Comments on CLH Report for Acetochlor, Version 2 (September 2013)

Monsanto Europe SA/NV
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Introduction

First of all, we would like to commend the author(s) of this report for a very thorough, comprehensive and well-written document.
Below you will find our detailed comments on the scientific evaluation of the data, more specifically on the human health hazard assessment.
Besides some comments on specific studies or endpoints, we are questioning the classification proposals listed below:

- Acute toxicity (oral) : Acute tox. 4; H302
- Skin corrosion/irritation : Skin Irrit. 2 ; H315
- Carcinogenicity : Carc. 2 ; H351
- Specific target organ toxicity – repeated exposure : STOTR RE 2; H373

The results of additional studies have been taken into consideration in our discussion.

- Zhang F., *et al* (2010) Acetochlor sec-methylsulfide : in-vitro metabolism by olfactory turbinate and liver microsomes of male Sprague Dawley rat
- Ahmed, FE (1980) CP 55097: 91-day Feeding Study in the Mouse
- Wagner VO, *et al* (2013) : MON52706 : Bacterial Reverse Mutation Assay.
- Beevers C. (2014). In vivo Muta™Mouse gene mutation assay with MON 52706

The Tier 2 summaries of these studies are presented in appendix to this document, and the full reports of 3 studies have been uploaded as confidential information. The size of the mouse feeding study didn't allow it to be uploaded. This study is available upon request.

It is also worth noting that, although acetochlor was not approved in the EU in December 2011, a new application for EU registration (under 1107/2009) will be submitted in 2014. This application will include additional studies and a revised GAP. Malta will be the RMS.

PART B : SCIENTIFIC EVALUATION OF THE DATA

4. HUMAN HEALTH HAZARD ASSESSMENT

A brief summary of our two major comments on this section are provided below.

1. **Comparison of rat nasal carcinogenic mode-of-action (MOA) data with CLP criteria for classification:**
   The CLH report appears to selectively cite the CLP guidance by stating that human relevance can be dismissed only if the MOA "is **conclusively** determined not to be operative in humans" (as stated in section 3.6.2.3.1 (k)) rather than "unless there is **strong** evidence that the mechanism of tumour formation is not relevant for humans" (as indicated in Annex I: 3.6.1.1) (emphasis added). It also does not appear to follow the CLP guidance suggesting that a comparative toxicokinetics/toxicodynamics evaluation should be conducted using a human relevance framework (HRF) such as that produced by the IPCS (IPCS, 2007; Boobis et al, 2006). The IPCS human relevance framework incorporates a weight-of-evidence approach to evaluate whether there are sufficient data to "**reasonably exclude**" the human relevance of the MOA on the basis of qualitative or quantitative species differences in toxicokinetics and/or toxicodynamics. We believe that such an evaluation would demonstrate that the MOA responsible for the rat nasal tumours is not relevant to humans on the basis of very large quantitative species differences in toxicokinetics and/or toxicodynamics, and thus these tumours should not trigger a Category 2 (CLP) cancer classification. It should also be noted that this IPCS Human Relevance Framework was published after the ECB classification of acetochlor and the USEPA evaluation of the acetochlor nasal MOA. Prior to this, the IPCS framework simply asked whether the MOA was plausible in humans, and took into account only qualitative, not quantitative, differences. Thus, the previous classifications by ECB and USEPA under the older framework may be outdated.

2. **Toxicological significance of marginal increases in mouse lung tumours and histiocytic sarcomas**
   Mouse lung tumours (alveolar/bronchiolar adenomas and/or carcinomas) and histiocytic sarcomas are very common spontaneous tumours in aged CD-1 mice. In fact, lung tumours are the most common tumour observed in female CD-1 mice and a very close second (to liver tumours) in male CD-1 mice. In addition, the incidence of both lung tumours and histiocytic sarcomas is highly variable in aged animals. Thus, extra caution should be utilized when evaluating the potential toxicological significance of slight increased incidences of these types of tumours, particularly when there is only marginal statistical significance and/or limited historical control data from the laboratory conducting the study. An independent Pathology Working Group, the European Chemical Bureau Group of Specialised Experts, and an independent panel of European pathologists and toxicologists have reviewed the

acetochlor data and available historical control information and concluded that the marginally increased incidences of both lung tumours and histiocytic sarcomas observed in the long-term mouse studies with acetochlor were not treatment-related. The USEPA has concluded that both of these tumours were only equivocally and/or possibly related to treatment with acetochlor, and that neither of them were a "tumor of concern for human exposure". Therefore, the mouse lung tumours and histiocytic sarcomas should not be considered relevant for the purposes of classification.

The following pages provide more details concerning these comments, as well as a number of additional comments, several of which are relatively minor and unlikely to impact the EChA conclusions or classifications but are provided to help improve the accuracy and/or completeness of the report.

4.1. Toxicokinetics

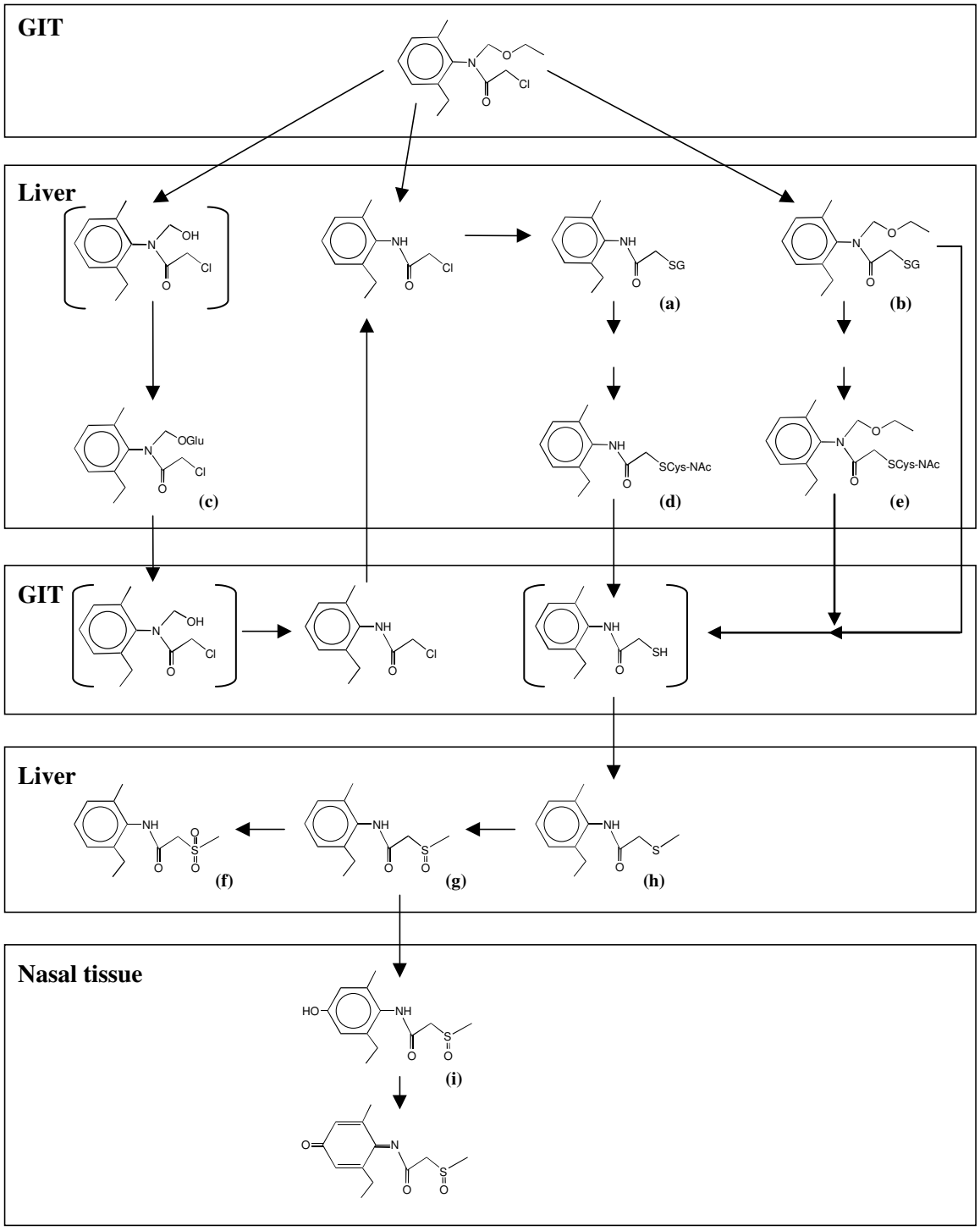
4.1.1. Non-human information

Although we generally agree with the information provided in this section, we believe that the discussion of p-OH sec-methyl sulfoxide should be emphasized as the primary route of quinone imine formation and that the discussion of p-OH EMA as a route of formation should be deemphasized. The weight-of-evidence indicates that ethylmethylaniline (EMA) is likely to be, at most, only a very minor precursor to DABQI formation relative to the sulfoxide metabolite. Although EMA was initially believed to be the key precursor responsible for DABQI formation, it was later determined that this was due to flawed experimental conditions that either favored EMA formation (*i.e*., *in vitro* studies conducted without an NADPH-regenerating system) or that resulted in the inadvertent removal of the sulfoxide moiety (*i.e.,* protein binding studies conducted using 6N HCl). Subsequent studies have demonstrated that the sulfoxide metabolite is the key precursor to DABQI formation following acetochlor administration and that EMA plays, at most, only a very minor role.

Further support for this position can be found in the report on the *in vitro* metabolism of acetochlor sec-methyl sulfide in rats (Zhang, Hansen, and Saghir 2010), which is included along with this submission (Tier summary in appendix to this document, and full report uploaded as confidential information). Results from the study strongly support a pathway involving p-OH sec-methyl sulfoxide as the primary route of quinone imine formation. We recommend that the metabolic pathway showing the major metabolic transformations of acetochlor in the rat in Figure 1 should be replaced with the attached modified pathway. The main difference between the revised figure and the original one is that formation of p-OH EMA and its subsequent conversion to quinone imine has been removed because we do not consider it to be a major pathway.

Figure 1 : Major metabolic pathway of acetochlor in the rat

(Simplified version of a very complex pathway. A number of minor metabolites, including those formed following hydroxylation of the alkyl group side chains, are not shown).



Metabolites with potential to exceed 0.1 µg/L only in exceptional circumstance (t-NCA)
We agree with the conclusion (page 29) that the weight of evidence suggests that the metabolite norchloroacetochlor (t-NCA) is not a clastrogenic agent *in vitro* and *in vivo*. However, to address the potential mutagenicity concerns previously raised during the EU review, an Ames assay was repeated, and a Muta™Mouse *in vivo* gene mutation assay just conducted with t-NCA. No evidence of genotoxicity was noted in either assay, which further supports the overall weight-of-evidence that t-NCA does not have any significant genotoxic potential. A summary of these studies is provided in appendix to this document, and the full reports have been uploaded as confidential information.

4.1.2 Human information

The weight of evidence indicates that ethylmethylaniline (EMA) is likely to be at most only a very minor precursor to DABQI formation relative to the sulfoxide metabolite (as discussed in 4.1). Based on an article by Coleman *et al* (2000), the CLH report concludes that human liver microsomes have similar rates of metabolism of acetochlor to CMEPA (dealkylated acetochlor, which we refer to as sec-acetochlor) as rat liver microsomes, and that the subsequent rate of metabolism from CMEPA to EMA is greater in humans than in rats. The CLH report then concludes that EMA is "plausibly carcinogenic" because it could be further metabolized to a quinoneimine (DABQI).

The fact that EMA may possibly be metabolized to trace levels of DABQI formation in humans does not necessarily mean that it should be considered carcinogenic to humans. Quantitative species differences in formation of EMA *in vivo* as well as its subsequent conversion to DABQI should be considered in the assessment, as indicated by the IPCS human relevance framework cited above.

In addition, there are several aspects to the Coleman *et al.* (2000) publication that should be noted:

- Coleman *et al.* suggest that the genotoxicity and carcinogenicity of the acetanilides is directly related to the rate of hepatic dealkylation of the parent chloroacetanilide to the corresponding 2-chloro-N-(2,6-dialkylphenyl)acetamide (CDAPA; CMEPA for acetochlor and metolachlor, CDEPA for alachlor and butachlor) and then subsequent metabolism to either DEA (for alachlor and butachlor) or EMA (for acetochlor and metolachlor). However, the rate of production of CDAPA or DEA/EMA in the liver does not appear to correlate with the *in vivo* toxicology findings. For example, none of the chemicals tested by Coleman *et al.* produced a genotoxic response in the *in vivo* rat liver UDS assay except at hepatotoxic dose levels. In addition, metolachlor was the only acetanilide tested that produced liver tumours below the MTD but it was ranked last by Coleman *et al.* in CDAPA formation. In contrast, acetochlor was reported to produce the most CDAPA by Coleman *et al.*, yet it produced an increased

incidence of liver tumours only at a dose level greatly exceeding the MTD. There was also no relationship to nasal tumour production since alachlor was the most potent nasal carcinogen but was ranked second to last in hepatic CDAPA formation. Finally, no nasal tumours were noted in two long-term feeding studies in mice despite the fact that CDAPA was the primary plasma metabolite.

- Coleman *et al.* (2000) apparently did not include an NADPH-regenerating system, which is generally considered obligatory for oxidative reactions catalyzed by mammalian cytochrome P-450 enzymes, when evaluating the formation of DEA and EMA from CDAPA. As a consequence, the conversion of the CMEPA (sec-acetochlor) to EMA by hepatic arylamidase was measured only in the absence of a competing metabolic pathway. In contrast, they reported that no EMA was detected after a 20-minute incubation of parent acetochlor in the presence of an NADPH-regenerating system and only very low levels were detected after a one-hour incubation. Thus, the results from all such *in vitro* experiments conducted in the absence of an NADPH-regenerating system (including several previously conducted by Monsanto) should be interpreted with extreme care.

4.2 Acute Toxicity:

4.2.1. Non-human information

4.2.1.1. Acute toxicity : oral

The acute oral LD50 for males in the Cummins study should be 4238 mg/kg, as reported in Table 14, not 2389 mg/kg as indicated in text.

4.2.4. Comparison with criteria

We disagree with the proposal to classify acetochlor for acute oral toxicity. The weight of evidence from two acute oral toxicity studies indicates that the rat oral LD50 is greater than 2000 mg/kg. There were no significant differences between sexes in either study and the combined LD50 of 2148 mg/kg from the cited study (Branch, 1982) as well as the male (4238 mg/kg) and female (4015 mg/kg) LD50 values from the other study (Cummins, 1986) do not meet the criteria for classification. Thus, acetochlor should not be classified for acute oral toxicity.

4.4. Irritation

4.4.1 Skin irritation

4.4.1.4. Comparison with criteria

Acetochlor was severely irritating in a 4-hr skin irritation study using test material produced by ICI (Barlow and Ishmael, 1989), but was essentially non-irritating in a study conducted using more stringent conditions (24-hour exposures to both abraded and unabraded skin) using test material produced by Monsanto (Branch, 1982). The fact that more stringent conditions were utilized in the Branch (1982) study, which was conducted prior to publication of OECD guidelines, should not result in it being considered unacceptable, as indicated in Table 15. The discrepancy in the results between the two studies may be due to the difference in purity of the test materials used (89.4% for the ICI material vs. 96.3% for the Monsanto material). It should be noted that ICI no longer produces acetochlor and that the test material used in the Branch study is more representative of the technical material currently being produced by Monsanto.

4.7. Repeated dose toxicity

4.7.1.1.1. Short term oral toxicity studies

For the Broadmeadow (1989) study, Tables 18 and 21, and text on pages 50 & 56, indicate that 2 mg/kg/day should be a LOAEL instead of a NOAEL because of the presence of granuloma in one male and one female, and reduced glycogen in one male. However, no granulomas were noted in males or females at either 10 or 50 mg/kg/day, which strongly indicates that the lesions at 2 mg/kg/day were not treatment related. In addition, single animals with reduced glycogen were also noted in the control and 10 mg/kg/day female groups. Thus, the weight of evidence supports the previous PRAPeR (and USEPA) conclusion that 2 mg/kg/day was the NOAEL for this study.

Oral short term toxicity studies in dogs :

Testicular effects in dogs: The paragraph immediately preceding Table 19 states, "At first, findings observed in testes were associated with lymphocytic orchitis …" . This should be clarified to indicate that Creasy concluded that only the testicular lesions in one of five dogs at 10 mg/kg/day were attributed to lymphocytic orchitis. This was the only animal in the study that exhibited multifocal tubular necrosis with associated lymphoid infiltrate. The testicular lesions in the high-dose animals (tubular degeneration, maturation arrest, spermatid giant cells, Epidimydes hypospermia) were all attributed to test article administration, but in absence of lymphocytic orchitis. The observation of lymphocytic orchitis, a pathology commonly encountered in beagle dogs, in one dog at 10 mg/kg and at none of all the other dose levels confirms that this lesion is incidental and not related to treatment . The fact that no testicular lesions were observed at 12 mg/kg/day in the other 1-yr dog study supports this conclusion.

4.7.1.10. Conclusions on classification for repeat dose toxicity

We agree that the kidney and liver are target organs following high doses of acetochlor. However, the conclusion that "severe effects on kidney (interstitial nephritis, chronic vasculitis and tubular basophilia) and liver (fatty infiltration) were observed after repeated exposure below the cut-off levels", and that these findings justify R48/22 classification, does not appear to be warranted based on the following:

- The interstitial nephritis and/or chronic vasculitis reported in the kidneys from a few male dogs at 10 mg/kg/day from one 12-month dog study (Broadmeadow, 1989) were reported to be of "minimal" severity (individual histopathology data). Minimal severity is generally considered to be a histologic change that barely exceeds the normal limits and that is unlikely to produce any functional impairment. As such, these findings should not trigger classification.

- An increased incidence of renal tubular basophilia was reported in male mice at 1.1 mg/kg/day in the 18-month mouse study (Amyes, 1989). However, the severity of the lesion in all animals at this level was minimal, there was no clear dose-response, and no such findings were reported even at ~500-fold higher dose levels in a 23-month mouse study (Ahmed, 1981). These findings were not considered evidence of a treatment-related adverse effect by either the USEPA or Japan FSC. Therefore, we believe that these findings were of questionable toxicological significance and should not trigger classification. This is further supported by the lack of renal effects in the mouse 90-day study (Ahmed, 1980). A Tier summary of this study is provided in appendix and the study is available upon request.
- Moderate diffuse fatty infiltration of the liver was reported in one male dog at 12 mg/kg/day in the other 12-month dog study (Ahmed, 1981) but was not accompanied by any treatment-related changes in liver weight or clinical pathology. The few statistically significant differences in LDH, SGOT or cholesterol at this dose level were sporadic (only seen occasionally despite being evaluated monthly throughout the study), were similar to the values seen in control animals at other time points in this study, and were well within the normal historical control ranges for beagle dogs of this age. In addition, the fatty infiltration observed in 1/6 animals at the higher dose was scored as “mild”. Thus, it is our opinion that the finding of fatty infiltration of the liver in the 12 mg/kg animal is of questionable toxicological significance and not sufficient to trigger classification,

4.9. Germ cell mutagenicity

4.9.1. Non-human information

In vitro gene mutation studies

- The text in the 3rd paragraph (page 65) states that "Nevertheless, the concentrations which induce[d] a mutagenic response in the 1st study were not tested in the 2nd." We believe this is erroneous as the 2nd study included concentrations that were higher than the 1st study (e.g., no mutagenic response at 150 or 200 µg/ml in the 2nd study compared to weakly positive responses at 125 and/or 150 µg/ml in the 1st study).
- Although the 3rd paragraph states that purity was unspecified, the purity of the test material used in the mouse lymphoma assay (Mitchell *et al*., 1982) was specified as 95.6% in the appendix of the study report. A similar statement about lack of purity information was also mentioned on page 79, section 4.9.1.1.2.

In vivo genotoxicity : Chromosomal aberrations

Page 68 of the CLH report (related to *in-vivo* studies) indicates that two of the three studies evaluating chromosomal aberrations "were not considered acceptable, either by the absence of toxicity or cytotoxicity at the highest dose tested (Farrow and Cortina, 1983) or because no certainty that the bone marrow has been exposed to the test substance (Cavagnaro and Cortina, 1985)." Similar statements were made elsewhere (Table 24, pages 85 and 87). Although a third study was considered acceptable, we believe that the rationales cited for rejection of the first two studies are incorrect for the following reasons:

- Farrow and Cortina (1983): The highest dose tested (500 mg/kg) in this rat bone marrow chromosomal aberration study caused a statistically significant decrease in body weight gain in both males and females over the 48-hr test period. In addition, this dose level was one-half of the dose (1000 mg/kg) that caused 75% mortality in the range-finding study. Thus, the highest dose level tested should be considered acceptable.

- Cavagnaro and Cortina (1985): The highest dose tested in this mouse bone marrow micronucleus study (2000 mg/kg) is the limit dose according to the current OECD guideline, and caused mortality in 43% (23/54) of the animals dosed, with an apparent decrease in the PCE/NCE ratio in surviving high-dose animals. In addition, clinical signs of toxicity were noted at both 2000 and 660 mg/kg. Furthermore, previous ADME studies have demonstrated that acetochlor is well-absorbed after oral administration to rodents and is found at significant levels in the blood and bone marrow. Since bone marrow is a highly perfused tissue, the levels of acetochlor and/or its metabolites in bone marrow should be similar to that observed in the blood (Guidance for Industry, S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use, US FDA, ICH, June 2012).

Therefore, based on the toxicity and ADME data, there should be high confidence that the bone marrow was indeed sufficiently exposed to the test material.

In-vivo genotoxicity : Comet assay

The CLH report indicates that the rat nasal comet assay was not considered acceptable due to an inappropriate rationale for the dose level used (1750 ppm). We disagree with this criticism for the following reasons:

- This study was intended to determine if there was any evidence of chromosomal or DNA damage in nasal turbinates at a dose level (1750 ppm) that slightly exceeded the chronic MTD and produced nasal tumors in rats. This would help determine if there was a genotoxic or non-genotoxic mode of action for the formation of these tumours.

- Although body weight data were not reported for this study, clear evidence of toxicity was noted at the same or similar dose levels in other studies. Decreased food consumption, decreased body weights, increased organ weights and/or nasal pathology (including tumors) were observed after a similar duration of exposure at 1750 ppm in two 2-generation rat reproduction studies (Milburn, 2001; Willoughby, 1989). In addition, significantly decreased food consumption and body weight gain and increased relative liver and kidney weights were noted at 2000 ppm in a previous 90-day rat study (Broadmeadow, 1986).

In vivo genotoxicity - Dominant lethal assays

The text and Table 25 indicates that no toxicity was observed in 3 of the dominant lethal studies. However, we believe this is inaccurate for 2 of the 3 studies. Decreases in body weight (~7.5%) and cumulative weight gain (~27%) were noted in the high-dose (2000 ppm) male rats after 9 weeks of dosing in the Naylor (1987) study. Initial weight loss followed by decreases (generally ~5%) in body weight were observed in high-dose (3500 ppm) male mice in the Milburn (1986b) study. In addition, although a minor detail, the rats used in the Naylor study were Charles River Sprague Dawley rats, not Alderly Park (Alpk AP (SD)) rats, and the high-dose level in this study was 2000 ppm, not 1500 ppm as reported near the top of page 102 (section 4.9.1.2.3).

4.10. Carcinogenicity

4.10.1. Non-human information

4.10.1.1.2. Oral carcinogenesis in mice

Non-neoplastic kidney findings in 78-wk mouse study (Amyes, S.J. (1989))

The last two sentences of text prior to Table 45.8 state, "Significantly increase[d] incidence of renal tubular basophilia was noted in males from the dose level of 10 ppm. In addition, there was a low incidence of hyperplasia of the renal tubular epithelium which achieved statistical significance in male which received 1000 ppm ($p \leq 0.05$)." Unfortunately, the last sentence may be misleading as it could be interpreted to mean that tubular hyperplasia was also noted at 10 ppm. In fact, as shown in the table below, there was no renal tubular hyperplasia observed in males at 10 ppm, and only a single occurrence at 100 ppm. In addition, although the incidence of hyperplasia at 1000 ppm was statistically significant ($p \leq 0.05$) when evaluating only those animals sacrificed at 78 weeks (4/34), it was not statistically significant when evaluating all 50 animals assigned to the oncogenic portion of the study (4/50). For clarity, we suggest that the following data for hypertrophy and hyperplasia be added to Table 45.8.

Selected Renal Tubular Lesions in 18-Month Mouse Oncogenicity Study (Amyes, 1989)

	Males				Females			
Dose (ppm)	**0**	**10**	**100**	**1000**	**0**	**10**	**100**	**1000**
# animals examined	50	50	50	50	50	50	50	50
hypertrophy	1	1	0	3	0	1	1	1
hyperplasia	0	0	1	4	0	0	0	0

** $p \leq 0.05$
** $p \leq 0.01$

4.10.3. Other relevant information

These pages cite USEPA conclusions from the fourth and fifth Cancer Assessment Review Committee (CARC) reports. More recent EPA wording regarding the mouse tumours varies slightly depending on the document, but all conclude that the evidence for mouse tumours being related to acetochlor treatment is weak or equivocal.[1][2][3]

[1] Human Health Risk Assessment (January 22, 2007) (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2006-0203-0005)
[2] Pesticide Tolerance, Final Rule (May 16, 2007) (http://www.gpo.gov/fdsys/pkg/FR-2007-05-16/pdf/E7-9430.pdf)
[3] Human Health Risk Assessment (July 1, 2009) (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2009-0002-0005)

Alachlor

- To avoid potential confusion, it should be noted that the two forestomach tumours noted in one of the acetochlor chronic rat studies are very different from the glandular stomach tumours observed with alachlor.

- It would be useful to also indicate the 2004 EFSA PPR conclusion for rat nasal tumors produced by alachlor: "While the mode of action could be relevant to humans, it is extremely unlikely that concentrations of the active metabolite would be achieved to initiate the chain of events terminating in cancer" [emphasis added].

- It might also be worth mentioning that the nasal tumour potency for acetochlor is significantly less than that of alachlor, which would suggest the likelihood of acetochlor being carcinogenic in humans is even less than "extremely unlikely".

- The last sentence in the 2nd paragraph states, "It was concluded to classify alachlor with R40 based on the **same tumours** found in rats and mice after administration of acetochlor (ECB, 2002)" [emphasis added]. That sentence appears to be misleading as alachlor did not produce forestomach tumours in rats or histiocytic sarcomas in mice. Although a slight increase in incidence of lung tumours was noted in mice, it was within the historical control range and was not considered treatment related by ECCO (Doc. SANCO/4330/2001 rev. 3-1 (18.09.2003)) or the USEPA.

Tumours findings for related acetanilides (Table 46)

- Both alachlor and butachlor produced the same type of tumors in the glandular stomach of the rat. These are very different than the two forestomach tumours noted in one of the three acetochlor chronic rat studies.

- Several rat nasal epithelial tumors were also produced by metolachlor, which is classified by the EPA as a Group C or Possible human carcinogen, not "probable" as is indicated in the table.

4.10.4. Summary and discussion of carcinogenicity

4.10.4.1 Carcinogenesis in rats

Mode of action for nasal tumours

The weight-of-evidence indicates that ethylmethylaniline (EMA) is likely to be, at most, only a very minor precursor to DABQI formation relative to the sulfoxide metabolite. Although EMA was initially believed to be the key precursor responsible for DABQI formation, it was later determined that this was due to flawed experimental conditions that either favored EMA formation (*i.e*., *in vitro* studies conducted without an NADPH-regenerating system) or that resulted in the inadvertent removal of the sulfoxide moiety (*i.e.,* protein binding studies conducted using 6N HCl). Subsequent studies have demonstrated that the sulfoxide metabolite is the key precursor to DABQI formation following acetochlor administration and that EMA plays, at most, only a very minor role. However, the fact that the EMA pathway plays no more than a minimal role is not always apparent in the CLH discussions.

For example:

- page 137, first paragraph: The relative importance of the two potential pathways leading to DABQI from acetochlor is not indicated. Although DABQI was originally believed to result from para-hydroxylation of EMA, it was later demonstrated that the vast majority of the DABQI produced from acetochlor was derived from p-hydroxylation of sulfoxide metabolite. Some studies have suggested that small amounts of EMA can be produced in the liver *in vitro* but this has not been confirmed *in vivo* (e.g., EMA was not identified in rat plasma). This is in contrast to alachlor where DEA phenol sulfate (a metabolite of the corresponding DEA analog of EMA) and several other metabolites that could potentially lead to DABQI were observed in addition to the corresponding alachlor sulfoxide.

- page 139, 4th and 5th paragraphs; bottom of page 141; and top of page 142: Initially, the acetochlor-protein adducts found in rat nasal tissue were believed to originate from 3-ethyl, 5-methyl-benzoquinoneimine (EMIQ). However, it was later determined that these adducts contained the sulfoxide side chain and thus probably resulted from binding with the sulfoxide, not EMA (Green, 2001). Unfortunately, the experimental conditions used in the initial experiment inadvertently removed this side chain. Thus, it may be more accurate to modify the text to replace "EMIQ" with "EMIQ-sulfoxide" or more simply "DABQI". In addition, the statement at the top of page 142 that "the analysis of adducts cannot distinguish whether it is originated from the sulfide or EMA" is misleading. As previously indicated, the analytical method used by Green (2001) did distinguish between the sulfoxide and EMA. Unfortunately, the method used in the previous assays did not.

- page 144, discussions regarding EMA: Based on the *in vitro* data, we cannot exclude the possibility that very small amounts of EMA may be produced by humans. However, the p-hydroxylation rate of EMA in primate nasal tissue is substantially (~24-fold) less than in rats.

Please also see additional comments regarding EMA in the previous Toxicokinetics section and in the subsequent section on Carcinogenicity - comparison with criteria.

4.10.4.2. Carcinogenesis in mice

Lung tumours in CD-1 mice

- There appear to be some minor errors in Table 49. The Life Science Research (LSR) data is based on 13 studies of 18-month duration, not 11. In addition, the data shown for 24-month studies at LSR is actually from Inveresk.

- It should be noted on pages 149-150 that the initial EPA comparison of "censored" data from the acetochlor chronic studies to "uncensored" historical control data was inappropriate as the denominators used for calculating the incidence are different (EPA excludes all animals dying prior to the first observation of a tumour, which generally results in an increase in the calculated incidence value). It is not possible to use the EPA approach for historical control data. Thus, while it may be appropriate to use censored incidence data for EPA calculations of cancer slope factor, these values should not be used for other purposes, esp. comparing to uncensored historical control data.

- The in-house LSR historical data was based on only 13 studies of 18-month duration. No historical control data were available from the contract laboratory conducting the 23-month study. Therefore, we believe that use of additional historical control data from studies conducted elsewhere at approximately the same time is appropriate for use with both studies.

We agree with the factors listed for the weight of evidence evaluation of the toxicological significance of the incidence of the lung tumours, except for the use of historical control data (as commented above). However, in our opinionthe results of this analysis strongly indicate that the association is too weak for classification.

4.10.5. Comparison with criteria:

The 7th paragraph on page 152 states, "The CLP guidance (chapter 3.6.2.3.2(k) states that "only if a mode of action of tumour development is conclusively determined not to be operative in humans then the carcinogenic evidence for that tumour may be discounted."

Although the quote is accurate, the use of the word "conclusively" in this sentence appears to be

somewhat inconsistent with the text in Annex I to CLP regulation point 3.6.1.1 which states, "Substances which have induced benign and malignant tumours in well performed experimental studies on animals are considered also to be presumed or suspected human carcinogens unless there is **strong** evidence that the mechanism of tumour formation is not relevant for humans" (emphasis added). The criteria for "strong" rather than "conclusive' evidence is more consistent with the rest of section 3.6.2.3.2 (k) which states that the mode of action evaluations should be conducted on a case-by-case basis taking into consideration "comparative toxicokinetics/toxicodynamics between the animal test species and humans to determine the relevance of the results to humans", and the recommendation that the IPCS Framework for Analyzing the Relevance of a Cancer Mode of Action for Humans (WHO, 2007; Boobis et al, 2006) should be used for the evaluation. The IPCS and similar frameworks are also cited in the introduction to section 3.6.2.3.

The IPCS Human Relevance Framework (HRF) asks the following 3 questions:

(1) Is the WOE sufficient to establish the carcinogenic MOA in animals?
(2) Can the human relevance of the MOA be reasonably excluded on the basis of qualitative differences in key events between animals and humans?
(3) Can the human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetics or dynamics between animals and humans?

For acetochlor, each of the three questions above is addressed below.

(1) The answer to the first question is YES - a non-genotoxic, threshold-mediated carcinogenic MOA for acetanilide-induced rat nasal tumours has been well-established. The key event is the formation of a reactive DABQI metabolite that forms adducts with nasal proteins and induces cytotoxicity, prolonged cell proliferation, and eventual development of nasal olfactory tumours. For acetochlor, the predominant pathway leading to DABQI is para-hydroxylation of the sulfoxide metabolite by the rat olfactory tissue. However, we cannot exclude the possibility that very small amounts of DABQI may be formed through the EMA pathway

(2) The answer to the second question is NO – we cannot exclude the theoretical possibility that the key event, formation of DABQI, would occur to some extent in humans. Both monkey and human nasal tissues were unable to para-hydroxylate the sulfoxide metabolite of acetochlor, which is the primary precursor to DABQI in the rat. However, a very low rate of para-hydroxylation (approximately 4% of the rate in rats) was observed when monkey nasal tissue was incubated with ethylmethylaniline (EMA) (Green, 1998). Although EMA was not identified in rat plasma, based on *in vitro* data, we cannot exclude the possibility that very minor amounts of EMA may be formed in rats and/or humans, and subsequently be para-hydroxylated to a DABQI.

(3) The weight of evidence indicates that the answer to the third question is YES – the carcinogenic MOA can be "**reasonably excluded**" in humans when taking into account toxicokinetics and toxicodynamics. Very large species differences in the overall metabolism of acetochlor indicate that the amount of DABQI produced in rats is significantly higher than in mice, which do not develop nasal tumours, and at least several orders of magnitude higher than in primates and humans. The low levels of DABQI produced in the mouse were not sufficient to lead to detectable accumulation in nasal tissues, formation of DABQI-protein adducts, nasal cell proliferation or nasal tumours. The amount of DABQI that theoretically may be produced in humans (from either sulfoxide or EMA) would be at least several orders of magnitude lower than in mice (due to much lower rate of para-hydroxylation in humans *vs.* mice), and thus would be **exceedingly unlikely** to initiate the chain of events terminating in cancer.

Based upon similar data for alachlor, the EFSA PPR panel concluded that "While the mode of action could be relevant to humans, it is **extremely unlikely** that concentrations of the active metabolite would be achieved to initiate the chain of events terminating in cancer" [emphasis added]. Since the carcinogenic potency of acetochlor is significantly lower than that of alachlor, and since the sulfoxide metabolite is much more prominent with acetochlor than with alachlor (for which evidence of DEA formation was observed in rat plasma), the likelihood that these events would occur following exposure to acetochlor would be even lower. Also, it should be noted that the PPR conclusion on alachlor was published in 2004, which was prior to the publication in 2006 & 2007 of the IPCS HRF, which asks whether the data are sufficient to "reasonably exclude" the human relevance of the mode of action instead of whether the occurrence of the key event would be "plausible" in humans, which was the language used in the earlier human relevance framework developed by IPCS or ILSI/RSI (Meek *et al.*, 2003). As discussed in the newer IPCS framework document, the change in wording was made to recognize that the requirement to demonstrate that a MOA was unequivocally not "plausible" in humans was too strict, and that "decisions about the adequacy of weight of evidence are not absolute".

Therefore, based on the substantial quantitative species differences, it can be concluded that rat nasal tumours produced by acetochlor are not relevant to humans and should not trigger classification.

The CLH report indicates that no mode of action data are available for the mouse lung tumours and histiocytic sarcomas. That is true since it is unlikely that these tumours are treatment related. Mouse lung tumours (alveolar/bronchiolar adenomas and/or carcinomas) and histiocytic sarcomas are very common spontaneous tumours in aged CD-1 mice. In fact, lung tumours are the most common tumour observed in female CD-1 mice and a very close second (to liver tumours) in male CD-1 mice. In addition, the incidence of both lung tumours and histiocytic sarcomas is highly variable in aged animals. Thus, extra caution should be utilized when evaluating the potential toxicological significance of slight increased incidences of these types of

tumours, particularly when there is only marginal statistical significance and/or limited historical control data from the laboratory conducting the study. An independent Pathology Working Group, the ECB Group of Specialised Experts, and a more recent independent panel of European pathologists and toxicologists have reviewed the data and concluded that both the marginal increases in incidences of lung tumours and histiocytic sarcomas observed in the long-term mouse studies with acetochlor were not treatment-related. The USEPA has also recently concluded that the increased incidences of mouse lung tumours and histiocytic sarcomas were equivocal and/or only possibly related to treatment, and represent no greater than a negligible risk of cancer.

Therefore, the mouse lung tumours and histiocytic sarcomas should not be considered relevant for the purposes of classification.

4.10.6. Conclusions on classification and labelling:

The current EPA cancer classification for acetochlor is "suggestive evidence of carcinogenic potential." The phrase "probable human carcinogen" was an older classification that was revised in January 2007 and should be deleted. However, as previously indicated, the most recent EPA evaluation of the potential human relevance of the MOA for the rat nasal tumours was conducted in 2004, prior to issuance of the 2007 IPCS Human Relevance Framework which incorporated quantitative species differences into the evaluation.

4.12. Other effects

4.12.1. Non-human information

4.12.1.2. Immunotoxicity

A 28-day mouse sheep red blood cell assay has recently been completed. No evidence of immunotoxicity or other toxicity was noted at any dose level. The NOAEL for that study was approximately 1535 mg/kg/day. This study will be submitted in 2014 along with a new application for EU Annex I approval.

4.12.2. Summary and discussion

52 weeks oral (capsule) study in dogs (Broadmeadow, 1989)

It would be useful to note that the brain lesions and clinical signs of neurotoxicity noted in one of the 1-yr dog studies was probably secondary to renal toxicity. The neurological effects were noted only at 50 mg/kg/day, a level which produced significant renal pathology (nephritis, hyperplasia of the collecting ducts and pelvic epithelium, cortical atrophy and /or fibrosis, vasculitis, and sometimes necrosis) in all animals. Surviving high-dose animals exhibited significantly increased levels of urea, creatinine and BUN. No evidence of brain or kidney pathology was noted at 40 mg/kg/day in the other 1-yr dog study. Thus, the brain lesions may have been the result of uremic encephalopathy. The renal toxicity also would have resulted in less efficient renal clearance and thus substantially higher blood levels of acetochlor and/or its metabolites, which could have contributed to the neurological effects observed.

Appendix

Tier summaries of studies

- Zhang F., *et al* (2010) Acetochlor sec-methylsulfide : in-vitro metabolism by olfactory turbinate and liver microsomes of male Sprague Dawley rat

- Ahmed, FE (1980) CP 55097: 91-day Feeding Study in the Mouse

- Wagner VO, *et al* (2013) : MON52706 : Bacterial Reverse Mutation Assay.

- Beevers C. (2014). In vivo Muta™Mouse gene mutation assay with MON 52706, Monsanto Study No. CV-2013-0032

Acetochlor *sec*-methylsulfide *in vitro* metabolism

Author(s):	Zhang F, Hansen SC, Saghir SA
Year:	2010
Title	Acetochlor *sec*-methylsulfide : *in vitro* metabolism by olfactory turbinate and liver microsomes of male Sprague-Dawley rats
Testing Laboratory:	Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan 48674
Report Number:	071129 (Monsanto report no Dow-2008-587)
Report Date:	4th August 2010
Test Guidelines:	There are no guidelines for this study
Deviations from guideline:	Not applicable
GLP Compliance:	All phases of this study were conducted in accordance with GLP (TSCA, 40 CFR, part 792) with following exceptions: - Purity of acetochlor sec. methylsulfide (ASMS) was determined during the course of the study - Concentration and stability of dosing solutions were not verified analytically

Executive Summary

The purpose of the study was to determine in rats the relative importance of the formation of iminoquinone metabolites through the 2-ethyl-6-methylaniline (EMA) metabolic pathway compared to other pathways, specifically the acetochlor *sec*-methylsulfoxide (ASMSO) pathway. Incubations of acetochlor *sec*-methylsulfide (ASMS) were conducted with rat liver and rat nasal turbinate microsomes in the presence of NADPH and glutathione (GSH). GSH served to trap reactive iminoquinone metabolites formed in the microsomal incubations by producing GSH conjugates. Identification and quantification of the non-conjugated metabolites and GSH conjugates formed in the incubations were conducted by LC/UV or LC/MS/MS analysis. For comparison purposes, rat liver and nasal turbinate microsomal incubations were also conducted using acetochlor *sec*-methylsulfoxide (ASMSO) as a substrate. Four major non-conjugated metabolites (ASMSO, p-OH-ASMSO, EMA and p-OH-EMA) were formed and identified in rat liver and nasal microsomal incubations of ASMS and ASMSO in the presence of NADPH and GSH. ASMSO was the major metabolite and was formed at comparable levels in liver and nasal incubations. EMA, derived by amidase hydrolysis of the amide function of ASMS was also formed at somewhat comparable levels in liver and nasal incubations, but at levels that are at least 10 times lower than ASMSO levels. The para-hydroxylated metabolites of EMA and ASMSO were formed from ASMS in nasal microsomes at initial velocities which were at least 40 times higher than in liver microsomes. Twelve glutathione conjugates resulting from trapping of reactive iminoquinone metabolites of para-hydroxylated ASMSO, ASMS or EMA were formed in rat nasal microsomal incubations of ASMS in the presence of NADPH and GSH. In contrast, only three detectable iminoquinone GSH conjugates were formed in rat liver microsomal incubations in the presence of NADPH and GSH. The initial velocities were much lower than those measured in nasal turbinate microsomal incubations. The p-OH-EMA GSH conjugate was the major GSH adduct formed in both liver and nasal turbinate incubations with ASMS. However, the rates of formation of p-OH-EMA GSH adducts in nasal microsomal incubations were more than 25-fold higher than those in rat liver microsomal incubations.

Similar non-conjugated and GSH conjugated metabolites were formed in a limited number of incubations of ASMSO in rat liver or nasal turbinate microsomes in the presence of NADPH and GSH. At similar substrate concentrations, the incubation of ASMSO with nasal turbinate microsomes formed almost 5 times more total GSH conjugates than the incubation of ASMS. The rate of formation of GSH adducts of p-OH ASMSO in nasal turbinate microsome incubations was found to be more than 10-fold higher when ASMSO was used as a substrate compared to ASMS. The rate of formation of p-OH EMA GSH adducts in nasal turbinate microsome incubations was more than 10-fold higher when ASMS was used as a substrate compared to ASMSO. The vast majority of GSH conjugates formed in nasal turbinate microsomal incubations of ASMSO are formed from p-OH-ASMSO (> 98%), unlike ASMS which formed reactive iminoquinone metabolites primarily via p-OH-EMA.
The toxicity of acetochlor to the olfactory epithelium is believed to result from site-specific formation of iminoquinone metabolites which can bind to cysteine residues of essential cell proteins in the olfactory epithelium. The level of GSH conjugate formation in nasal turbinate microsomes provides a measure of the relative contributions of the various iminoquinone metabolites to the observed toxicity. ASMSO is the predominant metabolite circulating in plasma of rats treated with acetochlor. Therefore, the results of this *in vitro* study, which show that >98% of iminoquinone metabolites formed from ASMSO in nasal turbinate microsomes are derived from p-OH-ASMSO, indicate that the p-OH-ASMSO pathway is the predominant pathway contributing to acetochlor nasal toxicity.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Materials:

Description:	Acetochlor *sec*-methylsulfide, *N*-(2-ethyl-6-methylphenyl)-2-(methylsulfanyl) acetamide (ASMS, CAS #: 34256-82-1)
Appearance:	Not reported
Lot/Batch #:	GLP-0705-18642-A
Purity/content:	99%
Stability:	Test substance was used before the recertification date of 31/05/2009
Description:	Acetochlor *sec*-methylsulfoxide, N-(2-ethyl-6-methylphenyl)-2-(methylsulfinyl) acetamide (ASMSO)
Appearance:	Not reported
Lot/Batch #:	GLP-0709-18584-A
Purity/content:	98%
Stability:	Test substance was used before the recertification date of 31/10/2009
Description:	Acetochlor *sec*-methylsulfoxide phenol, p-hydroxy acetochlor *sec*-methylsulfoxide, N-(2-ethyl-4-hydroxy-6-methylphenyl)-2-(methylsulfinyl) acetamide (p-OH ASMSO)
Appearance:	Not reported
Lot/Batch #:	GLP-0705-18644-A
Purity/content:	98%
Stability:	Test substance was used before the recertification date of 31/05/2009

Description:	2-Ethyl-6-methylaniline (EMA)
Appearance:	Not reported
Lot/Batch #:	12411AD
Purity/content:	99.2%
Stability:	Test substance was used before the recertification date of 31/05/2009

Description:	4-Amino-3-ethyl-5-methylphenol (p-OH EMA)
Appearance:	Not reported
Lot/Batch #:	GLP-0705-18643-A
Purity/content:	98%
Stability:	Test substance was used before the recertification date of 31/05/2009

2. Vehicle: Not applicable, *in vitro* study

3. Preparation of microsomes:

Species:	Rat
Strain:	Sprague-Dawley
Sex:	Male
Tissue:	Liver (from 50 rats) and nasal turbinates (from 150 rats)
Source:	Analytical Biological Services, Inc. , Wilmington, DE, USA
Shipping and storage:	Shipped on dry ice and stored at -80^{o}C until used.
Analysis:	All microsomal preparations were analyzed for CYP activity using standardized in-house procedures

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: Study initiation on 26th August 2008

2. Study design:

Part 1:

1 mM of ASMS was incubated for 30 minutes with rat liver or rat nasal olfactory microsomes in the presence of NADPH and GSH. After incubation at 37 ^{o}C, the enzymic reaction was stopped by cooling on ice and adding a stop solution (2% formic acid). Afterwards, the acidified solution was centrifuged for 5 min at 22000 x g. The supernatant was then analysed by LC/MS/MS or LC/UV to screen for non-conjugated metabolites and reactive iminoquinone metabolites which were trapped as glutathione conjugates using an excess GSH. After the identification of the metabolites formed, experiments were conducted to assess the linearity of metabolism with time. Aliquots of incubation solutions were quantitatively analyzed for the loss of test material and formation of metabolites or metabolite-related GSH conjugates.

1 mM ASMS was incubated for 1 hour with rat liver or rat nasal olfactory microsomes in the presence of NADPH and GSH. At different time points (0, 15, 30, 45, and 60 min), an aliquot was removed from the incubations and the enzyme reaction stopped by cooling on ice and adding a stop solution (2% formic acid). The acidified solution was then centrifuged for 5 min at 22000 x g and the supernatant stored at -80

°C pending quantitative analysis. For control experiments, incubations with or without substrate, NADPH, microsomes, or GSH were carried out. In addition, a control incubation of 1 mM ASMS without NADPH and duplicate incubations of 1 mM ASMSO in the presence or absence of NADPH were conducted under similar conditions to further verify the metabolites formed.

Part 2:
The results from the Part 1 time-course study showed that the formation rates of most of the non-conjugated and GSH-conjugated metabolites were linear through 30 min of incubation. Therefore, the microsomal incubations for determining the kinetics of metabolite formation were conducted for 30 minutes. Three replicates of rat liver or rat nasal olfactory microsomes were incubated at six different concentrations of ASMS (6.4, 3.2, 1.6, 0.8, 0.4, and 0.2 mM) in the presence of NADPH and GSH. The incubations were conducted under the conditions described in Part 1. After 30 minutes, the enzymic reaction was stopped by cooling on ice and adding a stop solution (2% formic acid). The respective solutions were then centrifuged for 5 min at 22000 x g and the supernatants analysed for non conjugated metabolites (ASMSO, EMA, p-OH-EMA, and p-OH-ASMSO) and various GSH conjugates. The rates of metabolism were calculated using Lineweaver-Burk plots.

II. RESULTS AND DISCUSSION

Four major non-conjugated metabolites i.e. EMA, p-OH-EMA, ASMSO, and p-OH ASMSO were identified in rat liver or rat nasal olfactory microsomal incubations of ASMS in the presence of NADPH and GSH. The molecular structures corresponding to the metabolite peaks were confirmed with enhanced product ion (EPI) mass spectral analysis of the authentic standards of EMA, p-OH EMA, ASMSO, and p-OH-ASMSO. However, using full-scan EPI-LC/MS/MS conditions, no metabolite-related GSH conjugates were detected in the Part 1 microsomal incubations. This is probably due to the low detection sensitivity of these low-level metabolites.
To further explore the possibility of ASMS metabolite-related GSH conjugate formation, p-OH-EMA and p-OH-ASMSO analytical standards were incubated with rat liver or rat nasal olfactory microsomes in the presence of NADPH and GSH. After full-scan EPI-LC/MS/MS analysis of the processed incubation solutions, four major GSH conjugates were detected. These were tentatively identified as isomers of GSH conjugates of p-OH-ASMSO and GSH conjugates of di-OH-ASMSO and p-OH-EMA. The identified GSH conjugate metabolites and non-conjugated metabolite standards were used to optimize the LC/MS/MS instrument to develop a sensitive LC/MS/MS method to detect and quantify the identified metabolites from microsomal incubations of ASMS. After optimization of the LC/MS/MS method, Multiple-Reaction-Monitoring (MRM) transitions were used to analyze metabolites formed from microsomal incubations.

Detection and identification of non-conjugated metabolites by LC/MRM
Four non-conjugated metabolites (p-OH ASMSO, ASMSO, p-OH-EMA, and EMA) were formed from both rat liver and nasal olfactory turbinate microsomal incubations of ASMS. Besides those four metabolites, four other novel metabolites were detected (some of them were isomers of parahydroxylated ASMSO and EMA).

Detection and identification of GSH conjugate metabolites by LC/MRM
Five p-OH ASMSO/di-OH ASMS GSH conjugates, 5 p-OH/di-OH ASMSO GSH conjugates, and 2 p-OH-EMA GSH conjugates were detected in incubations of ASMS with rat nasal olfactory microsomes in the presence of NADPH and GSH. In contrast, only 2 p-OH-ASMSO GSH conjugates and 1 p-OH EMA GSH conjugate were detected in rat liver microsomal incubations in the presence of NADPH and GSH. Among all GSH conjugates, 3 were identified as GSH conjugates formed from microsomal incubation of p-OH ASMSO and 1 was identified as a GSH conjugate of p-OH EMA. No further efforts were made to elucidate the structures of the other GSH conjugates.

Time course study of microsomal incubation of ASMS

The rates (Vi) of the formation of non-GSH adducts are summarized in Table 5.8.2.1/04-1. Rates were measured at 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 mM of substrate. Only the results obtained with 0.8 mM ASMS are discussed below to allow comparison with the rates measured with a similar substrate concentration of ASMSO (1 mM).

Table 5.8.2.1/04-1: V_i (nmol/mg protein/min) of the formation of non-GSH adducts at 0.8 mM ASMS (n=3) when incubated with rat liver and nasal turbinate microsomes in the presence of NADPH and GSH

Microsomes	ASMS →ASMSO	ASMS→→ p-OH ASMSO	ASMS→ EMA	ASMS→ p-OH EMA
Liver	2.499	<0.005	0.209	0.005
Nasal turbinates	4.308	0.222	0.082	0.206

The data in the table show that:

- the rate of para-hydroxylation in nasal microsomes is much greater than in liver microsomes (approx. 40-fold and more),
- the rates of formation of p-OH ASMSO and p-OH EMA are high and comparable in nasal turbinate microsomes,
- the rate of S-oxidation of ASMS to ASMSO is very high and comparable in liver and nasal microsomes,
- the rates of EMA formation from ASMS is somewhat higher (approx. 2.5-fold) in the liver than in nasal microsomes (approx. 2.5-fold) but these rates are approx. 10 (liver) to 50 (nasal turbinates) times lower than the rate of formation of ASMSO.

From the control incubation without NADPH (ASMS + microsomes + GSH), EMA is formed in a somewhat higher (approx. 2-fold) concentration in rat nasal microsomal incubations than in rat liver microsomal incubations. These data are consistent with higher activity of amidase (which hydrolyzes the ASMS to EMA) in rat nasal microsomes than in rat liver microsomes. Due to the limit of the replicate number (only one incubation was conducted for the control incubation), more incubations may be needed to confirm this conclusion.

The rates (Vi) of the formation of GSH adducts are summarized in Table 5.8.2.1/04-2.

Table 5.8.2.1/04-2: V_i (nmol/mg protein/min) of the formation of p-OH EMA GSH adducts at 0.8 mM ASMS (n=3) when incubated with rat liver and nasal turbinate microsomes in the presence of NADPH and GSH

Microsomes	ASMS→→→p-OH EMA GSH adducts
Liver	0.0014
Nasal turbinates	0.0347

The data in the table show that the rate of formation of EMA derived GSH adducts in nasal turbinate microsomes is much greater than in liver microsomes (approx. 25-fold). Also the formation of all other GSH conjugates is much greater than in liver microsomes (data not shown).

The ratio of p-OH-EMA GSH to all "other GSH conjugates" is fairly comparable in either microsomal incubation. Higher levels of EMA are formed in nasal microsomal incubation without NADPH, when compared to EMA formed in nasal microsomal incubation with NADPH. These results are consistent with NADPH reducing the level of EMA by enabling downstream oxidative metabolism.

Time course study of microsomal incubation of ASMSO

The rates (Vi) of the formation of non-GSH adducts are summarized in Table 5.8.2.1/04-3.

Table 5.8.2.1/04-3: V_i (nmol/mg protein/min) of the formation of non-GSH adducts at 1.0 mM ASMSO (n=3) when incubated with rat liver and nasal turbinate microsomes in the presence of NADPH and GSH

Microsomes	ASMSO→ p-OH ASMSO	ASMSO→ EMA	ASMSO→→ p-OH EMA
Liver	<LLQ	0.0044	0.0002
Nasal turbinates	↗↗*	0.0029	0.0027

*concentration of p-OH ASMSO formed higher than 200 ppm

The data in the table show that:

- the rate of formation of p-OH ASMSO is much greater in nasal turbinate microsomes than in liver microsomes,
- the rate of formation of EMA from ASMSO is small and comparable in liver and nasal microsomes,
- the rate of formation of p-OH EMA from ASMSO is greater in nasal turbinate than in liver microsomes (approx. 10-fold),
- the rate of formation of EMA and p-OH EMA from ASMSO is much less in liver (approx. 50- and 25-fold, respectively) and nasal turbinate (approx. 30- and 80-fold, respectively) microsomes when compared to incubations using ASMS as a substrate (Table 5.8.2.1/04-1).

The rate of EMA derived GSH adduct formation from ASMSO (1.0 mM) in the presence of NADPH and GSH in nasal turbinate microsomes is very small (0.003 nmol/mg protein/min) but still greater than in liver microsomes where the rate remained below the lower limit of quantification (LLQ).

The rates of formation of GSH adducts from ASMS and ASMSO incubated with rat nasal turbinate microsomes are compared in Table 5.8.2.1/04-4.

Table 5.8.2.1/04-4: V_i (nmol/mg protein/min) of the formation of GSH adducts at 0.8 mM and at 1.0 mM ASMSO (n=3) when incubated with rat nasal turbinate microsomes in the presence of NADPH and GSH

Substrate	p-OH ASMSO GSH adducts	p-OH EMA GSH adducts
ASMS	0.016	0.038
ASMSO	0.274	0.003

The data in the table show that:

- The rate of p-OH ASMSO GSH adduct formation is greater (approx. 17-fold) when ASMSO is the substrate,
- The rate of p-OH ASMSO GSH adduct formation is much greater (approx. 91-fold) than that of p-OH EMA GSH adduct formation when ASMSO is the substrate,
- The rate of formation of ASMSO and EMA derived adducts is low and comparable (only 2-fold difference) when ASMS is the substrate,
- The rate of formation of p-OH EMA GSH adducts from ASMS is greater (approx. 13-fold) than from ASMSO as substrate.

III. CONCLUSIONS

The vast majority of reactive metabolites (iminoquinone molecular species trapped as GSH conjugates) from ASMSO in nasal turbinates of the rat are formed from p-OH-ASMSO (>98%). In contrast, the lower amount of total reactive metabolites from ASMS arose primarily from p-OH-EMA. The rate of formation of GSH adducts of p-OH ASMSO in nasal turbinate microsome incubations was found to be more than 10-fold greater when ASMSO was used as a substrate compared to ASMS. The rate of formation of p-OH EMA GSH adducts in nasal turbinate microsome incubations was more than 10-fold greater when ASMS was used as a substrate.

ASMSO has been identified as the major acetochlor metabolite plasma in rats (species where nasal tumors are produced) which means that most of the hepatic ASMS is S-oxidized to ASMSO before it reaches the systemic circulation. ASMSO is virtually absent in plasma of mice, a species in which no nasal tumors are produced (CA 5.8.2/03). In consequence, ASMSO is the most important substrate that is offered to the nasal turbinate tissue for further metabolism into reactive molecular species. The results of this *in vitro* study show that virtually all of the ASMSO reactive metabolites in rat nasal microsomal incubations arise from initial hydroxylation of ASMSO, followed by oxidation to iminoquinone molecular species. Since Green *et al*.(CA 5.8.2/03) showed no measureable hydroxylation of ASMSO in primate or human nasal microsomes, there is little chance of reactive metabolite formation via this pathway in these two species. Green (CA 5.8.2/02) also showed that the formation of p-OH-EMA in microsomal incubation of EMA in primate nasal fractions is 23 times less than in rat nasal microsomal incubations. The rate of p-OH EMA formation in primates was also more than 20-fold less than in mice, a species which did not develop nasal tumors. The results of this *in vitro* metabolism study together with the ADME data demonstrating that ASMSO is the predominant circulating plasma metabolite, indicate that the vast majority of the reactive iminoquinone molecular species formed in nasal tissue in the rat as a result of acetochlor exposure is generated via the sulfoxide pathway and that any contributions from the EMA pathway are minimal.

Acetochlor 90-day mouse feeding study

Author(s):	Ahmed, FE
Year:	1980
Title:	CP 55097: 91-day Feeding Study in the Mouse
Testing Laboratory:	Pharmacopathics Research Laboratories Inc., Laurel, Maryland 20707, USA
Report Number:	7912 (sponsor's report number: PR-79-050)
Report Date:	12th November 1981
Test Guidelines:	No specific test guidelines were available at the time of the conduct of the study but the basic principles of the OECD test guideline for sub-chronic toxicity in rodents (TG 408) have been followed.
Deviations from guideline:	Deviations from OECD test guideline 408 (adopted on 21st September 1998) are: - No ophthalmological examination - No neurobehavioral observations - Organ weights not complete - No hematology measurements - No blood biochemistry measurements - No urinalysis - No recovery group
GLP Compliance:	A statement of the quality assurance unit of the laboratory has been added to the report.

Executive Summary

Random bred albino CD-1 mice of 21 days of age were put in quarantine for 9 days before the start of the study. Ten animals per sex were used for baseline histopathology and 40 animals were extra and were sacrificed on day 0. 20 animals per sex were treated with acetochlor at 0, 800, 2000 and 6000 ppm in the diet. No abnormal clinical findings were observed during the entire course of the study. Three mice died: one control female after 48 days, and 2 high dose females after 7 and 12 days since the start of the test. There were no test compound related changes in feed consumption and feed efficiency. A statistically significant decrease in body weight was observed in both sexes at the high dose group. Statistically significant changes in absolute and relative organ weights were observed in both sexes in various dose groups, however without any histopathological correlate. The NOAEL that can be derived from this study is 2000 ppm based on a significant decrease in body weight at the high dose level.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Materials:

Description: Acetochlor (MON 097, CP55097)

Appearance:	Dark brown liquid with an offensive odor
Lot/Batch #:	XHK-119
Purity/content:	91.3% w/w
Stability:	Expected to be stable for the duration of the study

2. Vehicle: Feed, provided *ad libitum*.

3. Test animals:

Species:	Mouse
Strain:	Swiss albino CD-1
Sex:	Males and females
Age:	The animals were received at the laboratory at the age of 21 days (weanlings) and put in quarantine for 9 days prior to the start of the study
Weight at dosing:	Mean body weight of the animals in the control group at the start of treatment was 20 g for males and 16 g for females
Source:	Charles River Breeding Laboratories, Wilmington, Mass., USA
Acclimation period:	9 days
Diet:	Charles River 19RF meal for rat, mouse and hamster, Agway of Syracuse, New York, USA
Water:	Howard county public water supply provided by Washington Suburban Sanitary Commission
Housing:	Individually housed in wire mesh cages
Environmental conditions:	Atmospheric change at 10 times per hour. The temperature was kept at approximately 22.8 ± 0.9^{o}C, with humidity at approximately 45% to 70%. The light/dark cycle was approximately 12 hours on and 12 hours off

4. Preparation of test diets:

Test diets were prepared weekly by mixing the given amount of test substance with the appropriate amount of basic diet in a Patterson-Kelley two cubic feet mixer. Fresh diets were prepared weekly.

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: Dosing started on 27th July, 1979.

2. Study design:

Dose levels:	0, 800, 2000, 6000 mg/kg diet (ppm)
Animals/dose level:	20 males and 20 females
Exposure time:	91 days
Route of exposure:	Oral
Vehicle:	Feed, provided *ad libitum*

Dose administration:	Diet, prepared weekly and based on the group weekly mean body weight and data taken two weeks ago
Analysis of diet:	Analysis of the diet indicated that the test substance was properly mixed in the feed and that it was stable when left at room temperature for at least a week
Clinical observations:	All animals were inspected daily for clinical signs. Positive findings were documented as they occurred. Negative findings were documented once a week. Any abnormal signs observed were routinely followed up either to clinical recovery or to moribund condition, whereupon the animal was sacrificed
Ophthalmology:	Not performed
Body weight:	Recorded every week during the study, starting at one week before treatment
Feed consumption:	Measured by weighing the feed containers full of feed at the beginning of the week (full weight) and the same feed containers with the remaining at the end of the week (empty weight). The difference between the full and empty weights represented the net feed consumption
Hematology:	Not performed
Blood biochemistry:	Not performed
Urinalysis:	Not performed
Gross necropsy:	All moribund animals were sacrificed within 16 hours so as to prevent autolysis of tissues. Prior to necropsy animals were fasted overnight, weighed and euthanized.
Organ weights:	Brain, heart, liver, kidney and gonads of all surviving animals were weighed at termination of the study
Histopathology:	Tissues that were examined microscopically: - from randomly selected 10 male and 10 female mice prior to initiation of the study, - from all mice in the control and high-dose groups at the end of the study, - from all gross lesions observed in the course of the complete necropsy, - from heart, lungs, liver and kidneys of all mice from the intermediate dose groups, and - in all other test groups when significant changes have been observed in these tissues in the high dose group Following tissues were examined in animals which received complete histopathology: adrenals, aorta, bladder (urinary), bone,

bone marrow, brain, cecum, colon, duodendum, esophagus, eye and optic nerve, gall bladder, heart, ileum, jejenum, kidneys, liver, lungs and bronchi, lymph nodes, mammary glands (if palpable), skeletal muscle, peripheral nerve (sciatic), ovaries, pancreas, parathyroids, penis, pituitary, prostate, salivary glands (submaxillary), skin, spinal cord, spleen, stomach, testes, thymus, thyroids, trachea, uterus and vagina.

II. RESULTS AND DISCUSSION

Diet data:

Analysis of the diet:	Not reported
Chemical consumption:	Not reported

Toxicology data:
No clinical abnormalities were observed throughout the course of the study.
One control female mouse was found dead after 48 days on test. One high-dose female mouse was sacrificed after seven days on test and one high-dose female mouse died after twelve days on test.
Statistically significant decreases in feed consumption have been observed in all dose groups but these are not considered to be compound related. The feed efficiency in both sexes at the various dose levels was not remarkably different from the control groups.
A statistically significant decrease in body weight as compared to the control group has been observed in the males of the mid dose group (weeks 10 and 12) and the high-dose group (from week 1 until the end of the study). In the females, no statistical significance was observed in either the low or the mid dose groups throughout the duration of the study. A statistically significant decrease was observed in the high-dose group from weeks 5 through 13. The changes in body weight at the high dose are considered to be test substance related.
Statistically significant changes in absolute mean organ weight were a decrease for brain in males at the high dose, an increase for liver in males at the mid and high dose and females at the low and mid dose, an increase for kidney in males at the low and the mid dose and a decrease in females at the high dose, and a decrease for gonads in females at the high dose.
Statistically significant changes in relative mean organ weight were an increase for brain in males at the high dose, an increase for heart in males at the mid and high dose, an increase for liver in males at the mid and high dose and in females at all dose levels (not dose related), and an increase for kidney in males at all dose levels and in females only at the mid dose.
No test substance related gross pathology findings were noted in any of the mice that died or were sacrificed in the study.
None of all the histopathological findings noted were found to be test substance related.

III. CONCLUSIONS

The decrease in body weight noted in males and females dosed at 6000 ppm acetochlor in the diet is considered to be related to treatment. The changes in organ weights at the lower dose levels remained without any histopathological correlates and should be considered to be of no toxicological significance. The NOAEL level that can be derived from this study is 2000 ppm.

t-Norchloroacetochlor - Ames Bacterial Mutation Assay (Repeat Study)

Report:	Wagner, III, V.O. and VanDyke, M.R. (2013) MON 52706: Bacterial Reverse Mutation Assay. Monsanto study No. BRL-2013-0134.
Guidelines:	This study meets the requirements of OECD test guideline 471 (1997)
GLP:	yes

Executive Summary

In a reverse gene mutation assay in bacteria, *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* tester strain WP2 *uvr*A were exposed to MON 52706 (>99% purity), using dimethylsulfoxide (DMSO) solvent. An initial toxicity-mutation assay was conducted where MON 52706 was tested up to the limit concentration, 5000 µg/plate. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Precipitate was observed at 5000 µg per plate. No toxicity was observed. Dose levels of 15, 50, 150, 500, 1500 and 5000 µg per plate ±S9 were used for the confirmatory mutagenicity assay using the plate incorporation method. Precipitate was observed at 5000 µg per plate. No toxicity was observed. The positive controls induced the appropriate responses in the corresponding strains.

No positive mutagenic responses were observed in any of the tests. It was concluded that MON 52706 was not mutagenic in the bacterial strains tested, either in the presence or absence of metabolic activation.

I. Materials and Methods

A. Materials:

1. **Test Material:** MON 52706
   Description: Pale yellow liquid
   Lot/Batch#: GLP-1212-22321-T
   Purity: >99 %

   CAS#: Not available

   Stability of test compound: The test substance was expected to be stable for the duration of testing.

   Solvent used: Dimethylsulfoxide (DMSO)

2. **Control Materials:**

   Negative: DMSO

   Solvent/ final concentration: DMSO at 0.1 mL/plate

Positive:

non-activation:	2-nitrofluorene: 1.0 µg/plate TA98 sodium azide: 1.0 µg/plate TA100, TA1535 9-aminoacridine: 75 µg/plate TA1537 methyl methanesulfonate: 1000 µg/plate WP2 *uvr*A
activation:	2-Aminoanthracene: 1.0 µg/plate TA98, TA1535, TA1537; 2.0 µg/plate TA100; 15 µg/plate WP2 *uvr*A

1. Activation

The S9 preparations were from livers of Aroclor 1254-induced rats (Lot Nos. 3017 and 3062 from Moltox, Boone, NC). The metabolic activation ability of the S9 was characterized using varying S9 and positive control concentrations. The composition of the S9 mix is shown below:

Component:	**Concentration:**
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
S9	10 % (v/v)

2. Test Concentrations:

a. Initial Toxicity-Mutation Assay:

Plate incorporation assay: 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate were evaluated with and without S9 activation in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* strain WP2 *uvr*A, in duplicate plates.

b. Confirmatory Mutagenicity Assay:

Plate incorporation assay: 15, 50, 150, 500, 1500 and 5000 µg per plate were evaluated in triplicate in the presence and absence of S9 activation in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* strain WP2 *uvr*A, in triplicate plates.

B. Test Performance:

The study was conducted during the period March to April, 2013.

1. Plate incorporation method

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 50 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test substance aliquot was replaced by a 50 µL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

2. Statistics

None.

3. Evaluation Criteria

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance.

Data sets for tester strains TA1535 and TA1537 were judged positive if the increase in mean revertants at the peak of the dose response was greater than or equal to 3.0-times the mean vehicle control value. Data sets for tester strains TA98, TA100 and WP2 uvrA were judged positive if the increase in mean revertants at the peak of the dose response was greater than or equal to 2.0-times the mean vehicle control value.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

II. Results and Discussion

A. Initial Toxicity-Mutation Assay

The maximum dose tested was 5000 µg per plate, which is the maximum concentration recommended by test guidelines (OECD 471, 1997). The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Precipitate was observed at 5000 µg per plate. No toxicity was observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

B. Confirmatory Mutagenicity Assay

No positive mutagenic responses were observed with any of the tester strains either the presence or absence of S9 activation. The dose levels tested were 15, 50, 150, 500, 1500 and 5000 µg per plate. Precipitate was observed at 5000 µg per plate. No toxicity was observed.

The vehicle controls and positive controls in the initial toxicity-mutation assay and confirmatory mutagenicity assays were within the acceptable historical ranges and fulfilled the requirements for a valid assay.

III. Conclusions

The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, MON 52706 did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

Mean Revertants Per Plate

STRAIN	DOSE LEVEL (µg/plate)	Initial Experiment				Confirmatory Experiment			
		+S9		-S9		+S9		-S9	
		Count	Ratio[1]	Count	Ratio	Count	Ratio	Count	Ratio
TA98	5000	18	0.6	13	0.7	20	1.0	6	0.5
	1500	17	0.6	18	1.0	20	1.0	7	0.6
	500	25	0.8	10	0.6	25	1.2	9	0.8
	150	16	0.5	9	0.5	22	1.0	8	0.7
	50	23	0.8	15	0.8	25	1.2	13	1.2
	15	34	1.1	14	0.8	19	0.9	8	0.7
	5	29	1.0	12	0.7	-	-	-	-
	1.5	19	0.6	20	1.1	-	-	-	-
	VEHICLE	30	-	18	-	21	-	11	-
	POSITIVE	502	16.7	170	9.4	466	22.2	182	16.5
TA100	5000	74	0.9	89	0.9	59	0.7	76	0.9
	1500	51	0.6	95	1.0	80	0.9	101	1.2
	500	76	0.9	73	0.8	78	0.9	92	1.1
	150	93	1.1	96	1.0	99	1.1	85	1.0
	50	91	1.1	84	0.9	85	1.0	95	1.2
	15	105	1.3	97	1.0	74	0.8	100	1.2
	5	77	0.9	74	0.8	-	-	-	-
	1.5	100	1.2	69	0.7	-	-	-	-
	VEHICLE	84	-	96	-	89	-	81	-
	POSITIVE	533	6.3	519	5.4	592	6.7	532	6.6

[1]Ratio of treated/vehicle control

STRAIN	DOSE LEVEL (µg/plate)	Initial Experiment				Confirmatory Experiment			
		+S9		-S9		+S9		-S9	
		Count	Ratio[1]	Count	Ratio	Count	Ratio	Count	Ratio
TA1535	5000	6	0.8	16	2.0	7	0.7	15	1.4
	1500	13	1.6	18	2.3	9	0.9	14	1.3
	500	7	0.9	10	1.3	11	1.1	14	1.3
	150	10	1.3	13	1.6	8	0.8	12	1.1
	50	12	1.5	11	1.4	9	0.9	9	0.8
	15	10	1.3	16	2.0	13	1.3	10	0.9
	5	14	1.8	16	2.0	-	-	-	-
	1.5	16	2.0	11	1.4	-	-	-	-
	VEHICLE	8	-	8	-	10	-	11	-
	POSITIVE	79	9.9	541	67.6	97	9.7	463	42.1
TA1537	5000	4	0.7	8	2.0	8	1.3	11	1.1
	1500	6	1.0	5	1.3	6	1.0	4	0.4
	500	10	1.7	2	0.5	8	1.3	6	0.6
	150	5	0.8	4	1.0	6	1.0	9	0.9
	50	4	0.7	5	1.3	7	1.2	9	0.9
	15	10	1.7	7	1.8	6	1.0	10	1.0
	5	6	1.0	6	1.5	-	-	-	-
	1.5	7	1.2	4	1.0	-	-	-	-
	VEHICLE	6	-	4	-	6	-	10	-
	POSITIVE	27	4.5	230	57.5	26	4.3	240	24.0

[1]Ratio of treated/vehicle control

STRAIN	DOSE LEVEL (µg/plate)	Initial Experiment				Confirmatory Experiment			
		+S9		-S9		+S9		-S9	
		Count	Ratio[1]	Count	Ratio	Count	Ratio	Count	Ratio
WP2*uvrA*	5000	22	1.1	20	1.0	20	0.6	15	0.8
	1500	17	0.9	17	0.9	21	0.7	16	0.8
	500	20	1.0	20	1.0	19	0.6	21	1.1
	150	16	0.8	21	1.1	17	0.5	22	1.2
	50	21	1.1	28	1.4	20	0.6	28	1.5
	15	18	0.9	19	1.0	26	0.8	19	1.0
	5	20	1.0	17	0.9	-	-	-	-
	1.5	21	1.1	20	1.0	-	-	-	-
	VEHICLE	20	-	20	-	32	-	19	-
	POSITIVE	305	15.3	285	14.3	312	9.8	299	15.7

[1]Ratio of treated/vehicle control

In Vivo Transgenic Gene Mutation Study in Mice with MON 52706

Author(s)	Beevers C.
Year:	2014
Title:	*In vivo* Muta™Mouse gene mutation assay with MON 52706
Testing Laboratory:	Covance Laboratories Ltd., North Yorkshire, England
Lab Report Number:	8279760
Monsanto Study Number:	CV-2013-0032
Monsanto Report Number:	MSL002544
Report Date:	January 16, 2014
Guidelines:	OECD Guideline 488 (2011), revised 2013
Deviations from Guidelines:	No
GLP Compliance:	Yes

Executive Summary

The objective of this study was to evaluate the potential of MON 52706 to induce gene mutations in mice following repeated oral administration. In this study, MON 52706 was administered for 28 days to 4 groups of 7 transgenic female Muta™Mice at dietary concentrations of 0, 600, 1800 and 6000 ppm (intended to achieve dose levels of 0, 100, 300 and 1000 mg/kg/day, respectively). A fifth group of 7 female mice was administered ethylnitrosourea (ENU), a known mutagen, for use as a positive control.

All animals were sacrificed on Day 31, three days after the last day of dosing, and subjected to a complete gross necropsy. Selected organs were weighed and examined microscopically. DNA was isolated from the lungs and bone marrow and evaluated for the presence of gene mutation in the *lacZ* gene.

No increase in gene mutation frequency was observed in MON 52706 treated animals. Therefore, MON 52706 is not considered to be mutagenic to female mice.

Introduction

The objective of this study was to evaluate the potential of MON 52706, a minor soil metabolite of acetochlor, to induce gene mutations in lung and bone marrow of female Muta™Mice. These two tissues were selected for evaluation since equivocal increases in the incidence of lung tumours and histiocytic sarcomas were observed in female mice in chronic feeding studies with parent acetochlor.

The Muta™Mouse (*lacZ/GalE*) assay is capable of detecting point mutations and small deletions in a wide variety of tissues from treated animals. The genome of the Muta™Mouse strain contains 40 copies of a transgenic lambda gt10 vector, each of which contains a bacterial *lacZ* gene, within every DNA-bearing cell of the animal. The *lacZ* gene encodes for the β-galactosidase enzyme, which is not present in the normal mouse. Treatments are performed *in vivo*, with sufficient treatment and expression time to permit any mutations to be expressed. Cellular DNA is extracted from selected tissues at necropsy and subsequently packaged into lambda bacteriophage. The packaged bacteriophage are then used to transfect a culture of *E. coli* C *lac*$^-$ *galE*$^-$ Kan^r (*galE*$^-$ Amp^r). Only bacteriophage units containing a *lacZ* gene from the transgenic vector in the mouse DNA are viable and capable of transfecting a bacterial cell. Successful transfection enables replication of the bacteriophage and transfection of neighbouring bacterial cells. This

results in the formation of a visible plaque, *i.e.* an area of bacterial cell lysis, in a lawn of uninfected viable bacterial cells. Furthermore, due to the *galE*⁻ status of the host bacterial strain cells, only cells infected with a non-functional (i.e. mutated) *lacZ* gene can form plaques on the positive selection plates.

Methods

Range-finding Study

A 14-day dose range-finding study was completed using 6 MutaMice to assess palatability and overt toxicity following dietary administration of MON 52706. Three female test mice were fed a diet containing 4500 ppm MON 52706 for a period of 7 days followed by additional 7 days of dosing with diet containing 6000 ppm MON 52706. A separate group of three control mice were fed normal diet for a period of 14 days. Transient slight decreases in food consumption were noted in the MON 52706-treated animals. However, there were no test substance related findings on survival, clinical observations or body weight.

Main Study

Species:	Mouse
Strain:	CD2-lacZ80/HazfBR (Muta™Mouse)
Sex:	Female
Source:	Harlan UK
Test Substance:	MON 52706 (norchloroacetochlor)
Lot/Batch#:	GLP-1212-22321-T
Purity:	>99%
Positive Control:	Ethylnitrosurea (ENU, Sigma-Aldrich, Poole, UK).
Negative (Vehicle) Control:	Diet
Study Design:	

Group Description	Number of Animals	Targeted Diet Concentration (ppm)	Targeted Dose (mg/kg/day)
Vehicle control	7	0	0
MON 52706 Low	7	600	100

	MON 52706 Intermediate	7	1800	300
	MON 52706 High	7	6000	1000
	Positive control (ENU)	7	NA	10 mL/kg/day (oral gavage)
Route of Exposure:	Oral, via the diet			
Dose Administration:	*Ad libitum* access to SQC Rat and Mouse Maintenance Diet No 1, expanded, ground fine (Special Diets Services Ltd. Witham).			
Dosing Period:	28 days			
Diet Preparation:	Prepared weekly			
Diet Analysis:	Diet batches of the highest (6000 ppm) and lowest (600 ppm) concentration of test article formulation were analysed for homogeneity and stability. All diet formulations used for dosing in Week 1 and Week 4 were analysed for achieved concentration.			
Physical Examinations:	Once weekly and day of necropsy			
Clinical Observations:	All animals were observed for ill health and overt toxicity. • Vehicle and test substance animals: Twice daily (AM and PM). • Positive control animals: Day 1: immediately after dosing, 2 and 4 hours post- dose. Day 2 – Day 28: prior to dosing, immediately after dosing, and 2 and 4 hours post-dose.			
Body Weight:	Prior to dosing on Day 1 and twice weekly thereafter			
Food Consumption and Food Efficiency:	Determined weekly for all vehicle control and test article animals. Not evaluated for positive control animals.			
Clinical Pathology:	Blood samples for clinical pathology evaluations (hematology and serum chemistry) were collected from the right ventricle of the heart at the scheduled necropsy from all vehicle control and test substance treated animals. However, due to difficulties in sample collection, an insufficient number of samples were available for a meaningful evaluation.			
Gross Necropsy:	A complete necropsy was conducted on all vehicle control and test substance treated animals on Day 31, three days after the end of the dosing period. However, the animals were inadvertently not sacrificed in a stratified or random manner. Instead, the animals were sacrificed by group, with the			

	vehicle control and 600 ppm groups being sacrificed in the morning, and the remaining groups being sacrificed in the afternoon. Although the order of sacrifice would not have affected any in-life data or the mutation results, this may have had an impact on one or more other parameters.
Organ Weights:	Adrenal glands, brain, heart, kidneys, liver, spleen, thymus, and uterus from all vehicle control and test substance treated animals
Tissue Collection:	About 30 tissues from all vehicle control and test substance treated animals were preserved in formalin for possible histopath evaluation. Samples of lung, bone marrow, liver and spleen were flash frozen in liquid nitrogen for possible gene mutation evaluation.
Histopathology:	Liver, kidney and gross lesions were evaluated microscopically for all animals. Sternum (with marrow), spleen, and lungs were examined from high-dose and vehicle control animals.
Gene Mutation Evaluation:	Genomic DNA from frozen lung and bone marrow samples was isolated using Recoverase™ DNA extraction kits. The isolated DNA was then packaged into bacteriophage heads, which were then transfected into *E. coli* C *lac*$^-$ *galE*$^-$ Kan^r (*galE*$^-$ Amp^r). The phage/bacteria were then suspended in top agar and plated for overnight incubation. Following overnight incubations, plates were manually scored for the number of clear plaques on each plate.
Statistics:	Mutant Frequency: The positive control group (Group 7) was compared to the vehicle control (Group 3) using a two-sample t-test. The test was interpreted with one-sided risk for increased response with increasing dose. Groups 3, 4, 5 and 6 were analyzed using one-way analysis of variance (ANOVA). An overall dose response test was performed along with Dunnett's test for pair wise comparisons of each treated group with the vehicle control. All tests were performed with a one-sided risk for increasing response. Levene's test for equality of variances between the groups was also performed and, in all cases, this showed no evidence of heterogeneity ($P>0.01$). Toxicology Parameters: The positive control group (Group 7) was excluded from statistical analysis. Food consumption, feed efficiency, body weight gains, terminal body weights, absolute organ weights, organ to body weight ratios, haematology and clinical chemistry variables were analyzed using one-way analysis of variance (ANOVA). Levene's test for equality of variances among the groups was performed. Where this showed no evidence of heterogeneity ($P>0.05$), and the group effect from the ANOVA was significant ($P\leq0.05$), pair wise comparisons with control were made using Dunnett's test.

Results and Discussion

Diet Analysis:

Diet analysis confirmed the homogeneity and target concentrations of MON 52706 at 600 and 6000 mg/kg diet. Concentrations after 9 days at room temperature were 14% and 12% lower than at Day 0, for the 600 and 6000 mg/kg diets, respectively. This was believed to be due, at least in part, to incomplete extraction rather than an indication of lack of stability of MON 52706. However, in either case, the slightly lower recovery after 9 days was not considered to be a concern since the achieved dose levels were slightly greater than intended.

Clinical Observations:

There were no test substance related deaths or clinical findings.

Body Weight:

There was no statistically significant effect on body weight observed in any group treated with **Error! Reference source not found.** compared to the concurrent vehicle control. However, Group 6 showed a statistically significant decrease (53%) in overall body weight gain (Day 1 to Day 29) compared to the vehicle control.

Table 1: Body Weight and Weight Gain (g)

Group	Day 1	Day 29	Cumulative Gain (Day 29 – Day 1)
0 ppm	20.6	23.8	3.2
600 ppm	21.7	25.1	3.3
1800 ppm	21.8	23.7	1.9
6000 ppm	20.5	22.0	1.5*

*p <0.05

Food Consumption:

There was a slight decrease in food consumption in the 6000 ppm animals, primarily during the first two weeks of the study.

Table 2: Average Weekly Food consumption

Group	Days 1-8	Days 8-15	Days 15-22	Days 22-29
0 ppm	31.3	30.2	29.5	29.1
600 ppm	28.5	29.6	31.2	30.3
1800 ppm	30.7	30.6	30.7	29.7
6000 ppm	26.4**	27.7	28.9	27.4

** p<=0.01

Test Substance Intake:

Mean test substance intake met or exceeded targeted doses throughout the course of the study.

Table 3: Mean Test Substance Intake Over 28 Days of Dosing

Target Dietary Level (ppm)	Mean Test Substance Intake[1] (mg/kg/day)
0	0
600	112
1800	345
6000	1130

[1]Based on nominal dietary concentrations.

Proof of Exposure:

Proof of test substance exposure to the bone marrow and lungs was not demonstrated as part of this study. However, a subsequent 5-day oral repeat dosing study (Study No. SW-2013-0572) demonstrated the presence of substantial amounts of MON 52706 in blood (plasma) after oral gavage administration to mice.

The bone marrow is a well-perfused tissue and the level of test article in the bone marrow is generally comparable to that in the blood or plasma. Accordingly, measurement of plasma levels of test material to confirm exposure to bone marrow is considered acceptable by the ICH guidelines (ICH S2(R1), 2012). The lung is also a highly perfused tissue. Thus, the detection of substantial amounts of MON 52706 in plasma after oral gavage administration is considered to be sufficient to demonstrate exposure to both the bone marrow and lungs.

Clinical Chemistry:

No statistically significant changes in clinical chemistry parameters were seen in animals dosed with **Error! Reference source not found.**, although it is noted that for many parameters data were available for too few animals to allow valid and reliable statistical analysis.

Macroscopic Observations:

No treatment related gross pathological findings.

Organ Weights:

There were no statistically significant differences in absolute or relative organ weights.

Table 4: Average Absolute Organ Weights

Group	Adrenal (g)	Brain (g)	Heart (g)	Kidney (g)	Liver (g)	Spleen (g)	Thymus (g)	Uterus (g)
0 ppm	0.009	0.365	0.130	0.323	1.378	0.122	0.062	0.146
600 ppm	0.009	0.365	0.128	0.328	1.391	0.122	0.065	0.244
1800 ppm	0.010	0.362	0.140	0.481	1.304	0.119	0.056	0.188
6000 ppm	0.010	0.354	0.125	0.312	1.273	0.113	0.055	0.148

None significantly different from control group

Table 5: Average Organ Weight-to-Body Weight Ratios

Group	Adrenal	Brain	Heart	Kidney	Liver	Spleen	Thymus	Uterus
0 ppm	0.0379	1.5101	0.5428	1.3326	5.6666	0.5120	0.2578	0.6175
600 ppm	0.0379	1.4391	0.5090	1.3029	5.5960	0.4939	0.2574	0.9932
1800 ppm	0.0403	1.5104	0.5815	1.9565	5.4452	0.4956	0.2315	0.7739
6000 ppm	0.0444	1.6031	0.5651	1.4090	5.7235	0.5119	0.2510	0.6668

None significantly different from control group

Microscopic Observations:

There were no treatment-related adverse microscopic findings.

An initial microscopic evaluation of liver sections showed a possible slight decrease in glycogen vacuolation in treated animals. However, this was considered likely to be an artifact of the necropsy order (which resulted in a longer period of fasting for the mid- and high-dose animals compared to the control and low-dose animals) and not a treatment-related adverse finding.

Focal nephropathy was found in the kidneys of two high-dose animals. However, in both cases, the lesion affected only a single tubule, and was therefore not considered to be test article related. There were no treatment related findings in the sternum (with marrow), spleen or lung.

Gene Mutation:

There was no indication of any increase in mutation frequency in either lungs or bone marrow from animals treated with MON 52706. Substantial increases in mutation frequency were observed in both lung and bone marrow from animals treated with ENU, a known mutagen, thus demonstrating the ability of this assay to detect gene mutations.

Table 6: Mutation Frequency in Lung and Bone Marrow

Group	Dose Level (ppm)	Mutation Frequency (x 10^{-6})					
		Lung			Bone Marrow		
		Mean	SD	n	Mean	SD	n
Neg. Control	0	43.30	12.2	6	32.79	17.5	6
MON 52706	600	48.56	9.5	7	35.40	17.8	7
MON 52706	1800	52.06	16.5	7	32.31	13.1	7
MON 52706	6000	49.40	22.9	7	36.35	15.2	5
Pos. Control	-	354.35***	97.2	7	883.23***	184.1	7

*** P≤0.001

Conclusions:

No evidence of any increase in gene mutation frequency was observed when MON 52706 was administered to female mice for 28 days at dose levels up to 6000 ppm (approximately 1000 mg/kg/day). Therefore, MON 52706 is not considered to be mutagenic to female mice.