DuPont 2015c)
- Evaluation of publically available *in vitro* oestrogen receptor binding studies (see Sections 3.3.1.2.1; DuPont 2015a)
- New *in vitro* oestrogen receptor binding studies (see Sections 3.3.1.2.2 (DuPont 2016a) and 3.3.1.2.3 (DuPont 2016b))
- Evaluation of repeated dose studies with respect to endocrine endpoints (see Section 3.3.1.3.1)
- New *in vivo* uterotrophic assay with thifensulfuron-methyl (See Section 3.3.1.3.2; DuPont 2015b)
- New *in vitro* dopamine receptor binding assay (see Section 3.3.2; DuPont 2015d) and assessment of structural alerts for dopamine receptor binding

3.3.1 Oestrogen receptor binding

3.3.1.1 QSAR assessments

(See Appendix 1.1 for full OECD summary)

The OECD QSAR Toolbox (v.3.3) was utilized to evaluate oestrogen receptor binding [through tool box components - Estrogen Receptor Binding Affinity Oasis software and the US EPA rtER expert System (v.1); (Appendix 1.1, OECD summary for DuPont 2015c).

No structural alerts for oestrogen receptor binding were identified for thifensulfuron-methyl or its metabolites (Appendix 1.1).

This is consistent with the absence of oestrogenic effects in existing thifensulfuron-methyl toxicological tests (see Section 3.3.1.3.1 below).

There are no specific OECD Toolbox components to evaluate dopamine receptor binding. However, based on recent publications, thifensulfuron-methyl lacks known D2 and D3 receptor binding structural alerts (e.g., a constrained nitrogen atom that is protonated at physiological pH; Salmas et al, 2015; Platania et al, 2012). Therefore, thifensulfuron-methyl is considered unlikely to induce rodent mammary tumours via binding to dopamine (D2 and D3) receptors.

3.3.1.2 In vitro assays

3.3.1.2.1 Oestrogen Receptor Binding, Androgen Receptor Binding, Thyroid Receptor Binding

EPA's Endocrine Disruptor Screening Program (EDSP) is designed to detect the intrinsic ability of chemicals to interact with the endocrine system, specifically for chemicals that can interact with the oestrogen, androgen, or thyroid systems. EPA researchers developed the EDSP21 Dashboard (an *in vitro* high-throughput screening system) to screen over 1800 chemicals of interest. Thifensulfuron-methyl was one of the chemicals screened as part of this program, and the results are summarized in Appendix 1.2 (DuPont 2015a). Of the 11 assays for potential androgenic activity, thifensulfuron-methyl was evaluated in 8 assays, and was negative for bioactivity in all 8 assays. Of the 18 assays for potential oestrogenic activity, thifensulfuron-methyl was evaluated in all 18 assays, and was negative for bioactivity in 17 of the 18 assays. While thifensulfuron-methyl was positive in the ATG_ERE_CIS_up assay, the AC_{50} (59 µM) occurred at a concentration that was approaching observed cytotoxicity. In addition, thifensulfuron-methyl was negative in all of the other ER assays. Of the 4 assays for potential thyroid activity, thifensulfuron-methyl was evaluated in 3 assays, and was negative for bioactivity in all 3 assays.

The weight of evidence from the EDSP21 assays demonstrates that thifensulfuron-methyl does not interact with the oestrogen, androgen, or thyroid signaling systems when evaluated in a wide variety of assay types with a comprehensive range of endocrine-related endpoints.

3.3.1.2.2 IN-A4098 Oestrogen Receptor Binding Assay

(See Appendix 1.3 for full OECD summary)

The objective of this study was to evaluate the ability of IN-A4098 (98.7% w/w) to compete with [^{14}H] ligand for binding oestrogen receptors (ERs) in rat uteri homogenate (DuPont-2016a).

The top concentration of IN-A4098 selected for use in binding assays was 10^{-3} M as no precipitation was observed.

In the first valid independent run the mean specific ligand binding against IN-A4098 was >98% (IN-A4098 could not displace radiolabeled ligand from the receptor) at every concentration tested classifying IN-A4098 as "not interactive" with the ERs for this run. In the second valid independent run the mean specific binding for IN-A4098 was >96% at every concentration tested, classifying IN-A4098 as "not interactive" with ERs for this run. In the third valid independent run the mean specific binding for IN-A4098 was ≥91% at every concentration tested, classifying IN-A4098 as "not interactive" with ERs for this run. Each run was performed in triplicate and included appropriate controls, including non-specific binding, a weak positive control (19-norethindrone) and a positive control (17β-estradiol).In conclusion, IN-A4098 does not interact with the oestrogen receptor based on the results in this assay.

3.3.1.2.3 IN-A4098 Oestrogen Receptor Transactivation Assay

(See Appendix 1.4 for full OECD summary)

The objective of this study was to evaluate the ability of IN-A4098 (98.7% w/w) to act as an agonist of human oestrogen receptor alpha (hERα) using the hERα-HeLa-9903 cell line (DuPont-2016b). Four reference compounds (17β-estradiol, 17α-estradiol, corticosterone, and 17α-methyltestosterone) were run each time the transcription activation assay was performed.

The maximum concentration of IN-A4098 selected for use was 10^{-4}M as test substance precipitation was observed at higher concentrations. There was no cytotoxicity (>20% reduction in cell viability) observed with IN-A4098 or the controls in any of the valid independent runs. In two independent runs of the transcriptional activation assay, IN-A4098 did not result in an increase in luciferase activity at any of the viable concentrations tested. Therefore, IN-A4098 is not an agonist of human oestrogen receptor alpha (hERα) in this system.

3.3.1.3 In vivo assays

3.3.1.3.1 No evidence of oestrogenic activity in existing repeated dose studies

Since the two-generation reproduction study with thifensulfuron-methyl was performed in 1983-1984, endocrine-specific parameters similar to those now included in the OECD 416 test guideline (adopted 2001) were not required endpoints when the study was performed. However, numerous endpoints evaluated in the guideline studies for thifensulfuron-methyl support the conclusion that thifensulfuron-methyl does not show effects consistent with potential endocrine activity.

In two reproductive studies, one a 90-day feeding with a 1-generation reproduction component (DuPont 1984a) and a 2-generation reproduction study (DuPont 1985), there were no test-substance related effects on fertility or on any reproduction or lactation parameter. While pathological examination of the parental rats was not performed for the 2-generation reproduction study, no test substance-related effects on gross or microscopic morphology of endocrine tissues (testes, epididymides, prostate, ovaries, cervix, uterus, vagina, pituitary, thyroid, para-thyroid, or adrenals) were observed in parental rats at dietary concentrations up to 7500 ppm in the one-generation study. In addition, no test substance-related gross or microscopic morphological changes were observed in the endocrine organs from the 2500 ppm F2B weanlings on lactation day 21 (testes, seminal vesicles, epididymides, prostate, ovaries, uterus, vagina, adrenals, pituitary, thyroid, parathyroids, and pancreas).

In the rat developmental toxicity study, there were no test substance-related effects on offspring development (DuPont 1984b) (UK CRD 2016). Finally, in the two-year rat study, there were no test substance-related effects on reproductive organ weight or on gross or microscopic changes to reproductive tissues in male or female rats (DuPont 1986a).

3.3.1.3.2 Uterotrophic assay

(See Appendix 1.5 for full OECD summary)

In order to closely examine the potential for thifensulfuron-methyl to cause an oestrogenic response *in vivo*, a uterotropic study was conducted according to OECD guideline 440 (DuPont 2015b). The highest dosage selected for this study was the highest dose associated with a putative increase in mammary tumours in the combined chronic toxicity and carcinogenicity study in rats, approximately 300 mg/kg/day (DuPont 1986a). In addition to the OECD guideline requirements, the potential for thifensulfuron-methyl to modulate prolactin secretion was evaluated by measurement of prolactin in serum from blood collected at the end of dosing. The oestrogen receptor agonist 17α-ethynyloestradiol and the dopamine (D2) receptor antagonist haloperidol were included as positive controls to verify test system performance.

Thifensulfuron-methyl did not induce changes on any parameters consistent with oestrogen receptor agonist activity in female rats, and did not modulate serum prolactin concentrations. Positive controls gave an appropriate response.

3.3.2 Dopamine receptor assay

(See Appendix 1.6 for full OECD summary)

The activity of thifensulfuron-methyl was evaluated in nine radioligand binding assays utilizing human recombinant CHO cell receptors (D_1, D_{2L}, D_{2S}, D_3, $D_{4.2}$, $D_{4.4}$, $D_{4.7}$, D_5, Dopamine Transporter (DAT) (DuPont 2015d). Methods employed in this study were adapted from the scientific literature to maximize reliability and reproducibility. Biochemical assay results were reported as the percent inhibition of specific binding or activity. No significant responses (>50% inhibition or stimulation for biochemical assays) were noted in any of the radioligand binding assays. These results confirm the *in silico* evaluation that thifensulfuron-methyl and its metabolites are unlikely to interact with the dopamine receptor. This result is confirmed by the absence of any effects on serum prolactin concentrations in the uterotropic assay (Section 3.3.1.3.2)

4.0 ADDITIONAL INFORMATION REGARDING THE RAT DEVELOPMENTAL TOXICITY (RAR ANNEX B.6A; MARCH 2015 UPDATE)

4.1 Developmental reproducibility study (new data)

(See Appendix 1.7 for full OECD summary)

The purpose of this study (DuPont 2015e) was to evaluate the reproducibility of the foetal renal papillary observations (small or absent renal papilla) reported at the highest dose level tested in the previous developmental toxicity study with thifensulfuron-methyl (DuPont 1984b) as described in the CLH report. The new study tested 0 and 800 mg/kg/day however, while in the previous study dams were dosed once daily via oral gavage beginning on gestation day (GD) 6 through 15 and caesarean sections were performed on GD 20 (the numbering of gestation days in the

original study has been brought into alignment with the current practice so as to enable easier comparison of data from the previous and current studies). In the new study (DuPont 2015e), dams were dosed in accordance with current guidelines, i.e. on GD 6 through GD 20, with caesarean sections performed on GD21. All live foetuses were examined externally and euthanized; following euthanasia, fresh visceral examination of the foetal kidneys was performed on all live foetuses.

In contrast to the original study (DuPont 1984b), no effects on offspring renal development were observed in offspring of maternal rats administered thifensulfuron-methyl during gestation days 6-20. Minimally decreased maternal body weight gain and food consumption occurred during gestation days 6-7 and 6-8, respectively in the 800 mg/kg/day group, which was consistent with the previous study. Minimally decreased offspring body weight was observed in the 800 mg/kg/day group, consistent with the original study.

These results demonstrate that the apparent increased incidences of small/absent renal papilla observed at 800 mg/kg/day in the original study were not compound-related, but were due to the greater biological variability occurring in offspring kidney development on gestation day 20, as compared to gestation day 21.

4.2 Supporting evidence from the rat multigeneration reproduction study

The studies by Woo and Hoar (1972), Bertram et al. (2000), and Schreuder et al. (2011) demonstrate that peak development of renal papilla occurs in the range of days 19-25 post conception (or gestation days 19 to day 3 postpartum assuming delivery on gestation day 22). However, in the original rat developmental study (DuPont 1984b), the last day of dose administration to the maternal animals was gestation day 15, and the foetuses were delivered by caesarean section on gestation day 20. Whereas, in the rat multigeneration reproduction study (DuPont 1985), maternal rats were administered dietary concentrations up to 2500 ppm continuously during gestation (days 0-22 post conception) and during lactation (days 0-21 postnatal or days 0-43 post conception).

Although detailed microscopic evaluations of the foetal rat kidneys were not undertaken in the original developmental toxicity study, kidneys from the 21-day old F2 generation weanlings of the reproduction study were evaluated microscopically, and no microscopic abnormalities were noted at any dietary concentration (DuPont 1985).

4.3 Dose reconstruction of the rat multigeneration reproduction study (DuPont 1985)

A summary of the multigeneration reproduction study is available in the CLH report (UK CRD 2016). However, DuPont now provides calculations of plasma concentrations (see Section 4.4).

In the original report (DuPont 1985), mean daily intake was reported for the premating periods for the parental generations only. Maternal body weight was reported only for the day of delivery, and maternal food consumption (and mean daily intake) was not reported for the gestation and lactation periods. The offspring were directly exposed to thifensulfuron-methyl continually during pregnancy (gestation days 0-22), and lactation (lactation days 0-21). Historical control food consumption

data from 18 multigenerational reproduction studies (Table 2) were used to estimate the maternal food consumption for DuPont 1985 for the interval of gestation days 14-21, and were subsequently used to calculate the mean daily intake value for the 2500 ppm group during gestation. The resulting mean values as calculated for the F1A, F1B, F2A, and F2B litters, are shown in Table 3. The mean daily intake value ascribed to the 2500 ppm F2B litter evaluated microscopically was 221 mg/kg/day. This value was used in Section 4.4 below in which the plasma concentration of thifensulfuron-methyl in the 2500 ppm F1b maternal rats (multigeneration study, DuPont 1985) is compared to the plasma concentration of thifensulfuron-methyl in the 800 mg/kg/day maternal rats from the developmental study (DuPont 1984b).

Table 2
Historical control data food consumption data (g/day) from reproduction studies conducted at DuPont Haskell Laboratory

DuPont Report No.	Year of Study Start	P1 GD 14-21	P1 LD0-4a	F1 GD14-21	F1 LD0-4a
30258	2010	23.5	NA	24.0	37.8
17692-1036	2009	31.8	34.5	NA	NA
26346	2008	26.4	30.9	NA	NA
22740	2007	25.4	31.0	NA	NA
28810	2007	27.5	32.8	NA	NA
22032	2007	26.8	32.6	25.4	35.0
21109	2007	25.9	25.5	NA	NA
21607	2006	25.8	30.7	NA	NA
21242	2006	26.1	30.5	NA	NA
21112	2006	22.1	25.4	NA	NA
20820	2006	26.9	NA	NA	NA
20964	2006	29.6	36.0	NA	NA
20813	2006	27.7	35.9	NA	NA
20699	2006	29.2	34	NA	NA
20308	2006	24.6	NA	NA	NA
18331	2006	28.9	37.8	NA	NA
19715	2006	27.1	NA	NA	NA
18827	2006	23.0	36.2	24.8	30.5
Mean		26.6	32.0	24.7	34.4
Minimum		22.1	25.4	24.0	30.5
Maximum		31.8	37.8	25.4	37.8

[a] If data for days 0-4 were not available, data for days 0-7 were used.

NA = Not available

Table 3
Mean daily intake calculation for 2500 ppm dams on the day of delivery from the multigeneration reproduction study with thifensulfuron-methyl technical

2500 ppm Dams	Generation	Mean Maternal BW on day of delivery (g)	Maternal FC (g/d)[a]	Mean Daily Intake (mg/kg bw/d)
P1	F1A litter	293.6	29.4	250
P1	F1B litter	287.8	29.4	255
F1B	F2A litter	309.3	29.4	238
F1B	F2B litter	332.1	29.4	221
Mean				241

[a] Overall mean food consumption from historical Gestation day 14-21 food consumption values for P1 and F1 generations and Lactation day 0-4 food consumption values for P1 and F1 generations (see Table 2).

4.4 Dose interpolation from the thifensulfuron metabolism studies, multigeneration reproduction study, and rat developmental study

(See Appendix 1.8 for full OECD summary)

The comparative dosimetry of the developmental and reproduction studies in rats with thifensulfuron-methyl is informative in assessing the association, whether causal or spurious, of the reported observations of small or absent renal papilla in the developmental study at 800 mg/kg/day as described in the CLH report. No changes were observed in the kidneys of F2B offspring in the multigeneration reproduction study after continuous dosing throughout gestation, including the period of rapid development of the renal papilla in rats (GD19 to about postnatal day 2) and thus providing a more robust assessment of kidney morphology in offspring (DuPont 1985).

Using the data from the rat metabolism studies (DuPont 1986b; DuPont 1986c) (non-pregnant females) to calculate the half-life of thifensulfuron-methyl, the internal dose of thifensulfuron-methyl in the blood of female rats can be estimated on gestation day 19 of the developmental study and compared to the internal dose on gestation day 19 of the multigeneration reproduction study (DuPont 2015f). The absence of changes in the renal papilla in the multigeneration study (DuPont 1985), which included a more robust assessment of the kidneys of offspring than was available in the developmental study (DuPont1984b), might putatively be the result of the higher dose level used in the developmental study (800 mg/kg/day) compared to the reproduction study (about 221 mg/kg/day). However, in the original developmental study, dams were dosed from gestation days (GD) 6-15, so the dosing protocol for the developmental study provided for approximately 4 days of clearance of the test material (effectively a 4-day recovery period) prior to GD19, the critical period for renal papillary development. In contrast, dams in the multigeneration study were continuously dosed (by the dietary route), including through both the late gestation and early post-natal phases of renal papillary development.

In the metabolism studies (DuPont 1986b, DuPont 1986c), the measured urinary excretion, and the blood concentrations indicated dose proportional and linear first-

order elimination kinetics (Appendix 1.8, Table 29). Blood concentrations at 6 hours after dose administration were estimated to be between 0.56-1.19 µg/g at the low (20 mg/kg bw) dose and 40-169 µg/g at the high (2000 mg/kg bw) dose. The mean measured blood concentrations at the two dose levels showed increases of 118 to 143-fold relative to the 100-fold increase in dose. Pretreatment with dietary (100 ppm) exposure did not alter the kinetics in the one ADME experiment with the [thiophene-2-^{14}C] radiolabel, indicating that repeated exposure would not be expected to appreciably alter uptake and elimination. The half-life for elimination of ^{14}C residues from blood ranged from 12 to 19 hours.

Since measured data from the metabolism studies indicated dose proportional and linear first-order elimination kinetics, it was appropriate to use a direct proportionality interpolation to predict the corresponding systemic (blood) exposure levels in the metabolism, developmental and reproductive toxicity studies. The metabolism studies were single dose studies, and in the developmental study, the last day of dosing was on GD15. Reconstructed blood concentrations declined over the following 96 hours for the metabolism and developmental toxicity studies. In contrast, the dosing protocol in the reproduction study involved continuous dosing (feeding) so that predicted blood concentrations at each 24 hour interval remained constant. Reconstructed blood concentrations under the differing dosing scenarios are given in Appendix 1.8, Table 30.

The key assumptions to perform this interpolation are as follows:

- Oral gavage and dietary administration would produce similar blood concentrations in rats.
- Peak kidney development occurs during post-conception days 19-25 (Woo and Hoar 1972).
- The 96 hours post dose blood and urine concentration in the metabolism studies corresponds to gestation day GD19 in the rat developmental study (DuPont 1984b) study. It is also the time that experimentally measured blood concentrations are available from the metabolism studies (Appendix 1.8, Table 30). Therefore, this blood concentration represents the residual internal dose on GD19 (DuPont 1984b), 4 days after cessation of exposure.
- The 24 hour post dose blood and urine concentration in the metabolism studies corresponds to an internal dose on GD19 (and any other day during gestation or lactation) following continuous dietary administration prior to parturition for the multigeneration reproduction study (DuPont 1985).

Maternal blood concentrations on GD19 in the developmental study (i.e., 96 hours following the last day of dosing on GD15), at the 800 mg/kg/day dose are predicted to have declined to 0.48 µg/g. In the F2B dams of the multigeneration study, where dosing was continuous at a dietary intake equivalent to 221 mg/kg/day, predicted blood concentrations are maintained at 4.4 µg/g (Appendix 1.8).

Therefore, systemic exposure was 10-fold greater for the multigeneration study relative to the developmental study, within the window of time most critical for kidney development.

In conclusion, in the multigeneration reproduction study (DuPont 1985), at the time of peak development for the renal papilla (approximately post conception days 19-25), the blood concentration of thifensulfuron-methyl was 10 times higher as compared to the blood concentration on gestation day 19 in the developmental study (DuPont 1984b), and no histopathological changes were observed in the kidneys of F2B weanlings evaluated on lactation day 21. In addition, if the kidney observations in the rat developmental toxicity study were not due to biological variability, it would be expected that an effect on the growth and survival of offspring in the rat multigeneration reproduction study would have been observed. However, there were no effects on growth or survival of 2500 ppm offspring observed in the rat multigeneration reproduction study. This outcome further underscores that the observations in the rat developmental study reflect biological variability.

5.0 CONCLUSIONS

5.1 Conclusions for evaluation of carcinogenicity and potential for endocrine mediated mode-of-action

Several strands of evidence lead to the conclusion that the increased incidences of mammary gland adenocarcinoma observed at 500 and 2500 ppm thifensulfuron-methyl are not treatment-related. However, the majority of the experts at the Expert Consultation Meeting agreed that it could not be excluded that the mammary gland tumours observed at 500 and 2500 ppm were treatment-related. The experts also agreed that there was insufficient evidence for classification. It is also noted that the TCC&L group in 1997 did not propose a carcinogen classification for thifensulfuron-methyl on the basis of the same data. -

Other observations relevant to a weight-of-evidence assessment of the thifensulfuron-methyl chronic rat study results are summarized in the following:

- Lack of Multi-Site Response and Response in only One Species and Sex: The female mammary tumour incidences from the thifensulfuron-methyl chronic rat study provided no evidence of a compound-related increase in tumour multiplicity in this tissue, in other organs, nor was there an increased tumour incidence in another sex or species.
- No Tumour Latency Reduction: The absence of a significant effect on tumour latency across the 100-fold dose range utilized for thifensulfuron-methyl further suggests the observed tumour incidences were within range of biological variation for this rat strain.
- Non-Mutagenic: Thifensulfuron-methyl is negative in an adequate, guideline-compliant battery of genetic toxicology studies; therefore, a potential genotoxic mode-of-action can be ruled out.

- No Common Pattern of Mammary Tumour Induction within Triazinyl Sulfonylurea Chemical Class: Although one triazine-containing sulfonylurea herbicide (tribenuron-methyl) was associated with an increase in mammary tumours at a dose exceeding the MTD, there is no pattern of mammary tumour induction with active substances in this chemical class.
- Mammary Tumours in Species, Sex and Strain with High Background Incidence: Female Sprague-Dawley rats are known for their high spontaneous rate of mammary tumours. The marginal observations with thifensulfuron-methyl are limited to a single sex and species and are within the historical control ranges published for Sprague-Dawley rats.
- No Progression of Lesion to Malignancy: The high incidence of mammary gland hyperplasia in control females (97%) and treated groups (69-82%) further illustrates the high spontaneous background of the common mammary tumours and precursor lesions in this rat strain.
- Endocrine-Mediated Mode-of-Action is not Supported: Substances may induce rodent mammary tumours by several non-genotoxic modes-of-action, principally: oestrogenicity; or substances that result in increased prolactin (e.g., via interaction with dopamine receptors – dopamine antagonists)
    - *In silico* (QSAR) evaluations conclude that thifensulfuron-methyl does not bind to oestrogen receptors and that there are no carcinogenic structural alerts. This is consistent with negative results from *in vitro* and *in vivo* tests for oestrogenicity. *In vitro* testing also demonstrates no binding of thifensulfuron-methyl to androgen and thyroid receptors. In *silico* evaluations also demonstrate that IN-A4098 and other metabolites do not bind to oestrogen or androgen receptors. *In vitro* testing of IN-A4098 confirms that it is non-oestrogenic.
    - Thifensulfuron-methyl was inactive *in vitro* in a dopamine binding assay and was inactive *in vivo* in the induction of prolactin secretion (modulated by the dopamine pathway), confirming *Q*SAR assessments.
    - Endocrine-Mediated Mode-of-Action is not Supported by the Overall Toxicology Profile: Results from existing subchronic, reproductive and chronic studies for thifensulfuron-methyl do not suggest an endocrine-mediated mode of action. For example, there are no significant compound-related effects on male and female reproductive organ weights or histopathology across several studies, no effects on reproductive function, and no effects on offspring suggestive of an endocrine-mediated mode-of-action.

Given the weight-of-evidence presented above, the decision not to propose carcinogen classification of thifensulfuron-methyl by the EFSA Expert Consultation group is further supported. This weight-of-evidence also supports the absence of an endocrine-mediated mode-of-action. The observed tumours are consistent with the known and highly variable background incidence for Sprague-Dawley rats.

5.2 Conclusion for evaluation of developmental toxicity

During the EFSA review, Member States considered the foetal kidney observations in the original rat developmental study to meet the criteria for classification. However, in the CLH Report, these incidences are considered spurious and a reflection of normal biological variability, a conclusion that DuPont considers to be correct. Factors reinforcing this conclusion include:

- Kidney observations were not reproduced in a more robust study: New data from a rat developmental reproducibility study in which there were no effects on foetal kidney development in rats administered 800 mg/kg bw/day on gestation days 6-20, consistent with the current EEC B.31 guidelines.
- No impact on kidney development in the related multigeneration rat reproduction study:

  A dosage comparison between the original rat developmental toxicity study and the multigeneration study demonstrated a 10-fold greater internal dose in the multigeneration study at the time of peak foetal kidney development. Histopathological evaluation of the F2B weanling kidneys from the multigeneration study demonstrated no adverse effect. Finally, the conclusion that the incidences of small/absent renal papilla represent normal biological variability is also supported by the literature that describes foetal kidney development.

All other foetal developmental variations noted in the study report were within laboratory historical control data ranges and were not test substance-related. The Expert Consultation meeting 125 considered that the NOELs for both maternal and foetal toxicity in the rat developmental toxicity study were 200 mg/kg/day based on the slight decreased maternal body weight gain during gestation days 7-9 and on slight decreased foetal weight at 800 mg/kg/day. No observations in this study meet the criteria for classification according to EU Regulation No. 1272/2008.

In conclusion, these additional data confirm that the requirements for carcinogenicity and reproduction classification under Regulation (EC) No. 1272/2008 have not been met. This weight-of-evidence also supports the conclusion that the observed distribution of mammary tumours in the 2-year rat study are neither related to thifensulfuron-methyl administration nor are associated with an endocrine-mediated mode-of-action.

6.0 REFERENCES

Allison, R.H., Capen, C.C., and Prentice, D.E., 1994. Neoplastic Lesions of Questionable Significance to Humans. Toxicologic Pathology, 22, 179-186.

Bertram, J.F., Young, R.J., Spencer, K., and Gordon, I. (2000) Quantitative Analysis of the Developing Rat Kidney: Absolute and Relative Volumes and Growth Curves. The Anatomical Record 258: 128-135.

Cooper, R.L., Laws S.C, Das, P.C, Narotsky, M.G., Goldman, J.M., Tyrey, E. L. and Stoker, T. E. Atrazine and Reproductive Function: Mode and Mechanism of Action Studies. Birth defects Research (Part B) 80:98-112 (2007).

DuPont 1984a. Ninety-Day Feeding and One-generation Reproduction Study in rats with 2-thiophenecarboxylic acid, 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-amino]carbonyl]amino]sulfonyl]-, methyl ester (IN-M6316). DuPont Unpublished Report HLR-89-84.

DuPont 1984b. Developmental Toxicity Study in Rats Given INM-6316 by Gavage on Days 7-16 of Gestation. DuPont Unpublished Report HLR 146-84

DuPont 1985. Two-generation, four litter reproduction study in rats with IN-M6316. Unpublished DuPont report No., HLR-432-85.

DuPont 1986a. Long-Term Feeding Study in Rats with IN-M6316. DuPont Unpublished Report HLR-261-86.

DuPont 1986b. Metabolism of [thiophene-2-^{14}C]Thifensulfuron-Methyl by rats. DuPont Unpublished Report HLR-91-86.

DuPont 1986c. Metabolism of [Triazine-2-^{14}C]Thifensulfuron-Methyl in male and female rats. DuPont Unpublished Report HLR-234-86.

DuPont 2015a. Thifensulfuron-Methyl: Assessment of Potential Endocrine Activity from the U.S. Environmental Protection Agency (US EPA) Endocrine Disruptor Screening Program (EDSP) for the 21st Century Dashboard. Unpublished Report DuPont-45948.

DuPont 2015b. Thifensulfuron-Methyl (DPX-M6316) Technical: 6 Day Uterotrophic Assay for Detecting Estrogenic Activity and Prolactin Changes in Ovariectomized Rats. Unpublished Report DuPont-44801.

DuPont 2015c. *In Silico* QSAR Evaluation of Thifensulfuron-Methyl, and Metabolites for Potential Endocrine Activity. Unpublished Report DuPont-44803.

DuPont 2015d. Thifensulfuron-Methyl (DPX-M6316) Technical: Receptor Binding Assay. Unpublished Report DuPont-44799.

DuPont 2015e. Thifensulfuron-Methyl (DPX-M6316) Technical: Developmental Reproducibility Toxicity Study in Rats. Unpublished Report DuPont-44802

DuPont 2015f. Thifensulfuron-Methyl: Dosimetry Assessment for the Female Sprague-Dawley Rat during Gestation. Unpublished Report DuPont-45278

DuPont 2016a. IN-A4098: Estrogen receptor binding Assay Using rat Uterine Cytosol (ER-RUC). Unpublished Report DuPont -45572.

DuPont 2016b. IN-A4098: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903)). Unpublished Report DuPont-45573.

ECHA, 2013. Guidance on the Application of CLP Criteria. Guidance to Regulation (EC) No. 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. Version 4.0; November, 2013.

ECHA, 2014. Opinion Development Document proposing harmonised classification and labelling at EU level of Chlorsulfuron. Adopted 4 December 2014. CLH-O-0000001412-86-48/F.

EFSA, 2004. Conclusion regarding the peer review of the pesticide risk assessment of the active substance tribenuron. European Food Safety Authority, EFSA Scientific Report, 15, 1 – 52, 2004. 19 October 2004.

EFSA, 2008a. Conclusion on the peer review of the pesticide risk assessment of the active substance, chlorsulfuron. EFSA Scientific Report, 201, 1-107. 26 November 2008.

EFSA, 2008b. Conclusion regarding the peer review of the pesticide risk assessment of the active substance triflusulfuron (evaluated variant triflusulfuron-methyl). EFSA Scientific Report, 195, 1-115. 30 September 2008.

EFSA, 2014. Conclusion regarding the peer review of the pesticide risk assessment of the active substance ethametsulfuron (evaluated variant ethametsulfuron-methyl). EFSA Journal, 12(7), 3787.

EFSA, 2015a. Conclusion on the peer review of the pesticide risk assessment of the active substance metsulfuron-methyl. European Food Safety Authority, EFSA Journal, 13(1), 3936.

EFSA, 2015b. Conclusion on the peer review of the pesticide risk assessment of the active substance triasulfuron. European Food Safety Authority, EFSA Journal, 13(1), 3958.

EFSA, 2015c. Conclusion on the peer review of the pesticide risk assessment of the active substance thifensulfuron-methyl. European Food Safety Authority, EFSA Journal, 13(7), 4201.

Eldridge, J.C., Wetzel, L.T., Stevens, J.T., and Simpkins, J.W., 1999. The mammary tumour response in triazine-treated female rats: A threshold-mediated interaction with strain and species-specific reproductive senescence. Steroids, 64, 672-678.

European Commission, 2001a. Commission Directive 2001/99/EC of 20 November 2001. Amending Annex I to Council Directive 91/4114/EEC concerning the placing of plant protection products on the market to include glyphosate and thifensulfuron-methyl as active substances. Official Journal of European Communities, L304/14, 21/11/2001.

European Commission, 2001b. Summary Record. Meeting of the Commission Working Group on the Classification and Labelling of Dangerous substances. Pesticides – Environmental effects; ECB – Ispra, 28-29 September 2000. ECBI/73/00 Rev.2, 21 May 2001.

European Commission, 2001c. Review Report for the Active Substance Substance Thifensulfuron-methyl. Thifensulfuron-methyl, SANCO/7577/VI/97-final.12 December 2001.

European Commission, 2003. Iodosulfuron. SANCO/10166/2003-Final. 3 July 2003.

European Commission, 2004. Commission Directive 2004/73/EC

European Commission, 2010. Regulation No 1141/2010

European Commission, 2011. Implementing Regulation No 540/2011

European Commission, 2013a. Draft Renewal Assessment Report. Prosulfuron. Volume 3 – Annex B.6. May 2013.

European Commission, 2013b. Draft Renewal Assessment Report. Triasulfuron, Volume 3 – Annex B.6. August, 2013

Germany, 2002. Monograph. Tritosulfuron. Report and Proposed Decision. Vol 1, 20 August 2002.

Harvey, P.W., 2005. Human relevance of rodent prolactin-induced non-genotoxic mammary carcinogenesis: prolactin involvement in human breast cancer and significance for toxicology risk assessments.

Platania, C.B.M., Salomone, S., Leggio, G.M., Drago, F., Bucolo, C., 2012. Homology Modeling of Dopamine D2 and D3 Receptors: Molecular Dynamics Refinement and Docking Evaluation., 7:9(9), e44316.

Salmas, E.S., Yurtsever, M., Stein, M., Durdage, S., 2015. Modeling and protein engineering studies of active and inactive states of human dopamine D2 receptor (D2R) and investigation of drug/receptor interactions. 19:2, 321-332.

Schreuder, M.F., Bueters, R.R., Huigen, M.C., Russel, F.G.M., Masereeuw, R., and van den Heuvel, L.P., 2011. Effect of Drugs on Renal Development. American Society of Nephrology 6: 212-217.

Simpkins, J.W., Swenberg, J.A., Weiss, N., Brusick, D., Eldridge, J.C., Stevens, J.T., Handa, R.J., Hovey, R.C., Plant, T.M., Pastoor, T.P., and Breckenridge, C.B., 2011. Atrazine and Breast Cancer: A Framework Assessment of the Toxicological and Epidemiological Evidence. Toxicological Sciences, 123(2), 441-459.

UK CRD, 2014. Reporting table, Thifensulfuron-methyl (Hb). Section 2 – Mammalian toxicology (B.6).

UK CRD, 2015a. Thifensulfuron-methyl – Annex B (Volume 3); B.6a Toxicology and Metabolism. Renewal Assessment Report, July 7, 2014, updated following PRAPeR expert consultation at meeting 125 (March, 2015).

UK CRD, 2015b. Report of Pesticides Peer Review Meeting 125 (25-27 February, 2015). Thifensulfuron-methyl. 27 February 2015.

UK CRD, 2016. CLH Report Proposal for Harmonized Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2 Thifensulfuron-methyl March 2016

Woo DC and Hoar RM (1972) "Apparent Hydronephrosis" as a Normal Aspect of Renal Development in Late Gestation of Rats: The Effect of Methyl Salicylate. Teratology 6: 191-196.

Appendix 1
OECD Summaries of New Studies

Appendix 1.1
DuPont-44803 (QSAR)

Report: *In Silico* QSAR Evaluation of Thifensulfuron-Methyl (DPX-M6316) and Metabolites for Potential Endocrine and Dopaminergic Activity

DuPont Report No.: DuPont-44803

Guidelines: Not applicable – position paper

GLP: No

Executive summary:

The technical active thifensulfuron-methyl (DPX-M6316), and its rat and ground water metabolites were evaluated as target molecules for potential endocrine (oestrogenic and androgenic) and dopamine (agonist or antagonist) activities using the OECD QSAR Toolbox v3.3.5, OASIS TIMES v2.27.16, MedChem Studio v4.0, and ADMET Predictor v7.2 software programs. These *in silico* assessments were conducted to determine the potential for mammary tumour induction by thifensulfuron-methyl in a susceptible rodent strain, Sprague-Dawley rats. Specifically, these assessments were conducted to determine whether oestrogenic or dopamine antagonistic activities, which are most commonly associated with mammary tumour induction in this rat strain, represented a potential mode of action for thifensulfuron-methyl and its metabolites.

No structural alerts for oestrogen receptor binding were found for these target molecules by the OECD QSAR Toolbox for the USEPA rtER Expert System ver1. Furthermore, thifensulfuron-methyl and its metabolites were labeled “Non binder, without OH or NH2 group” for the Toolbox Estrogen Receptor Binding alert.

Models from OASIS TIMES and ADMET Predictor were used to evaluate thifensulfuron-methyl and its metabolites for androgen and oestrogen binding potential. The predictions from the models and alerts are in good alignment, indicating that binding to androgen or oestrogen receptors is unlikely.

There are no specific components of the OECD Toolbox or other available software for evaluation of potential dopamine receptor binding. In order to better understand the potential for dopamine receptor binding, the common pharmacophores were investigated. Furthermore, MedChem Studio v4.0 was used to perform similarity analyses on thifensulfuron-methyl and its metabolites against known dopamine agonists and antagonists. The weight of evidence from the results of these evaluations indicates that the target molecules will not bind to dopamine D_1, D_2 or D_3 receptors.

In summation, the weight of evidence from these *in silico* analyses supports that there are no significant concerns with thifensulfuron-methyl or its metabolites for oestrogen- or androgen-mediated activities, or dopamine antagonistic activities. The absence of oestrogenic and dopamine antagonistic activities, the most common modes-of-action for mammary tumour induction in Sprague-Dawley rats, is consistent with the view that thifensulfuron-methyl and its metabolites are not likely to induce mammary tumours.

Introduction

This position paper presents a QSAR evaluation of thifensulfuron-methyl, as well as its potential ground water metabolites and rat metabolites (Table 4) with respect to potential oestrogenic or androgenic activity, and for potential effects on the dopaminergic pathway. Oestrogenic compounds and dopamine antagonists represent the most common modes for induction of mammary gland tumours in the highly sensitive Sprague-Dawley rat strain.[1,2]

1 Alison, RH, Capen CC, Prentice DE. Neoplastic Lesions of Questionable Significance to Humans. Tox. Path. **1994**, 22(2), 179-186

2 O’Connor JC, Plowchalk DR, Van Pelt CS, Davis LG, Cook JC. Role of prolactin in chloro-s-triazine rat mammary tumorigenesis. Drug Chem. Toxicol. **2000** 23(4), 575-601.

Table 4
Thifensulfuron-methyl and associated metabolites

Target Compound	Function
Thifensulfuron-methyl (DPX-M6316)	Technical active
IN-A4098, IN-A5546, IN-L9223, IN-L9225, IN-W8268	Rat metabolite, ground water metabolites
IN-L9226	Rat metabolite
IN-JZ789, IN-V7160, IN-USF72 (2-acid-3-triuret)	Ground water metabolites

A. QSAR ASSESSMENT OF POTENTIAL FOR DOPAMINERGIC ACTIVITY

1. *Assessing the Dopaminergic Pharmacophores*

While no QSAR model for the dopamine receptor was available for evaluation, a literature search was performed to identify dopaminergic pharmacophores. Thifensulfuron-methyl and its metabolites (Table 4) were found to lack the common sub-structural moieties that would make them candidates for binding to dopamine D_1, D_2, and D_3 receptors.

Dopamine is the most abundant catecholamine in the basal ganglia. It participates in the regulation of motor functions and the cognitive processes, including learning and memory. Neuropsychiatric disorders including addiction, Parkinson's disease, and Schizophrenia are thought to be caused by abnormalities in the dopaminergic systems.

Dopamine receptors are found in the brain, peripheral nervous system, blood vessels, gastrointestinal tract and the kidney. Five known types of dopamine receptors exist in the human body. They can be broken down into two sub-classes: D_1-like and D_2-like. The D_1-like receptor family includes the D_1 and D_5, which have short third intercellular peptide loops and long carboxyl terminal tails. The D_2-like family includes D_2, D_3, and D_4, which have long third intercellular peptide loops and short carboxyl terminal tails. Antagonists for the D_2 receptor have been studied intensively within the pharmaceutical industry and are known to stimulate prolactin release.[3] The latter has been implicated as a mode of action for mammary tumour induction in Sprague-Dawley rats[2] and is relevant for assessments of the potential for tumour induction by thifensulfuron-methyl and its metabolites.

Structural requirements for the dopamine D_1 receptor are more demanding than for D_2. As a rule, a catecholic system is required for D_1, and these receptors are usually less tolerant of nitrogen substitution.[4]

All human dopamine D_2 receptor ligands known to date include a constrained nitrogen atom, which is protonated under physiological conditions (Figure 1).[3] An examination of known dopamine D_2 and D_3 receptor binding ligands shows that they usually have protonated nitrogen or can act as a hydrogen bond donor.[5]

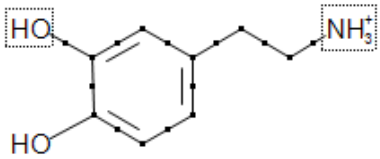
3 Salmas ES, Yurtsever M, Stein M, Durdage S Modeling and protein engineering studies of active and inactive states of human dopamine D2 receptor (D2R) and investigation of drug/receptor interactions. **2015** 19:2, 321-332.

4 Kohli JD, Glock D, Goldberg LI, Differential antagonism of postsynaptic (DA1) and presynaptic (DA2) peripheral dopamine receptors by substituted benzamides. **1982** Adv BioChem Psychopharmacol 35:97-108.

5 Platania CBM, Salomone S, Leggio GM, Drago F, Bucolo C Homology Modeling of Dopamine D2 and D3 Receptors: Molecular Dynamics Refinement and Docking Evaluation. **2012**, 7:9(9), e44316.

The meta-hydroxyphenethylamine moiety can be thought of as the dopaminergic pharmacophore with the nitrogen interacting with the receptor in the ammonium form. N-substitution profoundly influences the mode of interaction with the pharmacophore and receptor.[6]

Figure 1
Dopamine pharmacophore: meta-hydroxyphenethylamine moiety



Thifensulfuron-methyl and its metabolites do not contain catechol-like moieties nor do they contain a protonated nitrogen atom (constrained or otherwise) at or near physiological pH similar to that of dopamine.[7] Therefore, the target molecules will not likely bind to the D_1, D_2, or D_3 receptors.

2. Assessing Chemical Similarity with the Jaccard (Tanimoto) Index

Based on a thorough similarity analysis against 72 known dopamine agonists and antagonists, it is unlikely that thifensulfuron-methyl and its metabolites will bind to the dopamine receptors.

Twenty agonists and 52 antagonists were obtained from Wikipedia[8,9] and confirmed through MeSH[10,11] (Medical Subject Headings), the NLM controlled vocabulary thesaurus used for indexing articles for PubMed.

Chemical structures were identified for all dopaminergic compounds utilizing name to structure searches from ChemSpider[12] and PubChem.[13]

The potential for read-across between the target compounds (thifensulfuron-methyl and its metabolites) and the known dopaminergic compounds based on chemical similarity was assessed using MedChem Studio v4.0.[14] MedChem Studio is a cheminformatics/data-mining platform that can be used to clustering and classify compounds.

Jaccard indices (Tanimoto similarity coefficients) were calculated for the aforementioned compounds to identify the most similar dopaminergic compound with respect to each target compound. The Tanimoto coefficients can range from 0 to 1, where 1 indicates the highest level of similarity between two compounds for a given set of chemical fingerprints. Tanimoto similarity coefficients were calculated for the target compounds (thifensulfuron-methyl and its metabolites) and the list of dopamine

6 Seiler FM, Markstein R Further characterization of the structural requirements for agonists at the striatal dopamine D-1 receptor. Studies with a series of monohydroxaminotetralins on a dopamine-sensitive adenylate cyclase and comparison with dopamine receptor binding. **1982** Mol. Pharmacol. 22:281-289.

7 pKa Confirmed through ADMET Predictor v7.2

8 https://en.wikipedia.org/wiki/Dopamine_agonist accessed 8/14/2015

9 https://en.wikipedia.org/wiki/Dopamine_antagonist accessed 8/14/2015

10 https://www.ncbi.nlm.nih.gov/mesh/82018491 accessed 8/17/2015

11 https://www.ncbi.nlm.nih.gov/mesh/82018492 accessed 8/17/2015

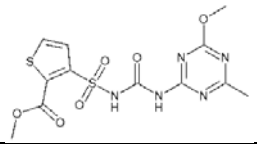
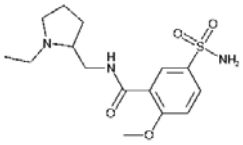
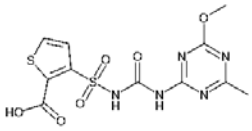
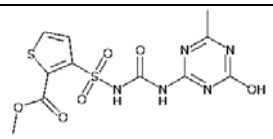
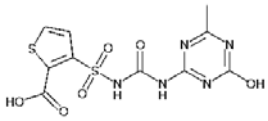
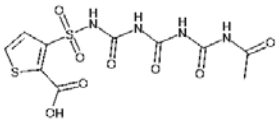
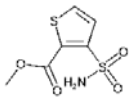
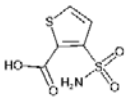
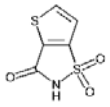
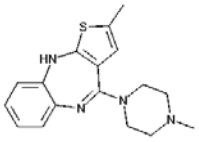
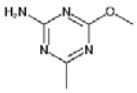
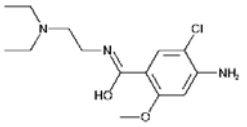
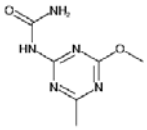
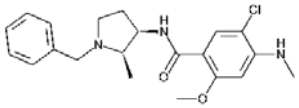
12 http://www.chemspider.com/ accessed 8/14-17/2015

13 https://pubchem.ncbi.nlm.nih.gov/ accessed 8/14-17/2015

14 http://www.simulations-plus.com/Products.aspx?pID=12 accessed on 10/8/2015

agonists/antagonists using MedChem Studio v4.0 fingerprints. The coefficients ranged from 0.313 to 0.600 (Table 5).

Table 5
Tanimoto similarity analysis of target molecules and their most similar dopaminergic counterpart

Target Compound	Structure	Most Similar Dopamine Agonist/Antagonist	Tanimito Coefficient
DPX-M6316		 Sulpiride (Antagonist)	0.391
IN-L9225			0.41
IN-L9226			0.387
IN-JZ789			0.407
2-Acid-3-Triuret			0.52
IN-A5546			0.553
IN-L9223			0.6
IN-W8268		 Olanzapine (Antagonist)	0.36
IN-A4098		 Metoclopramide (Antagonist)	0.313
IN-V7160		 Nemonapride (Antagonist)	0.375

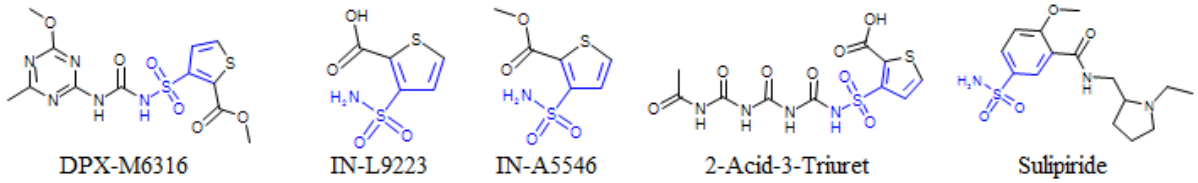
MedChem Studio v4.0 fingerprints were used to perform the Tanimoto similarity analysis.

Notably, the similarity profile between sulpiride and the remainder of the dopaminergic compounds yielded 7 compounds with a Tanimoto index higher than 0.80 and the remainder of the scores ranged from 0.03 to 0.638. This indicates a high level of variance among the dopaminergic compounds, a very low degree of selectivity among the dopamine receptors, or a deficiency of the fingerprint to adequately differentiate between actives and inactives. In this case, the fact that there are 5 different dopamine receptors must be considered.

The highest Tanimoto similarity coefficients, implying greatest similarity, were encountered when comparing IN-L9223, IN-A5546, and 2-Acid-3-Triuret to sulpiride, a known dopamine antagonist.[15] Here, the maximum common substructure (MCS) between these compounds is depicted in Figure 2. This motif was also found to exist in the dopamine antagonist thiothixene, the only other compound containing a sulfonamide moiety connected to an aromatic ring dopamine agonists/antagonists dataset. The structural differences between the other target compounds (thifensulfuron-methyl, IN-L9225, IN-L9226, IN-JZ789, IN-W8268, IN-A4098, and IN-V7160) and their most similar respective dopamine agonists/antagonists are even more significant.

While these structural differences do not definitively exclude the potential binding to the D1-D5 dopamine receptors, they strongly indicate that the target compounds will not bind to the dopamine D1-D5 receptors. Therefore, read-across cannot be applied to infer dopaminergic activity and due to the significant structural dissimilarities between the target and dopaminergic compounds, it is unlikely that thifensulfuron-methyl and its metabolites will exhibit any dopaminergic activities.

Figure 2
Maximum common substructure: Thifensulfuron-methyl (DPX-M6316), IN-L9225, IN-L9226, IN-JZ789, 2-acid-3-triuret, IN-A5546, IN-L9223, IN-W8268 and known dopamine antagonist sulpiride.



The SMARTS representation of maximal common substructure ccc(c)S(=O)(=O)N is highlighted in blue.

3. Assessing Chemical Similarity with Clustering

MedChem Studio (v4.0) was used to cluster the target compounds with the dopaminergic data set (Table 5) using four independent methodologies. Clusters contain similar compounds based on the classification methodology. The four methodologies include:

1. Frameworks
2. Ring systems
3. Topogeneric fragments
4. Fingerprints

Clustering analysis on this data set was performed on the data set comprised of thifensulfuron-methyl, its metabolites, and the dopamine agonists/antagonists data using MedChem Studio v4.0 classification methodologies: frameworks, ring systems, topogeneric fragments, and fingerprints. In all cases, neither

15 http://www.ncbi.nlm.nih.gov/pubmed/7756714 accessed 8/19/2015: Caley CF, Weber SS. "Sulpiride: an antipsychotic with selective dopaminergic antagonist properties." Ann Pharmacother. 1995; 29(2): 152-60.

thifensulfuron-methyl (DPX-M6316) nor any of its metabolites were assigned to any of the classes that included compounds with dopaminergic activity. Therefore it is unlikely that thifensulfuron-methyl or any of its metabolites will follow the known action of any of the compounds in the dopaminergic data set.

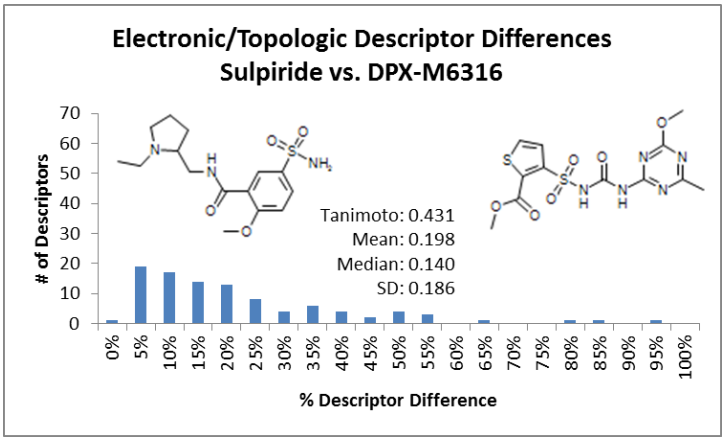
4. Assessing Chemical Similarity within a Defined Descriptor Space

The fundamental idea behind QSAR and read-across is that structurally similar compounds will behave similarly. The basic theory behind the OECD QSAR toolbox is that read-across is reinforced when two compounds have similar physicochemical property, toxicity, metabolic profiles, and QSAR profiles. QSARs are reliant upon chemical descriptors including sub-structural fragments, topological indices, hydrogen bonding properties, atom based electronic indices, etc. Therefore, since QSARs are derived using chemical descriptors, similarities between the chemical descriptor profiles are also suitable reinforcement to read-across. Furthermore, chemical descriptor profiles based on topological indices and those derived from electronic properties are even stronger support of read-across as they can identify potential areas where chemicals of significantly different structures will exhibit the same type of activity/toxicity profiles. Alternatively, if two compounds of similar structure have significantly different topological and electronic profiles, they will also not share the same activity, toxicological, or metabolic profiles.

ADMET Predictor was then used to calculate chemical descriptors for topology, hydrogen bonding, and derived from the electronic properties for the diversity set plus the 72 known dopamine agonists/antagonists from the similarity analysis, thifensulfuron-methyl and its metabolites. This descriptor space was used to establish a means to more thoroughly interpret the similarity/dissimilarity of pairs of compounds. This descriptor space is based on continuous descriptors, unlike the Tanimoto coefficient which is based upon on categorical pairings, where two compounds either share or do not share sub-structural fragments.

In order to establish a baseline for interpretation, histograms of the distribution of all descriptors were created to give an overview of the differences between two chemicals in this framework of chemical/descriptor space using dopamine receptor antagonist sulpiride and other chemicals with a wide variance of Tanimoto scores. The mean, median, and standard deviation (D-scores) were calculated for each chemical pairing. The score ranges from 0 to 1, where 0 is an exact match and 1 would indicate the highest level of difference possible based on the chemical space defined by the subset of chemicals being investigated. In Figure 3, the comparison of the reference compound sulpiride to thifensulfuron-methyl is shown. Both the tanimoto score and the D-scores for this comparison indicate the structural dissimilarity between the compounds.

Figure 3
ΔD *norm* profile between Sulpiride (left) and thifensulfuron-methyl (right)



The results from the similarity analysis utilizing descriptor space indicate that it is likely that thifensulfuron-methyl and its metabolites will not bind to the dopamine receptors or have dopaminergic activities.

B. QSAR ANALYSIS OF ENDOCRINE ACTIVITY

The OECD QSAR Toolbox v3.3.5 was used to identify any potential oestrogen alerts. The Toolbox is software primarily used to enhance and identify read-across opportunities and fill data gaps in toxicity profiles needed for assessing the hazards of chemicals. The Toolbox incorporates information and tools from various sources into a logical workflow. It functions to identify relevant structural characteristics and potential mechanism or mode of action of a target chemical, identify other chemicals that have the same structural characteristics and/or mechanism or mode of action, and use existing experimental data to fill in data gaps.[16] The Toolbox indicated that thifensulfuron-methyl and its metabolites would not bind to the oestrogen receptor as the compounds either lacked or had impaired alcohol and primary amine groups (Table 6). No scope or applicability domain is offered for this endpoint by the OECD QSAR Toolbox. No alerts were raised for Oestrogen Receptor binding by the USEPA's rtER Expert System ver.1 (Table 3).

OASIS TIMES v2.27.16, a product of the Laboratory of Mathematical Chemistry, was used to evaluate oestrogen both with and without the presence of S9. TIMES (TIssue MEtabolism Simulator) is a heuristic algorithm used to generate plausible metabolic maps from a comprehensive library of bio-transformations and abiotic reactions. It allows prioritization of chemicals according to the toxicity of their metabolites.[17] OASIS TIMES predictions were negative for androgen and oestrogen receptor binding with the exception of IN-JZ789 (no prediction could be made) (Table 6).

ADMET Predictor v7.2, a product of Simulations Plus Inc., was used to evaluate androgen and oestrogen binding potential. Both artificial neural network classification models use the ensemble approach, which is equivalent to taking a consensus. In this case, the resulting predictions was derived from a consensus of 33 independent models derived from the same training pool, but using varied training and validation sets, but always the same external test set. All ADMET Predictor androgen binding predictions were made with high confidence (≥84%) indicating that there was substantial data and significant agreement in the 33 models that constitute the individual predictions considered in the consensus model. All predictions for androgen and oestrogen receptor binding were negative (Table 6). However over half of the oestrogen receptor binding predictions lacked confidence values, indicating that these compounds were outside the applicability domain of the model.

16 http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm accessed on 10/8/2015

17 http://oasis-lmc.org/products/software/times.aspx accessed on 10/8/2015

Table 6
QSAR analysis of thifensulfuron-methyl (DPX-M6316) and its metabolites for endocrine binding

Models Endocrine Receptor Binding	Androgen Receptor		Estrogen Receptor Binding			
	ADMET Predictor	OASIS TIMES	ADMET Predictor	OASIS TIMES	OECD QSAR	rtER expert System ver.1.
DPX-M6316	- (95%)	-	-	-	-	No Alert
IN-L9225	- (95%)	-	-	-	-	No Alert
IN-L9226	- (95%)	-	-	-	-	No Alert
IN-JZ789	- (95%)	CP	-	-	-	No Alert
2-acid-3-triuret	- (95%)	-	-	-	-	No Alert
IN-A4098	- (95%)	-	- (96%)	-	-	No Alert
IN-V7160	- (95%)	-	-	-	-	No Alert
IN-A5546	- (84%)	-	- (75%)	-	-	No Alert
IN-L9223	- (84%)	-	- (75%)	-	-	No Alert
IN-W8268	- (95%)	-	- (58%)	-	-	No Alert

Minus (-) – non-binder
CP – can't predict
(%) – confidence score in prediction based on consensus of 33 sub-models

Based on the above QSAR analysis, there is no indication that thifensulfuron-methyl or any of its metabolites will bind to the androgen or oestrogen receptors. However, it is necessary to keep in mind that many of the predictions are made outside of the model applicability domains, indicating that the models were extrapolating. The reliability and confidence in a QSAR model is significantly higher when the model is interpolating, working inside of a known chemical domain, as opposed to extrapolation, working outside of a known chemical domain. Although, predictions are outside of the model applicability domain, empirical data demonstrating non-binding of thifensulfuron-methyl to the oestrogen receptor *in vitro* is consistent with the QSAR results for this active substance.[18]

18 Bitsch, N., Korner, W., Failing, K, and Brunn, H. In vitro Screening of the Estrogenic Activity of Active Components in Pesticides. Umweltwissenshaften und Schadstoff-Forschung, **2002** 14(2), 76-84.

CONCLUSIONS

Thifensulfuron-methyl technical active substance (DPX-M6316), and its rat and ground water metabolites were evaluated for potential endocrine (oestrogenic and androgenic) and dopamine (agonist or antagonist) activities using OECD QSAR Toolbox v3.3.5, OASIS TIMES v2.27.16, MedChem Studio v4.0, and ADMET Predictor v7.2 software programs.

No structural alerts for oestrogen receptor binding were found for these target molecules by the OECD QSAR Toolbox for the USEPA rtER Expert System ver1. Furthermore, thifensulfuron-methyl and its metabolites were labeled "Non binder, without OH or NH_2 group" for the Toolbox Oestrogen Receptor Binding alert.

Models from OASIS TIMES and ADMET Predictor were used to evaluate thifensulfuron-methyl and its metabolites for androgen and oestrogen binding potential. The predictions from the models and alerts are in good alignment, indicating that binding to androgen or oestrogen receptors is unlikely.

There are no specific components of the OECD Toolbox or other available software for evaluation of potential dopamine receptor binding. In order to better understand the potential for dopamine receptor binding, the common pharmacophores were investigated. Furthermore, similarity analyses on thifensulfuron-methyl and its metabolites were performed against known dopamine agonists and antagonists. The weight of evidence from the results of these evaluations indicates that the target molecules will not bind to dopamine D_1, D_2 or D_3 receptors.

In summation, the weight of evidence from these *in silico* analyses supports that there are no significant concerns with thifensulfuron-methyl or its metabolites for oestrogen- or androgen-mediated activities or dopamine agonistic or antagonistic activities. The absence oestrogenic and dopamine antagonistic activities, the most common modes-of-action for mammary tumour induction in Sprague-Dawley rats, is consistent with the view that thifensulfuron-methyl and its metabolites are not likely to induce mammary tumours in this rat strain.

(DuPont 2015c)

Appendix 1.2
DuPont-45948 (EDSP)

Report: Thifensulfuron-methyl: Assessment of Potential Endocrine Activity From the U.S. Environmental Protection Agency (US EPA) Endocrine Disruptor Screening Program (EDSP) for the 21st Century Dashboard

DuPont Report No.: DuPont-45948

Guidelines: Not applicable – position paper

GLP: No

Executive summary:

EPA's Endocrine Disruptor Screening Program (EDSP) is designed to detect the intrinsic ability of chemicals to interact with the endocrine system, specifically for chemicals that can interact with the oestrogen, androgen, or thyroid systems. In order to aid in screening the large number of chemicals subject to regulation in the U.S., EPA researchers developed the EDSP21 Dashboard to provide access to new chemical data on over 1800 chemicals of interest. Of the 11 potential assays for androgenic activity, thifensulfuron-methyl was evaluated in 8 assays, and was negative for bioactivity in all 8 assays. Of the 18 potential assays for oestrogenic activity, thifensulfuron-methyl was evaluated in all 18 assays, and was negative for bioactivity in 17 of the 18 assays. While thifensulfuron-methyl was positive in the ATG_ERE_CIS_up assay, the AC_{50} (59 uM) occurred at a concentration that was approaching observed cytotoxicity. In addition, thifensulfuron-methyl was negative in all of the other ER assays. Of the 4 potential assays for thyroid activity, thifensulfuron-methyl was evaluated in 3 assays, and was negative for bioactivity in all 3 assays.

Considered collectively, the weight of evidence from the EDSP21 assays demonstrates that thifensulfuron-methyl does not interact with the oestrogen, androgen, or thyroid signaling systems when evaluated in a wide variety of assay types with a comprehensive range of endocrine-related endpoints.

Methods

EPA's Endocrine Disruptor Screening Program (EDSP) is designed to detect the intrinsic ability of chemicals to interact with the endocrine system, specifically for chemicals that can interact with the oestrogen, androgen, or thyroid systems. In order to aid in screening the large number of chemicals subject to regulation in the U.S., EPA researchers developed the EDSP21 Dashboard to provide access to new chemical data on over 1800 chemicals of interest. However, activity of a chemical in a specific assay does not necessarily mean that it will cause toxicity or an adverse health outcome. There are many factors that determine whether a chemical will cause a specific adverse health outcome, and these are not included in the evaluation presented on the EDSP21 Dashboard. The results summarized below indicate only whether a chemical has potential to interact with a particular signaling cascade, and do not indicate whether a substance would cause adverse effects *via* that signaling pathway in experimental animals and/or humans.

The data for the Dashboard comes from various sources:

- Rapid, automated (or in vitro high-throughput) chemical screening data generated by the EPA's Toxicity Forecaster (ToxCast) project and the federal Toxicity Testing in the 21st century (Tox21) collaboration.
- Chemical exposure data and prediction models (ExpoCastDB).
- High quality chemical structures and annotations (DSSTox).
- Physchem Properties Database (PhysChemDB).

Results

The bioactivity summaries for thifensulfuron-methyl in each of the EDSP21 assays are summarized in Table 7. Of the 11 potential assays for androgenic activity, thifensulfuron-methyl was evaluated in 8 assays, and was negative

for bioactivity in all 8 assays. Of the 18 potential assays for oestrogenic activity, thifensulfuron-methyl was evaluated in all 18 assays, and was negative for bioactivity in 17 of the 18 assays. While thifensulfuron-methyl was positive in the ATG_ERE_CIS_up assay (Figure 4), the AC_{50} (59 uM) occurred at a concentration that was approaching observed cytotoxicity. In addition, thifensulfuron-methyl was negative in all of the other ER assays. Of the 4 potential assays for thyroid activity, thifensulfuron-methyl was evaluated in 3 assays, and was negative for bioactivity in all 3 assays.

Considered collectively, the weight of evidence from the EDSP21 assays demonstrates that thifensulfuron-methyl does not interact with the oestrogen, androgen, or thyroid signaling systems when evaluated in a wide variety of assay types with a comprehensive range of endocrine-related endpoints.

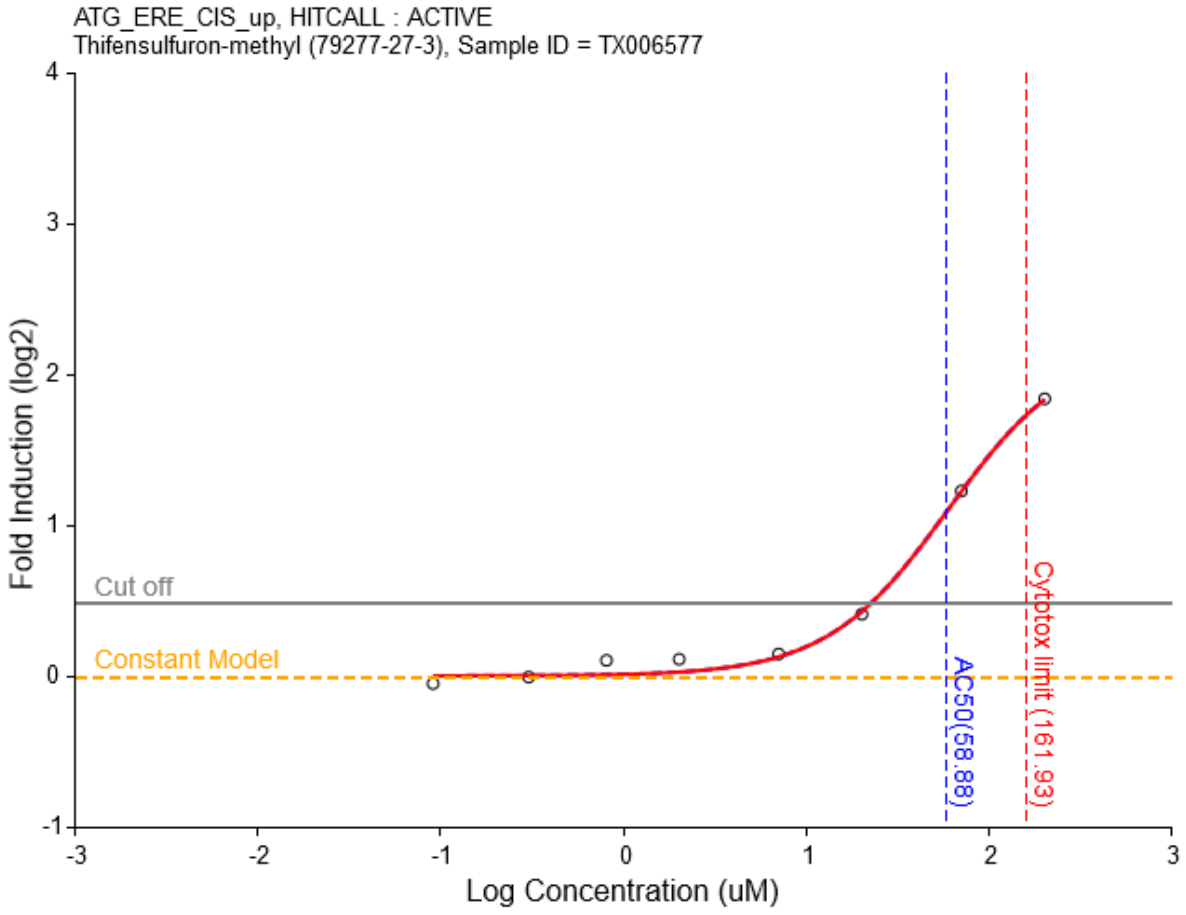
Table 7
Summary of results of endocrine activity screening assays from EDSP21 Dashboard

Receptor System (Organism)	Assay Component Endpoint Name and Assay Description	Assay Result
Androgen (human)	ATG_AR_TRANS_up	Inactive
Androgen (chimpanzee)	NVS_NR_cAR	Not Tested
Androgen (human)	NVS_NR_hAR	Not Tested
Androgen (rat)	NVS_NR_rAR	Not Tested
Androgen (Chinese hamster)	OT_AR_ARELUC_AG_1440	Inactive
Androgen (human)	OT_AR_ARSRC1_0480	Inactive
Androgen (human)	OT_AR_ARSRC1_0960	Inactive
Androgen (human)	Tox21_AR_BLA_Agonist_ratio	Inactive
Androgen (human)	Tox21_AR_BLA_Antagonist_ratio	Inactive
Androgen (human)	Tox21_AR_LUC_MDAKB2_Agonist	Inactive
Androgen (human)	Tox21_AR_LUC_MDAKB2_Antagonist	Inactive
Estrogen 1 (human)	ACEA_T47D_80hr_Positive	Inactive
Estrogen 1 (human)	ATG_ERE_CIS_up	Active
Estrogen 1 (human)	ATG_ERa_TRANS_up	Inactive
Estrogen 1 (bovine)	NVS_NR_bER	Inactive
Estrogen 1 (human)	NVS_NR_hER	Inactive
Estrogen 1 (mouse)	NVS_NR_mERa	Inactive
Estrogen 1 (human)	OT_ER_ERaERa_0480	Inactive

Table 7
Summary of results of endocrine activity screening assays from EDSP21 Dashboard (continued)

Receptor System (Organism)	Assay Component Endpoint Name and Assay Description	Assay Result
Estrogen 1 (human)	OT_ER_ERaERa_1440	Inactive
Estrogen 1 (human)	OT_ER_ERaERb_0480	Inactive
Estrogen 1 (human)	OT_ER_ERaERb_1440	Inactive
Estrogen 1 (human)	OT_ER_ERbERb_0480	Inactive
Estrogen 2 (human)	OT_ER_ERbERb_1440	Inactive
Estrogen 2 (human)	OT_ERa_EREGFP_0120	Inactive
Estrogen 1 (human)	OT_ERa_EREGFP_0480	Inactive
Estrogen 1 (human)	Tox21_ERa_BLA_Agonist_ratio	Inactive
Estrogen 1 (human)	Tox21_ERa_BLA_Antagonist_ratio	Inactive
Estrogen 1 (human)	Tox21_ERa_LUC_BG1_Agonist	Inactive
Estrogen 1 (human)	Tox21_ERa_LUC_BG1_Antagonist	Inactive
Thyroid (human)	ATG_THRa1_TRANS_up	Inactive
Thyroid (human)	NVS_NR_hTRa	Not Tested
Thyroid (rat)	Tox21_TR_LUC_GH3_agonist	Inactive
Thyroid (rat)	Tox21_TR_LUC_GH3_antagonist	Inactive

Figure 4
Response of thifensulfuron-methyl in the ATG_ERE_CIS_up assay for oestrogenicity



(DuPont 2015a)

Appendix 1.3
DuPont-45572 (A4098 ER binding)

Oestrogen receptor binding

DuPont-45572 OECD Summary

IN-A4098: Estrogen receptor binding assay using rat uterine cytosol (ER-RUC)

Willoughby, J. A.

Executive summary:

IN-A4098 (98.7% w/w) was evaluated for its ability to bind to the oestrogen receptors in rat uterine cytosol. The *in vitro* oestrogen receptor binding assay using rat uterine cytosol is part of Endocrine Disruptor Screening Program (EDSP). This assay was intended to be used in conjunction with other guidelines in the OPPTS 890 series that comprise the full screening battery under the EDSP.

Saturation binding assays measure the affinity of a radiolabelled oestrogen ligand, 17β-oestradiol ([^{3}H]17β-oestradiol), (K_d) for the oestrogen receptor and the concentration of the oestrogen receptors (B_{max}) present in the cytosol. This is determined by measuring specific binding of increasing concentrations of radioligand under conditions of equilibrium. The K_d was approximately 0.078 nM [^{3}H]17β-oestradiol, and the B_{max} was approximately 57.70 fmol/100 μg protein, which is consistent with the acceptable range listed in the test guideline.

Competitive binding assays measure the binding of the radioligand to the receptors with increasing concentrations of a test substance. The concentration at which the test substance displaces half of the bound radioligand is the IC_{50} (often expressed as $logIC_{50}$). Three independent runs were performed to evaluate IN-A4098 for its ability to compete with [^{3}H]17β-oestradiol in binding to rat uterine oestrogen receptors *in vitro*. IN-A4098 was evaluated at eight concentrations ranging 10^{-10} to 10^{-3} M. 17β-oestradiol, the oestrogen receptor agonist reference standard, 19-norethindrone, a weak oestrogen receptor agonist used as the positive control, and octyltriethoxysilane, a non-oestrogen receptor agonist used as the negative control, were used to verify test system performance. As expected, 17β-oestradiol and 19-norethindrone showed effects consistent with strong and weak competitive binding, respectively, and octyltriethoxysilane did not compete for binding to the oestrogen receptor in all three runs. The $logIC_{50}$ values for 17β-oestradiol for each of the three runs were -8.8, -9.0, and -9.0 logM [^{3}H]17β-oestradiol with an average of -8.9 logM [^{3}H]17β-oestradiol. The $logIC_{50}$ values for 19-norethindrone for each of the three runs were -4.9, -5.0, and -5.0 logM with an average of -5.0 logM. The relative binding affinity (RBA) of 19-norethindrone as compared to 17β-oestradiol for each of the three runs was 0.0121, 0.0101, and 0.0089% with an average of 0.0104%. As expected, neither a $logIC_{50}$ nor RBA were able to be determined for octyltriethoxysilane as no competitive binding was observed. A $logIC_{50}$ was not determined for the test substance since there were no test substance-related effects on oestrogen receptor binding up to the concentration of 10^{-3} M which represents the highest concentration required by the test guideline.

The $logIC_{50}$ value for the 17β-oestradiol and 19-norethindrone is consistent with those previously reported in the EPA's Integrated Summary Report for the validation of the oestrogen receptor binding assay; no $logIC_{50}$ values for octyltriethoxysilane were reported.

Under the conditions of the study, the test substance, IN-A4098 was classified as "not interacting" in all three valid independent runs. Therefore, IN-A4098 is classified as "not interacting" in the oestrogen receptor binding assay.

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	IN-A4098
	Lot/Batch #:	050942-015
	Purity:	98.7%, by analysis
	Description:	Solid
	CAS #	1668-54-8
	Stability of test compound:	Not determined
	Solvent/ final concentration:	Dimethyl Sulfoxide (DMSO)/ 4%
2.	Radioactive ligand:	[3H] Oestradiol
	Lot/Batch Number:	2088404
	Source:	Perkin Elmer, Boston, MA
	Specific Activity:	120 Ci/mmol
	Production Date:	02-Nov-2015
3.	Positive control:	17β-oestradiol
	Lot number:	SLBL7310V
	Purity:	100%
	Source:	Sigma-Aldrich, St. Louis, MO
	CAS #:	50-28-2
	Solvent/ final concentration:	DMSO/ 4%
4.	Weak positive control:	19-norethindrone
	Lot number:	SLBF1672V
	Purity:	100%
	Source:	Sigma-Aldrich, St. Louis, MO
	CAS #:	68-22-4
	Solvent/ final concentration:	DMSO/ 4%
5.	Negative control:	Octyltriethoxysilane
	Lot number:	SHBD3756V
	Purity:	98.90%
	Source:	Sigma-Aldrich, St. Louis, MO
	CAS #:	2943-75-1
	Solvent/ final concentration:	DMSO/ 4%
6.	Test system (oestrogen receptor):	Uterine cytosol from ovariectomised adult female Sprague-Dawley rats
	Source:	Charles River Laboratories, Inc., Kingston, NY
	Age	85 – 100 days on date of uteri collection

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   03-December-2015 to 11-December-2015

2. Rat uterine cytosol preparation

   Uteri were added to a beaker of TEDG + PI (10 mM Tris, 1.5 mM EDTA, 1 mM DTT, 0.5% Protease Inhibitor (v/v), 10% glycerol, pH approximately 7.4) in an ice bath at 10 mL of buffer/g tissue, minced until tissues were 1-2 mm cubes, then homogenised using a pre-chilled homogeniser. The homogenate was then centrifuged for 10 minutes at approximately 2500 × g at 4°C, the supernatant from all samples was pooled and centrifuged at approximately 105000 × g for 60 minutes at approximately 4°C. The resulting supernants were then aliquoted and stored frozen (approximately -80°C) until use.

   The protein concentration of the uterine cytosol was determined prior to performing the receptor assays. Samples for the standard curve were prepared using bovine serum albumin with final concentrations

ranging from 0.03–3 nM. The protein concentration of the uterine cytosol was determined to be 3.1 mg/mL.

3. Dose preparations and analysis

The reference standard, 17β-oestradiol (not adjusted for purity), was used to verify test system performance at seven final concentrations ranging from 1×10^{-11} to 1×10^{-7} M. The weak positive control, 19-norethindrone (not adjusted for purity), was used to verify test system performance at eight final concentrations ranging from $1 \times 10^{-8.5}$ to 1×10^{-4} M. The negative control, octyltriethoxysilane (not adjusted for purity), was used to verify test system performance at final concentrations ranging from 1×10^{-10} to 1×10^{-3} M. The test substance (adjusted for purity) was evaluated at eight targeted final concentrations ranging from 1×10^{-10} to 1×10^{-3} M.

Preparations of the initial solutions for the reference standard, positive control, negative control, and test substance were prepared by serial dilution from a stock solution, which was also the respective high initial concentration. No indication of precipitation of the reference standard, positive control, negative control, or test substance was observed during the assay.

4. Verification of scintillation counting efficiency accuracy

Standards (^{3}H, ^{14}C and background) were used to verify accurate counting, and the liquid scintillation analyser has an enhanced Instrument Performance Assessment (IPA) for monitoring efficiencies, backgrounds, E2/B and Chi-square values for ^{3}H and ^{14}C over the life of the instrument.

5. Oestrogen receptor saturation assay

Briefly, [^{3}H]17β-oestradiol was diluted in cold TEDG + PMSF buffer to final concentrations of 0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, and 3 nM. Non-labelled 17β-oestradiol was prepared in a similar manner to final concentrations of 3, 6, 8, 10, 30, 60, 100 and 300 nM, respectively, (*i.e.*, 100× the concentration of [^{3}H]-17β-oestradiol).

Uterine cytosol was diluted in cold TEDG + PMSF buffer to a concentration of approximately 1.033 mg protein/mL, which was shown previously to bind to approximately 13.5% of 1 nM [^{3}H]-E_2.

After addition of all assay components, tubes were incubated at approximately 4°C with gentle vortexing for approximately 18-20 hours, with the exception of the total [^{3}H]17β-oestradiol tubes, which were counted without further processing. Hydroxyapatite (HAP) slurry was washed twice in cold TEDG + PI buffer and resuspended to a ratio of 60% HAP in TEDG + PI buffer by volume. The bound [^{3}H]17β-oestradiol was separated from free by adding 250 µL of cold resuspended HAP slurry to each assay tube, vortexing (3 times with 5-minute intervals), centrifuging for approximately 10 minutes at 4°C at 1000 × g, and then discarding the supernatant. The tubes were washed three times by adding 2.0 mL of cold (approximately 4°C) TEDG + PI buffer, briefly vortexing, and centrifuging at 4°C for 10 minutes at 1000 × g. The supernatant from each wash was discarded. The bound [^{3}H]17β-oestradiol was extracted by adding 1.5 mL ethanol and vortexing. The tubes were then centrifuged for 10 minutes at 1000 × g, and 1 mL of the supernatant was added to 10 mL scintillation cocktail (Perkin Elmer Opti Fluor, catalogue # 6013199, lot # 47-15131). The vial was capped and shaken. The vials were placed in a scintillation counter (Perkin Elmer Tri-Carb 2910TR Liquid Scintillation Analyser Model B2910) and each vial was counted for at least one minute with quench correction for determination of disintegrations per minute (DPM) per vial.

6. Oestrogen receptor competitive binding assay

The positive control was evaluated in triplicate at final concentrations of 1×10^{-11}, 1×10^{-10}, 3.16×10^{-10}, 1×10^{-9}, 3.16×10^{-9}, 1×10^{-8}, and 1×10^{-7} M. The weak positive control was evaluated in triplicate at final concentrations of $10^{-8.5}$, $10^{-7.5}$, 10^{-7}, $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, $10^{-4.5}$, and 10^{-4} M. The negative control was evaluated in triplicate at final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. The test

substance was evaluated in triplicate at final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M.

Uterine cytosol was diluted in cold TEDG + PMSF buffer to approximately 1.033 mg protein/mL, which was shown previously to bind to approximately 13.5% of 1 nM [^{3}H]-17β-oestradiol. The average estimated K_d of [^{3}H]17β-oestradiol for the batch of uterine cytosol used in this study was 0.078 nM as determined by the saturation binding assay.

The final volume per assay tube was 500 µL. Each assay tube contained 380 µL of 1 nM [^{3}H]17β-oestradiol in cold TEDG + PI buffer, 20 µL of either solvent or pre-diluted reference standard, positive control, negative control, or test substance, and 100 µL of diluted rat uterine cytosol. Each total [^{3}H]17β-oestradiol tube contained 50 µL of 1 nM [^{3}H]17β-oestradiol for a total volume of 50 µL and were counted without further processing.

After addition of all assay components, the tubes were incubated at approximately 4°C with gentle mixing for approximately 19 hours, with the exception of the total [^{3}H]17β-oestradiol tubes, which were counted without further processing. HAP was washed three times in cold TEDG + PMSF buffer and resuspended to a ratio of 60% HAP by volume. The bound [^{3}H]17β-oestradiol was separated from free by adding 250 µL of cold resuspended HAP slurry to each assay tube, vortexing for 10 seconds every 5 minutes for 15 minutes, centrifuging for approximately 10 minutes at 4°C and 1000 × g and discarding the supernatant. The tubes were washed three times by adding 2.0 mL of cold (approximately 4°C) TEDG + PMSF buffer, vortexing, and centrifuging at 4°C for 10 minutes at 1000 × g. The supernatant from each wash was discarded. The bound [^{3}H]17β-oestradiol was extracted by adding 1.5 mL ethanol and vortexing for approximately 10 seconds at 5-minute intervals for approximately 20 minutes at room temperature. The tubes were then centrifuged for 10 minutes at 1000 × g and 1 mL of supernatant was added to 10 mL of scintillation cocktail and radioassayed by scintillation counting.

7. Calculations

The dissociation constant (K_d), maximal binding capacity (B_{max}), and the concentration that inhibits 50% of maximum radioligand binding ($logIC_{50}$) values were determined as appropriate using Origin 8.5.1.

The K_d and B_{max} values for the uterine cytosol were determined by fitting the saturation curves using the following equation:

$$Y = \frac{B_{max} \times X}{X + K_d} + (a \times X)$$

where Y = total binding, a = ratio between non-specific bound ligand and free ligand, and X = concentration of [^{3}H]-E_2.

The IC_{50} values were determined by fitting the competitive curves using the following equation:

$$Y = Bottom + \frac{Top - Bottom}{1+10^{(Log\ IC50-X)HillSlope+log[(Top-Bottom)/(50-Bottom)-1]}}$$

where X is the logarithm of the concentration of test substance and Y is the percent of radioligand bound to the receptor. $LogIC_{50}$ is X at Y = 50%. "Top" and "Bottom" refer to the value of Y when there is minimal binding by the test substance, and when there is maximal binding by the test substance, respectively. A concentration-response model was fitted for each test run for each curve generated.

For the Saturation Binding Experiment, total binding and non-specific binding data were modelled *via* non-linear regression using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA), incorporating

automatic outlier elimination according to the method of Motulsky and Brown (2006) implemented by using the ROUT procedure in Prism v. 5 with a Q value of 1.0. Scatchard plots were also generated using Graph Pad Prism v. 5. Receptor binding data plots were corrected for ligand depletion using the method of Swillens (1995). Parameters reported from the Saturation Binding Experiment (K_d and B_{max}), means and standard deviations, were calculated for each run and the means and standard errors were calculated for the composite three runs using Microsoft Excel 2007 (Redmond, WA; version 12.0.6557.5000).

8. Performance criteria

The following criteria were applied to assess the performance of the competitive binding assays. Increased concentrations of 17β-oestradiol displace [^{3}H]17β-oestradiol from the receptor in a manner consistent with one-site competitive binding. Ligand depletion was no greater than 15%. The parameter values (top, bottom, and slope) for reference standard and positive control were within the tolerance bounds provided with two exceptions. The bottom plateau level for 19-norethindrone was 2.8%, 7.0% and 4.4% for the first, second and third valid run, respectively, which is outside of the range of -5 to 1. In this assay, the bottom plateau level is consistently above the recommended parameters. This may be may be due to mathematical modelling of the curves. The Hill Slope was -1.16 for 19-norethindrone, which is marginally outside of the range of -0.7 to -1.1. These deviations were minor and not considered to affect the study outcome. The solvent control did not alter the sensitivity or reliability of the assay. The negative control substance (octyltriethoxysilane) did not displace more than 25% of the radioligand from the oestrogen receptor on average across all concentrations. The test substance was tested over a concentration range that fully defined the top of the curve (*i.e.*, a range that showed that a top plateau was achieved), and the top is within 25% of either the solvent control or the value for the lowest concentration of the 17β-oestradiol standard for that run. In addition to meeting the criteria for individual runs, consistency occurs across runs of the top plateau level, Hill slope, placement along the X-axis, and the bottom plateau (where defined).

II. RESULTS AND DISCUSSION

A. OESTROGEN RECEPTOR SATURATION BINDING ASSAY

Three independent oestrogen receptor saturation binding runs were completed using [^{3}H]17β-oestradiol as the radioligand and 17β-oestradiol as the ligand. The K_d values for each of the three runs were 0.085, 0.068, and 0.082 nM [^{3}H]17β-oestradiol with an average of 0.078 nM [^{3}H]17β-oestradiol. The B_{max} values for each of the three runs were 55.15, 62.86, and 55.10 fmol/100 µg protein with an average of 57.70 fmol/100 µg protein. Confidence in these numbers is high as the adjusted coefficient of determination (adjusted R^2) was 0.978, 0.983, and 0.975 for each of the respective runs, with small variations between runs. The K_d and B_{max} were within the range provided by the test guideline.

Table 8
Saturation binding assay: K_d and B_{max} values for uterine cytosol

Parameter	Run S1[a]	Run S2[a]	Run S3[a]	Mean[b]
Adjusted R^2 (unweighted)	0.978	0.983	0.975	0.979 ± 0.004
B_{max} (nM)	0.011	0.014	0.012	0.012 ± 0.001
B_{max} (fmol/100 µg protein)	55.15	62.86	55.10	57.70 ± 4.47
K_d (nM)	0.085	0.068	0.082	0.078 ± 0.009
Time since specific activity certification (days)	31	36	38	NA[c]

[a] N = 3

[b] Mean ± SEM for all three runs

[c] NA = not applicable

B. OESTROGEN RECEPTOR COMPETITIVE BINDING ASSAY

Three independent oestrogen receptor competitive binding runs were completed using [^{3}H]17β-oestradiol as the radioligand and 17β-oestradiol as the reference standard, 19-norethindrone as the weak positive control, octyltriethoxysilane as the negative control or the test substance.

The logIC_{50} values for 17β-oestradiol for each of the three runs were -8.8, -9.0, and -9.0 logM [^{3}H]17β-oestradiol with an average of -8.9 logM [^{3}H]17β-oestradiol. The logIC_{50} values for 19-norethindrone for each of the three runs were -4.9, -5.0, and -5.0 logM with an average of -5.0 logM. The relative binding affinity (RBA) of 19-norethindrone as compared to 17β-oestradiol for each of the three runs was 0.0121, 0.0101, and 0.0089% with an average of 0.0104%. As expected, neither a logIC_{50} nor RBA were able to be determined for octyltriethoxysilane as no competitive binding was observed.

A logIC_{50} was not determined for the test substance since there were no test substance-related effects on oestrogen receptor binding up to the concentration of 10^{-3} M represents the highest concentration required by the test guideline.

Table 9
Competitive binding assay: Mean ± standard error of the percent [^{3}H]17β-oestradiol bound for each concentration

Competitor	Final conc.	Run C1[a]		Run C2[a]		Run C3[a]	
	Log(M)	Mean	SD[b]	Mean	SD[b]	Mean	SD[b]
17β-oestradiol (NSB)	-7	0.0	0.1	0.0	1.1	0.0	0.1
	-8	12.5	0.5	8.9	0.3	8.3	0.1
	-8.5	32.9	0.8	22.8	0.9	22.6	0.8
	-9	63.4	1.8	50.3	0.9	47.1	1.6
	-9.5	87.0	1.3	75.9	1.8	73.5	0.7
	-10	99.4	2.1	92.8	0.7	90.4	2.0
	-11	101.6	1.5	98.7	1.6	94.1	1.9
Octyltriethoxysilane	-3	89.4	4.9	86.9	2.5	85.3	2.7
	-4	97.2	4.7	95.2	1.2	94.5	0.5
	-5	103.7	0.1	100.1	2.5	96.7	0.9
	-6	103.6	3.8	101.8	3.2	98.9	2.4
	-7	99.3	6.4	99.5	0.3	95.5	1.1
	-8	104.4	7.3	100.7	0.6	96.1	2.4
	-9	100.0	1.4	100.6	0.9	97.3	1.5
	-10	101.7	5.7	99.7	2.6	96.1	1.3
19-Norethindrone	-4	11.3	2.7	11.4	0.2	11.7	0.4
	-4.5	29.8	0.3	25.3	3.0	26.9	0.8
	-5.5	81.5	0.8	75.7	0.9	73.1	1.3
	-6	96.1	2.0	92.9	1.5	87.1	1.5
	-6.5	101.2	2.8	99.8	1.1	94.1	0.2
	-7	103.2	2.8	100.2	1.5	93.9	3.1
	-7.5	99.3	1.7	94.3	1.4	91.9	3.7
	-8.5	93.5	8.7	96.6	1.2	90.8	4.5
IN-A4098	-3	101.6	0.9	97.2	0.9	94.9	0.9
	-4	102.2	3.1	96.6	3.2	91.0	2.4
	-5	103.7	1.6	99.7	1.2	96.3	1.8
	-6	103.8	7.1	100.1	5.6	100.8	9.7
	-7	103.5	3.7	101.0	1.7	94.7	1.9
	-8	103.1	4.1	98.2	5.5	94.3	1.3
	-9	98.6	7.8	98.9	1.4	94.6	2.9
	-10	100.5	0.5	98.6	0.1	97.4	2.5

[a] N = 3
[b] SD = standard deviation

C. ASSAY PERFORMANCE

The performance parameters for 17β-oestradiol, as listed in Table 10, were within the acceptable ranges as specified in the test guideline. The performance parameters for 19-norethindrone, as listed in Table 10, were within the acceptable ranges as specified in the test guideline, with the exception of the bottom of the curve (%) of 2.8, 7.0, and 4.4% for runs C1, C2, and C3, which was marginally outside the bottom plateau level the suggested range as specified in the test guideline. All runs were within or very close to the suggested performance criteria, and therefore the assay was considered valid. The second deviation from the parameters is in the run C2, the Hill Slope was -1.16 for 19-norethindrone, which is marginally outside of the range of -0.7 to -1.1.

Confidence in these numbers is high due to the small variation between runs. There was no observed precipitation at any of the concentrations tested. The solvent control responses indicated no drift in any of the runs. The $logIC_{50}$ value for the 17β-oestradiol and 19-norethindrone is consistent with those previously reported in the EPA's Integrated Summary Report for the validation of the oestrogen receptor binding assay; no $logIC_{50}$ values for octyltriethoxysilane were reported.

Table 10
Competitive binding assay: $LogIC_{50}$ values, relative binding affinity, performance standards, and assay drift

Competitor	Parameter	Lower limit[a]	Upper limit[a]	Run C1[b]	Run C2[b]	Run C3[b]	Mean[c]
17β-oestradiol	Top (%)	94	111	103	100	96	0.979 ± 0.004
	Bottom (%)	-4	1	-1	0	0	-0.333 ± 0.577
	Slope	-1.1	-0.7	-1.1	-1.0	-1.1	-1.07 ± 0.058
	Log_e (S_{yx})	NA	2.35	0.4	0.2	0.5	0.367 ± 0.153
	$LogIC_{50}$ (M)	NA	NA	-8.8	-9.0	-9.0	-8.93 ± 0.115
19-norethindrone	Top (%)	90	110	100	98	93	97.0 ± 3.61
	Bottom (%)	-5	1	3[f]	7[f]	4[f]	4.67 ± 2.08
	Slope	-1.1	-0.7	-1.1	-1.1	-1.1	-1.10 ± 0.000
	Log_e (S_{yx})	NA	2.60	1.5	1.0	1.0	1.17 ± 0.289
	$LogIC_{50}$ (M)	NA	NA	-4.9	-5.0	-5.0	-4.97 ± 0.058
	RBA (%)[h]	NA	NA	0.0121	0.0101	0.0089	0.0104 ± 0.002
IN-A4098[i]	NA	NA	NA	NA	NA	NA	NA
Time since specific activity certification (days)							
		NA	NA	31	36	38	NA

[a] Suggested acceptable range according to test guideline
[b] N = 3
[c] Mean ± SE for all three runs
[d] With no weighting as bottom of the curve was below 0
[e] NA = Not applicable
[f] Parameter was outside the performance criteria
[g] With no weighting
[h] Relative binding affinity (RBA) relative to 17β-oestradiol
[i] No competitive binding observed, therefore no parameters could be calculated

III. CONCLUSION

Under the conditions of the study, the test substance, IN-A4098 was classified as "not interacting" in all three valid independent runs. Therefore, IN-A4098 is classified as "not interacting" in the oestrogen receptor binding assay.

(Willoughby, J. A., 2016)

Appendix 1.4
DuPont-45573 (A4098 ER transactivation)

Oestrogen receptor transactivation assay

DuPont-45573 OECD Summary

IN-A4098: Estrogen receptor transcriptional activation (human cell line (HeLa-9903))

Willoughby, J. A.

Executive summary:

IN-A4098 was evaluated for its ability to act as an agonist of human oestrogens receptor alpha (hERα) using the hERα-HeLa-9903 cell line. Preliminary assessments of cytotoxicity and precipitation were conducted in order to identify a suitable top concentration of IN-A4098 for use in the transcriptional activation assays. Dimethyl sulfoxide (DMSO) was selected as the vehicle for the test substance and did not have a significant effect on the assay. The final concentrations of IN-A4098 tested in the transcriptional activation assays were: 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M for the first and second runs. All concentrations were tested in replicates of six per plate. In addition, for each concentration, two replicates per plate were prepared that incorporated the hERα antagonist ICI 182,780. Replicates incorporating the hERα antagonist allow for the identification of non-specific (*i.e.*, non-hERα-mediated) induction of the luciferase gene. The duration of exposure was 24 hours. A complete concentration response curve for each of four reference compounds (17β-oestradiol, 17α-oestradiol, corticosterone, and 17α-methyltestosterone) was run each time the transcriptional activation assay was performed.

The maximum concentration of IN-A4098 selected for use in the transcriptional activation assays was 10^{-4} M as higher concentrations exhibited problems with solubility (test substance precipitation observed). There was no cytotoxicity (≥20% reduction in cell viability) observed with IN-A4098 or the controls in any of the valid independent runs. In two independent runs of the transcriptional activation assay, IN-A4098 did not result in an increase in luciferase activity (RPC_{max} <10%) at any of the viable concentrations tested.

Based on the results of this study, IN-A4098 is not considered an agonist of human oestrogen receptor alpha (hERα) in the HeLa-9903 model system.

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	IN-A4098 technical
	Lot/Batch #:	A4098-005
	Purity:	98.7%, by analysis
	Description:	Solid, powder
	CAS #	1668-54-8
	Stability of test compound:	Not determined
	Vehicle/final concentration:	Dimethyl sulfoxide (DMSO)/0.1% (v/v)
2.	Cell line:	hERα-HeLa-9903
	Source:	Japanese Collection of Research Bioresources (JCRB) Cell Bank, Ibaraki-shi, Osaka, Japan

B. STUDY DESIGN AND METHODS

1. Experimental start/completion

   19-November-2015 to 01-December-2015

2. Cell culture and plating conditions

Cells were maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with 60 mg/L of Kanamycin (antibiotic) and 10% dextran-coated-charcoal-treated foetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37 ± 1°C. When the cells reached 75–90% confluency, they were subcultured. The cells were suspended with 10% DCC-FBS in EMEM and plated into wells of a 96-well cell culture plate at a density of about 1×10^4 cells/100 µL/well. The cells were then placed into a 5% CO_2 incubator approximately 37°C for approximately 4 hours 25 minutes prior to chemical exposure. This time overage prior to dosing was described in a deviation to the study protocol.

3. Chemical exposure and assay plate organisation

The reference chemicals and IN-A4098 were dissolved in DMSO. The reference controls and test substance were then serially diluted in DMSO before further dilution in medium to prepare 2× concentrated stock solutions. The final concentration of DMSO in the medium was held constant at 0.1% (v/v).

After the 4-hour 25 minute post-seeding incubation, the plates were removed from the incubator, and the media was aspirated. A 75-µL aliquot of fresh media, followed by 75 µL of the 2× concentrated stock solutions were added to wells containing about 1×10^4 cells/well for a final volume of 150 µL/well. All test substance and reference control assay plates were organised in 96-well plates with 12 columns and 8 rows.

After adding the reference chemicals/test substance, the plates were incubated in a 5% CO_2 incubator at approximately 37°C for 24 hours 04 minutes. The time overage for the incubation period was also described in a deviation to the study protocol.

All concentrations were tested in replicates of six per plate. In addition, for each concentration, two replicates per plate were prepared that incorporated the hERα antagonist ICI 182,780. Replicates incorporating a hERα antagonist allow for the identification of non-specific (*i.e.*, non-hERα-mediated) induction of the luciferase gene as true hERα-mediated induction is inhibited by addition of an antagonist whereas non-specific induction is not.

4. Cytotoxicity assay

Cell viability was monitored by propidium iodide (PI) uptake. As PI is a light sensitive compound, all procedures were conducted under low light conditions.

Cells were seeded and exposed to the test substance in replicates of six while the last two rows received 125 µM digitonin as a positive control for cell death. Following chemical exposure, the growth medium was removed, and 50 µL of a PI working solution (44 µM in cell culture medium) was added to each well. Background fluorescence was evaluated by measuring fluorescence immediately on a BioTek Synergy H4 plate reader at an excitation wavelength of 544 nm and an emission wavelength of 612 nm. Following this determination, 50 µL of a 2% (v/v) Triton X-100 solution was added to each well and the plate was incubated at room temperature for a minimum of 15 minutes to fully lyse all cells in the wells before measuring fluorescence at the same wavelengths.

The background-corrected fluorescence was calculated for each well by subtracting the results of the first read from the results of the second read. The change in cell viability was determined by comparing treated wells to the vehicle control wells. A ≥20% reduction in cell viability was considered evidence of cytotoxicity.

5. Precipitation assay

Limit of solubility was determined by laser-based light scattering using a NEPHELOstar nephelometer and visual inspection.

6. Transcriptional activation assay

A luciferase assay was performed as described in Cyprotex Standard Operating Protocol SOP-2041. Luciferase assay reagent was prepared as described in Cyprotex SOP-2041 (proprietary information).

7. Preliminary range finding

In order to identify a suitable top concentration for use in the transcriptional activation assays, preliminary cytotoxicity and precipitation assays were conducted with IN-A4098. These preliminary assays assessed cytotoxicity and precipitation, respectively, at the following: 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M, while solubility was assessed at the same concentrations including 10^{-3} M.

8. Analysis

The test substance was considered negative if the maximum response relative to the positive control (RPC_{Max}) was <10% in at least two runs of the transcriptional activation assay. The test substance was considered positive if RPC_{Max} was ≥10% in at least two runs of the transcriptional activation assay.

II. RESULTS AND DISCUSSION

A. CONCENTRATION RANGE FOR THE TEST SUBSTANCE

In order to identify a suitable top concentration for use in the transcriptional activation assays, preliminary assessments of cytotoxicity and precipitation were conducted over a concentration range of 10^{-11} M to 10^{-3} M. Results of the cytotoxicity assay and precipitation assessment for IN-A4098 are shown in Table 11. On the basis of this preliminary assessment, the suitable top concentration of IN-A4098 for use in the transcriptional activation assays was 10^{-4} M as higher concentrations exhibited problems with solubility (test substance precipitation observed). There was no cytotoxicity (≥20% reduction in cell viability) observed with the test substance or controls in any valid run of the assay.

The final concentrations of IN-A4098 tested in the transcriptional activation assays were: 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M for the first and second runs. The results for IN-A4098 and the four reference compounds (17β-oestradiol, 17α-oestradiol, corticosterone, and 17α-methyltestosterone) are shown in Table 12 and Table 13.

Table 11
Results of the preliminary cytotoxicity and precipitation assays

Concentration of IN-A4098 (M)	Cell viability (% of VC[a])		Precipitation
	Mean	SD[b]	
10^{-11}	94.5	19.1	—[c]
10^{-10}	100.3	17.8	—
10^{-9}	107.2	20.8	—
10^{-8}	109.7	20.5	—
10^{-7}	100.4	15.2	—
10^{-6}	110.8	16.6	—
10^{-5}	105.4	17.7	—
10^{-4}	105.8	10.8	—
10^{-3}	ND[d]	ND	+[e]

[a] Vehicle control
[b] Standard deviation
[c] —; No precipitation observed
[d] No data
[e] +; Precipitation observed

Table 12
Results of the first transcriptional activation assay

Chemical	Concentration (M)	RTA[a] (% of PC[b])		RTA with ICI (% of PC)		Cell viability (% of VC[c])		Precipitation
		Mean	SD[d]	Value 1	Value 2	Mean	SD	
IN-A4098	10^{-11}	0.5	0.4	0.2	0.1	96.5	29.5	—[e]
	10^{-10}	0.3	0.5	0.8	0.0	103.6	25.7	—
	10^{-9}	0.4	0.5	0.0	-0.4	95.1	20.1	—
	10^{-8}	0.3	0.4	-0.2	-0.6	100.9	21.9	—
	10^{-7}	1.0	0.4	0.6	0.6	100.0	26.7	—
	10^{-6}	0.8	0.3	0.8	-0.2	100.9	26.6	—
	10^{-5}	1.2	0.4	0.9	1.0	99.0	26.8	—
	10^{-4}	0.7	0.5	0.9	0.6	101.7	21.6	—
17β-oestradiol	10^{-15}	0.1	0.3	0.5	0.6	99.4	14.5	—
	10^{-14}	0.1	0.4	0.7	0.5	95.6	13.4	—
	10^{-13}	0.4	0.6	1.0	0.3	98.3	18.4	—
	10^{-12}	1.1	0.8	1.0	0.5	101.1	8.1	—
	10^{-11}	12.7	1.9	1.2	0.9	103.6	16.6	—
	10^{-10}	80.4	19.0	1.0	0.4	109.3	8.8	—
	10^{-9}	155.3	36.4	-0.1	1.1	109.2	13.7	—
	10^{-8}	120.2	27.0	1.2	1.2	110.6	19.8	—
17α-oestradiol	10^{-13}	-1.7	0.4	1.0	0.8	102.6	14.0	—
	10^{-12}	-1.5	0.5	1.4	0.4	102.9	15.4	—
	10^{-11}	-1.4	0.7	0.2	0.3	102.3	12.9	—
	10^{-10}	-0.7	1.4	0.6	0.5	103.5	13.1	—
	10^{-9}	17.8	5.0	3.2	1.5	100.4	12.0	—
	10^{-8}	87.6	7.9	0.8	0.9	105.2	16.3	—
	10^{-7}	94.4	29.4	1.3	0.6	104.9	13.3	—
	10^{-6}	65.2	10.3	2.1	1.4	99.9	16.3	—
Corticosterone	10^{-11}	0.1	0.5	0.2	0.3	95.8	6.9	—
	10^{-10}	0.2	0.5	0.5	0.5	97.1	15.9	—
	10^{-9}	0.4	0.3	0.1	0.0	99.7	3.0	—
	10^{-8}	-0.1	0.3	0.8	0.2	102.0	5.8	—
	10^{-7}	0.7	0.2	0.8	0.6	98.3	10.3	—
	10^{-6}	0.3	0.4	0.6	0.3	106.0	10.6	—
	10^{-5}	0.6	0.2	0.4	0.2	93.1	16.6	—
	10^{-4}	0.5	0.6	-0.1	-0.4	88.9	10.7	—
17α-methyltestosterone	10^{-12}	0.6	0.5	0.4	0.5	96.1	12.4	—
	10^{-11}	0.4	0.7	0.0	0.7	97.2	15.6	—
	10^{-10}	0.4	0.4	0.3	0.1	90.0	8.0	—
	10^{-9}	0.2	0.4	0.9	-0.3	90.1	15.2	—
	10^{-8}	0.9	0.3	1.4	-0.1	85.4	10.5	—
	10^{-7}	1.4	0.9	0.9	0.3	84.7	8.4	—
	10^{-6}	6.0	3.6	0.7	0.3	91.6	8.5	—
	10^{-5}	33.0	11.8	0.1	-0.4	81.8	11.7	—

Table 12
Results of the first transcriptional activation assay

Chemical	Concentration (M)	RTA[a] (% of PC[b])		RTA with ICI (% of PC)		Cell viability (% of VC[c])		Precipitation
		Mean	SD[d]	Value 1	Value 2	Mean	SD	

[a] Relative transcriptional activation
[b] Positive control (1nM 17β-oestradiol)
[c] Vehicle control
[d] Standard deviation
[e] —; No precipitation observed

Table 13
Results of the second transcriptional activation assay

Chemical	Concentration (M)	RTA[a] (% of PC[b])		RTA with ICI (% of PC)		Cell viability (% of VC[c])		Precipitation
		Mean	SD[d]	Value 1	Value 2	Mean	SD	
IN-A4098	10^{-11}	0.2	0.6	0.8	-0.5	94.2	18.8	—[e]
	10^{-10}	0.3	0.9	0.3	-0.7	90.9	16.1	—
	10^{-9}	0.3	1.0	0.3	-0.6	92.5	17.9	—
	10^{-8}	0.2	0.6	0.4	-0.8	91.6	17.0	—
	10^{-7}	0.8	0.9	0.8	-0.5	96.1	22.4	—
	10^{-6}	0.6	0.7	0.6	-0.1	86.9	6.5	—
	10^{-5}	1.5	1.4	1.3	-0.6	91.9	21.1	—
	10^{-4}	0.3	0.8	1.0	-0.8	100.3	26.5	—
17β-oestradiol	10^{-15}	0.5	0.7	0.4	0.2	104.0	19.0	—
	10^{-14}	0.9	0.9	0.1	-0.5	104.7	13.7	—
	10^{-13}	1.0	1.0	-0.3	-0.6	106.7	18.3	—
	10^{-12}	1.3	1.1	0.8	0.0	100.6	12.9	—
	10^{-11}	12.8	8.5	0.7	0.1	103.1	19.4	—
	10^{-10}	62.8	10.0	1.0	0.6	104.9	20.6	—
	10^{-9}	153.5	37.5	1.8	0.6	104.9	14.2	—
	10^{-8}	97.4	27.8	3.0	0.1	107.9	12.0	—
17α-oestradiol	10^{-13}	0.4	0.6	0.1	-0.2	96.8	26.0	—
	10^{-12}	0.6	0.6	-0.3	0.0	97.6	22.4	—
	10^{-11}	0.6	0.5	0.2	0.1	97.7	22.3	—
	10^{-10}	0.9	0.6	0.5	-0.5	90.8	23.0	—
	10^{-9}	12.9	3.1	-0.5	0.5	92.3	30.2	—
	10^{-8}	79.4	27.3	0.0	-0.4	96.6	22.4	—
	10^{-7}	131.5	33.5	0.7	0.3	85.8	22.6	—
	10^{-6}	109.3	15.3	1.4	0.3	85.4	20.4	—
Corticosterone	10^{-11}	0.8	0.7	0.6	0.1	100.2	24.6	—
	10^{-10}	0.3	0.4	0.4	-0.4	102.4	13.5	—
	10^{-9}	0.9	0.6	1.0	-0.3	109.3	16.1	—
	10^{-8}	0.3	0.7	0.6	-0.3	104.8	11.5	—
	10^{-7}	1.0	0.9	0.6	0.4	108.9	14.9	—
	10^{-6}	0.9	0.4	0.5	0.2	108.3	17.9	—
	10^{-5}	1.2	0.9	0.1	-0.2	103.4	9.8	—
	10^{-4}	1.3	0.8	-0.8	-0.9	90.1	9.8	—
17α-methyltestosterone	10^{-12}	0.7	0.4	0.8	-0.5	80.8	16.6	—
	10^{-11}	0.3	0.5	0.6	0.0	95.6	19.5	—
	10^{-10}	0.7	0.7	0.8	-0.4	98.5	14.8	—
	10^{-9}	0.4	0.7	0.7	-0.8	95.2	18.0	—
	10^{-8}	0.9	0.6	1.0	0.1	91.0	13.7	—
	10^{-7}	1.6	0.9	0.8	-0.1	85.1	20.8	—
	10^{-6}	6.4	3.6	0.9	-0.2	86.4	11.9	—
	10^{-5}	29.0	11.1	0.4	-0.8	89.6	15.5	—

Table 13
Results of the second transcriptional activation assay

Chemical	Concentration (M)	RTA[a] (% of PC[b])		RTA with ICI (% of PC)		Cell viability (% of VC[c])		Precipitation
		Mean	SD[d]	Value 1	Value 2	Mean	SD	

[a] Relative transcriptional activation
[b] Positive control (1nM 17β- oestradiol)
[c] Vehicle control
[d] Standard deviation
[e] —; No precipitation observed

B. TRANSCRIPTIONAL ACTIVATION ASSAY RESULTS

The suitable top concentration of IN-A4098 for use in the transcriptional activation assays was 10^{-4} M, as higher concentrations of the test substance exhibited precipitation. There was no cytotoxicity (≥20% reduction in cell viability) observed with IN-A4098 or the controls in any of the valid independent runs.

In two independent runs of the assay, IN-A4098 did not result in an increase in luciferase activity at any of the non-precipitating concentrations tested as the RPC_{max} values in both independent runs of the assay were <10% (mean values of 1.2 ± 0.4% and 1.5 ± 1.4% in the first and second independent runs, respectively) (Table 12 and Table 13).

III. CONCLUSION

Based on the results of this study, IN-A4098 is not considered an agonist of human oestrogen receptor alpha (hERα) in the HeLa-9903 model system.

(Willoughby, J. A., 2016)

Appendix 1.5
DuPont-44801 (uterotrophic)

Report: Thifensulfuron-methyl (DPX-M6316) technical: 6 day uterotrophic assay for detecting oestrogenic activity and prolactin changes in ovariectomized rats

DuPont Report No.: DuPont-44801 OECD Summary

Guidelines: U.S. EPA Health Effects Test Guidelines, OPPTS 890.1600 (2009); OECD, Section 4 (Part 440): Uterotrophic Bioassay in Rodents, Guideline for the Testing of Chemicals (2007)

GLP: Yes

Executive summary:

Thifensulfuron-methyl technical was evaluated for its potential oestrogenic effects when administered by oral gavage to ovariectomised rats for 5 consecutive days. Three groups of young adult ovariectomised Crl:CD(SD) rats (15/group) were dosed by oral gavage with 0, 150, or 300 mg/kg/day of the test substance for 5 consecutive days and sacrificed approximately 24 hours after the last administered dose. Additionally, two separate ovariectomised positive control groups, one administered 0.1 mg/kg/day of the oestrogen receptor agonist 17α-ethynyl oestradiol, and the other administered 2 mg/kg/day of the dopamine (D2) receptor antagonist haloperidol were included to verify test system performance. Body weights and clinical observations were recorded daily. Food consumption was recorded on test day 1 and test day 6. Vaginal cytology was evaluated daily to assess the potential of the test substance to induce cytological changes consistent with those observed with the 17α-ethynyl oestradiol positive control. At scheduled necropsy, uterine weights were collected in order to assess the ability of the test substance to induce uterine growth. Blood was collected at the time of sacrifice from all animals for serum prolactin concentration analysis.

No test substance-related instances of mortality occurred, and there were no clinical observations noted during the in-life phase of the study. No test substance-related effects on body weight or nutritional parameters were observed. There were no test substance-related effects indicative of oestrogenic activity. All animals receiving the test substance remained in dioestrus for the duration of the study, and at necropsy, there were no gross observations noted or test substance related effects on uterine weight. There were no test substance related effects on serum prolactin concentration at any dose level.

In rats administered the positive control chemical, 17α-ethynyl oestradiol, no instances of mortality occurred, and there were no clinical observations noted during the in-life phase of the study. Mean body weight gain and mean final body weights were decreased compared to the negative control group. The body weight effects were accompanied by decreased food consumption and food efficiency. As expected, rats administered 17α-ethynyl oestradiol showed effects consistent with an oestrogen receptor agonist. All 15 rats administered 17α-ethynyl oestradiol showed effects on the stage of oestrous, and on test Day 4, all 15 rats administered 17α-ethynyl oestradiol showed cytological markers indicative of either pro-oestrus or oestrus. At necropsy, all rats showed the presence of uterine fluid within the uterus. The increased presence of fluid within the uterus was accompanied by increased uterine weights. Absolute uterine wet weight and blotted weight were increased to 162% and 150% of the negative control, respectively. Relative (to final body weight) uterine wet weight and blotted weight were increased to 188% and 175% of control, respectively. Serum prolactin levels were increased by 505% when compared to the negative control values. The results seen with 17α-ethynyl oestradiol are consistent with an oestrogen receptor agonist.

In rats administered the positive control chemical, haloperidol, no mortality occurred during the study and there were no clinical observations noted during the study. Mean body weight gain and mean final body weights were decreased as compared to negative control group. The body weight effects were accompanied by decreased food consumption and food efficiency. All animals receiving haloperidol remained in dioestrus for the duration of the study, and at scheduled euthanasia, there were no gross observations noted nor were there any treatment related effects on uterine weight. Serum prolactin levels were increased by 250% when compared to the negative control values. The results with haloperidol are consistent with a D2 receptor antagonist.

In conclusion, the test substance, thifensulfuron-methyl, did not induce changes on any parameters consistent with the potential to act as an oestrogen receptor agonist in ovariectomised adult female rats, and did not modulate serum prolactin concentrations. Under the conditions of this study, thifensulfuron-methyl did not induce oestrogenic effects or modulate serum prolactin concentrations in the uterotrophic assay when administered up to 300 mg/kg/day for 5 consecutive days.

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	Thifensulfuron-methyl (DPX-M6316) technical
	Lot/Batch #:	M6316-293
	Purity:	99.3 % of a.s. in technical used, by analysis
	Description:	Solid powder
	CAS #:	79277-27-3
	Stability of test compound:	The 5-day room temperature stability of the formulations from 10 to 200 mg/mL in the vehicle has been established in a previously conducted study (DuPont-31771).
	Vehicle:	0.5% methylcellulose
2.	Positive control:	17α-ethynyl oestradiol
	Lot number:	028K1411
	Purity:	>99%
	Source:	Sigma-Aldrich, St. Louis, Missouri, USA
	CAS #:	57-63-6
	Positive control:	Haloperidol
	Lot number:	MKBR1073V
	Purity:	100%
	Source:	Sigma-Aldrich, St. Louis, Missouri, USA
	CAS #:	52-86-8
3.	Test animals	
	Species:	Rat
	Strain:	Crl:CD(SD)
	Age at initial dosing:	Approximately 61 days old
	Weight at initial dosing:	240.7–299.8 g
	Source:	Charles River Laboratories International, Inc., Raleigh, North Carolina, USA
	Acclimation period:	At least 17 days
	Diet:	Harlan Teklad certified feed (2016), *ad libitum*
	Water:	Filtered tap water, *ad libitum*
	Housing:	Animals were housed in 2 to 3 per cage in solid bottom caging with bedding mixed with nestlets provided as enrichment.
4.	Environmental conditions	
	Temperature:	20–26°C
	Humidity:	30–70%
	Air changes:	Not reported
	Photoperiod:	Alternating 12-hour light and dark cycles

B. STUDY DESIGN AND METHODS

1. In-life initiated/completed

   18-July-2015 to 23-July-2015

2. Animal assignment and treatment

Three groups of 15 animals/concentration were dosed by oral gavage with 0, 150, and 300 mg/kg/day of the test substance for 5 consecutive days. A separate ovariectomised positive control group, administered 0.1 mg/kg/day of the oestrogen receptor agonist 17α-ethynyl oestradiol, was included to verify test system performance. Another separate ovariectomised positive control group, administered 2 mg/kg/day of the D2 receptor agonist haloperidol, was included to verify test system performance. Animals were assigned to dose groups based on adequate body weight gain and freedom from any clinical signs of disease or injury, and were distributed by computerised, stratified randomisation so that there were no statistically significant differences among group body weight means. Animal housing and husbandry were in accordance with the provisions of the *Guide for the Care and Use of Laboratory Animals* (NRC 2011).

Table 14
Study design: 6-Day uterotrophic assay for detecting oestrogenic activity in female rats

Group no.	No./ group	Daily dose (mg/kg/day)[a]	Dose volume (mL/kg/day)[a]	Test substance	Vehicle
1	15	0 (control)	5	None (negative control)	0.5% MC
2	15	150	5	Thifensulfuron-methyl	0.5% MC
3	15	300	5	Thifensulfuron-methyl	0.5% MC
4	15	0.1	2	17α-Ethynyl oestradiol (positive control)	Corn Oil With 1% Ethanol
5	15	2	5	Haloperidol (Positive Control)	0.5% MC

[a] Test substance or positive control administered once daily by oral gavage on test Days 1–5.

MC = Methylcellulose

3. Dose preparation and analysis

The dose volume for the groups receiving the test substance was 5 mL/kg bw/day. The test substance was suspended in the vehicle (0.5% methylcellulose) and was adjusted for purity. The same volume of vehicle was given to the negative control group. The dosing solutions for the test substance were prepared twice during the dosing period.

The dose concentration of the positive control, 17α-ethynyl oestradiol, selected for this study, 0.1 mg/kg/day, was based on the recommend dosage specified by the test guideline. The dose volume for the group receiving the positive control, 17α-ethynyl oestradiol, was 2 mL/kg bw/day. The positive control was dissolved in ethanol prior to dilution in corn oil and was not adjusted for purity. The final concentration of ethanol in the vehicle was 1%. The dosing solution for the 17α-ethynyl oestradiol was prepared once and used within the period of established stability.

The dose concentration of the D2 receptor antagonist, haloperidol selected for this study, 2 mg/kg/day, was based on information obtained in the published literature. The dose volume for the group receiving haloperidol was 5 mL/kg body weight/day. The haloperidol was suspended in the vehicle (0.5% methylcellulose, and was not adjusted for purity). The dosing solution for the haloperidol D2 receptor antagonist was prepared daily for the duration of the study and within the expiration date of the certificate of analysis. Stability of the positive control substance in the vehicle was not evaluated.

Samples of each test substance dosing preparation were collected near the beginning of the study. Analysis of the samples verified the concentration. At the time of analysis, the samples were diluted with an appropriate solvent and analysed by ultra high performance liquid chromatography (UHPLC) with ultraviolet (UV) detection.

4. Statistics

Table 15
Statistics: 6-Day uterotrophic assay for detecting oestrogenic activity in female rats

Parameter	Preliminary test	If preliminary test is not significant	If preliminary test is significant
Body weight Body weight gain Food consumption Food efficiency Organ weight Hormonal evaluation	Levene's test for homogeneity and Shapiro-Wilk test for normality	One-way analysis of followed by Dunnett's test	Transforms of the data to achieve normality and variance homogeneity were used. The order of transforms attempted was log, square-root, and rank-order. If the log and square-root transforms failed, the rank-order was used.

C. METHODS

1. Observations

Animals were observed at least twice daily for mortality and morbidity and for signs of abnormal behaviour and appearance. Once during weighing, and at least 2 hours post-dosing, each animal was individually handled, examined for abnormal behaviour and appearance, and subjected to detailed clinical observations. On test Day 6, careful clinical observations were performed once.

2. Body weights

All animals were weighed daily to the nearest 0.1 g.

3. Food consumption and food efficiency

The amount of food consumed by each cage of animals over test Days 1–6 was determined by weighing each feeder at the beginning and end of the interval and subtracting the final weight. Cage food consumption was divided by the number of animals in the cage to calculate individual animal food consumption. From these measurements, mean daily food consumption over the interval was determined. From the food consumption and body weight data, the mean daily food efficiency was calculated.

4. Oestrous cycle evaluation

All rats were evaluated for vaginal cytology on test Days -5 through 6. Vaginal washes were collected using saline and evaluated using established cytological markers for evidence of conversion out of dioestrus. All rats were in dioestrus throughout the acclimation period, indicating complete ovariectomy of all animals.

5. Hormonal evaluation

Blood was collected at the time of sacrifice from all animals. The blood was placed in a serum separator tube on ice until the serum was prepared. Serum was stored between -60°C and -80°C for analysis for serum prolactin concentrations. Serum prolactin concentrations were measured using a commercially-available radioimmunoassay kit.

6. Sacrifice and pathology

Approximately 24 hours after the last dose, animals were sacrificed by exsanguination under carbon dioxide anaesthesia. Gross examinations were performed on all main study animals. Organs that were weighed are listed in Table 16. Relative uterine weights (% final body weight) were calculated.

Table 16
6-Day uterotrophic assay for detecting oestrogenic activity in female rats: Organs/tissues collected for pathological examination

Organ	Organs weighed	Tissues saved for potential microscopic evaluation
Uterus (including uterine horns and cervix)	X	X
Vagina		X
Ovarian stumps		X

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Thifensulfuron-methyl was present at acceptable concentrations in the dosing solutions (within 88.8 to 89.0% of nominal concentrations). Thifensulfuron-methyl was previously shown to be stable in the dosing solutions under the conditions of the study in a separate study. The positive and solvent control(s) fulfilled the requirements for a valid test. The test substance was not detected in the negative control sample.

B. OBSERVATIONS

1. Clinical signs of toxicity

   No adverse, test substance-related clinical signs of toxicity were observed for any treatment group, including those administered the test substance or 17α-ethynyl oestradiol or haloperidol (positive controls).

2. Mortality

   No instances of mortality occurred during the course of this study.

C. BODY WEIGHT AND BODY WEIGHT GAIN

There were no test substance-related effects on body weights or body weight gains.

Rats administered 0.1 mg/kg/day 17α-ethynyl oestradiol (positive control group) showed a statistically significant decrease in mean final body weight, which was accompanied by a statistically significant decrease in mean body weight gain over the duration of the study (test Days 1–6). Mean final body weight was approximately 9% lower than the negative control group. Rats in the 17α-ethynyl oestradiol group lost an average of 4.6 g body weight compared to a gain of 22.8 g in the negative control group.

Rats administered 2 mg/kg/day haloperidol (positive control group) showed a statistically significant decrease in mean final body weight which was accompanied by a statistically significant decrease in mean body weight gain (test Days 1-6). Mean final body weight was 8% lower than the negative control group. Rats in the haloperidol group lost an average of 1.7 g body weight compared to a mean gain of 22.8 g in the negative control group.

Table 17
6-Day uterotrophic assay for detecting oestrogenic activity in female rats: Body weights (g)

Day	0 mg/kg bw/day	150 mg/kg bw/day	300 mg/kg bw/day	0.1 mg/kg bw/day 17α-ethynyl oestradiol	2 mg/kg bw/day haloperidol
Day 1	270 ± 15.6	270.4 ± 13.2	269.8 ± 14.5	270.1 ± 14.3	270.2 ± 13.5
Day 2	273.4 ± 15.9	272.4 ± 13.0	272.0 ± 14.2	271.1 ± 15.0	256.4 ± 14.9[a]
Day 3	279.6 ± 15.4	277.7 ± 13.5	277.9 ± 15.8	267.1 ± 12.9	253.6 ± 17.5[a]
Day 4	283.3 ± 15.8	282.3 ± 13.0	281.7 ± 15.9	264.4 ± 12.3[a]	255.1 ± 18.6[a]
Day 5	286.8 ± 16.6	286.8 ± 13.7	284.6 ± 15.9	264.5 ± 12.4[a]	263.0 ± 17.6[a]
Day 6	292.8 ± 17.2	290.8 ± 15.2	292.0 ± 16.7	265.4 ± 12.1[a]	268.5 ± 16.8[a]

[a] Significantly different from control by the Dunnett 2-sided test criteria, p <0.05.

Table 18
6-Day uterotrophic assay for detecting oestrogenic activity in female rats: Body weight gain (g)

Day	0 mg/kg bw/day	150 mg/kg bw/day	300 mg/kg bw/day	0.1 mg/kg bw/day 17α-ethynyl oestradiol	2 mg/kg bw/day haloperidol
Days 1–6	22.8 ± 5.1	20.4 ± 3.4	22.2 ± 4.0	-4.6 ± 8.3[a]	-1.7 ± 8.3[a]

[a] Significantly different from control by the Dunnett non-parametric 2-sided test criteria, p <0.05.

D. FOOD CONSUMPTION AND FOOD EFFICIENCY

There were no test substance-related effects on food consumption or food efficiency.

Rats administered 0.1 mg/kg/day 17α-ethynyl oestradiol (positive control group) had statistically significant decreased mean daily food consumption and mean daily food efficiency compared to the negative control group. Mean daily food consumption was 32% lower than the negative control group.

Rats administered 2 mg/kg/day haloperidol (positive control group) had statistically significantly decreased mean daily food consumption and mean daily food efficiency compared to the negative control group. Mean daily food consumption was approximately 19% lower than the negative control group.

Table 19
6-Day uterotrophic assay for detecting oestrogenic activity in female rats: Food consumption/ food efficiency

Parameter	0 mg/kg bw/day	150 mg/kg bw/day	300 mg/kg bw/day	0.1 mg/kg bw/day 17α-ethynyl oestradiol	2 mg/kg bw/day haloperidol
Food consumption, Days 1–6 (g/animal/day)	24.5	23.8	24.1	16.7[a]	20.0[a]
Food efficiency, Days 1–6 (avg wt gain/avg food consumed)	0.187	0.170	0.185	-0.059[a]	-0.023[a]

[a] Significantly different from control by the Dunnett non-parametric 2-sided test criteria, p <0.05.

E. OESTROUS CYCLE EVALUATION

There were no test substance-related effects on the stage of oestrous over the duration of the study.

Rats administered 0.1 mg/kg/day 17α-ethynyl oestradiol (positive control group) showed effects on the stage of oestrous on test Days 3 through 6. On test Day 3, six of fifteen rats administered 17α-ethynyl oestradiol showed cytological markers indicative of pro-oestrus. On test Day 4, fourteen rats administered 17α-ethynyl oestradiol showed cytological markers indicative of oestrus, and one rat showed cytological markers indicative of pro-oestrus. All rats administered 17α-ethynyl oestradiol showed cytological markers indicative of oestrus on test day 5 and 6. Cytological evidence of increased conversion out of dioestrus was consistent with an oestrogen receptor agonist.

F. SACRIFICE AND PATHOLOGY

1. Organ weights and gross observations

There were no test substance-related effects on mean absolute uterus (wet weights or blotted weights) or uterus wet or blotted weights relative to body weight. There were no test substance-related gross observations.

Rats administered 0.1 mg/kg/day 17α-ethynyl oestradiol (positive control group) showed a statistically significant increase in mean absolute uterine weight and mean relative (to final body weight) uterine weights. Absolute uterine wet weight and blotted weight were increased to 162% and 150% of the negative control group, respectively. Relative (to final body weight) uterine wet weight and blotted weight were increased to 188% and 175% of negative control group, respectively. The effects on uterine weight with the positive control chemical were consistent with an oestrogen receptor agonist.

Table 20
Mean terminal body and organ weights in female rats

Sex: Female		0 mg/kg/day	150 mg/kg/day	300 mg/kg/day	0.1 mg/kg/day	2 mg/kg/day
Terminal Body Wt (g)	Mean	292.8	290.8	292.0	265.4 #[1]	268.5 #[1]
	SD	17.2	15.2	16.7	12.1	16.8
	N	15	15	15	15	15
Uterus Wt- wet (g)	Mean	0.0978	0.0892	0.0869	0.2558 @[2]	0.0886
	SD	0.0110	0.0119	0.0079	0.0463	0.0111
	N	15	15	15	15	15
Uterus wet/ Term BW (%)	Mean	0.034	0.031	0.030	0.097 #[3]	0.033
	SD	0.005	0.004	0.003	0.019	0.004
	N	15	15	15	15	15
Uterus Wt. Blotted (g)	Mean	0.0952	0.0865	0.0841	0.2379 #[3]	0.0853
	SD	0.0108	0.0112	0.0080	0.0330	0.0108
	N	15	15	15	15	15
Uterus blot Term BW (%)	Mean	0.033	0.030	0.029	0.090 #[3]	0.032
	SD	0.005	0.004	0.003	0.014	0.004
	N	15	15	15	15	15

1 [# - Test: Dunnett 2 Sided p < 0.05]
2 [@ - Test: Dunnett Non-Parametric 1 Sided positive (Treatment > Control) p < 0.05]
3 [# - Test: Dunnett 1 Sided positive (Treatment > Control) p < 0.05]

0 mg/kg/day = control
150 mg/kg/day = 150 mg/kg/day Thifensulfuron-methyl
300 mg/kg/day = 300 mg/kg/day Thifensulfuron-methyl
0.1 mg/kg/day = 17α-ethylnyloestradiol
2 mg/kg/day = haloperidol

G. HORMONAL EVALUATION

There were no test substance-related effects on serum prolactin concentrations at any dose level.

Rats administered 0.1 mg/kg/day 17α-ethynyl oestradiol (positive control group) and 2 mg/kg/day haloperidol (positive control group) had statistically significantly increased serum prolactin concentrations as compared to the negative control group. The effects on serum prolactin concentrations with the positive control chemicals were consistent with an oestrogen receptor agonist and a D2 receptor antagonist.

Table 21
Summary of prolactin concentrations

Day: 19 Relative to Start Date

Sex: Female		0 mg/kg/day	150 mg/kg/day	300 mg/kg/day	0.1 mg/kg/day
Prolactin (ng/mL)	Mean	8.3	11.1	9.4	50.2 @1
	SD	6.5	6.9	7.7	57.0
	N	15	15	15	15

1 [@ - Test: Dunnett Non-Parametric 2 Sided p < 0.05]

Day: 19 Relative to Start Date

Sex: Female		2 mg/kg/day
Prolactin (ng/mL)	Mean	29.1 @1
	SD	25.1
	N	15

1 [@ - Test: Dunnett Non-Parametric 2 Sided p < 0.05]

III. CONCLUSION

In conclusion, the test substance, thifensulfuron-methyl, did not induce changes on any parameters consistent with the potential to act as an oestrogen agonist in ovariectomised adult female rats, and did not modulate serum prolactin concentrations. Under the conditions of this study, thifensulfuron-methyl did not induce oestrogenic effects or modulate serum prolactin in uterotrophic assay when administered up to 300 mg/kg/day of the test substance for 5 consecutive days.

(DuPont 2015b)

Appendix 1.6
DuPont-44799 (dopamine binding)

Report: Data report for radioligand binding assays

DuPont Report No.: DuPont-44799 OECD Summary

Guidelines: Not applicable

GLP: No

Executive summary:

The activity of Thifensulfuron-methyl (DPX-M6316) was evaluated in nine radioligand binding assays utilizing human recombinant CHO cell receptors. Methods employed in this study were adapted from the scientific literature to maximize reliability and reproducibility. Biochemical assay results are presented as the percent inhibition of specific binding or activity. Under the conditions of this study, no significant responses (≥50% inhibition or stimulation for biochemical assays) were noted in any of the radioligand binding assays.

I. MATERIALS AND METHODS

A. MATERIALS

1.	Test material:	Thifensulfuron-methyl technical
	Lot/Batch #:	M6316-293/AUG06MA076
	Purity:	99.3%, by analysis
	Description:	Not stated in the report
	CAS #:	79277-27-3
	Stability of test compound:	Not determined
	Solvent:	Dimethyl sulfoxide (DMSO), 1.00%
2.	Control materials	
	Reference Compounds:	R(+)-SCH-23390 for Dopamine D_1 and D_5 Spiperone for Dopamine D_{2L}, D_{2S}, D_3, $D_{4.2}$, $D_{4.4}$, and $D_{4.7}$ GBR-12909 for Dopamine Transporter (DAT)
3.	Incubation buffer (except as noted)	
	Tris-HCl (pH 7.4):	50 mM
	Ascorbic acid:	1.4 mM
	BSA:	0.001%
	NaCl:	150 mM/100 mM for Transporter (DAT)
	Leupeptin	1 µM for Transporter (DAT)
	PMSF	10 µM for Transporter (DAT)

B. STUDY DESIGN AND METHODS

1. Study initiated/completed

   04-June-2015 to 15-June-2015

2. Radioligand binding assay

   This study consisted of nine individual radioligand binding assays. The methods employed in this study were adapted from the scientific literature to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Individual assays were performed under the conditions described in the following section.

Dopamine D_1 (Eurofins catalogue number: 219500)

Source:	Human recombinant CHO cells
Incubation time/temperature:	2 hours at 37°C
Ligand:	1.40 nM [^{3}H] SCH-23390
Non-specific ligand:	10.0 μM (+)-Butaclamol
Specific binding (historical value):	90%
K_d (historical value):	1.40 nM
B_{max} (historical value):	0.63 pmole/mg protein

Dopamine D_{2L} (Eurofins catalogue number: 219600)

Source:	Human recombinant CHO cells
Incubation time/temperature:	2 hours at 25°C
Ligand:	0.16 nM [^{3}H] Spiperone
Non-specific ligand:	10.0 μM Haloperidol
Specific binding (historical value):	85%
K_d (historical value):	0.080 nM
B_{max} (historical value):	0.48 pmole/mg protein

Dopamine D_{2S} (Eurofins catalogue number: 219700)

Source:	Human recombinant CHO cells
Incubation time/temperature:	2 hours at 25°C
Ligand:	0.16 nM [^{3}H] Spiperone
Non-specific ligand:	10.0 μM Haloperidol
Specific binding (historical value):	90%
K_d (historical value):	0.090 nM
B_{max} (historical value):	1.60 pmole/mg protein

Dopamine D_3 (Eurofins catalogue number: 219800)

Source:	Human recombinant CHO cells
Incubation time/temperature:	2 hours at 37°C
Ligand:	0.70 nM [^{3}H] Spiperone
Non-specific ligand:	25.0 μM S(-)-Sulpiride
Specific binding (historical value):	85%
K_d (historical value):	0.36 nM
B_{max} (historical value):	1.10 pmole/mg protein

Dopamine $D_{4.2}$ (Eurofins catalogue number: 219900)

Source:	Human recombinant CHO-K1 cells
Incubation time/temperature:	2 hours at 25°C
Ligand:	0.50 nM [^{3}H] Spiperone
Non-specific ligand:	10.0 μM Haloperidol
Specific binding (historical value):	90%
K_d (historical value):	0.32 nM
B_{max} (historical value):	0.55 pmole/mg protein

Dopamine $D_{4.4}$ (Eurofins catalogue number: 220000)

Source:	Human recombinant CHO-K1 cells
Incubation time/temperature:	2 hours at 25°C
Ligand:	1.20 nM [^{3}H] Spiperone
Non-specific ligand:	10.0 µM Haloperidol
Specific binding (historical value):	85%
K_d (historical value):	0.46 nM
B_{max} (historical value):	0.63 pmole/mg protein

Dopamine $D_{4.7}$ (Eurofins catalogue number: 220100)

Source:	Human recombinant CHO-K1 cells
Incubation time/temperature:	2 hours at 25°C
Ligand:	1.50 nM [^{3}H] Spiperone
Non-specific ligand:	10.0 µM Haloperidol
Specific binding (historical value):	85%
K_d (historical value):	0.48 nM
B_{max} (historical value):	0.77 pmole/mg protein

Dopamine D_5 (Eurofins catalogue number: 220200)

Source:	Human recombinant CHO cells
Incubation time/temperature:	2 hours at 25°C
Ligand:	2.0 nM [^{3}H] SCH-23390
Non-specific ligand:	10.0 µM Flupentixol
Specific binding (historical value):	85%
K_d (historical value):	0.73 nM
B_{max} (historical value):	0.47 pmole/mg protein

Transporter, Dopamine (DAT; Eurofins catalogue number: 220320)

Source:	Human recombinant CHO-S cells
Incubation buffer:	Tris-HCl (pH 7.4): 50 mM NaCl: 100 mM Leupeptin: 1 µM PMSF: 10 µM
Incubation time/temperature:	3 hours at 4°C
Ligand:	0.15 nM [^{125}I] RTI-55
Non-specific ligand:	10.0 µM Nomifensine
Specific binding (historical value):	90%
K_d (historical value):	0.58 nM
B_{max} (historical value):	0.047 pmole/mg protein

3. Calculations and statistics

Where presented, IC_{50} values were determined by a non-linear, least squares regression analysis using MathIQ™ (ID Business Solutions Ltd., UK). Where presented, inhibition constant (K_i) values were calculated using the equation of Cheng and Prusoff (Cheng, Y., Prusoff, W.H., Biochem. Pharmacol. 22:3099–3108, 1973) using the observed IC_{50} of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the K_d of the ligand (obtained experimentally at

Eurofins Panlabs, Inc.). Where presented, the Hill coefficient (n_H), defining the slope of the competitive binding curve, was calculated using MathIQ™. Hill coefficients significantly different than 1.0 may suggest that the binding displacement does not follow the laws of mass action with a single binding site. Where IC_{50}, K_i, and/or n_H data are presented without standard error of the mean (SEM), data are insufficient to be quantitative, and the values presented (K_i, IC_{50}, n_H) should be interpreted with caution.

Biochemical assay results are presented as the percent inhibition of specific binding or activity. All other results are expressed in terms of that assay's quantitation method.

4. Evaluation criteria

The criterion for significance was ≥50% inhibition or stimulation in the biochemical assay.

For primary assays, only the lowest concentration with a significant response judged by the assays' criteria is shown in this summary. Where applicable, either the secondary assay results with the lowest dose/concentration meeting the significance criteria or, if inactive, the highest dose/concentration that did not meet the significance criteria is shown. Primary screening in duplicate with quantitative data (*e.g.*, $IC_{50} \pm$ SEM, Ki ± SEM, and nH) are shown where applicable for individual requested assays. In screening packages, primary screening in duplicate with semi-quantitative data (*e.g.*, estimated IC_{50}, K_i, and n_H) are shown where applicable (concentration range of 4 log units); available secondary functional assays are carried out (30 mM) and MEC or MIC determined only if active in primary assays >50% at 1 log unit below initial test concentration.

II. RESULTS AND DISCUSSION

A. RADIOLIGAND BINDING ASSAYS

A summary of results for the test substance and reference compounds are presented in Table 22 and Table 23, respectively. For Thifensulfuron-methyl, no significant responses (≥50% inhibition or stimulation for biochemical assays) were noted.

Reference standards were run as an integral part of each assay to ensure the validity of the results obtained.

Table 22
Summary of radioligand binding assay results for thifensulfuron-methyl

Compound	Assay name (Eurofins catalogue number)	Conc. (μM)	Batch[a]	Species	Replicates	% Inhibition	IC_{50}	K_i	n_H	R
DPX-M6316	Dopamine D_1 (219500)	10	372631	Human	2	-14	—	—	—	—
	Dopamine D_{2L} (219600)	10	372767	Human	2	2	—	—	—	—
	Dopamine D_{2S} (219700)	10	372649	Human	2	-15	—	—	—	—
	Dopamine D_3 (219800)	10	372692	Human	2	-11	—	—	—	—
	Dopamine $D_{4.2}$ (219900)	10	372651	Human	2	-4	—	—	—	—
	Dopamine $D_{4.4}$ (220000)	10	372651	Human	2	1	—	—	—	—
	Dopamine $D_{4.7}$ (220100)	10	372651	Human	2	1	—	—	—	—
	Dopamine D_5 (220200)	10	372632	Human	2	7	—	—	—	—
	Transporter, Dopamine (DAT) (2200320)	10	372623	Human	2	-14	—	—	—	—

[a] Represents compounds tested concurrently in the same assay(s).

Table 23
Summary of radioligand binding assay results for reference compounds

Reference compound	Assay name (Eurofins catalogue number)	Historical			Concurrent	
		IC_{50} (nM)	K_i (nM)	n_H	Batch[a]	IC_{50} (nM)
R(+)-SCH-23390	Dopamine D_1 (219500)	1.40	0.70	0.90	372631	1.41
Spiperone	Dopamine D_{2L} (219600)	0.26	0.082	1.20	372767	0.14
Spiperone	Dopamine D_{2S} (219700)	0.25	0.089	1.0	372649	0.097
Spiperone	Dopamine D_3 (219800)	0.36	0.12	0.90	372692	0.16
Spiperone	Dopamine $D_{4.2}$ (219900)	0.50	0.20	0.90	372651	0.24
Spiperone	Dopamine $D_{4.4}$ (220000)	0.76	0.21	0.80	372651	0.31
Spiperone	Dopamine $D_{4.7}$ (220100)	0.93	0.23	0.90	372651	0.50
R(+)-SCH-23390	Dopamine D_5 (220200)	1.50	0.40	0.90	372632	1.62
GBR-12909	Transporter, Dopamine (DAT) (2200320)	1.70	1.30	0.90	372623	1.13

[a] Represents compounds tested concurrently in the same assay(s).

III. CONCLUSION

For Thifensulfuron-methyl, no significant responses (≥50% inhibition or stimulation for biochemical assays) were noted in any of the radioligand binding assays.

(DuPont 2015d)

Appendix 1.7
DuPont-44802 (developmental)

Report: Thifensulfuron-methyl (DPX-M6316) technical: Developmental reproducibility toxicity study in rats

DuPont Report No.: DuPont-44802 OECD Summary

Guidelines: U.S. EPA Health Effects Test Guidelines, OPPTS 870.3700 (1998); OECD Guideline for the Testing of Chemicals, Section 4 (Part 414) (2001); EC Methods for the Determination of Toxicity and Other Health Effects, Directive 440/2008/EC Method B.31 (2008); MAFF Japan Test Guidelines for Agricultural Chemicals, 12-Nousan-8147 (2000)

GLP: Yes

Executive summary:

In a developmental toxicity study, thifensulfuron-methyl (DPX-M6316) technical was administered by oral gavage to time-mated Crl:CD (SD) female rats (22/dose group) on gestation Days 6–20. Gavage doses in 0.5% methylcellulose were 0 or 800 mg/kg bw/day. The dose volume was 5 mL/kg bw. Parameters evaluated in dams were body weight, body weight gain (absolute and adjusted for the products of conception), food consumption, survival, clinical signs, reproductive outcomes, gross pathology, kidney weight, and liver weight. Parameters evaluated in foetuses were body weight, incidences of dead foetuses and/or foetal resorptions, and incidences of external and visceral (kidney evaluation only) observations.

Under the conditions of this study which was conducted using a contemporary exposure protocol, the findings that were reported previously (small or absent renal papilla) were not reproduced. At 800 mg/kg bw/day, there were no adverse test substance-related effects on any maternal or foetal endpoint. Specifically, there were no alterations observed in the foetal kidneys; all foetal kidneys appeared normal at the fresh visceral examinations.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Thifensulfuron-methyl
   Lot/Batch #: M6316-293
   Purity: 99.3%, by analysis
   Description: Off-white solid
   CAS #: 79277-27-3
   Stability of test compound: The test substance was present at target concentrations and was assumed stable under the conditions of the study. Dosing solutions/suspensions were prepared at least weekly and stored refrigerated until used.
2. Vehicle and/or positive control: 0.5% Aqueous methylcellulose
3. Test animals
   Species: Rat
   Strain: Crl:CD (SD) female rats
   Age at initial dosing: Approximately 63 days old
   Weight at initial dosing: 218.8–297.5 g for females
   Source: Charles River Laboratories International, Inc., Kingston, New York, USA
   Acclimation period: 2–5 days
   Diet: PMI® Nutrition International, LLC Certified Rodent LabDiet® (#5002), *ad libitum*.
   Water: Tap water, *ad libitum*
   Housing: Animals were housed in groups in solid bottom caging with bedding mixed with enrichment (Nestlets™).
4. Environmental conditions
   Temperature: 20–26°C
   Humidity: 30–70%
   Air changes: Not reported
   Photoperiod: Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. In-life initiated/completed

   14-June-2015 to 30-June-2015

2. Animal assignment and treatment

   A previous developmental toxicity study of thifensulfuron-methyl (HLR 146-84) found foetal renal papillary observations (small or absent renal papilla) at the highest dose level tested. In this developmental toxicity study, thifensulfuron-methyl was administered by oral gavage to mated Crl:CD (SD) female rats (22/dose group) on gestation Days 6–20. Based on the previous study (HLR 146-84), doses of 0 or 800 mg/kg bw/day were selected. Animals were assigned to control and experimental groups using a computerised randomisation procedure designed to produce a homogeneous distribution of body weights across groups within each breeding lot. The test substance was administered in 0.5% methylcellulose at a volume of 5 mL/kg bw based on the most recent body weight. A negative control group received 0.5% methylcellulose alone. Animal housing and husbandry were in accordance with the provisions of the *Guide for the Care and Use of Laboratory Animals* (NRC 2011).

Table 24
Study design: Prenatal developmental toxicity test by the oral route in the rat

Group no.	Exposure (mg/kg bw/day)[a]	Test formulation concentration (mg/mL)[b]	Number of time-mated females
1	0[b]	0 (control)	22
4	800	160	22

[a] Formulations of test substance in 0.5% methylcellulose were administered once daily by oral gavage on gestation Days 6–20 at a dosing volume of 5 mL/kg bw.

[b] To achieve these concentrations of thifensulfuron-methyl, the formulations were adjusted for sample purity.

[c] The control group received 0.5% methylcellulose only at 5 mL/kg.

3. Dosing suspensions, preparation, and analysis

Suspensions of test substance in 0.5% methylcellulose were prepared at least weekly and stored refrigerated until used. The homogeneity and concentration of thifensulfuron-methyl in the dosing suspensions were checked by analyses using HPLC at beginning and end of study. The test substance was at target concentrations ±12%, homogeneous (RSD = 0.0 in the vehicle under the conditions of the study. Based on this information, it can be concluded that the animals received the targeted concentrations of test substance during the study. The stability of the dosing formulations at concentrations bracketing the concentrations administered to the animals was demonstrated for up to 5 days in a previously conducted study.

4. Statistics

Table 25
Statistics: Prenatal developmental toxicity test by the oral route in the rat

Parameter	Preliminary test	If preliminary test is not significant	If preliminary test is significant
Maternal body weight, maternal body weight gain, maternal food consumption, corpora lutea, live foetuses, dead foetuses, resorptions, implantations, foetal weight	Levene's test for homogeneity and Shapiro-Wilk test for normality	One-way analysis of variance followed with Dunnett's test	Transforms of the data to achieve normality and variance homogeneity were used. The order of transforms attempted was log, square-root, and rank-order. If the log and square-root transforms fails, the rank-order was used.
Sex ratio	None	Transforms of arcsine square-root of percent ÷ 100, one-way analysis of variance, followed by Dunnett's test	
Incidence of foetal alterations	None	Exact Mann-Whitney with a Bonferroni-Holm adjustment	

Significance was judged at $p < 0.05$. For litter parameters, the proportion of affected foetuses per litter of the litter mean was used as the experimental unit for statistical evaluation.

C. METHOD

1. Observations

Clinical signs were recorded once daily on gestation Day 4, twice daily on gestation Days 6–20, and once on gestation Day 21.

2. Body weights

All dams were weighed on gestation Day 4 and then daily on gestation Days 6–21.

3. Food consumption

Food consumption was measured on gestation Days 4, 6, 8, 10, 12, 14, 16, 18, 20, and 21.

4. Sacrifice and pathology

At termination (gestation Day 21), females were sacrificed by isoflurane anaesthesia and exsanguination. Foetuses were sacrificed by decapitation. Gross examinations were performed on all animals. Abdominal and thoracic viscera were examined. The uterus of each pregnant rat was removed and dissected to permit examination of the uterine contents. Livers and kidneys were weighed (kidneys paired). Microscopic examination of gross lesions was not performed since it was not considered necessary to meet the objective of the study.

5. Reproductive outcomes

The following reproductive parameters were evaluated: pregnancy rate, early delivery rate, total resorption rate, mean number of implantations, live foetuses, dead foetuses, corpora lutea counts, and pup sex ratio.

6. Evaluation of foetuses

Parameters evaluated in foetuses were body weight, incidences of dead foetuses and/or foetal resorptions, and incidences of external and visceral (limited to an examination of kidney alterations only). Kidneys from all live foetuses were saved for possible future microscopic evaluation.

II. RESULTS AND DISCUSSION

A. MATERNAL EFFECTS

1. Clinical signs of toxicity

There were no clinical signs of toxicity noted on this study.

2. Mortality

There was no effect on survival.

3. Body weight and body weight gain

There were no effects on body weights. There were slight, test substance-related reductions in cumulative absolute/adjusted (minus products of conception) body weight gains which were 8%/6% lower than the control group, respectively. These differences were not statistically significantly different from control and were of minimal magnitude and, therefore, not considered adverse. In the previous developmental toxicity study, slight non-adverse effects on maternal body weight were observed at 800 mg/kg bw/day so these current data are consistent with the previous study.

Table 26
Mean maternal body weight gain (g) during gestation

Interval (Days)	0 mg/kg/day	800 mg/kg/day
6-7	4.8 (3.6)	3.0 (3.2) -37.1%
7-8	3.6 (2.7)	5.2 (4.4) 43.8%
8-9	6.0 (3.2)	6.0 (8.0) -1.3%
9-10	4.9 (3.3)	5.0 (7.1) 1.6%
10-11	6.6 (3.0)	6.3 (2.6) -5.8%
11-12	4.6 (3.3)	3.9 (4.4) -16.1%
12-13	4.6 (3.7)	3.4 (3.7) -26.1%
13-14	6.3 (3.4)	6.7 (2.6) 7.3%
14-15	7.5 (2.9)	7.0 (2.2) -6.8%
15-16	9.3 (3.9)	9.1 (4.8) -2.7%
16-17	12.1 (3.9	10.3 (3.7) -14.7%
17-18	14.2 (5.0)	13.1 (4.6) -7.1%
18-19	13.3 (4.0)	10.5 (5.4) -21.3
19-20	16.4 (5.1)	15.8 (3.6) -3.9%
20-21	17.4 (6.8)	16.0 (5.1) -7.9%
6-21	131.7 (19.8)	121.3 (22.2) -7.9%
6-21 adjusted for products of conception	30.84 (11.92)	32.80 (11.74) 6.3%

Mean (standard deviation)

(*) Statistically significant by Anova & Dunnett's ($p<0.05$)

4. Food consumption

There were no adverse, test substance-related effects on maternal food consumption at any level tested. Mean food consumption was statistically reduced (6%) as compared to controls at 800 mg/kg bw/day during the first few days of dosing (gestation Days 6–8). This reduction correlated to the aforementioned effects observed on body weight gains after the first day of test substance administration at 800 mg/kg bw/day. However, this reduction did not impact overall (gestation Days 6–21) mean food consumption at this level and was therefore considered non-adverse. This reduction in food consumption is consistent with the effect on maternal body weights and is therefore consistent with the previous study.

5. Reproductive outcomes

There were no adverse, test substance-related effects on reproductive parameters during the course of this study. One animal (#459) at 800 mg/kg/day delivered a litter prior to scheduled euthanasia on gestation Day 21. This early delivery was not considered treatment-related since it only occurred for a single animal in the treatment group. Therefore, this single instance was considered spurious.

6. Gross pathology

No test substance-related gross lesions were observed at necropsy. There was a statistically significant increase in relative (% kidney/ terminal body weight) kidney weights as compared to concurrent controls at 800 mg/kg/day. However, this increase was not considered adverse since the magnitude of affect was minimal (8% higher than control group mean) and there were no effects on absolute kidney weights.

Table 27
Mean maternal organ weight

Parameter	0 mg/kg/day	800 mg/kg/day
Terminal body weight (g)	4.8 (3.6)	3.0 (3.2) -37.1%
Liver weight (g)	3.6 (2.7)	5.2 (4.4) 43.8%
Liver/body wt ratio	6.0 (3.2)	6.0 (8.0) -1.3%
Kidney weight (g)	4.9 (3.3)	5.0 (7.1) 1.6%
Kidney/body wt ratio	6.6 (3.0)	6.3 (2.6) -5.8%

Mean (standard deviation)

(*) Statistically significant by Anova & Dunnett's ($p<0.05$)

B. FOETAL EFFECTS

There was a statistically significant reduction in mean foetal weight observed at 800 mg/kg bw/day. Mean foetal weight was 3% lower than control group at this level. Although this reduction was statistically significant, it was not considered adverse since the magnitude of effect was low. The mean foetal weight was lower than historical control means for treated group and the concurrent control group mean was on the lower end of the historical control mean range (mean foetal weight: 5.85 g; range: 5.56–6.01 g). Current study control mean foetal weight was 5.66 g compared to treated group (800 mg/kg bw/day) mean of 5.49 g. This reduction is consistent with the previous study in which a similar non-adverse reduction in mean foetal weight was observed.

There were no foetal external malformations or variations observed at 800 mg/kg bw/day. All foetuses appeared normal at the external examinations. There were no visceral findings of the foetal kidney observed at 800 mg/kg bw/day. All foetal kidneys appeared normal at the visceral examinations.

Table 28
Mean foetal body weight

Parameter	0 mg/kg/day	800 mg/kg/day
Foetal wt (M) (g)	5.85 (0.28)	5.65 (0.32)
Foetal wt (F) (g)	5.50 (0.23)	5.33 (0.29)
Combined M&F wt (g)	5.66 (0.26)	5.49* (0.29) -3.1%

Mean (standard deviation)
M = male; F = female
*Dunnett's (two-sided)($p<0.05$)

III. CONCLUSION

Under the conditions of this study which was conducted using a contemporary exposure protocol, the findings that were reported previously (small or absent renal papilla) were not reproduced. At 800 mg/kg bw/day, there were no adverse test substance-related effects on any maternal or foetal endpoint. Specifically, there were no alterations observed in the foetal kidneys; all foetal kidneys appeared normal at the fresh visceral examinations.

(DuPont 2015e)

Appendix 1.8
DuPont-45278 (dosimetry)

Report: Thifensulfuron-methyl: Dosimetry Assessment for the Female Sprague-Dawley Rat During Gestation

DuPont Report No.: DuPont-45278

Guidelines: Not applicable – position paper

GLP: No

Executive summary:

The comparative dosimetry of the developmental and reproduction studies in rats with thifensulfuron-methyl is relevant in assessing the significance, whether causal or spurious, of the reported observations of small or absent renal papilla in the developmental study at 800 mg/kg/day administered during gestation days 6-15. However, no changes were observed in the kidneys of F_{2b} offspring in the multigeneration reproduction study which included continuous dosing throughout the period of rapid development of the renal papilla in rats (GD19 to about post natal day 2 and a more robust assessment of kidney morphology in offspring.

The disparity in renal papillary findings between the developmental and reproductive studies could be the result of the lower dose used in the feeding study (approximately 220 mg/kg/day compared to 800 mg/kg/day in the developmental study). However, in the developmental study, the last day of dose administration was dosing was GD15 so that by GD19, the beginning of the rapid phase of renal papillary development, maternal blood concentrations were predicted to decline to less than 0.5 µg/g. In contrast, the continuous dosing protocol of the reproduction study resulted in a predicted blood concentration of 4.4 µg/g on GD19 (and actually throughout gestation and lactation). Thus, while the dose level (800 mg/kg/day) in the developmental study was approximately 4-fold higher than that in the reproduction study, the differing dosing protocols, along with elimination kinetics of the test material, result in an approximately 10-fold higher predicted systemic exposure in the reproduction study compared to the developmental study. While these differences in predicted blood concentrations do not account for differences in route of administration between the two studies, it is unlikely that the gavage versus dietary route of administration in the developmental and reproductive studies, respectively, would negate the 10-fold difference in blood concentrations predicted to occur by GD19.

In conclusion, estimates of maternal systemic dosimetry in the developmental and reproduction studies with thifensulfuron-methyl indicate that by GD19, the beginning of the rapid growth phase of the renal papilla, systemic blood concentrations in the developmental study had declined to very low concentrations (less than 0.5 µg/g) and were approximately 10-fold less than those predicted for the reproduction study which followed a continuous exposure dosing regimen. No changes were observed in the kidneys of offspring in the reproduction study that provided a more robust assessment of the kidney, including a microscopic evaluation. These results support the conclusion that the observation of small or absent renal papilla in the developmental toxicity study with thifensulfuron-methyl were not test substance-related but were spurious findings resulting from the high variability in renal papillary development in late gestation rat foetuses.

INTRODUCTION

In a developmental toxicity study in rats with thifensulfuron-methyl, the incidence of the foetal variation "small renal papilla" was reported to be increased at 800 mg/kg/day, the highest dose tested (DuPont 1984).[19] The incidence of absent renal papilla, which was diagnosed in treated and control groups, was also reported as slightly increased at 800 mg/kg/day; however, this putative increase was not statistically significant, and the diagnosis of absent renal papilla was not confirmed by microscopic evaluation as recommended by current criteria. In addition, the incidences of small and absent papilla in the developmental study fell within the range of relevant test facility historical control data for small renal papillae. In a subsequent two-generation, four litter reproduction study in rats

19 DuPont (1984). Developmental Toxicity Study in Rats Given INM-6316 by Gavage on Days 7-16 of Gestation. Unpublished report, DuPont HLR 146-84.

with TSM (DuPont 1985)[20], parameters evaluated included a gross and microscopic examination of the kidneys of male and female weanling rats from the F_{2b} generation. There were no gross or microscopic changes in the kidney of these weanling rats at maternal dietary concentrations of up to 2500 ppm. This dietary concentration was equivalent to an intake during gestation of 221 mg/kg/day.[21]

The absence of changes in the renal papilla in the reproduction study, which included a more robust assessment of the kidneys of offspring than was available in the developmental study, could be the result of the higher dose level used in the developmental study (800 mg/kg/day) compared to the reproduction study (about 221 mg/kg/day). However, the dosing protocols for the two studies had important differences critical to an assessment of the comparative dosimetry of the respective studies and especially relevant to assessing the significance of the renal papillary findings in the developmental study. Specifically, in the developmental study, dams were dosed from gestation days (GD) 6-15[22,23], the dosing protocol for the developmental study provided for approximately 4 days of clearance of the test material (effectively a 4-day recovery period) prior to GD19, the critical period for renal papillary development. In contrast, dams in the reproduction study were continuously dosed (by the dietary route) through both the late gestation and early post-natal phases of renal papillary development. Thus, determining the significance of the renal papillary findings in the developmental study with thifensulfuron-methyl, and their absence in the reproduction study, requires consideration of several factors in addition to the dose levels used in those studies. These include differences between the respective dosing protocols, the late gestation morphogenesis of the rat renal papilla, and the elimination half-life of the test material. The objective of the analysis discussed below was to estimate the comparative maternal systemic exposure (blood concentrations) to thifensulfuron-methyl under the differing dosing protocols used in the developmental and reproduction studies with this material. These estimates were derived from measured data from two metabolism studies with thifensulfuron-methyl [24,25]. The analysis focused on blood concentration calculations beginning on GD19, as this comparison is the most relevant in assessing the significance of the observation of small or absent renal papilla that was observed in offspring in the developmental study but not in the reproduction study with thifensulfuron-methyl.

METHODS

A. METABOLISM STUDIES WITH THIFENSULFURON-METHYL

The absorption, distribution, metabolism and elimination (ADME) of [^{14}C]thifensulfuron-methyl in male and female rats has been studied in two separate studies .[6,7] These are summarized below. Only the data for female rats were used for the current dosimetry evaluation.

Study	Radiolabel	Dose (mg/kg bw)	Number of animals		Endpoint evaluated
			Male	Female	
DuPont HLR 91-86[6]	[Thiophene-2-^{14}C]	20	2M	2F	Urine, Blood
		20[a]	2M	2F	Urine, Blood
		2000	2M,2M	2F,2F	Urine, Blood
DuPont HLR 234-86[7]	[Triazine-2-^{14}C]	2000	5M	5F	Urine, Blood

[a] Rats were pretreated with non-radiolabeled thifensulfuron-methyl (100 ppm) in diet for 21 days prior to ^{14}C single oral dose administration

20 DuPont (1985). Two-Generation, Four-Litter Reproduction Study in Rats with INM-6316. Unpublished report, DuPont HLR 432-85

21 Intake calculations are summarized in DuPont-44635 EU.

22 Based on day of confirmed mating designated as Day 0, consistent with Woo and Hoar (1972) and common practice for developmental toxicity studies. HLR 146-84 considered day of confirmed mating as GD1 and thus reported the dosing period to be GD7 – GD16

23 Woo D.C. and Hoar R.M. (1972). "Apparent hydronephrosis" as a normal aspect of renal development in late gestation of rats: The effect of methyl salicylate. Teratology, 6(2), 191-196

24 DuPont (1986a). Metabolism of [Thiophene-2-^{14}C] DPX-M6316 by Rats. Unpublished report, DuPont HLR 91-86

25 DuPont (1986b). Metabolism of [Triazine-2-^{14}C] DPX-M6316 in Male and Female Rats. Unpublished report, DuPont HLR 234-86

For both metabolism studies, the kinetic data available included the rate and extent of radioactivity recovered in urine at 0-6, 6-24, 24-48, 48-72, and 72-96 hours following single oral dose administration. The concentration of ^{14}C residues in blood was measured at 96 hours after dose administration. The urinary excretion data were reported as percent of dose per collection interval (Table 29). The results from both of these metabolism studies, which used different ^{14}C labels, showed that the majority of the radioactivity was eliminated in the urine within 96 hours. Total mean recoveries in urine ranged from 66.5 to 89.2% of the dose through 96 hours after dose administration. Thus, while more detailed pharmacokinetic studies are not available, the rapid and urine-predominant elimination of the test substance, along with the measured blood values at 96 hours post-dosing, allow for a reasonable prediction of the elimination half-life of the test material in blood. The blood half-life in turn can be used to estimate and compare maternal blood concentrations under the respective dosing protocols of the developmental toxicity study[1] and the reproduction study[2] in rats with thifensulfuron-methyl.

Table 29
Summary of urinary excretion and ^{14}C residues in blood from female rats

			[Thiophene-2-^{14}C][a]					[Triazine-2-^{14}C][b]	
		Time	20[c]	20[d]	2000[c]			2000[c]	
Sample	Endpoint	(hour)	Mean	Mean	Mean	SD		Mean	SD
			(n = 2)	(n = 2)	(n = 3)			(n = 5)	
Urine	%	6	10.2	5.6	2.2	2.0		5.9	4.1
		24	51.6	60.2	24.1	0.8		26.4	11.5
		48	8.85	19.8	37.2	1.8		26.8	8.7
		72	1.45	2.8	2.3	1.9		12.0	5.3
		96	0.8	0.9	0.6	0.3		4.3	3.0
	Cumul%[e]	6	10.2	5.6	2.2	2.0		5.9	4.1
		24	61.8	65.7	26.3	1.3		32.3	8.1
		48	70.7	85.5	63.5	1.1		59.1	14.6
		72	72.1	88.3	65.8	2.6		71.1	14.5
		96	72.9	89.2	66.5	2.9		75.4	14.6
	ARE%[f]	6	72.9	89.2	66.5	2.9		75.4	14.6
		24	62.7[g]	83.6[g]	64.2	2.7		69.5	18.1
		48	11.1[g]	23.5[g]	40.1[g]	2.4		43.1[g]	10.2
		72	2.25[g]	3.7[g]	2.9[g]	2.2		16.3[g]	8.1
		96	0.8[g]	0.9[g]	0.6[g]	0.3		4.3[g]	3.0
	kel (/hr)		0.061	0.064	0.086			0.048	
	t1/2 (hr)		11.4	10.8	8.1			14.4	
Blood	ke (/hr)[h]		0.044	0.057	0.057			0.036	
	t1/2 (hr)		15.6	12.1	12.1			19.1	
	µg eq/g	6[i]	0.57	1.19	167	39.0		39.9	10.5
	µg eq/g	24[i]	0.26	0.43	59.6	13.9		20.8	5.5
	µg eq/g	48[i]	0.089	0.11	15.1	3.5		8.7	2.3
	µg eq/g	72[i]	0.031	0.028	3.8	0.9		3.7	1.0
	µg eq/g	96[j]	0.011	0.007	0.973	0.227		1.53	0.40

[a] DuPont HLR 91-86

[b] DuPont HLR 234-86

[c] Single oral dose administration (mg/kg bw)

[d] Pretreatment for 21 days with non-labeled test substance (100 ppm in diet) followed by single oral gavage dose (mg/kg bw)

[e] Cumulative urinary excretion

[f] Amount-remaining-for-excretion

[g] Points used to estimate urinary excretion slope (kel)

[h] Blood slope (ke) = Cumulative fraction excreted (Cumul%/100 at 96 h)*kel

[i] Estimated as $C(96h) = C(t)\exp^{(-slope*delta(t))}$, solve for C(t) where t = 6, 24, 48 or 72 hours

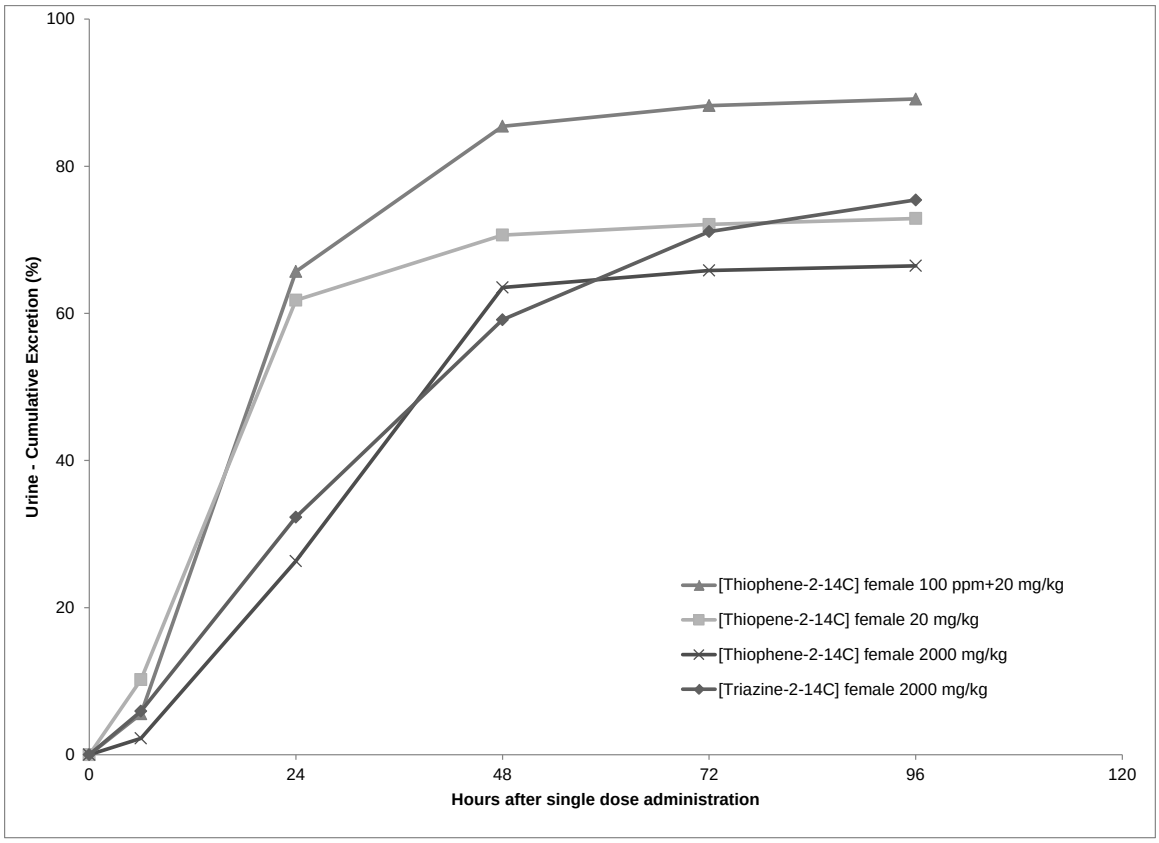
[j] Experimentally measured

B. CALCULATION OF URINE ELIMINATION KINETICS

For the current evaluation, the urine values were summed at the various collection intervals to obtain cumulative excretion (Table 29). Mean cumulative values were calculated for n = 2 to 5 female rats and graphed relative to time (Figure 5). Urinary excretion reached a plateau and was thus substantially completed by 72 hours after dose administered dose eliminated (fe) by the urinary route.

The cumulative sample collection also provided kinetic information about the rate of whole body elimination. These data support an "amount-remaining-for-excretion" calculation using the end-point of each sample collection interval and the declining percent of dose remaining (Figure 6). Log-linear elimination slopes (kel) were obtained from 3-4 time points per treatment group (Table 1 and Figure 3). The amount-remaining-for-excretion calculation (also referred to as a "Sigma-minus plot") is a recognized method for analysis of cumulative urinary excretion data (Wagner 1975; Ritschel and Kearns, 1999; Bourne, 2014).[26,27,28] This approach assumes that the rate of elimination follows first-order kinetics, which is reasonable given the strong regression analysis fit (R^2 = 0.9778-0.9953) to the data (Figure 7). Also, this approach is technically sound since urine was the predominant excretory route, and un-metabolized thifensulfuron-methyl represented the major component of radioactivity identified in urine .[6,7]

Figure 5
Cumulative excretion of radioactivity in urine of female rats following single oral dose administration of ^{14}C-thifensulfuron



26 Wagner J.G. (1975). Fundamentals of Clinical Pharmacokinetics, Drug Intelligence, Hamilton, Illinois.

27 Ritschel W.A. and Kearns G.L. (1999). Handbook of Basic Pharmacokinetics…including Clinical Applications. American Pharmaceutical Association, Washington, DC.

28 Bourne D.W.A (2014). PHAR 7633 Chapter 5 Analysis of Urine Data - Boomer http://www.boomer.org/c/p4/c05/c05.html, p 1-23.

Figure 6
Cumulative excretion expressed as amount (%) of dose remaining for excretion in urine

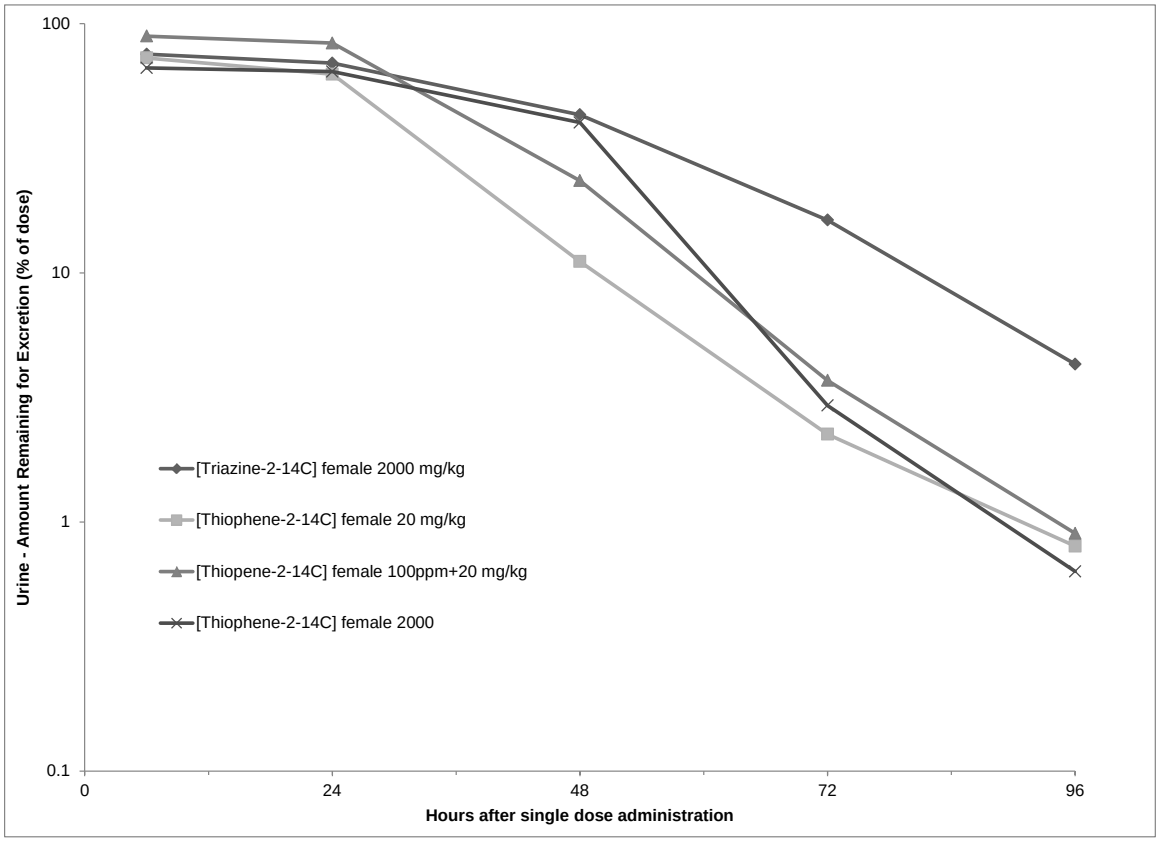
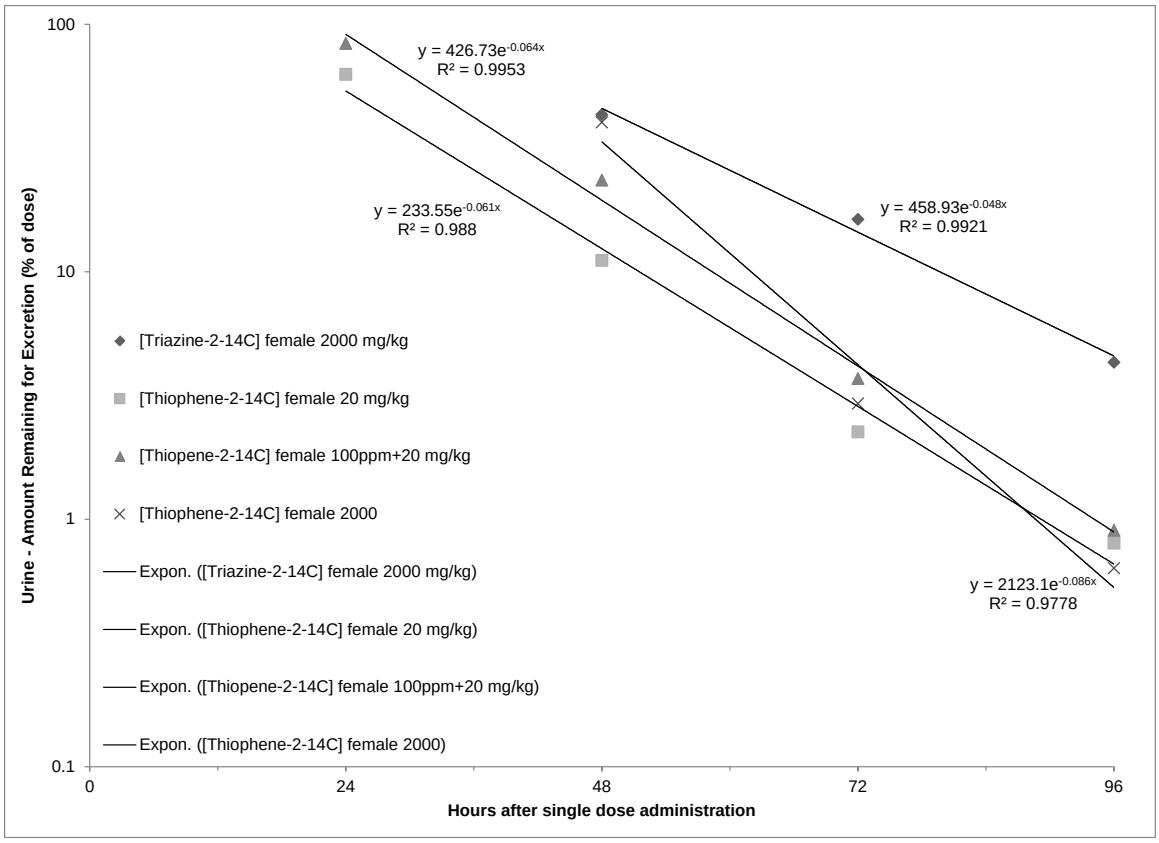


Figure 7
Calculation of log-linear half-life slopes from selected values for amount (%) of dose remaining for excretion in urine



C. ESTIMATION OF HALF-LIFE AND CONCENTRATION IN BLOOD

Blood collected at study termination provided a single time point measure of circulating systemic exposure. From the previous studies, mean blood concentrations at 96 hours ranged from 0.007 to 0.011 and 0.973 to 1.53 µg equivalents/g after low (20 mg/kg bw) and high (2000 mg/kg bw) dose administration, respectively (Table 29 and Figure 8).

The urinary "kel" rate constant was used to estimate the total body (blood) elimination slope (ke) and thus a half-life (t1/2) in blood using the following equations and definitions:

Parameter	**Abbreviation**
Urinary excretion slope	kel
Cumulative fraction eliminated	fe
Blood elimination slope (derived)	ke
Measured blood concentration at 96 hours	C(96 hr)
Predicted blood concentration at time (t)	C(t)
Half-life	t1/2
Natural logarithm of 2 (50% decline)	0.693
Natural logarithm exponent	exp

fe (cumulative urine total%) × kel (urine slope, 1/hr) = ke (blood, 1/hr),

and

0.693 ÷ ke = t1/2 (blood, hr).

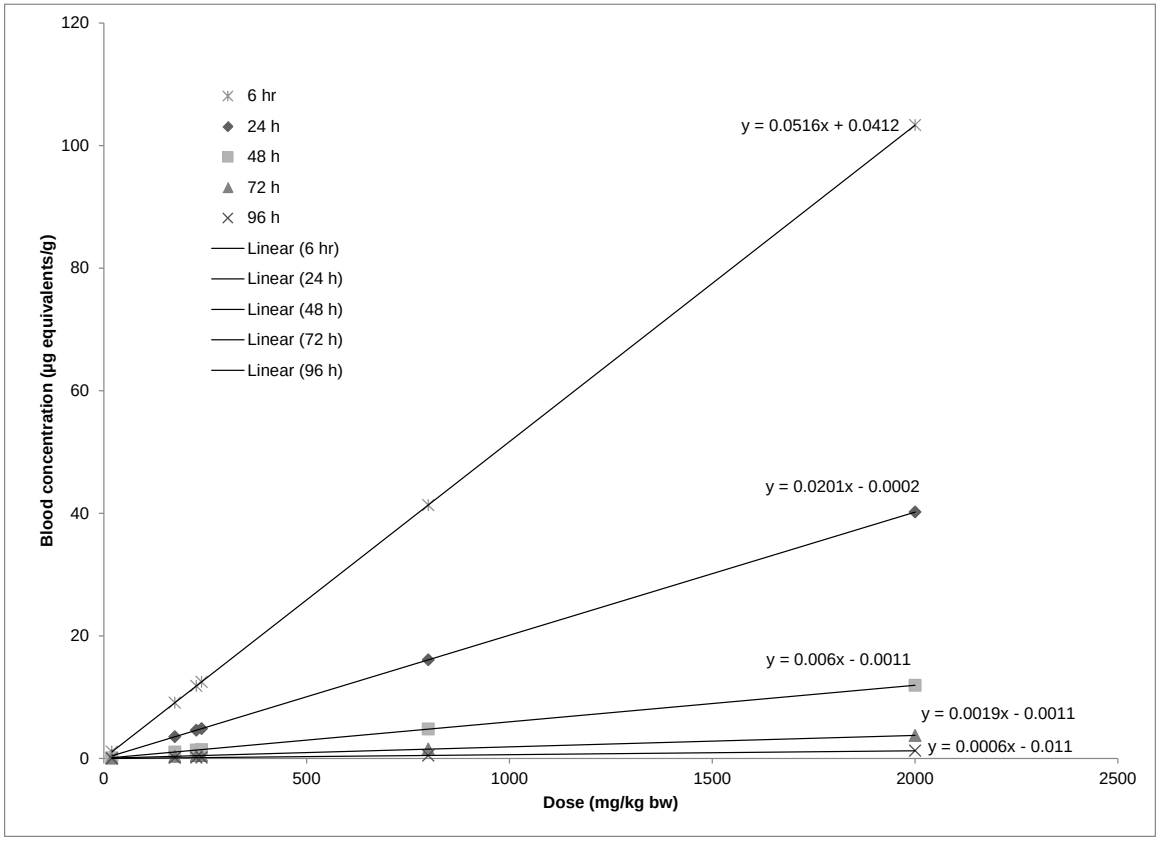
The measured blood concentrations "C(96 hr)" and the derived blood elimination slope (ke) were used to predict blood concentrations (C(t)) at earlier time points using the following equation:

C(96 hr) = C(t) × exp(-ke × (96 hr - t)),

where "exp" is the natural exponent.

Using the above calculations, blood concentrations of thifensulfuron-methyl equivalents were estimated at 6, 24, 48, 72 and 96 hours, based on reverse dosimetry from the measured 96 hour concentrations and the blood elimination half-life derived from the urinary excretion data.

Figure 8
Estimated blood concentrations of thifensulfuron in rats based on pharmacokinetic evaluation of measured urinary excretion (0-96 hr) and measured blood concentrations (96 hr)



RESULTS

The measured urinary excretion and the measured and predicted blood concentrations from the metabolism studies indicated dose proportional and linear first-order elimination kinetics (Table 29 and Table 30). Blood concentrations at 6 hours after dose administration were estimated to be between 0.56-1.19 µg/g at the low (20 mg/kg bw) dose and 40-169 µg/g at the high (2000 mg/kg bw) dose. The mean measured and predicted blood concentrations at the two dose levels showed increases of 118 to 143-fold relative to the 100-fold increase in dose. Additionally, pretreatment with dietary (100 ppm) exposure did not alter the kinetics in the one ADME experiment with the [thiophene-2-^{14}C] radiolabel, indicating that repeated exposure during the toxicity studies would not be

expected to appreciably alter uptake and elimination. The half-life for elimination of ^{14}C residues from blood ranged from 12 to 19 hours.

Since measured data from the metabolism studies indicated dose proportional and linear first-order elimination kinetics, it was appropriate to use a direct proportionality interpolation to predict the corresponding systemic (blood) exposure levels in the metabolism, developmental and reproductive toxicity studies. The metabolism studies were single dose studies, and in the developmental study, the last day of dosing was on GD15. Therefore, as expected, predicted blood concentrations declined over the following 96 hours for the metabolism and developmental toxicity studies. In contrast, the dosing protocol in the reproduction study involved continuous dosing (feeding) so that predicted blood concentrations at each 24 hour interval remained constant. Predicted blood concentrations under the differing dosing scenarios are given in Table 30.

Table 30
Summary of estimated thifensulfuron-methyl blood concentrations (µg/g) in the developmental, reproduction and metabolism studies in rats

Study	Dose (mg/kg/day)	Blood concentration (µg/g)				
		6 hr[a]	**24 hr**	**48 hr**	**72 hr**	**96 hr**
Metabolism study (HLR 91-86)(DuPont 1986b)	20	0.9	0.3	0.11	0.03	0.009
		GD15[b]	**GD16**	**GD17**	**GD18**	**GD19**
Reproduction Study (HLR 432-85)(DuPont 1985)	221	11	4.4	4.4	4.4	4.4
Developmental Study (HLR 146-84)(DuPont 1984b)	800	41	16	4.8	1.5	0.48
		6 hr[a]	**24 hr**	**48 hr**	**72 hr**	**96 hr**
Metabolism Studies (HLR 91-86 and HLR 234-86) (DuPont 1986b, DuPont 1986c)	**2000**	**103**	**40**	**12**	**3.7**	**1.3**

[a] Hours following a single gavage dose in the metabolism studies

[b] GD = Gestation day; dosing ended on gestation day 15 in the developmental study but was continuous throughout gestation (and lactation) in the reproduction study

From 2, it can be seen that by GD19 in the developmental study (i.e., 96 hours following the last day of dosing on GD15), blood concentrations at the 800 mg/kg/day dose are predicted to have declined to 0.48 µg/g. In the reproduction study, where dosing was continuous at a dietary intake of 221 mg/kg/day, predicted blood concentrations are maintained at 4.4 µg/g.

DISCUSSION AND CONCLUSIONS

The comparative dosimetry of the developmental and reproduction studies in rats with thifensulfuron-methyl is relevant in assessing the significance, whether causal or spurious, of the reported observations of small or absent renal papilla in the developmental study at 800 mg/kg/day. No changes were observed in the kidneys of F_{2b} offspring in the reproduction study which included continuous dosing throughout the period of rapid development of

the renal papilla in rats (GD19 to about post natal day 2 (Woo and Hoar, 1972)[5] and a more robust assessment of kidney morphology in offspring.

The disparity in renal papillary findings between the developmental and reproductive studies could be the result of the lower dose used in the feeding study (approximately 220 mg/kg/day compared to 800 mg/kg/day in the developmental study). However, in the developmental study, dosing was discontinued on GD15 so that by GD19, the beginning of the rapid phase of renal papillary development, maternal blood concentrations were predicted to decline to less than 0.5 µg/g. In contrast, the continuous dosing protocol of the reproduction study resulted in a predicted blood concentration on GD19 (actually throughout gestation and lactation) of 4.4 µg/g. Thus, while the dose level (800 mg/kg/day) in the developmental study was approximately 4-fold higher than that in the reproduction study, the differing dosing protocols, along with elimination kinetics of the test material, result in an approximately 10-fold higher predicted systemic exposure in the reproduction study compared to the developmental study. While these differences in predicted blood concentrations do not account for differences in route of administration between the two studies, it is unlikely that the gavage versus dietary route of administration in the developmental and reproductive studies, respectively, would negate the 10-fold difference in blood concentrations predicted to occur by GD19.

In conclusion, estimates of maternal systemic dosimetry in the developmental and reproduction studies with thifensulfuron-methyl indicate that by GD19, the beginning of the rapid growth phase of the renal papilla, systemic blood concentrations in the developmental study had declined to very low concentrations (less than 0.5 µg/g) and were approximately 10-fold less than those predicted for the reproduction study which followed a continuous exposure dosing regimen. No changes were observed in the kidneys of offspring in the reproduction study that provided a more robust assessment of the kidney, including a microscopic evaluation. These results support the conclusion that the observation of small or absent renal papilla in the developmental toxicity study with thifensulfuron-methyl were not test substance-related but were spurious findings resulting from the high variability in renal papillary development in late gestation rat foetuses (Woo and Hoar, 1972).[5]

(DuPont 2015f)