8-31-04

MODE OF ACTION ASSESSMENT DOCUMENT

EVALUATION OF THE MODE OF ACTION OF

ACETOCHLOR

FOR

NASAL OLFACTORY EPITHELIUM TUMORS IN RATS

AND ITS

RELEVANCE TO HUMAN CANCER RISK ASSESSMENT

FINAL REPORT

August 31, 2004

CANCER ASSESSMENT REVIEW COMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

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EXECUTIVE SUMMARY

On April 21 and 22, 2004, the Cancer Assessment Review Committee (CARC) of the Health Effects Division of the Office of Pesticide Programs met to:

1. Reevaluate the carcinogenic potential of acetochlor
2. Determine the adequacy of the database to support a proposed mode of action for thyroid tumorigenesis
3. Determine the adequacy of the database to support a proposed mode of action for nasal tumors in rats and the relevance of such tumors to human cancer risk assessment.

This document covers the conclusions of the CARC meeting concerning Item No. 3: the adequacy of the acetochlor database to support a proposed mode of action for nasal tumors in rats and the relevance of such tumors to human cancer risk assessment. Items Nos. 1 and 2 are covered in a separate document: Evaluation of the Carcinogenic Potential of Acetochlor (Fourth Evaluation, June 2004).

Acetochlor is structurally related to other chloroacetanilide herbicides, including alachlor, propachlor, butachlor and metolachlor. Alachlor and butachlor also induce nasal epithelial tumors and thyroid follicular cell tumors. On the basis of preliminary reviews of mechanistic data for acetochlor and butachlor, the FIFRA SAP concluded on March 19, 1997 that alachlor, acetochlor and butachlor may be grouped together for common mode of action (MOA) for induction of nasal and thyroid tumors (USEPA 1997, 2001).

The mechanistic data for acetochlor have been now fully reviewed. Based on these data the committee concluded:

1. MOA for nasal tumors.

The data supporting the mechanism of action for nasal olfactory epithelium tumors in rats by acetochlor have been evaluated. It is concluded that the non-genotoxic MOA for nasal olfactory epithelium tumors in rats, discussed in this document, is supported by the data.

The MOA includes the he following steps:

i) Acetochlor conjugates with glutathione (GSH) and is excreted in the bile.
ii) The conjugate is biotransformed to a series of sulfur-containing products. Enterohepatic circulation of these products creates a pool of metabolites that are delivered to the nose.
iii) Biotransformation to tissue-reactive and toxic metabolites. Metabolism by nasal enzymes results in formation of a benzoquinoneimine, an electrophile and redox-active molecule.
iv) Binding to cellular proteins plus possible generation of oxidative stress.
v) Cytotoxicity (respiratory metaplasia)
vi) Regenerative cell proliferation.

vii) Sustained cytotoxicity and cell proliferation that results in neoplasia.

The following data are consistent with the MOA:

a. The absence of a demonstrated positive mutagenic effect of the chemical.

b. Acetochlor administration results in dose related increases in the binding of the quinone imine metabolite in the target tissue. This metabolite is considered to be the putative active species.

c. There is respiratory metaplasia of the nasal olfactory epithelium, an indication of cytotoxicity to the original olfactory tissue and its being replaced by respiratory epithelium, which originates from undifferentiated cells in the epithelium.

d. Lipofucsin pigment was observed to increase in a dose related manner in the nasal olfactory epithelium of rats that show nasal olfactory tumors at the high dose. Lipofucsin pigment is associated with oxidative damage to lipids and lipoproteins, which is consistent with the redox alterations known to be produced by quinones and quinone imines.

e. Acetochlor administration results in dose related increases in cell proliferation in cells of the target tissue.

f. The absence of nasal epithelial tumors in mice correlated with their inability to form adducts of the quinone imine at the target site. This evidence of no quinone imine binding was confirmed autoradiographically.

g. Rats administered the sulfoxide metabolite of acetochlor (a proximate precursor of the toxic metabolite, the quinone imine) show nasal olfactory mucosa adenomas after 26 weeks of treatment (MRID 46081801).

The data on the non-genotoxic MOA for acetochlor are supported by the entire database for the analog alachlor, in particular:

i. Reversibility of cell proliferation in rats treated with alachlor for 60 days at a tumorigenic dose, after placement on basal diet for 60 days (MRID 42852102)

ii. Rats treated with the analog alachlor for 1 month at a tumorigenic dose (126 mg/kg/day) did not have detectable neoplasms when examined after a 5-month holding period on basal diet. No detectable olfactory mucosal lesions were observed in any of the "stop study" rats (Genter et al. 2002b)

The weight of the evidence in support for the mode of action evaluated in this document is high. The evidence would have been strengthened if corroborative experiments, such as prevention or reversal of a precursor event (e.g. cell proliferation) by appropriate administration of a chemical (e.g. N-acetylcysteine) known to interfere with a key step (e.g. formation of quinone imine), had been available. Although dimethylaniline (DMA) and diethylaniline (DEA) [analogs of ethylmethyl aniline (EMA)] have been found to form *in vivo* DNA adducts in rat nasal mucosa,

concerns about a genotoxic mechanism for acetochlor are mitigated by several factors. These include absence of formation of DNA adducts in nasal mucosa in parallel experiments in rats using the analog alachlor and the reversibility of cell proliferation of olfactory epithelium observed with alachlor.

2. Relevance of rat nasal olfactory epithelium tumors to human health risk assessment.

The Registrant's data in support of the idea that rat nasal olfactory epithelium tumors have no relevance to human health risk assessment has been evaluated. It is concluded that the Registrant's argument that there is no relevance to humans cannot be sustained.

This conclusion is supported by:

- The realization that production of a metabolite (EMA) with the capacity of undergoing biotransformation to a quinone imine is possible for humans (Coleman et al. 2000).

- *In vitro* studies of p-hydroxylation of EMA using olfactory epithelium enzymes indicate that rat-to-monkey ratios of activities are not as large as 23.7 but could be as small as 7 or 8.

- In *in vitro* studies, the ratio of rat to monkey for p-hydroxylation of the sulfoxide metabolite of acetochlor may be not astronomically large, as initially postulated, but as small as 88.

- Although nasal tissue was not included in the Coleman et al. (2000) study, the data indicate that human liver has the potential to produce EMA (Figure 13), a plausibly carcinogenic metabolite of acetochlor, which would then be available to all organs via the circulatory system.

Comments by one of the presenters (B. Dementi OPP/HED/TOX) are discussed as a minority dissenting opinion in Part 3 of the final document.

I. INTRODUCTION

On April 21-22, 2004, the Cancer Assessment Review Committee (CARC) of the Health Effects Division of the Office of Pesticide Programs reviewed the recommendations of the toxicology reviewer for acetochlor with regard to the proposed mechanism of action of acetochlor in the production of nasal olfactory epithelium tumors. The conclusions drawn at this meeting are presented in this report.

On October 30 of 1996 the FIFRA SAP was presented a postulated mode of action (MOA) for alachlor for nasal epithelium tumors in rats similar to the one to be discussed in this document. Concerning the relevance of the presented mechanism for human cancer risk assessment, the SAP concluded that " .. the argument that limitation in the metabolism of alachlor in the human precludes alachlor being considered as a human carcinogen can not be supported."

On March 19 of 1997, on the basis of preliminary reviews of mechanistic data for acetochlor and butachlor, the FIFRA SAP concluded that alachlor, acetochlor, and butachlor share a common MOA for nasal epithelium tumors and for thyroid follicular tumors in rats (USEPA 1997, 2001).

II. BACKGROUND INFORMATION

1. Introduction: Acetochlor is a herbicide used both pre- and post-emergence for killing grasses and some broad leafed weeds in corn. Joint USA registration was granted to the Acetochlor Registration Partnership (ARP) in March 1994. Acetochlor is sold in the USA as *Surpass* (Zeneca) and *Harness* (Monsanto).

2. Chemical Identification:

Table 1 summarizes the identification data for acetochlor. Table 2 summarizes the physicochemical properties of acetochlor.

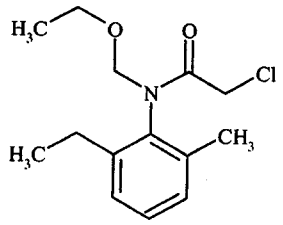
Table 1. Acetochlor Nomenclature	
Chemical structure	
Common name	Acetochlor
Molecular Formula	$C_{14}H_{20}ClNO_2$
Molecular Weight	269.8
IUPAC name	2-chloro-N-ethoxymethyl-6'-ethylacet-o-toluidide
CAS name	2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide
CAS #	34256-82-1
PC Code	121601

TABLE 2. Physicochemical Properties of Acetochlor.		
Parameter	Value	Reference
Boiling point/range	163 °C at 10 mm Hg; decomposition occurs before the boiling point at atmospheric pressure; (calculated by extrapolation of vapor pressure at lower temperature)	DEB 7474, 2/6/91, M. Flood
pH	4.41, 1% solution in acetone:water (1:1, v:v)	DEB 7474, 2/6/91, M. Flood
Density at 20 °C	1.123 g/mL	DEB 7474, 2/6/91, M. Flood
Water solubility at 25 °C	223 mg/L	2001 Farm Chem Handbook
Solvent solubility at 25 °C	Infinitely soluble in acetone, benzene, carbon tetrachloride, ethanol, chloroform, and toluene	HED Memo, 1/21/94, M. Flood
Vapor pressure at 25 °C	0.045 · Hg (4.5 x 10^{-5} mm Hg)	DEB 7474, 2/6/91, M. Flood

Dissociation constant, pK_a	Not applicable because acetochlor is neither an acid nor a base.	DEB 7474, 2/6/91, M. Flood
Octanol/water partition coefficient	970 or 1082	DEB 7474, 2/6/91, M. Flood
UV/visible absorption spectrum	Not available	

III. DATA PRESENTATION

1. Listing of Available Data

Data from the following documents were examined by the Committee in its deliberations:

Table 3. List of Documents used in the Cancer Assessment Review Committee Meeting for evaluation of Acetochlor MOA and Carcinogenicity status.

No.	Type of Study	Comments	Reference
1	Chronic Rat	1983 Sprague-Dawley Rat Study	MRID 00131089 ❄
2	Chronic Rat	1986 Sprague-Dawley Rat Study	MRID 400770601 ❄
3	Chronic Rat	1988 Sprague-Dawley Rat Study	MRID 41592004 ❄
4	2-Gen Repro Rat.	2001 Sprague-Dawley Rat Study	MRID 45357503 ❄
5	Chronic Mouse.	1983 CD-1 Mouse Study (24-months)	MRID 00131088 ❄
6	Chronic Mouse.	1989 CD-1 Mouse Study (18-months)	MRID 41565119 ❄
7	CARC Doc.	Details MOA for Alachlor nasal tumors	
8	CARC Doc.	Pathology Report-Rats & Mice J. Pletcher	❄
9	Mechanism Rat	1998 SD Rat quinone imine-protein binding; autoradiography	MRID 44496210
10	Mechanism Mouse	1998 CD-1 Mouse quinone imine-protein binding	MRID 44496211
11	Mechanism Rat; (aceto.sec.sulfide)	1998 SD Rat quinone imine-protein binding; autoradiography	MRID 44496212
12	Mechanism Monkey	1998 Rhesus Monkey quinone imine-protein binding	MRID 44496213
13	Mechanism Rat	1996 SD Rat nasal cell proliferation	MRID 44496207
14	Mechanism Mouse	1996 CD Mouse nasal cell proliferation	MRID 44496209
15	Mechanism Rat	1996 SD Rat thyroid parameter effects	MRID 44496208 ❄
16	Metabolism	1998 Comparative metabolism Rat & Mouse	MRID 44496203
17	Metabolism	1998 Protein Adducts Rats	MRID 46009402
18	Review	"Acetochlor Mechanism of Nasal Tissue Carcinogenicity" (Dementi, 1/26/04)	

Table 3 (Continued) List of Documents used in the Cancer Assessment Review Committee Meeting for evaluation of Acetochlor MOA and Carcinogenicity status.

No.	Type of Study	Comments	Reference
19	Review/concerns	2/4/04 Email B. Dementi (BD)/Colleagues expressing Acetochlor issues of concern	
20	Review/concerns	3/2/04 Email BD/Colleagues expressing additional Acetochlor issues of concern	
21	Comments	3/9/04 Email BD/Colleagues comments on SD Rat Repro. Study DER	
22	Correspondence	12/19/03 Email J. Kronenberg (JK)/BD; nasal cytotoxicity	
23	Comments	1/14/04 Email BD/Gentlepersons; comments on Monsanto's 1996 assessment of alachlor for SAP	
24	Review	10/1/96 Monsanto: Executive Summary from review of info. on carcinogenicity of alachlor for SAP 1996.	
25	Review	1998 Clapp et al., Monsanto document assessing carcinogenic potential	MRID 44496201
26	Review	1992 Pathology: rat nasal tissue mapping	MRID 44496214
27	Review	2003 *In Vitro* Metabolism; Multiple Species Plus Humans	MRID 46081802
28	Review	2003 *In vitro* metabolism; rat, mouse, primate	MRID 44530002
29	Review	1998 *In vitro* metabolism (sulfoxide metabolite), rat and mouse	MRID 44530001
30	Review	1996 Mouse thyroid study *(mechanism)*	MRID 44496208
31	Review	9/5/03 Monsanto Justification for Reclassification of Carcinogenic Potential. "White Paper"	MRID 46081801
32	Comments	10/24/03 Dementi on "White Paper"	

33	Correspondance	5/28/03 Email: BD/JK; comments on Clapp et al.	
34	Correspondance	10/8/03 Email: JK/BD;	
35	Correspondance	10/20/03 Email: JK/BD	

Table 3 (Continued). List of Documents used in the Cancer Assessment Review Committee Meeting for evaluation of Acetochlor MOA and Carcinogenicity status.

No.	Type of Study	Comments	Reference
36	Correspondence	11/17/03 Memorandum BD/Jim Jones; concerns about PWGs	
37	SAP	12/6/96 report of 10/30/96 SAP mtg.	❄
38	Review	6/27/97 Report of 2/5/97 CARC meeting addressing 10/30/96 SAP/SAB	
39	SAP	4/28/97 report of 3/19-20 SAP meeting	
40	Phone call/notes	10/20/03 Email BD/L.Hansen (LH) & A. Protzel (AP); record of 10/20/03 conference with Dr. Genter	
41	Phone call/notes	10/8/03 Email BD/JK	
42	Phone call/notes	12/17/03 Email BD/JK	
43	Phone call/notes	12/17/03 Email BD/Colleagues; comments on reproduction study	
44	Phone call/notes	1/14/04 Email BD/AP; phenacetin cytotoxicity	
45	Phone call/notes	1/14/04 Email BD/W.Burnam; CARC report - are nasal tumors respiratory as so recorded?	
46	Phone call/notes	1/21/04 Email LH/BD; lung tumors in both alachlor and acetochlor mouse studies	
47	Phone call/notes	5/21/03 Email BD/BD record of May 20 request of JK for "White Paper"	
48	Review	3/16/96 Cancer Peer review Alachlor 3th	❄
49	Review	2/5/97 Cancer Peer review Alachlor 4th	❄
50	Review	3/30/87 Peer Review Acetochlor	❄

Table 3 (continued). List of Documents used in the Cancer Assessment Review Committee Meeting for evaluation of Acetochlor MOA and Carcinogenicity status.

No.	Type of Study	Comments	Reference
51	Review	5/31/89 2nd Peer review- Acetochlor	❄
52	Review	1/27/92 3rd Peer review- Acetochlor	❄
53	Correspondence	2/25/04 D Wolf to A Protzel	
54	Correspondence	3/15/04 D Wolf to A Protzel	
55	Article	Coleman et al., 2000	
56	Review	Common Mechanism Document	
57	Review	ACETOCHLOR QUALITATIVE RISK ASSESSMENT	❄
58	Correspondence	3/17/04 J Pletcher to N McCarroll	
59	Correspondence	3/18/04 J Pletcher to N McCarroll	

❄ Document for CARC

2. Presentation of Available Data

a. Introduction

The Acetochlor data presented below were submitted by the Registrant in support of a non genotoxic mode of action (MOA) for the production of tumors of the nasal olfactory epithelium in rats. The genotoxic data are discussed below under **Mutagenicity Results**, and are summarized in Table 5.

The postulated MOA for the induction of nasal tumors by acetochlor in rats (See Figure 1) proposes that acetochlor conjugates with glutathione (GSH) and is excreted in the bile. Subsequent biotransformation of the conjugate to a series of sulfur-containing products, followed by enterohepatic circulation of these products creates a pool of metabolites that are delivered to the nose where they undergo further biotransformation to tissue-reactive and toxic metabolites. Metabolism by nasal enzymes results in formation of benzoquinoneimine, an electrophile, which binds to cellular proteins and produces oxidative damage, producing cytotoxicity and regenerative cell proliferation. If cytotoxicity and cell proliferation is sustained, neoplasia eventually results.

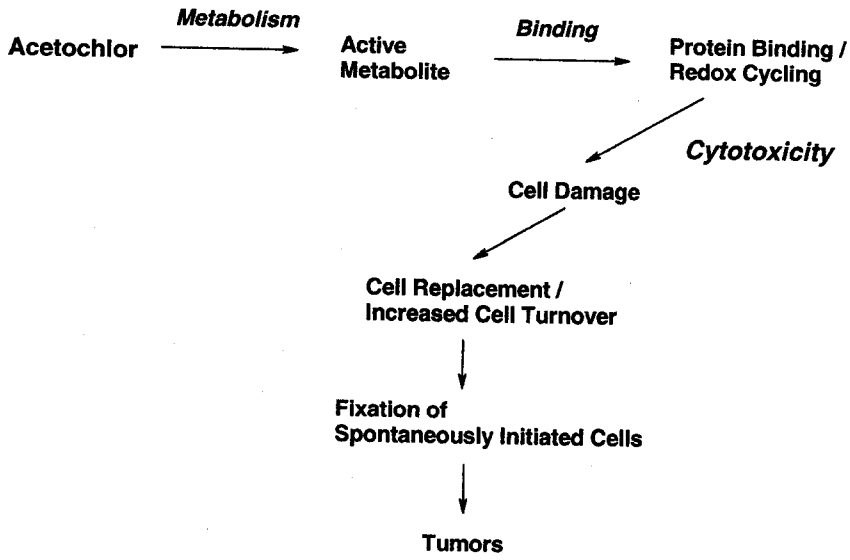
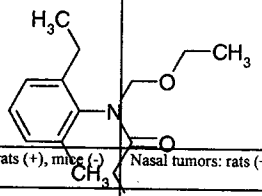
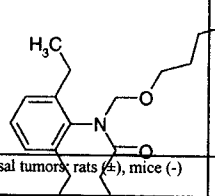
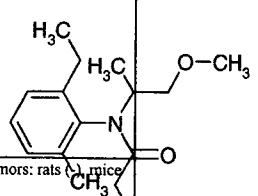


Figure 1. Postulated mode of action of acetochlor in the induction of nasal tumors in rats

Because the MOA database for acetochlor is very large, the data have been briefly summarized, as an overview, in Table 4 and keyed to the MOA steps shown in Figure 1.

Additionally, because the chloroacetanilides have been studied as a common mechanism grouping, data are given in Table 4 for four additional chloroacetanilides. These include alachlor and butachlor, which have been grouped together with acetochlor, as a common mechanism group based on nasal turbinate tumors in rats by the FIFRA SAP in a meeting dated March 19 of 1997.

Table 4. Summary of data supporting the MOA for nasal tumors in rats.

MOA Step	Data for Acetochlor and Analogs				
	Acetochlor	**Alachlor**	**Butachlor**	**Metolachlor**	**Propachlor**
					
EndPoint	Nasal tumors: rats (+), mice (-)	Nasal tumors: rats (+), mice (-)	Nasal tumors: rats (+), mice (-)	Nasal tumors: rats (±), mice (-)	Nasal tumors: rats (-), mice (-)
Metabolism					
Oral Absorption	>70%	Extensive	Extensive	Extensive	>68%
Tissue Distribution	Extensive. Including binding to nasal epithelium in rats but not mice or other sp.	Extensive. Including binding to nasal epithelium in rats but not mice or other sp.	No Data	Extensive. Including binding to nasal epithelium in rats but not mice or other sp.	No Data
Biotransformation	Extensive, including *in vivo* precursors of quinone imine: sec-sulfoxide and sec-chloramide metabolites . *In vitro* p-OH-metabolite of sec-sulfoxide metabolite.	Extensive, including precursors of quinone imine: sec-methylsulfide, 4-amino-3,5-diethylphenol (as sulfate)	Extensive, including a precursor of quinone imine: 4-amino-3,5-diethylphenol (as sulfate)	Extensive. A possible quinone imine precursor: 2-methyl-6-ethylaniline (at less than 0.00055% of the dose.	Extensive, including 4-aminophenol (a quinoneimine precursor) and its glucuronide.
Active Metabolite	Derivatives diagnostic of dialkyl-benzoquinoneimine (DBZQ) identified in urine of dosed rats by Jeffries et al. (1998)	Derivatives diagnostic of DBZQ identified in urine of dosed rats by Jeffries etal. (1998)	Derivatives diagnostic of DBZQ identified in urine of dosed rats by Jeffries et al. (1998)	Derivatives diagnostic of DBZQ identified in urine of dosed rats by Jeffries et al. (1998)	No Data

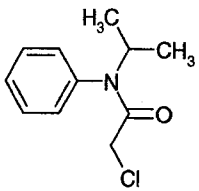


Table 4. Summary of data supporting the MOA for nasal tumors in rats.

MOA Step	Data for Acetochlor and Analogs				
	Acetochlor	**Alachlor**	**Butachlor**	**Metolachlor**	**Propachlor**
Binding					

Binding to Nasal Protein	Dosing with C^{14}acetochlor or its C^{14} sec-sulfide showed concentration of label in nasal turbinates. Analysis of adduct indicated origin from binding of a benzo-quinone imine to protein	Dosing with C^{14}alachlorr, its C^{14} sec-sulfide, or DEA[1] showed concentration of label in nasal turbinates. Analysis of adduct indicated origin from binding of a benzo-quinone imine to protein.	No Data.	Dosing with C^{14}metolachlor showed concentration of label in nasal turbinates.	No Data.
Redox Cycling	Limited data. GSH depleted in liver ($p<0.05$). Lipofucsin pigment seen in olfactory epithelium in rats	Limited data. GSH depleted in liver ($p<0.05$). Genomic data on oxidative damage.	GSH depletion in stomach tissue and other tissues.	No data	No data
Cytotoxicity	Histopathology: Olfactory epithelial respiratory metaplasia.	In vitro cytotoxicity found using nasal tissue explants. In vivo histopathological: respiratory metaplasia of olfactory epithelium	Histopathology: Olfactory epithelial respiratory metaplasia	No data.	No data.
Cell damage	Lipofucsin pigment seen in olfactory epihelium	Ggenomic data on oxidative damage.	No data	No data	No data
Cell Replacement/ Increased cell turnover	Significantly increased cell proliferation in the olfactory epithelium of rats but not mice.	Significantly increased cell proliferation in the olfactory epithelium of rats	Significantly increased cell proliferation in the olfactory epithelium of rats	No data	Nodata
Nasal Tumors	See Above	See Above	See Above	See Above	See Above

[1] DEA = Diethylaniline

b. Mutagenicity Results

Acetochlor was evaluated in a variety of genetic toxicology assays submitted to HED which were summarized along with published articles by Dearfield et al. (1999) and are discussed below (and summarized in Table 5) according to endpoint.

GENE MUTATIONS

Results from published assays and studies submitted to HED in support of FIFRA registration were generally negative for gene mutations in bacteria (*Salmonella typhimurium*). There was a reproducible increase in mutant colonies (<2-fold increase) of strain TA1538 in one study (MRID 41565121). The response was, however, confined only to this strain. It was not observed in strain TA98 (the more sensitive derivative of TA1538) and was not concentration-related (observed only at 1000 and 2500 ·g/plate +S9).

Hill et al. (1997) reported that a metabolite of acetocholor, diethylquinoneimine was "weakly" positive without S9 activation in a pre-incubation assay with *S. typhimurium* TA100. This finding, however, should be viewed with caution because the increase (2.1-fold) was limited to a single concentration (30 ·g/plate -S9) that also induced cytotoxicity. Similarly, the "weak positive effect" seen in a Chinese hamster ovary (CHO)/HPRT gene mutation assay (~4x increase at 125 ·g/plate -S9, with 71% cell survival) (MRID 00131395) was not observed in an independent second assay with this same cell line (MRID 42713106). There was, however, a positive response in the mouse lymphoma L5178Y forward gene mutation assay (MRID 00131394). In contrast to the weak effect observed in one of two CHO assays, the response in mouse lymphoma cells was seen in the presence of S9 activation with concentration dependency (*i.e.*, >2-fold ↑at 30 ·L/L with 45% survival to 4-fold ↑at 40 ·L/L with 12 % survival). At the time this assay was performed, mouse lymphoma colony size distribution was not preformed. Hence, it can not be ascertained whether the increased mutant colonies resulted from mutagenic or clastogenic action.

In keeping with the Agency's conclusions regarding the structural and toxicological similarities between acetochlor and **alachlor**, relevant genetic toxicology studies on **alachlor** are also included in this review. Wetmore et al. (1999) conducted a series of gene mutation assays in *S. typhimurium* TA100 on **alachlor** using S9 homogenates from the liver, olfactory mucosal or the respiratory mucosal of Long-Evans rats. Results showed an ~2- to 3-fold increases in revertant colonies of TA100 at 15,000 or 8430 ·g/plate, respectively with rat liver S9; no response with 1500-15,000 ·g/plate plus rat olfactory mucosal S9 but a 2-fold increase at 1250 ·g/plate with rat olfactory mucosal S9 in a repeat experiment; and no response in the presence of rat respiratory. These findings should be considered equivocal since the positive data in the presence of rat liver S9 was not dose-related, was accompanied by cytotoxicity (thinning of the background lawn of growth), and the concentrations eliciting a ≥2-fold response were excessive. Similarly, the response in the presence of olfactory S9 homogenates was not reproducible. Kier et al. (1996) also tested *alachor* in the Ames assay using rat olfactory S9 homogenates and found only negative results. Wetmore et al. also reported a 2.9-fold increase in the mutation frequency of L5178Y mouse lymphoma cells at 5.6 ·g/mL + rat olfactory S9. At this concentration, total cell

survival was reduced to 8.3%. From results for gene mutation assay with bacteria or mammalian cells, Wetmore et al. concluded that positive results were only obtained in mouse lymphoma and TA 100 over a narrow concentration range immediately below levels that were lethal. "Thus, there appears to be a close association of a narrow concentration range that induces gene and chromosomal mutations and concentrations that induce toxicity." Such findings, without support, are not considered by the genetic toxicology community to be valid evidence of a positive response.

Overall, the results from gene mutation assays are conflicting, show only sporadic positive findings, and, therefore, do not provide a convincing picture of gene mutation.

CHROMOSOME ABERRATIONS

In vitro test systems

Acetochlor induced a clastogenic effect in human lymphocytes (in whole blood cultures) at concentrations of 50 and 100 ·g/mL -S9 and at 100 ·g/mL +S9 (MRID 41565122). Cytotoxicity [i.e, ≥50%↓ in the mitotic index, MI was seen at 100 ·g/mL -S9 (60-65% ↓MI). Induced chromosome aberrations were predominantly breaks, fragments and minutes. Ashby et al . (1996) (MRID 44496215) reported on the above study and presented data from two other assays with acetochlor demonstrating that acetochlor of analytical grade was a confirmed clastogen in human lymphocytes in whole blood cultures or in isolated human lymphocytes. Types of induced chromosome aberrations were breaks and fragments/minutes. The study authors stated that acetochlor appeared to be "more toxic to the isolated cells" than lymphocytes in treated-blood cultures and that the greater toxicity was probably due to "the absence of glutathione (GSH) in the isolated lymphocytes as compared to its presence in the whole blood cultures". Ashby et al. presented additional data showing that two other chloroacetyl non-carcinogens that were negative in the Ames assay (2-chloroacetophenone and 4-chloroacetylacetanilide) were also clastogens in human lymphocytes as was the N-butyl analogue of acetochlor. However, the des-chloro analogue was not clastogenic, which lead the authors to conclude that, "These results establish the chloro substituent in acetochlor as the clastogenic entity and indicate the protective cellular effect afforded by the free thiol (SH) group of glutathione".

In vivo test systems

There was no evidence of a clastogenic or aneugenic response in micronucleus assays in the bone marrow of CD-1 or C57 mice (MRID 00164941 or 41565123, respectively), and in a Sprague-Dawley rat bone marrow cytogenetic assay (MRID 00131392) up to toxicologically overt doses or cytotoxic levels causing reductions in the ratio of polychromatic to normochromatic erythrocytes (PCE:NCE) in mice or up to the limit dose using the oral route of exposure in rats. Similarly, acetochlor was not clastogenic in a series of dominant lethal mutation assays in rats (MRID 44069502 or 44093703) or mice (MRID 44093701) using either a dietary exposure or oral gavage up to toxicologically overt doses or the limit dose. Findings from one of the rat dominant lethal assays (MRID 44093703) showed significantly ($p < 0.025$) reduced enzyme levels for glutathione (GSH) in the testes, caput and cauda of the epididymis and the vas deferens of males 12 hours after treatment with 2000 mg/kg.

The overall results from chromosome aberration assays indicate that acetochlor is a confirmed clastogen in cultured mammalian cell test systems and the N-butyl analogue of acetochlor, but not the des-chloro analogue, was also clastogenic *in vitro*, as well as two chloroacetyl non-carcinogens. However, acetochlor's clastogenic activity in cultured mammalian cells results in breaks, fragments and minutes, which are generally associated with cytotoxicity because the types of induced aberrations are asymmetric and, therefore, not consistent with cell survival (Galloway 2000). Furthermore, the induction of chromosome aberrations is not expressed *in vivo* in either somatic or germinal cells of rats or mice.

OTHER MUTAGENIC MECHANISMS

In vitro test systems

Hill et al. (1997) reported suggestive evidence of sister chromatid exchange (SCE) induction in human lymphocytes at 10 ·M acetochlor while 0.3 and 0.1 ·M of a dialkylquinoneimine metabolite of acetochlor, (ethylmethylquinoneimine) induced a significant increase in SCE which was only 1.2-1.3-fold over background suggesting a weak response. Acetochlor was negative for unscheduled DNA synthesis (UDS) in primary rat hepatocytes up to cytotoxic concentrations (≥10.6 ·g/mL) (MRID 00131393).

In vivo test systems

A weak positive UDS response was revealed in hepatocytes harvested from Sprague-Dawley rats treated for 12 hours with 2000 mg/kg acetochlor. Ashby et al. (1996) pointed out, however, that the weak UDS response was accompanied by major hepatic pathology [(e.g. necrosis, 60-fold increases in alanine transaminase and aspartate transaminase and 70% depletion of glutathione (MRID 44496215)]. A negative comet assay was reported in which DNA was harvested from the nasal, olfactory or respiratory tissue of male Sprague-Dawley rats administered dietary preparations of 1750 ppm (~88 mg/kg/day) acetochlor for either 7days or 18 weeks (MRID 44069503). Finally, a time- and dose-related significant ($p<0.05$-0.01) increase in cell proliferation (S-phase induction) was noted in the nasal turbinates of the olfactory region but not the respiratory region of male Sprague-Dawley rats receiving dietary dosages of 1750 or 5000 ppm acetochlor (~88 or 250 mg/kg/day, respectively) for 60, 90 or 160 days (MRID 44496207). This finding is of great importance since the increase in cell proliferation, which was time and dose-dependent, is most likely due to cytotoxicity to the nasal turbinates. Additionally, the sustained increase in cell proliferation is proposed a key event in the induction of nasal olfactory tumors in rats. It should be noted that the doses used in the comet assay or the cell proliferation assays caused nasal olfactory tumors in the Sprague-Dawley rat while the dose used in the UDS assay (2000 mg/kg) was in excess of the both the tumorigenic doses and the MTD (1000 ppm, ~50 mg/kg/day for 104 weeks).

CONCLUSIONS

Results for gene mutation assays are conflicting and provide no clear evidence of a positive effect in either bacteria or mammalian cell test systems. Similarly, the evidence from *in*

vitro and *in vivo* UDS assays, *in vitro* SCE studies, and an *in vivo* comet test provide no convincing pattern of genotoxic activity. Although there is suggestive or weak evidence of *in vitro* SCE induction (only 1.3-fold higher than control for acetochlor), the impact of increased SCE induction is not well understood and this phenomena has not been linked to a cancer risk (Albertini et al., 2000). Thus, the results from these various assays are mixed and without confirmation. By contrast, results from chromosome aberration assays indicate that acetochlor is a confirmed clastogen in cultured human lymphocytes. There is also the possibility that the increased mutant colony counts observed in the positive mouse lymphoma assay resulted from a clastogenic rather than mutagenic response since this test system can detect chromosome breakage. Nevertheless, clastogenicity is confined to *in vitro* mammalian cell test systems and the types of induced aberrations suggest cytotoxicity. Based on data from three bone marrow assays in either mice or rats and three dominant lethal mutation studies also in rats or mice, acetochlor-induced clastogenicity is not expressed in either somatic or germinal cells of whole animals. This finding is consistent with a similar profile of *in vitro* but not *in vivo* clastogenicity for the chloroacetanilides. Like acetochlor, **alachlor** has also been widely tested in a variety of assays either submitted to the Agency or published in the open literature. In agreement with the acetochlor data, alachlor is generally negative in gene mutation assays, clastogenic in CHO cells and human lymphocytes *in vitro* but negative in whole animal studies.

Ashby et al. (1996) claim that the clastogenicity of acetochlor results from a preferential reaction with GSH as opposed to the nitrogen or oxygen atoms of DNA, and "when these DNA-protective cellular nucleophiles are depleted (with increasing doses of acetochlor), a reaction will occur with chromatin sulphydryl groups leading to clastogenicity". Binding to macromolecules is characteristic of this chemical class as indicated by the preferential binding of acetochlor to sulfhydryl groups such as GSH and proteins. This would also appear to explain the weak UDS response in hepatocytes harvested from Sprague-Dawley rats treated for 12 hours with 2000 mg/kg acetochlor since non-protein sulfhydryl levels (consisting of >95% GSH in the liver) were reduced by 67%. The response was accompanied by liver necrosis which "reached such levels in some of the rats that panlobular destruction was observed." However, these findings conflict with the comet assay results showing that acetochlor did not produce DNA damage in Sprague-Dawley rat nasal olfactory tissue after dietary exposure to a tumorigenic dietary level (1750 ppm) for 18 weeks. The implications from these data have great impact since nasal olfactory tumors induced by acetochlor are considered by the Agency to be the neoplasia of toxicological importance. At this time, however, only a very weak case can be made for mutagenicity as the primary driver in the development of nasal olfactory tumors.

Mutagenicity studies with acetochlor are summarized below in Table 5.

Table 5. Summary of mutagenicity studies with acetochlor

MUTAGENICITY STUDIES WITH ACETOCHLOR		
GENE MUTATIONS		
870.5100 Bacterial Gene Mutation Assay *Salmonella typhimurium*	00050930 (1978) Acceptable/guideline 0.001-1 ·l/plate - /+ S9	**Negative** up to the highest dose tested (1 ·l/plate - /+ S9); higher concentrations (≥10 ·l/plate - /+ S9) were cytotoxic

870.5100 Bacterial Gene Mutation Assay *Salmonella typhimurium*	41565121 (1989) Acceptable/guideline 1.6-5000 ·g/plate - /+ S9	**Equivocal positive** in TA 1538 at 2500 and 1000 ·g/plate + S9; reproducible at 1000 ·g/plate but <2-fold, not dose-related and not seen in TA98
870.5100 Bacterial Gene Mutation Assay *Salmonella typhimurium* TA1538 only	44863202 (1989) Acceptable/nonguideline 100-5000 ·g/plate - /+ S9 (Arochlor 1254 or Pheno-barbital /β-naphthoflavone induced rat livers)	**Negative** in TA1538 using 3 different Batches (89.8-99.6%) in two separate tests
870.5300 *In vitro* mammalian cell gene mutations Chinese Hamster Ovary (CHO) cells	00131395 (1983) Acceptable/guideline 25-150 ·g/mL - S9 25-125 ·g/mL + 10% S9	**Positive** ≥2-fold in mutation frequency (MF) at 125 or 150 ·g/mL - S9 & 125 ·g/mL + S9 accompanied by cytotoxicity (61% or 93%↓in cell survival - /+S9)
870.5300 *In vitro* mammalian cell gene mutations CHO cells	42713106 (1989) Acceptable/guideline 50-200 ·g/mL -S9 50-300 ·g/mL + 1, 2, 5 or 10% S9	**Negative** up to cytotoxic levels (≥200 ·g/mL -/+ 10% S9)

870.5300 *In vitro* mammalian cell gene mutations Mouse lymphoma L5178Y cells	00131394 (1982) Acceptable/guideline 20-400 ·L/mL -S9 5-250 ·L/mL +S9	**Positive** 30-50 ·L/mL +S9 2.2-5.2 fold increase accompanied by cytotoxicity (<10% survival at ≥50 ·L/mL +S9)
MUTAGENICITY STUDIES WITH ACETOCHLOR		
CHROMOSOME ABERRATIONS		
870.5375 Cytogenetics *In vitro* mammalian cell chromosomal aberration assay human lymphocytes	41565122 (1989) Acceptable/guideline 0, 10, 50 100 ·g/mL -/+ S9	**Positive** at 50 and 100 ·g/mL -S9 and 100 ·g/mL +S9 accompanied by marked reduction in mitotic indices at 100 ·g/mL (≥59%↓). Types of aberrations: breaks, fragments and minutes
870.5375 Cytogenetics *In vitro* mammalian cell chromosomal aberration assay human lymphocytes (whole blood vs separated blood)	44863204 (1998) Acceptable/guideline 0, 10, 75 150 ·g/mL -/+ S9 0, 100 ·g/mL - S9 (whole blood) 0, 75 ·g/mL - S9 (separated blood)	Whole Blood: **Positive** at 75 and 150 ·g/mL -S9 and 150 ·g/mL +S9 accompanied by slight reduction in mitotic indices at 150 ·g/mL (31% -S9; 13 % +S9 ↓). Types of aberrations: breaks, fragments and minutes Whole Blood: **Positive** 9-fold↑ in aberrations at 100 ·g/mL Separated Blood: **Positive** 26-fold↑ in aberrations at 75 ·g/mL
SOMATIC CELLS		
870.5385 Mammalian Bone Marrow Chromosomal Aberration Test Rat	00131392 (1983) Acceptable/guideline 0, 40, 150, 500 mg/kg IP injection	**Negative** up to overt toxicity (significant ↓body weight gain)
870.5395 Mammalian Erythrocyte Micronucleus Test CD-1 Mice	00164941 (1986) Acceptable/guideline 0, 200, 660, 2000 mg/kg oral gavage	**Negative** up to overt toxicity (mortality) & cytotoxicity (significant ↓ PCE:NCE ratio at 2000 mg/kg, both sexes combined)
870.5395 Mammalian Erythrocyte Micronucleus Test CD-1 Mice	41565123 (1989) Acceptable/guideline 0, 898 or 1436 mg/kg ~ 0, 1075 or 1719 mg/kg~	**Negative** up to a cytotoxic dose (significant ↓ PCE:NCE ratio) seen at both doses in ~~
GERMINAL CELLS		

870.5450 Cytogenetics Dominant Lethal Rat	44069502 (1996) Unacceptable/guideline 0, 200, 1000, 1500 ppm for 10 weeks	**Negative** for dominant lethal mutations but dosage was insufficient
870.5450 Cytogenetics Dominant Lethal Mouse	44093701(1996) Unacceptable/guideline 0, 200, 1000, 3500 ppm for 8 weeks	**Negative** for dominant lethal mutations but dosage was insufficient
870.5450 Cytogenetics Dominant Lethal Rat	41963309/44093703 (1991/1996) Acceptable/guideline 0, 200, 1000, 2000 mg/kg oral gavage	**Negative**; earlier report of positive results now considered to be due to reproductive (infertility) toxicity
OTHER MUTAGENIC MECHANISMS		
Other Genotoxicity *In vitro* sister chromatid exchange assay Human Lymphocytes	Hill et al. (1997) 10 ·M (2.7 ·g/mL)	**Weak evidence of positive** response (1.5-fold ↑) in one of two donor cells
870.5550 Other Genotoxicity *In vitro* UDS in Primary Rat Hepatocytes	00131393 (1983) Acceptable/guideline 0.032-320 ·g/well	**Negative** up to cytotoxic concentrations (≥10.6 ·g/well)
Other Genotoxicity *In vivo* UDS in Primary Rat Hepatocytes	41565124 (1989) 0, 500, 1000, 2000 mg/kg oral gavage	**Weak positive response** accompanied by major hepatic pathology (necrosis, 70% ↓ GSH, 60-fold increase in aspartate transaminase
Other Genotoxicity *In vivo* Comet Assay in Rat Olfactory and respiratory cells	44863208 (1999) 1750 ppm (175 mg/kg/day) 7 days	**Negative** at a tumorigenic dose *in vivo*

c. Mode of Action Data

1. Nasal tumor observations

As summarized in Table 6, three rat chronic studies reported statistically significant incidences of nasal tumors in rats at acetochlor dietary levels of ≥1000 ppm. Additional data indicate that these tumors are confined to the olfactory epithelium region. No nasal tumors were seen in two acceptable mouse carcinogenicity studies.

Table 6. Incidence of nasal tumors in rat chronic studies.

	Study (MRID)	Dose Level (ppm)							
		Males				Females			
#1	**PR-80-006 (00131088, 40484801)**	**0**	**500**	**1500**	**5000**	**0**	**500**	**1500**	**5000**
	papillary adenoma	0/69	1/70	6/69*	18/69**	0/69	0/68	2/70	1/69
	pap. adenocarcinom.	0/69	0/70	0/69	2/69	0/69	0/69	0/70	0/69
	Combined	ND	ND	ND	ND	ND	ND	ND	ND
#2	**ML-83-200 (40077601)**	**0**	**40**	**200**	**1000**	**0**	**40**	**200**	**1000**
	papillary adenoma	1/58 **	0/54	0/58	12/59**	0/69**	0/69	0/67	19/68**
#3	**88/SUC017/0348 (41592004)**	**0**	**18**	**175**	**1750**	**0**	**18**	**175**	**1750**
	papillary adenoma	0/69 **	0/59	0/59	35/70**	0/69**	0/57	0/58	36/63**
	carcinoma.	0/69	0/59	0/59	2/70	0/69	0/57	0/58	1/63
	Combined	0/69* *	0/59	0/59	37/70**	0/69**	0/57	0/58	37/63**
#4	Sulfoxide Metabolite 1 year[a](Special study)	**0**	**300**	-	-	**0**	**300**	-	-
	polypoid adenoma	0 7/32 **	-	-	-	-	-	-	-

* = p≤0.05; ** = p≤0.01. ; [a] Sex unspecified in this special study. Incidence at 26 weeks .

To determine if there was a similarity in morphology, origin, and location of proliferative lesions , the Registrant conducted a review (See MRID 44496214) of hematoxylin/eosin slides of nasal tissue of rats treated with acetochlor, alachlor or butachlor in previously conducted long-term oral studies. In the case of acetochlor, slides from study #3 (88/SUC017/0348, MRID 41592004) of Table 1 were used. In the case of alachlor, slides from an alachlor special rat chronic study (EHL 93049, MRID 43590001); and in the case of butachlor, slides from a butachlor rat chronic study (Biodynamics 79-2388, MRID 42244901) were used.
It was found that:

- All three of the chloracetanilides induced morphologically similar lesions confined

almost entirely to the olfactory epithelium lining in specific regions of the posterior nasal passages.

• These lesions and tumors were found within all six ethmoturbinates, but were almost entirely absent from the dorsal septum and dorsal medial regions of the ethmoturbinates with the exception of foci of basal cell hyperplasia in female rats (16/69) exposed to acetochlor, of which 9/16 were mapped to those regions.

• Most of the benign tumors exhibited ciliation of the olfactory epithelial cells and were associated with **respiratory metaplasia of adjacent olfactory mucosa.** Many lesions were close to the olfactory-respiratory epithelial junctions (See pp. 14-16 in MRID 44496214).

• For all three compounds, these changes were apparently identical in nature and location and differed only in frequency of occurrence (comparison of frequency compromised by differences in animal sacrifice times, strains used and number of animals evaluated for each compound).

It was also concluded in this study that the similar morphology suggests that the same processes may be responsible for these preneoplastic and neoplastic changes for all three acetanilides.

These results are supported by observations with the rats from study #1 (MRIDs 00131088 and 40484801) in Table 6. In study #1 (conducted at 0, 500, 1500, & 5000 ppm) no nasal tumors were initially found, in spite of the higher doses used, because sections had been taken from the anterior part of the nasal cavity. When new histological sections were taken so as to include the posterior region of the nasal cavity, the tumors reported in Table 6 for study #1 were seen. The posterior region of the nasal cavity in rats is essentially olfactory epithelium, in contrast to respiratory epithelium, which is found in the more anterior part of the nasal cavity, Young (1981).

2. Metabolism

i. Absorption.

Bile-duct cannulated rats were administered C^{14}-alachlor excreted over 80% of a radioactive dose in the bile, in contrast to similarly dosed non-cannulated rats, that excreted 70% of the dose in urine. These values are consistent with high absorption by the oral route followed by extensive enterohepatic circulation of the metabolites.

ii. Tissue distribution

Sprague-Dawley rats were administered ^{14}C-Acetochlor in the diet at levels of 1750 or 5000 ppm. The animals were sacrificed after 14 days on the diet for examination by whole body autoradiography (WBA) and microautoradiography. WBA revealed significant localization of radioactivity in the nasal turbinates. Micro radioautography in high-dose rats showed intense

localization in Bowman's glands, a lower degree in the olfactory surface, and no evidence of localization in the respiratory epithelium. In low-dose animals only slight to moderate localization was seen in the Bowman's glands (MRID 44496210).

Male Sprague-Dawley rats received 5 consecutive daily doses of the ^{14}C-secondary sulfide metabolite of Acetochlor by gavage. Rats were sacrificed at 1 or 5 days after the last dose for examination by WBA. Examination of radioautographs from animals sacrificed one day after dosing show high levels of radioactivity in the intestinal contents & liver, nasal turbinates, and lining of the tongue. At 5 days after dosing in addition to residual radioactivity in the stomach and intestinal contents, there was clear localization in the nasal turbinates, radioactivity in surrounding areas was greatly diminished. Micro radioautography showed that the label was concentrated in the Bowman glands of the nasal turbinates (MRID 44496212).

iii. Biotransformation

As summarized in Figure 2, acetochlor in rats undergoes extensive biotransformation involving enterohepatic recirculation (MRIDs 41565125, 41565126, 41565127, 41592006, 41592007) leading to the precursors of the quinone-imine (e.g. the sulfoxide metabolite, Figure 2). Following conjugation with GSH or glucuronic acid in the liver, the conjugates are excreted in the bile. The GSH conjugate undergoes partial degradation in the gut and is reabsorbed resulting in the appearance of the sulfoxide metabolite (U11, Figure2) and its precursors in the blood.

The metabolites identified in Figure 2 amounted to about 77% of the dose. Other possible metabolites such as 2-ethyl-6-methylaniline (EMA) that would lead to a quinone imine were not discussed.

In more recent work (MRID 44530002), it was shown in vitro that microsomal fractions from rat liver and olfactory epithelium can metabolize the S-methyl sulfide metabolite of acetochlor to EMA (Figure 3). Thus, the Registrant summarized (MRID 46081801, and Green et al., 2000) the formation of precursors of the quinone imine by way of two paths (A and B) in the rat. Path A proposed amidase hydrolysis of the secondary methyl sulfide metabolite to 2-ethyl-6-methylaniline (EMA), hydroxylation of EMA to pOH-EMA and the subsequent formation of the quinone-imine. This pathway has been well characterized for **acetochlor**. Path B proposes oxidation of the secondary methyl sulfoxide metabolite to the sulphoxide (Acetochlor sulfoxide), hydroxylation of acetochlor sulfoxide to p-hydroxy sulfoxide leading to the formation of the sulfoxide quinone imine. Path B is an alternative pathway, which proposed a methyl sulfoxide as the major intermediate in the formation of DABQI as opposed to the aniline metabolite, formed by **alachlor**. In support of Path B, Green et al. found that acetochlor sulfoxide was the major metabolite in the plasma of rats fed 1750 ppm acetochlor in the diet for 6 months (~700 dpm sulfoxide, 20 minutes) or in rats 17 hours after being given a single oral dose of 200 mg/kg acetochlor (~190 dpm.sulfoxide, 20 minutes). In contrast, very little acetochlor sulfoxide (~75 dpm sulfoxide, 20 minutes) was detected in CD-1 mice administered single a oral dose of 200 mg/kg acetochlor.

However, results from the earlier comparative *in vitro* metabolism study in the rat and

mouse (MRID 44530002) indicate that this is not a significant route of metabolism of acetochlor in the mouse because high levels of acetochlor sulfoxide were found in rat plasma as opposed to mouse plasma which contained very little acetochlor sulfoxide. Unlike the rat, therefore, acetochlor sulfoxide would not be considered a significant circulating metabolite for the mouse.

Overall, the data suggest that Path B is a plausible metabolic pathway for metabolism of acetochlor to the reactive quinone imine in the rat and that acetochlor sulfoxide is the major circulating metabolite available *in vivo* to the rat nasal tissues.

It should be pointed out, however, that while Path A is well established for **alachlor**, the Path B pathway has not been evaluated for this chloroacetanilide. Furthermore, the "accepted" Path A pathway has not been ruled out for acetochlor. Regardless of the pathway, however, the data presented by the Registrant do suggest that the secondary methyl sulphide substrate may not be available to the mouse because the major route of acetochlor metabolism in the mouse is through glucuronide conjugation. This is supported by data showing that acetochlor sulfoxide is not found in mouse plasma, and is, therefore, not a circulating metabolite. Nevertheless, there are concerns regarding these conclusion based on an independent analysis of radiochromatograms of the rat and mouse urine samples because not all of the rat or mouse urinary metabolites have been taken into account.

Either way, either quinone imine will then bind to tissue proteins and other nucleophiles such GSH.

Based on work by Green (1998) (MRID 44496203) conjugation with GSH plus path B seems the major source of quinone imine in the rat. When the mouse was studied (MRID 44496203) it was found that the major *in vivo* metabolic route was glucuronidation plus and excretion of the chloramide. Glutathione conjugation, enterohepatic circulation and formation of quinone imine precursors was not a major route. The author considered this interspecies difference to be consistent with the absence of nasal tumors in the mouse.

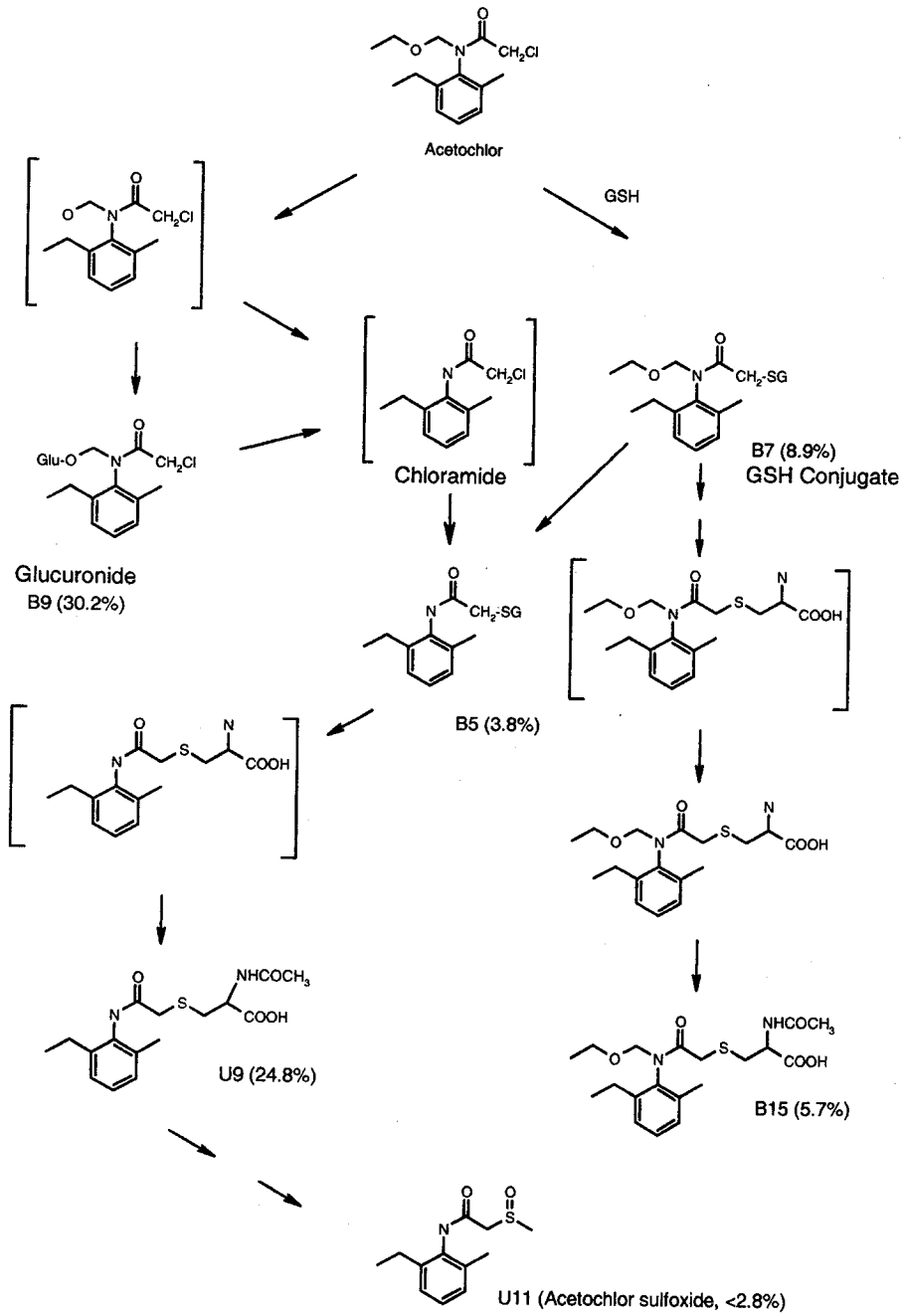

Acetochlor
GSH
Glucuronide
B9 (30.2%)
Chloramide
B7 (8.9%)
GSH Conjugate
B5 (3.8%)
U9 (24.8%)
B15 (5.7%)
U11 (Acetochlor sulfoxide, <2.8%)

Figure 2. Biotransform ation of acetochlor in SD rats (MRID 41565127). This pathway covers biotransformation from parent acetochlor up to formation of U11, the sulfoxide metabolite of acetochlor, one of the possible precursors of the quinone imine. (Percentages of metabolites, as percent of dose, are in parentheses).

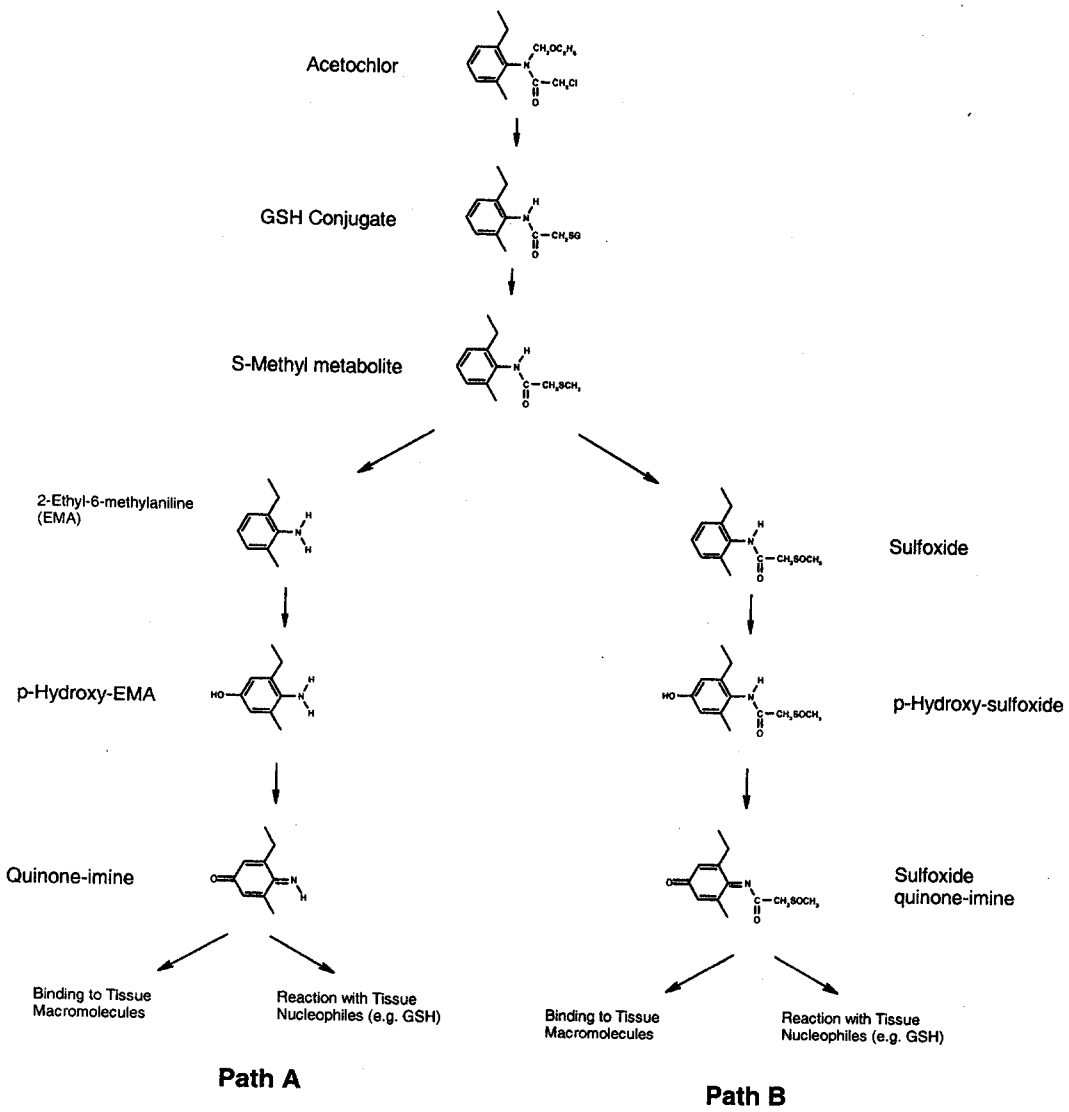

Acetochlor
GSH Conjugate
S-Methyl metabolite
2-Ethyl-6-methylaniline (EMA)
p-Hydroxy-EMA
Quinone-imine
Binding to Tissue Macromolecules
Reaction with Tissue Nucleophiles (e.g. GSH)
Path A
Sulfoxide
p-Hydroxy-sulfoxide
Sulfoxide quinone-imine
Binding to Tissue Macromolecules
Reaction with Tissue Nucleophiles (e.g. GSH)
Path B

Figure 3. Biotransformation of the S-methyl metabolite of acetochlor, leading to either methylethylquinone imine (MEQI) or methylethylquinone imine sulfoxide (Adapted from MRID 46081801).

3. Binding

i. Introduction.

The registrant has submitted a series of studies analyzing different aspects of binding of acetochlor and its metabolites to nasal tissue proteins.

In an initial study (MRID 44496210) the authors found a dose-related increase in adduct formation in SD rats administered ^{14}C-acetochlor in the feed at 1710 and 5170 ppm for 14 days. Nasal tissue proteins were extracted, subjected to acid hydrolysis and the released products were analyzed. As shown in Figure 4, two products were seen: EMIC-cysteine and EMA. One product, EMIC-cysteine, was formed from a quinone imine. EMIC-cysteine is formed regardless of whether the adduct comes from binding of EMA-quinone imine or sulfoxide metabolite-quinone imine. The second product (bottom reaction in Figure 4) EMA is formed if the adduct originated from direct binding of acetochlor or a metabolite still retaining the chlorine atom. Binding was confirmed by autoradiography. The level of binding was dose-related (Table 7). Notice that the level of product originating quinone imine binding (EMIQ-Cysteine) is smaller than that originating from binding from direct chlorine displacement.

TABLE 7 : Concentration of EMIQ-cysteine and EMA in nasal protein hydrolysate of rats fed with ^{14}C-acetochlor for 14 days[a]

Group (ppm in diet)	EMIQ-Cysteine (pmole/mg Protein)	EMA (pmole/mg Protein)
1710	119 ± 14.7	440 ± 273
5170	206 ± 64	1060 ± 445

[a]Data from Table 1, p. 32, MRID 44496210

Another pair of studies confirmed formation of nasal tissue adducts in rats dosed with two acetochlor metabolites which are quinone-imine precursors (see Figure 3 for structures): acetochlor sulfoxide metabolite (MRID 46009402) and acetochlor secondary sulfide metabolite (MRID 44496212). By dosing separately with [phenyl-^{14}C]-acetochlor and [carbonyl-^{14}C]-acetochlor, it was confirmed that the sulfoxide moiety was retained in the formed adduct.

Binding to nasal tissues of the mouse and rhesus monkey after ^{14}C-acetochlor administration was investigated in another pair of studies. In the case of the mouse (MRID 44496211) no EMIQ-cysteine was found after adduct analysis, only EMA was found, bound at dose related levels Table 8). EMA originated from binding of parent acetochlor or one of its metabolites still containing chlorine. In the case of the rhesus monkey (MRID 44496213) neither EMIQ-cysteine or EMA were found.

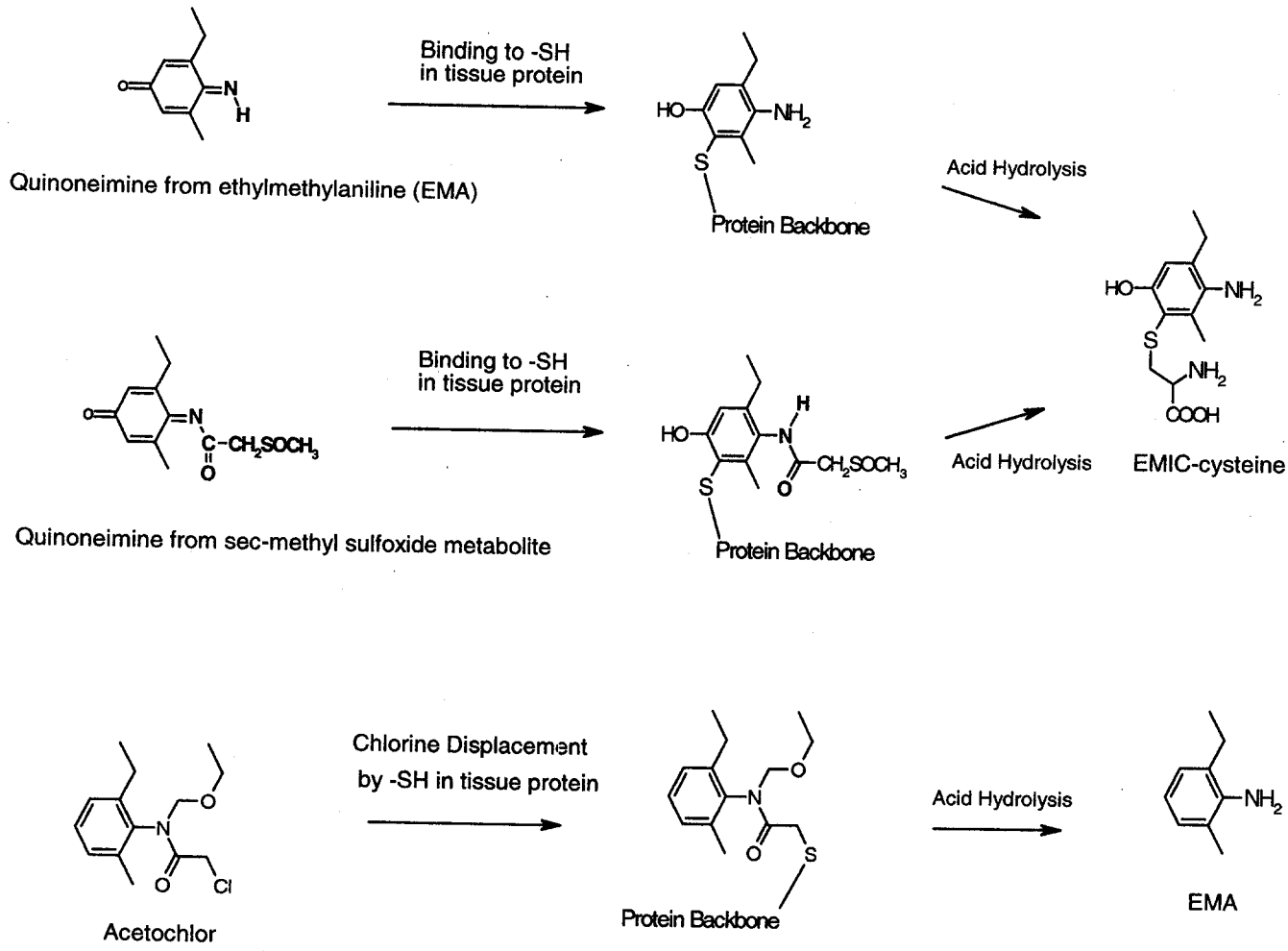


Figure 4. Identification of bound moieties in adducts to nasal epithelium proteins [adapted from MRID 44496212].

Microradioautography indicates that radioactivity is highest in the Bowman gland part of the olfactory epithelium for acetochlor (MRID 44496210) and acetochlor sulfoxide metabolite (MRID 46009402). Furthermore, HPLC chromatographic analysis of nasal protein hydrolysates after dosing with C^{14}-phenyl acetochlor show the label largely confined to hydrolysates from the olfactory epithelium (MRID 46009402).

TABLE 8: Concentration of EMIQ-cysteine and EMA adducts in nasal protein hydrolysate of mice fed ^{14}C-acetochlor for 14 days[a]

Group (ppm in feed)	EMIQ-cysteine (pmole/mg protein)	EMA (pmole/mg protein)
1800	<LOD[b]	128 ± 26.7
4750	<LOD	197 ± 54.4

[a]Data from Table 1, p. 26, MRID 44496211,
[b]Limit of detection

ii. Adduct formation in rats (Executive Summaries)

a) In a protein binding study (MRID 44496210), as explained by the investigators (p. 13 of the Study Report), acetochlor (95.2% a.i.) was administered to male Sprague Dawley rats in the diet at concentrations of 1710 ppm and 5170 ppm for 14 days to determine and characterize the nasal localization of ^{14}C-acetochlor. The results in this study address the potential binding of the putative metabolic adduct of acetochlor, 3-ethyl, 5-methylbenzoquinone-4-imine (EMIQ) to rat nasal proteins. The binding of acetochlor adducts to rat nasal turbinates was determined by an acid hydrolysis technique followed by HPLC analysis. HPLC analysis of the protein hydrolysate from both groups of animals showed a significant and dose-dependent formation of the 3-ethyl, 5-methyl-benzoquinoneimine-cysteine (EMIQ-cysteine) adduct *in vivo*. The average level of the EMIQ-cysteine adduct in rat nasal turbinates from rats fed 1710 and 5170 ppm was 119 pmole/mg protein and 206 pmole/mg protein, respectively. In addition to EMIQ binding, direct binding of acetochlor to nasal tissues was identified by the investigators.

The results of the whole body autoradiography showed significant localization of radioactivity in nasal turbinates at both dose levels. Microautoradiography studies showed intense localization of radioactivity within the Bowman's glands in high dose rats. There was a lower degree of localization of radioactivity in the olfactory surface epithelium and no evidence of localization within the respiratory epithelium. In low-dose rats, only slight to moderate localization of radioactivity was found in the Bowman's glands.

This protein-binding characterization study is **Unacceptable/Nonguideline**. This study may be upgraded to Acceptable/Nonguideline if the following data/information are submitted and are deemed to be satisfactory by the Agency: 1) the investigators did not present the rationale in support of the structure of the synthesized EMIQ-cysteine marker (p. 25 of the Study Report). The investigators should submit such rationale; 2) the investigators discussed the binding of EMIQ to nasal proteins in the context of a mechanism of action for acetochlor. In addition to EMIQ binding, the investigators identified binding of acetochlor to rat nasal proteins after "non-enzymatic direct chlorine displacement of acetochlor by the sulfhydryl group of cysteine in rat

nasal proteins" (p. 27 of the Study Report). However, the significance of these adducts is not included in the context of discussion of mechanism of action. The investigators should discuss this since this interaction with proteins may also underlie or contribute to the mechanism(s) of carcinogenicity, particularly since according to Table 1 (p. 32 of the Study Report) this form of binding to nasal protein is more extensive in terms of pmole/mg protein (as assessed by EMA release) than is EMIQ binding; 3) a clear statement describing the methodology employed in quantifying the concentration (pmole/mg protein) of EMIQ-cysteine and EMA in nasal protein hydrolysates, as reported, for example, in Table 2 of the Results section in this review; 4) citations to specific reference materials identified as needed in various sections of this review.

b) In a protein binding study (MRID 44496212), as explained by the investigators (p. 14 of the Study Report), acetochlor secondary sulfide (>99% radiochemical purity) was administered to male Sprague Dawley rats to determine and characterize the nasal localization of ^{14}C-acetochlor secondary sulfide. Four males were used in group M1, 2 males in group M2, 3 males in group M3, and 2 males were used in group M4. Groups M1, M2 and M3 were given five consecutive daily doses of approximately 7 mg/kg body weight and M4 group was given a single oral dose. In groups M1 and M2, half the animals were sacrificed one day after the final dose, and the other half were sacrificed five days after the final dose. In groups M3 and M4, all animals were sacrificed one day after the final dose.

The binding of acetochlor secondary sulfide to rat nasal turbinates was determined by an acid hydrolysis technique followed by HPLC analysis. HPLC analysis of the protein hydrolysate from treated animals showed the formation of a cysteine conjugate derived from 3-ethyl, 5-methyl-benzoquinoneimine (EMIQ-cysteine). The average level of this EMIQ-cysteine adduct was 19.3 pmole/mg protein following oral administration of acetochlor secondary sulfide for 5 days at approximately 7 mg/kg/day. The results of the whole body autoradiography showed significant localization of radioactivity in the nasal turbinates. Microautoradiography studies showed intense localization of radioactivity in the Bowman's glands of treated animals.

In summary, the study supported the hypothesis that rat nasal tissue is capable of metabolizing acetochlor secondary sulfide to EMIQ [p. 14 of the Study Report]. It is noteworthy that Green et al. (2000) indicate that the nasal metabolism of acetochlor proceeds through a secondary sulfide, with subsequent branching through either EMA or sulfoxide pathways, both of which result in protein adducts via a reactive intermediate quinone-imine. The final hydrolysis step in the analytical procedure to assay for protein binding yields the same final quinone imine protein metabolite in both pathways, i.e. the EMIQ and other quinone imine pathway analytical end products are the same.
Principally, through the use of the acetochlor secondary sulfide, which precludes the direct binding to nasal tissue proteins via chlorine displacement that occurs with acetochlor in addition to the EMIQ, or other quinone-imine pathway (per Green et al.), this study nonetheless demonstrated the presence of a quinone-imine protein adduct in rat nasal tissue following administration of acetochlor secondary sulfide. This study thus serves as further support for a quinone-imine protein binding mechanistic explanation for acetochlor induced nasal toxicity.

This study on secondary sulfide binding is **Unacceptable/Nonguideline**. This study may be

upgraded to Acceptable/Nonguideline if the following data are submitted and are deemed to be satisfactory by the Agency: 1) a reference, preferably the best reference, wherein the rationale may be found for the hypothesis that the formation of EMIQ is critical to the induction of nasal tumors by acetochlor; 2) a reference to the chronic/carcinogenicity study upon which dose selection was based; 3) if possible, some indication of the comparative magnitude of localization of radioactivity in nasal tissue, versus those of liver, kidney, lining of tongue, for example; 4) any information that may be available on the nasal tissue cytotoxicity and carcinogenicity of acetochlor secondary sulfide; 5) any available information on the question of acetochlor metabolic conversion to EMIC in the liver.

c) In a special nonguideline mechanistic study (MRID 46009402), localization of protein adducts formed in the nasal cavity of rats by binding of [^{14}C-labeled] acetochlor sulfoxide or its metabolites was assessed in male Crl:CD(SD)BR rats by the following three techniques: (1) *In vivo* protein binding - comparison by HPLC of the total radioactivity bound to nasal cavity proteins in hydrolysates prepared from the olfactory mucosa vs. the respiratory mucosa of 4 rats given a single gavage dose of [^{14}C phenyl-labeled] acetochlor sulfoxide (10 mg/kg bw in 4 mL/kg bw PEG 600; 100 MBq/kg bw); (2) *In vitro* protein binding - comparison using SDS-PAGE, western blot and phosphor imaging of radioactivity bound to proteins following incubation of microsomal fractions derived from rat nasal cavity olfactory tissues with either [^{14}C phenyl- labeled]acetochlor sulfoxide or [^{14}C carbonyl-labeled] acetochlor sulfoxide (0.4 mM; 407-458 Kbq per incubation mixture) and (3) Histoautoradiographic localization of protein binding - localization of protein-bound radioactivity in sections of the olfactory and respiratory regions of the nasal cavities of rats exposed to a single gavage dose of either [^{14}C phenyl-labeled] acetochlor sulfoxide or [^{14}C carbonyl-labeled] acetochlor sulfoxide (10 mg/kg bw in 4 mL/kg bw PEG 600; 100 Mbq/kg bw) and sacrificed at 8 or 24 hr postdosing (1 rat/dose group/time point). Washed and unwashed slides were processed for autoradiography to compare levels of total and tightly bound radioactivity.

HPLC profiles of fractions eluted from the nasal olfactory tissue protein hydrolysates of rats treated with [^{14}C phenyl-labeled] acetochlor sulfoxide showed significantly higher levels of bound radioactivity than fractions from the nasal respiratory tissue protein hydrolysates. In addition to the largest peak, which appeared to elute as a doublet at 7.5 minutes, at least 3 other major peaks were identified along with numerous minor peaks (none were further characterized). No major peaks were isolated from the respiratory tissue fractions. [^{14}C]-labeled protein adducts formed by incubation *in vitro* of rat nasal olfactory mucosa microsomes with [^{14}C-carbonyl]- or [^{14}C- phenyl]-labeled acetochlor sulfoxide showed similar patterns when compared by SDS-PAGE/western blot/phosphor imaging, indicating that the sulfoxide moiety was retained in much of the bound radioactivity, although quantitative or qualitative comparisons of these proteins were not performed. Histoautoradiography of the olfactory and respiratory regions of the rat nasal cavity at 8 and 24 hrs postdosing showed the highest level of bound radioactivity occurring over Bowman's glands in the olfactory mucosa (as determined by visual inspection, not quantitative grain count). The radioactivity in Bowman's glands was determined to be tightly bound by comparing washed (bound radioactivity) vs. unwashed (total radioactivity) slides. No tightly

bound radioactivity was reported in the slides of the respiratory region of the nasal cavity, although histoautoradiograms of these sections were not presented in the study report. Although the highest grain count at 24 hr was observed over Bowman's glands, reported binding over the olfactory epithelial mucosal surface was less clearly visible due to background labeling.

This special nonguideline metabolism study in the rat is classified as **Unacceptable/non-guideline (upgradable)**. It was not conducted to satisfy guideline requirements for reregistration of acetochlors, but to provide supplemental data addressing the mechanism of nasal carcinogenicity of acetochlor in the rat. Although the study appeared to be well-conducted, reporting of the histoautoradiographic findings was incomplete (photos of the autoradiographs were not provided for some of the sections evaluated; see "Study Deficiencies", in Discussion section). The study may be upgraded to acceptable/nonguideline with submission of this confirmatory data.

iii) Binding in the mouse and monkey

a) In a protein binding study (MRID 44496211), as explained by the investigators (p. 13 of the Study Report), acetochlor (95.2%) was administered in the diet of female CD-1 mice at concentrations of 1800 and 4750 ppm to determine and characterize the nasal protein binding of ^{14}C-acetochlor. Acetochlor binding to mouse nasal turbinates was determined by acid hydrolysis followed by HPLC analysis. HPLC profiles of the protein hydrolysate from both treatment groups showed no significant formation of the 3-ethyl, 5-methyl-benzoquinoneimine-cysteine (EMIQ- cysteine) adduct *in vivo*. For both treatment groups, significant amounts of radioactive components were consistent with the 2-ethyl-6-methylaniline (EMA) standard. The concentration of EMIQ-derived cysteine adducts was below the limit of detection. The only detectable protein adduct formed between acetochlor and mouse nasal protein likely resulted from the chlorine displacement of acetochlor by the sulfhydryls of mouse nasal proteins. For whatever reason, on exposure to acetochlor via the diet, mouse (unlike rat) nasal mucosa does not yield the EMIQ-protein adduct, which may support the hypothesis that mouse nasal tissue lacks the capacity to metabolize acetochlor to the putative reactive metabolite, EMIQ. To the extent the formation of EMIQ *in vivo,* and its subsequent binding to nasal tissue protein, is critical as believed for the induction of nasal tumors by acetochlor, failure to detect EMIQ-cysteine adducts in the mouse after dietary exposure supports the hypothesis that the carcinogenic mechanism for acetochlor is not operative in the mouse, as contrasted with the rat. (p. 13 of the Study Report)

This study on the characterization of acetochlor protein binding in the mouse is **Unacceptable/Nonguideline**. This study may be upgraded to Acceptable/Nonguideline if the following data/information are submitted and are deemed to be satisfactory by the Agency: 1) the characterization of the structure of the EMIQ-cysteine marker as requested for the rat study (MRID 44496210), 2) a reference, preferably the best reference, wherein the rationale for the hypothesis that the formation of EMIQ is critical to the induction of nasal tumors by acetochlor, 3) comment on the question as to whether the EMA pathway of direct protein binding by chlorine displacement of acetochlor that occurred in this study would be associated with nasal cytotoxicity in the mouse.

b) In a 14-day oral toxicity study (MRID 44496213), as explained by the investigators (pp. 9, 13 of the Study Report), acetochlor (95.2%) was administered to 3 male Rhesus monkeys by gavage to determine and characterize the nasal localization of ^{14}C-acetochlor. The monkeys were administered ^{14}C-acetochlor at a dose level of 126 mg/kg body weight for 14 days. The results in this study address the potential binding of the putative metabolite of acetochlor, 3-ethyl, 5-methylbenzoquinone-4-imine (EMIQ), to monkey nasal tissue proteins. The binding of acetochlor to monkey nasal turbinates was determined by an acid hydrolysis technique followed by HPLC analysis. HPLC analysis of the protein hydrolysate showed no significant formation of the 3- ethyl, 5-methyl-benzoquinoneimine-cysteine (EMIQ-cysteine) adduct *in vivo*. The lack of detection of EMIQ-cysteine adducts in the monkey after oral dosing of acetochlor, according to the investigators, supports the hypothesis that the carcinogenic mechanism for acetochlor is species specific and among species tested appears to be restricted to the rat.

This Non-guideline oral toxicity study on nasal cell adduct formation is **Acceptable/ Nonguideline** and contributes toward satisfying the intent of the study.

4. Cytotoxicity

There is data supporting cytotoxicity of acetochlor to the rat nasal olfactory epithelium. This data consists of observations of respiratory metaplasia of the olfactory mucosa and lipofucsin granules in the olfactory mucosa of rats treated with acetochlor. Additionally, supportive data from the analog alachlor.

a. Cytotoxicity data for acetochlor.

i. Respiratory metaplasia of the olfactory mucosa:

In a nonguideline study evaluating the distribution of rat nasal tissue proliferative lesions (MRID 44496214), hyperplasia, adenoma and adenocarcinoma of the nasal passages of rats exposed orally to alachlor, acetochlor or butachlor were mapped to determine site(s) of origin. Slides of nasal cavity tissue sections from the high dose male and female test groups of the 2-year dietary guideline studies on acetochlor (1750 ppm; 69 male and 70 female Sprague-Dawley rats) and butachlor (3000 ppm; 12 male and 13 female Long Evans rats) and from a one year oral non-guideline gastric initiation-promotion study (126 mg/kg; 10 male and 10 female Sprague-Dawley rats) that were previously determined to have preneoplastic lesions were reexamined by an experienced veterinary pathologist.

All three of these chloracetanilides induced morphologically similar lesions confined almost entirely to the olfactory epithelium lining in specific regions of the posterior nasal passages. Most of the benign tumors exhibited ciliation of the olfactory epithelial cells and **were associated with respiratory metaplasia of adjacent olfactory mucosa**. Many lesions were close to the olfactory-respiratory epithelial junctions. For all three compounds, these changes were apparently identical in nature and location and differed only in frequency of occurrence (comparison of frequency compromised by differences in animal sacrifice times, strains used and number of animals evaluated for each compound).

The appearance of respiratory metaplasia, described above was discussed by the two pathologists present at the meeting (J. Pletcher and D. Wolf) and they agreed that such metaplasia constituted a manifestation of cytotoxicity of acetochlor to the olfactory epithelium. Death (and loss) of the original olfactory epithelium cells results in their being replaced by the respiratory epithelium. cells, originating from differentiating stem cells.

ii. Lipofucsin pigment in the olfactory epithelium

In a 2-generation reproduction toxicity study (MRID 45357503), acetochlor was administered continuously in the diet to CD (SD) IGS BR (Sprague-Dawley) rats (26/sex/dose) at nominal dose levels of 0, 200, 600, or 1750 ppm (equivalent to 0, 21.2, 65.6, and 196.4 mg/kg/day in F1 males and 0, 22.4, 70.9, and 215.9 mg/kg/day in F1 females). F0 animals were given test article diet formulations for 10 weeks prior to mating to produce the F1 litters. On postnatal day (PND) 29, F1 animals (26/sex/dose) were selected to become the F1 parents of the F2 generation and were given the same concentration test formulation as their dams. F1 animals were given test formulations for 10 weeks prior to mating to produce the F2 litters. Histopathological evaluation (Table 9) revealed treatment-related incidences of benign proliferative lesions (focal epithelial hyperplasia and polypoid adenomata) in the epithelial lining of the ethmoid region of the nasal cavity in F0 and F1 adult animals receiving 1750 ppm acetochlor and in F1 animals at the 600 ppm level. Minimally increased brown pigment (lipofuscin) was observed in the olfactory mucosa, mainly in the lamina propria and occasionally in the basal epithelium in most animals receiving 600 and 1750 ppm in both F0 and F1 generations and also in F1 females at the 200 ppm dose level.

Table 9: Selected histopathology findings in the nasal cavity [a]

Finding and severity	Sex	Dietary Concentration (ppm)							
		F0				F1			
		Control	200	600	1750	Control	200	600	175
Nasal Cavity Examined No Abnormalities Detected	M	265	2612	263	250	2614	264	262	260
Nasal Cavity Examined No Abnormalities Detected	F	2510	254	250	250	2615	267	260	220
Nasal cavity - Polypoid adenoma (Benign)	M	0	0	0	4	0	0	3	8 [c]
	F	0	0	0	6 [b]	0	0	1	17 [d]
Nasal cavity - Hyperplasia of the olfactory epithelium (Minimal to slight)	M	0	0	0	3	0	0	0	7
	F	0	0	0	7	0	0	4	14
Nasal cavity - Hyperplasia of the respiratory epithelium (Minimal)	M	0	0	0	2	0	0	0	1
	F	0	0	0	2	0	0	0	0
Nasal cavity - Increased lipofuscin of the olfactory mucosa (Minimal to slight)	M	0	0	21	25	0	0	15	26
	F	0	11	25	25	0	9	25	22
Nasal cavity - Hyperplasia, squamous epithelium (Minimal)	M	0	0	0	0	0	0	0	0
	F	0	0	0	1	0	0	0	0

a Data extracted from Table 9 of DER for MRID 45357503.
b Includes 4 animals with single and 2 with multiple lesions.
c Includes 5 animals with single and 3 with multiple lesions.
d Includes 7 animals with single and 9 with multiple lesions.
e Minimal to moderate severity for F1 findings.

Lipofucsins are yellow-brown to reddish-brown pigments that occur widely throughout the body and are thought to be produced by an oxidation process of lipids and lipoproteins. The oxidation process occurs slowly and progressively and therefore, the pigments exhibit variable staining reactions (Bancroft and Stevens, 1996)

b. Cytotoxicity data for alachlor.

i. Alachlor *In vitro* data.

The registrant used a modification of the method of Trela and Bogdanffy (1991) to assess *in vitro* cytotoxicity. Olfactory and respiratory epithelial explants from the nasal cavity and were placed in tissue culture plates and incubated in Williams E media containing alachlor or its metabolites [2,6 diethylaniline (DEA), secondary sulfide or secondary amide]. Cytotoxicity was determined in terms of acid phosphatase released into the medium.

- Alachlor produced a statistically significant (vs controls) release of acid phosphatase with olfactory epithelium but not with respiratory epithelium.
- DEA produced a statistically significant release of acid phosphatase with olfactory and respiratory epithelium
- Secondary sulfide or amide did not produce statistically significant releases of acid phosphatase.

ii. Alachlor *In vivo* data.

Genter et al. (2002b) studied the progression of alachlor-induced olfactory tumors in rats. Male long-Evans rats were administered alachlor in the diet at levels of 0 or 126 mg/kg/day. The rats were sacrificed at 3, 4, or 5 months on the diet. Sections were taken through the ethmoid turbinates (Young's levels 3 & 4 or Mery's levels 22 & 30) and stained with H&E or Wright-Giemsa for light microscopic evaluation. Histological changes were seen in the olfactory mucosa after 3 months. "These changes consisted of respiratory **metaplasia** (i.e. replacement of olfactory mucosa by respiratory epithelium), increased cellularity and epithelial disorganization, with no evidence of cytotoxicity." Alachlor-induced neoplasms were first detected after 5 months of exposure. One of the 5 rats treated for 5 months had two neoplasms.

The pathologists present at the meeting (J. Pletcher and D. Wolf) noted at the meeting that even though Genter et al. (2002b) indicate "no evidence of cytotoxicity" in their study, the presence of "respiratory metaplasia" is indicative of prior cytotoxicity to the original olfactory epithelium.

As summarized below, studies of shorter duration than 3 months fail to show histopathological evidence of cytotoxicity.

In a cell proliferation study for alachlor (MRID 42852102) the authors found a dose-related increase in cell proliferation in the nasal turbinate epithelium, but did not find evidence of cytotoxicity in spite of looking for it.

• In an initial experiment (EHL 87112), female LE rats were administered via the diet at 0, 1, 126, or 252 mg alachlor/kg/day for 10, 30 or 60 days. The rats were administered ^{3}H- thymidine (ip) for evaluation of cell proliferation in nasal turbinates. Tissue sections were processed for microautoradiography and stained with nuclear Fast Red. Although, dose- related and statistical significant increases in cell proliferation were seen [e.g 0, 1.46, 4.10** , and 8.58** labeled cells/field at 60 days], no signs of cytotoxicity were seen at any dose level.

• In another experiment (EHL 90059), groups of Female LE rats were administered via the diet 0, 0.5, 2.5, 15, 42, or 126 mg alachlor/kg/day for 60 days and evaluated with ^{3}H-thymidine (ip) for cell proliferation in nasal turbinates. Two other groups of rats were treated with 0 and 126 mg alachlor/kg/day for 60 days and then placed on basal diet for another 60 days. Separately from processing for microautoradiography, nasal tissue from 3 rats/dose/time point was embedded, sectioned, and stained with H&E, nuclear Fast Red or thionin for histological examination of cytotoxicity. Although, dose-related and statistical significant increases in cell proliferation were seen in the olfactory epithelium, no signs of cytotoxicity were seen at any dose level in any section examined.

In connection to study EHL 90059, the authors noted that "the respiratory and olfactory portions of the nasal epithelium as well as the respiratory/olfactory junction were **specifically examined** for evidence of cytotoxicity by histological procedures. In no animal were cytological changes detected by the techniques employed. The nasal tumors induced by alachlor in the chronic rat feeding studies were generally very small (microscopic in size and only one or two occurred in the entire nasal mucosa. Therefore the likelihood of finding a cell with significant cytotoxicity or preneoplastic changes in this short term study is very small."

Wetmore et al. (1999) evaluated histopathology and cell proliferation nasal olfactory epithelium of rats treated ip with alachlor. Male Long-Evans rats were administered alachlor (ip, 0 or 126 mg/kg/day) for 1, 4, and 28 days (5 days per week). The animals were sacrificed 24 hours after the last dose. Two hours prior to sacrifice the rats received BrdU (ip) for evaluation of cell proliferation. There was no evidence of cytotoxicity (histological, H&E stain) or increased cell proliferation (immunohistochemical evaluation of S-phase cells) following examination of level III sections of nasal ecto- or endoturbinates.

iii. Genomics data for alachlor (oxidative damage)

In addition to the *in vitro* and *in vivo* cytotoxicity data presented above for alachlor, there is information that alachlor affects the redox status of the cell, leading to oxidative stress and which may result in DNA damage. Oxidative damage to DNA may lead to cytotoxicity followed by regenerative cellular proliferation (Clayson et al. 1994).
Based on a genomic analysis of **alachlor**-induced tumors in the olfactory mucosa of Long-Evans rats exposed to 126 mg/kg/day and sampled at various intervals from 1 day to 18 months, Genter et al. (2002b) proposed the following steps in the **alachlor**-mediated carcinogenesis model:

- **Initial progression from histologically normal olfactory mucosa to foci of abnormal**

mucosa

This step, which is regulated by genes in the acute phase of exposure, is accompanied by "upregulation" (≥2-fold increase) of genes consistent with a mutagenic response possibly as a result of oxidative damage to DNA (**↑GADD 45, apurinic/apyramidinic endonuclease).** While the exact role of GADD (growth arrest and DNA-damage inducible) gene products is not known, this gene group is upregulated in response to stress to allow cells time to repair macromolecular damage or to lead cells into apoptosis so that a genetic defect is not propagated. Types of environmental stress that induce GADD genes include UV irradiation, alkylating agents and glucose starvation (Takahashi et al. 2001; Jackman et al. 1994). Stokes et al. (2002) also demonstrated that GADD 45 gene induction occurs in response to reactive oxygen species (ROS) and quinones and is abolished in the presence of the antioxidant, ascorbic acid. It is of note that quinones, which are operationally non-genotoxic (Clayson et al., 1994), are highly redox active molecules which can redox cycle with their semiquinone radicals, leading to formation of ROS, including superoxide, hydrogen peroxide, and ultimately the hydroxyl radical. Production of ROS can cause severe oxidative stress within cells through the formation of oxidized cellular macromolecules, including lipids, proteins and DNA (Bolton et al. 2000). Supporting the hypothesis of oxidative stress, Genter et al.(2002a), also observed upregulation of other genes associated with oxidative stress, [***i.e.,*** **heme oxygenase** (Otterbein et al. 2000), **glutathione synthase and metallothionein** (Andrews 2000)].

- **Progression from histologically altered olfactory mucosa to the development of adenomas**

This step was accompanied by expression of genes indicating inhibition of apoptosis **[Bid3(AI102299)]** and enhancement of cell proliferation **(zyxin)**. It is of note that Sarafian and Bredesen (1994) state that ROS can serve as common mediators of apoptosis.

- **Progression to a malignant adenocarcinoma phenotype**
This phase was indicated by induction of genes related to the **wnt signaling pathway**, which are generally upregulated late in the carcinogenesis process.

- **Transformation to adenocarcinomas**

In the late stages of tumor progression, the activation of **nuclear ß-catenin genes,** which is critical for tumor formation in other organs and is associated with mutations in the **wnt pathway**.

Several other studies support a role for oxidative stress in **Alachlor**-induced toxicity. Burman et al. (2003) show that dietary exposure of Long-Evans rats to 126 mg/kg/day for 1 day caused an ~20% depletion of the olfactory mucosa antioxidant, GHS followed by a significantly ($p<0.001$) increased expression of genes associated with increased GHS production after 2 and 4 days of treatment. A return to control values was seen by 10 days of treatment. A pattern somewhat similar to GSH was observed for ascorbate in the olfactory tissue of 126-mg/kg/day

male rats (*i.e.,* initially, a significant decrease 1 day post-treatment, followed by significant increases 2 and 4 days after dosing). In contrast to the GSH data, there was a reduction in ascorbate at 10 days. We noted, however, that the response with either antioxidant was not dose related. From these results, the investigators concluded that, "Despite the fact that GSH levels recovered, acute antioxidant perturbations may have been sufficient to trigger other steps in the carcinogenic process. Therefore, acute depletion of GSH and ascorbate may trigger more sustained events involved in both the initiation and promotion of the carcinogenic process."

There is also evidence of the ability of **alachlor** to induce oxidative stress in other tissues. Bagchi et al.(1995) evaluated the potential of **alachlor** to induce oxidative stress and oxidative tissue damage, as measured by production of lipid peroxidation and DNA-single strand breaks (SSB), in the liver and brain of Sprague-Dawley rats administered two equal oral doses (at 0 and 21 hours) of 300 mg/kg. As noted by Clayson et al. (1994), SSB are considered by to be a good indicator of oxygen damage to DNA. Results from the study of Bagchi et al. (1995) show that **alachlor** induced moderate lipid peroxidation in liver and brain tissues and SSB in brain but not liver DNA in samples harvested 24 hours after exposure to the first dose. The same authors also conducted *in vitro* studies of chemiluminescence on liver and brain homogenates, and found that 1nmol/mL **alachlor** induced 3-fold increases in chemiluminescence in both tissues further suggesting that **alachlor** induced ROS. Finally, the results from *in vitro* studies with cultured PC-12 neuroactive cells exposed to 100 nM **alachlor** illustrate the sequence of early events postulated for this MOA (generation of ROS →DNA damage →tissue damage) with a 2-fold increase in DNA-SSB and a 3-fold increase in LDH leakage. Although olfactory nasal tissue was not examined in this series of assays, the ability of **alachlor** to generate ROS with subsequent DNA damage and tissue damage both *in vivo* and *in vitro* has been established. Finally, Bagchi et al. cite the work of Akubue and Stohs (1991) showing that the oral administration of 800 mg/kg **alachlor** to rats caused the increased urinary excretion of the "oxidative lipid metabolites, malondialdehyde, formaldehyde, acetaldehyde and acetone".

Based on the above considerations, the postulated MOA (generation of ROS →DNA damage →tissue damage → cell proliferation→olfactory nasal tumors) in rats is plausible and coherent. An additional factor favoring this MOA is the evidence of weak and sporadic mutagenic effects, generally seen only at concentration near or at cytotoxic concentrations. Nevertheless, these conclusions are based solely on data for **alachlor**. Similarly, the characterization of the hypothesized early events for this MOA draws heavily on the cDNA microarray findings of Genter et al. (2002a) for **alachlor** which have not as yet been independently confirmed. Additionally, there are no data available on acetochlor to fully test the plausibility of oxidative stress as an early and critical event leading to frank tumor formation in rat nasal olfactory tissue. Since oxidative damage to DNA induced by ROS is considered a mechanism related to carcinogenesis especially by operationally non-mutagenic carcinogens (Clayson et al. 1994), it may be prudent to test acetochlor for ROS formation. A possible analysis could be the production of increased 8-hydroxy-2'-deoxyguanosine (8-OHdG), one of the known markers of cellular oxidative stress during carcinogenesis (Kasai et al. 1997).

5. Cell Damage

The observation of respiratory metaplasia of the olfactory epithelium in rats treated with acetochlor, alachlor or butachlor (MRID 44496214) is indicative of death of olfactory cells and replacement of these cells by respiratory epithelial cells, differentiating from stem cells. The presence of lipofucsin granules in olfactory epithelium of rats treated with acetochlor in the diet for 3 months at 200 -1750 ppm (MRID 45357503) is a reflection of oxidative damage to cell membranes. This data are supported by observations of respiratory metaplasia produced by the analog alachlor in rats treated with alachlor in the diet at 126 mg/kg/day for 3 months in a study by Genter et al. (2002b).

6. Cell Replacement/Increased cell turnover

The following data indicate that Acetochlor significantly increased cell proliferation in the olfactory region of the nasal turbinates in rats but not in mice. Acetochlor, however, significantly increased the rate of cell proliferation in mouse liver.

In a nasal cell proliferation study (MRID 44496207), acetochlor (95.2%a.i.) was administered to male Sprague-Dawley rats in the diet at concentrations of 0, 1750, and 5000 ppm. The two higher levels of acetochlor were carcinogenic to rats in a chronic study (see Table 6). Cell proliferation was measured after 60, 90 and 160 days of treatment in nasal turbinate respiratory and olfactory epithelium by measuring the DNA incorporation of ^{3}H-thymidine. ^{3}H-thymidine was administered IP for three consecutive days prior to sacrifice. In a separate study, nasal cell proliferation was also measured ~160 days with bromodeoxyuridine incorporation in rats receiving 0, 200, 1750 and 5000 ppm acetochlor in the diet.

Acetochlor (Table 10) significantly increased cell proliferation in the olfactory region of the nasal turbinates in rats administered 5000 ppm acetochlor in the diet for 60 days. Cell proliferation was also significantly increased at 90 and 160 days in the 1750 ppm and 5000 ppm treatment groups. There were no significant increases in cell proliferation in the respiratory region at any of the time points or doses tested.
In a non-guideline nasal olfactory and respiratory epithelial cell proliferation study (MRID 44496209), acetochlor (95.2% ai, lot/batch # T940059, MUS-9308-5458-T) was administered to 26 male CD-1 mice/dose in the diet at concentrations of 0, 1000, or 5000 ppm (equivalent to 0, 166.6, or 887.9 mg/kg bw/day) for 60 and 90 days. None of the mice died during the study. Acetochlor had no effect on nasal cell proliferation in mice administered acetochlor in the diet at 1000 or 5000 ppm for 60 days

These cell proliferation studies on acetochlor are supported by previous work done with the analog alachlor. As summarized in Table 11, alachlor produced dose-related increases in nasal cell proliferation in rats, with statistical significance after 60 days at the higher doses and after 30 days at the highest dose. Table 12 shows dose-related increases in nasal cell proliferation reaching statistical significance at the highest dose of alachlor (126 mg/kg/day) after 60 days on the diet. Table 12 shows that the cell proliferation effect of alachlor is limited to the olfactory epithelium and is reversible in rats returned to the basal diet after 60 days of treatment.

Table 13 (from Tables 6 and 10) compares doses for nasal adenoma formation in rats with doses for cell proliferation.

Table 10. Nasal cell proliferation in male SD rats treated for 60, 90, or 160 days with **acetochlor** (MRID 44496207).

Treatment (ppm)	No. of labeled cells/0.2 mm of basement membrane							
	Respiratory epithelium				Olfactory epithelium			
	^{3}H-Thymidine label			BrdU label	^{3}H-Thymidine label			BrdU label
	60-day	90-day	160-day	160-day	60-day	90-day	160-day	160-day
0	2.80 ± 2.37	1.37 ± 0.63	0.52 ± 0.23	2.43 ± 0.54	4.23 ± 2.37	4.75 ± 1.03	3.48 ± 0.55	7.17 ± 1.92
250	-	-	-	2.23 ± 0.34	-	-	-	6.25 ± 1.73
1750	1.37 ± 1.01	1.28 ± 0.59	0.79 ± 0.82	2.53 ± 1.12	3.81 ± 1.42	6.37 ± 1.06**	5.24 ± 0.92**	9.78 ± 1.80**
5000	2.07 ± 1.71	1.14 ± 0.38	0.86 ± 0.29	2.61 ± 0.73	6.66 ± 2.08*	7.90 ± 1.07**	7.06 ± 1.39**	11.46 ± 1.85**

Table 11. Cell proliferation at the respiratory-olfactory junction in the nasal turbinates of rats fed **alachlor** (MRID 42852102).

Dose (mg/kg/day)	Mean No. labeled cells/field ± SEM at various days on the diet			
	1 day	10 days	30 days	60 days
0	0.74 ± 0.2	0.30 ± 0.17	2.60 ± 0.08	0.76 ± 0.27
1	0.64 ± 0.17	3.48 ± 0.60*	2.02 ± 0.25	1.46 ± 0.28
126	0.82 ± 0.30	4.54 ± 1.15**	3.44 ± 1.03	4.10 ± 0.92**
252	0.34 ± 0.14	6.06 ± 0.94**	6.80 ± 0.48**	8.58 ± 0.59**

* = $p<0.05$, ** = $p<0.01$

Table 12. Effect of 60 day **alachlor** diet or 60 day **alachlor diet** followed by a 60 day recovery diet on cell proliferation in the respiratory and olfactory epithelia of the nasal turbinates of L-E female rats (MRID 42852102).

Dose (mg/kg/day)	Mean No. labeled cells/field ± SEM at various periods on the diet			
	Respiratory region		Olfactory region	
	60 days	60 days + 60 days recovery[1]	60 days	60 days + 60 days recovery
0	1.0 ± 0.2	0.8 ± 0.4	1.0 ± 0.3	0.8 ± 0.2
0.5	1.8 ± 0.6	-	0.6 ± 0.2	-
2.5	0.5 ± 0.1	-	1.4 ± 0.3	-
15	1.5 ± 0.7	-	1.2 ± 0.4	-
42	1.7 ± 0.5	-	2.9 ± 0.9*	-
126	0.7 ± 0.2	0.4 ± 0.0	3.2 ± 1.1*	1.0 ± 0.2

* = $p<0.05$, ** = $p<0.01$

[1] The animals were maintained in the alachlor diet for 60 days and then placed in alachlor-free diet for another 60 days.

III. DATA EVALUATION FOR MODE OF ACTION

In its evaluation of the MOA for the subject chemical, the CARC applied the 'IPCS Conceptual Framework for Evaluating a Mode of Action for Chemical Carcinogenesis', developed by the International Programme on Chemical Safety, Geneva, Switzerland (Sonich-Mullin et al., 2001) The results of such an evaluation are summarized below.

1. Introduction

Previous pages have summarized acetochlor data submitted in support of a non-genotoxic MOA for the induction of tumors of the nasal olfactory epithelium in rats.

Although acetochlor also produces tumors at other sites in rats such as thyroid follicular cells and liver, this document covers only the MOA for nasal tumors in rats. The MOA for thyroid follicular tumors is discussed in the Cancer Assessment Document for Acetochlor (Fourth Evaluation) for the meeting dated April 21 and 22, 2004 (Part 1).

As summarized in Table 6, the endpoint of nasal tumors is clearly attained at the higher doses in three separate rat chronic rat studies, with the unexplained exception of females in Study #1. Although the Registrant's Study Report tables do not state explicitly that the nasal tumors originate from the nasal olfactory epithelium, there is evidence that the tumors originate in the olfactory portion of the nasal epithelium of the rats.

- To determine if there was a similarity in morphology, origin, and location of proliferative lesions (MRID 44496212), the Registrant conducted a review of hematoxylin/eosin slides of nasal tissue of rats treated with acetochlor, alachlor or butachlor in previously conducted long-term oral studies. In the case of acetochlor, slides from study #3 (88/SUC017/0348, MRID 41592004) of Table 6 were used. Among other findings , it was determined that all three of the chloracetanilides induced morphologically similar lesions confined **almost entirely to the olfactory epithelium lining** in specific regions of the posterior nasal passages.

- In rat chronic study #1 in Table 6 (conducted at 0, 500, 1500, & 5000 ppm) (MRIDs 00131088 and 40484801) no nasal tumors were initially found because sections had been taken from the anterior part of the nasal cavity. When new histological sections were taken so as to include the posterior region of the nasal cavity, the tumors reported in Table 6 for study #1 were seen. The posterior region of the nasal cavity in rats is essentially olfactory epithelium, in contrast to respiratory epithelium, which is found in the more anterior part of the nasal cavity (Young,1981).

Nasal tumors were also seen in rats treated with the sulfoxide metabolite of acetochlor. No nasal tumors were seen in acetochlor-treated mice in two acceptable cancer studies.

2. Postulated mode of action (theory of the case)

The postulated MOA for the induction of nasal tumors by acetochlor in rats involves the following steps:

i) Acetochlor conjugates with glutathione (GSH) and is excreted in the bile.
ii) The conjugate is biotransformed to a series of sulfur-containing products. Enterohepatic circulation of these products creates a pool of metabolites that are delivered to the nose.
iii) Biotransformation to tissue-reactive and toxic metabolites. Metabolism by nasal enzymes, results in formation of a benzoquinoneimine, an electrophile and redox-active molecule.
iv) Binding to cellular proteins plus possible generation of oxidative stress .
v) Cytotoxicity
vi) Regenerative cell proliferation.
vii) Sustained cytotoxicity and cell proliferation that results in neoplasia.

These steps are summarized in Figure 5 (same as Figure 1).

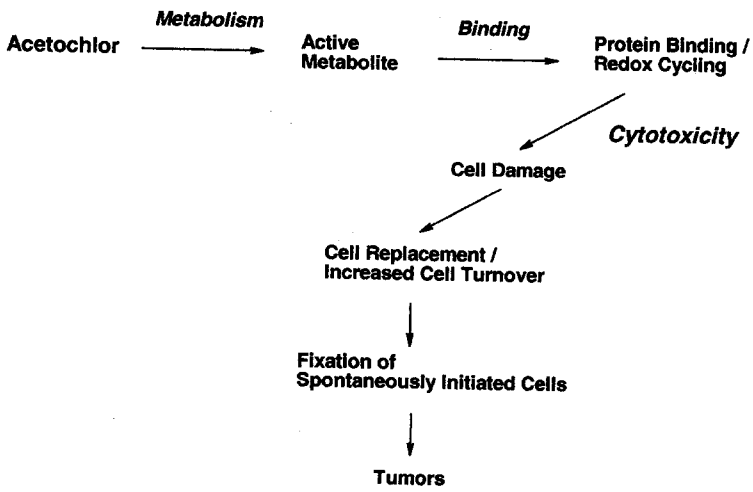


Figure 5. Postulated MOA of acetochlor in the induction of nasal tumors in rats

3. Initial events

As discussed in the metabolism section, there is strong evidence that the compound is well absorbed (high urinary excretion and other facts), is conjugated with GSH (major metabolites can be traced to GSH conjugation, e.g. mercapurates, sulfoxide etc.), undergoes enterohepatic recirculation (80% biliary excretion & over 70% urinary excretion), appears in plasma (e.g. the sulfoxide metabolite is found in rat plasma). Additional information (autoradiography) indicates that acetochlor and/or its metabolites distribute to the nasal turbinates, in particular to the olfactory epithelium (site of the nasal tumors) with no label at the respiratory epithelium. Autoradiographic evidence indicates that although, initially, radioactivity from acetochlor and its sulfide metabolite distributes widely, there is still significant residual radioactivity in the nasal turbinates and low background radioactivity in surrounding tissues, several days after a single oral dose. The mouse and other species do not show this pattern of nasal epithelium labeling.

4. Key events

The following three events are considered key for formation of nasal tumors by the proposed MOA:

QUINONE IMINE- FORMATION (PROTEIN BINDING) → CYTOTOXICITY → CELL PROLIFERATION

There is ample evidence that Acetochlor is metabolized to precursors of the quinone-imine.:

-After dosing with acetochlor, analysis of protein adducts obtained from nasal olfactory tissues reveals that hydrolysis of these adducts releases EMIC-cysteine (Figure 4). This product is consistent with a nucleophilic attack by an SH group in a protein on a quinone-imine [formed from EMA (path A) or sec-methyl sulfoxide metabolite (path B)].

- Administration *in vivo* of sec-methyl sulfide or sulfoxide metabolite of acetochlor (Putative precursors of nasal protein adducts) produces adducts that release EMIC-cysteine.

- Incubation of rat nasal tissue microsomal preparations will produce precursors of quinoneimines given appropriate substrates (e.g. sec sulfide, MEA)

- Administration of the sulfoxide metabolite of acetochlor (the postulated quinone imine precursor) to rats (see Figure 10b) produces statistically significant incidences of nasal polypoid adenomas after 26 weeks of treatment. Similar incidences were seen after 52 weeks.

Although it is likely that in the rat the quinone-imine is formed from the sulfoxide metabolite, the possibility that EMA might also be a precursor cannot be ruled out. Figure 2 indicates that more than 20% of the metabolites of acetochlor are not identified. Furthermore, the analysis of adducts

cannot distinguish whether the adduct originated from the sulfide or EMA. This issue is important since *in vitro* work of Coleman et al. (2000) indicates that human liver microsomes are as effective as the rat in forming EMA from acetochlor.

There is support for the idea that the quinone imine has to be generated locally (within the cell)

Consideration of high reactivity of the quinone imine coupled to the localized distribution of the label indicates that the active species must be formed at the site. If the active species were not locally activated at or within the cell, one would observe a very spread out pattern of labeling. This contention is supported by *in vivo* studies with N-acetyl-p-benzoquinone imine (NAPQI, the putative active species in the production of liver toxicity in mice dosed with acetaminophen). BALB/c mice were administered pure NAPQI by the ip route. At necropsy, liver cells were normal, with no apparent necrosis, and SGPT levels were normal. However, the blood was extensively coagulated, indicating that little or nothing reaches the liver, most of the material is used up in the intervening tissue fluids (Dahlin and Nelson (1982)).

There is support that the quinone imine is formed in the rat nasal epithelium in a dose related manner, but not in the mouse.

As summarized in Table 7, EMIQ-cysteine (indicator of quinone imine binding, or in general of its presence) is formed in a dose related manner. These levels of binding were seen in rats administered the test diet for 14 days at tumorigenic doses. There are no acetochlor data, however, to determine if there is a NOAEL for this binding. In experiments conducted in mice no EMIQ-cysteine was found, but EMA (indicative of direct Chlorine substitution) was found. The absence of quinone imine binding in the mouse (Table 8) is consistent with the negative autoradiography studies with the mouse and the negative nasal tumor results seen in the mouse chronic studies.

There is support for Cytotoxicity.

Re-reading of the slides (See MRID 44496214) for the 1988 acetochlor rat chronic study (MRID 41592004) and for butachlor (MRID 42244901) and alachlor (MRID 43590001) studies indicated that most of the benign tumors were associated with respiratory metaplasia of adjacent olfactory epithelium. This effect implies disappearance (death) of olfactory epithelium and replacement with respiratory epithelium. Furthermore, in a 2-generation reproductive toxicity study (MRID 45357503), F_0 and F_1 adult rats showed lipofucsin granules in the olfactory epithelium. These results are consistent with an oxidative process affecting cellular lipids and lipoproteins.

There is also additional supportive evidence for cytotoxicity in studies reported in the literature.

In a study by Genter et al. (2002b) with rats dosed with acetochlor at 126 mg/kg/day, it was noted that "Histological changes were seen in the olfactory mucosa after 3 months of dietary alachlor exposure. These changes consisted of respiratory metaplasia (i.e. replacement of olfactory mucosa by respiratory epithelium), increased cellularity and epithelial disorganization, **with no**

evidence of cytotoxicity." Although these authors note "**no evidence of cytotoxicity**", their remarks suggest that there is actually evidence of cytotoxicity if:

- One interprets the phrase "epithelial disorganization" as a sign of cytotoxicity. In fact the expression "disorganization of the epithelial cell layer" was used as one of the characterizing features of the lesions seen in nasal mucosa of rats dosed with phenacetin in a study by Bogdanffy et al.(1989).

- One notes that there was "respiratory metaplasia", which requires disappearance of the olfactory epithelium cells to be replaced by respiratory epithelium cells.

Additionally, Wetmore et al. (1999) observed from their study of Long-Evans male rats dosed intraperitoneally with 126 mg/kg/day **alachlor** (prepared in dimethyl sulfoxide) for 1, 4 or 28 days that the lack of a cytotoxic and regenerative cell proliferation response in the nasal mucosa at an **alachlor** dose equivalent to a tumorigenic dose distinguishes **alachlor** from other nasal carcinogens. While Wetmore et al. considered the finding to be unusual, they state, "this observation is not unprecedented for chemical carcinogens in general". The authors further indicated that a recent survey of the carcinogenic compounds identified by the National Toxicology Program (NTP) revealed that approximately 44% do not cause cytotoxicity or enhance cell proliferation in the target tissue.

This is perhaps not unexpected for certain nasal carcinogens since D. Wolf (2004, private communication) indicates that equating cytotoxicity with the presence of dead cells in the epithelium lining of the nasal cavity is almost impossible because of the architecture of the lumenal structure. The entire nasal cavity is lined with a single layer of pseudostratified epithelium. The nasal cavity appears to be a couple of cell layers thick, however, all cells within the structure are attached to the basement membrane. When cells die, they pull away from the basement membrane and slough off. Since they are in a lumenal structure, they fall off into the air. Hence, one does not always see cells undergoing necrosis or apoptosis in tissue sections. It is, therefore, assumed that cell proliferation parameters such as BrdU labeling indices only increase when lost cells are being replaced. In general, increased labeling without significant hyperplastic expansion is considered secondary to loss of cells through necrosis or apoptosis. This is seen in the urinary bladder and kidney as well as the nose. With chronic treatment, the only indication of cytotoxicity is the increased proliferation rates. Data presented earlier from the nasal cell proliferation study with dietary administrations of 1750 or 5000 ppm acetochlor to Sprague-Dawley rats (MRID 44496207) support Wolf's position. Since the increase in cell proliferation, which was time and dose-dependent, is most likely due to cytotoxicity to the nasal turbinates, the sustained increase in cell proliferation is a key event in the induction of nasal olfactory tumors in rats.

Finally, if one accepts that a quinone imine has been formed inside of the nasal epithelium and the very high reactivity of the quinone imines [as electrophiles and oxidants, See Monks and Jones, 2002), then cytotoxicity is to be expected. Several studies found in the open literature suggest that DABQI may induce damage to DNA through oxidative stress. This is a reasonable effect to pursue since oxidative damage to DNA may lead to cytotoxicity followed by regenerative

proliferation (Clayson et al., 1994).

There is support for nasal olfactory epithelium cell proliferation.

As summarized in Table 10, and graphically in Figures 6 and 7 acetochlor induces nasal olfactory cell proliferation. Figure 6 (using H^3- thymidine label) shows a statistically significant increase in cell proliferation vs controls at 1750 and 5000 ppm after 90 or 160 days of treatment. The statistically significant increase in cell proliferation vs controls is evident only at 5000 ppm after 60 days of treatment, with a NOAEL of 1750 ppm for this time period of treatment. Figure 7 (using BrdU label) shows a statistically significant increase in cell proliferation vs controls at 1750 and 5000 ppm after 160 days of treatment, with a NOAEL at 250 ppm. Taken together, Figures 6 and 7 point to a time relation for the NOAEL for cell proliferation: As the time of treatment increases, the NOAEL decreases (See Figure 8).

Although cell proliferation NOAEL values are defined for cell proliferation at two dose levels, there is no direct cell proliferation data to assure that the lowest of them (250 ppm) will remain a NOAEL for longer treatment periods than 160 days.

Examination of Table 10 indicates that the response is limited to the olfactory epithelium, consistent with the binding data. Table 13 lists nasal tumors and cell proliferation to facilitate comparisons.

Table 13. Values for nasal adenoma formation[a] and nasal olfactory epithelium cell proliferation at various dietary ppm levels of acetochlor. (This table was compiled from Tables 6 and 10 as an aid in determining a POD.) Data are for male rats only.

Effect	**No. adenomas/total examined** or **No. labeled cells/0.2 mm of basement membrane**										
	0	18	40	175	200	250	500	1000	1500	1750	5000
NasalTumors:: (3 studies) :											
PR-80-006	0/69	-	-	-	-	-	1/70	-	6/69**	-	18/69**
ML-83-200	1/58		0/54		0/58	-	-	12/59**	-	-	-
CTL/C/2191	0/69	0/59	-	0/59	-	-	-	-	-	35/70**	-
Cell Prolif. 60Days	4.2±2.4	-	-	-	-	-	-	-	-	3.81±1.4	6.66±2.1*
Cell Prolif. 90D	4.75±1	-	-	-	-	-	-	-	-	6.37±1.1**	7.9±1.1**
Cell Prolif. 160D (^{3}H-Thymidine	3.48±0.6	-	-	-	-	-	-	-	-	5.24±0.9**	7.06±1.4**
Cell Prolif. 160D (BrdU)	7.17±1.9	-	-	-	-	6.25±1.7	-	-	-	9.78±1.8**	11.46±1.9**

[a] The tumor data above refer to Chronic rat studies in Table 6.
B " - " means not tested ; * = p≤0.05; ** p≤0.01

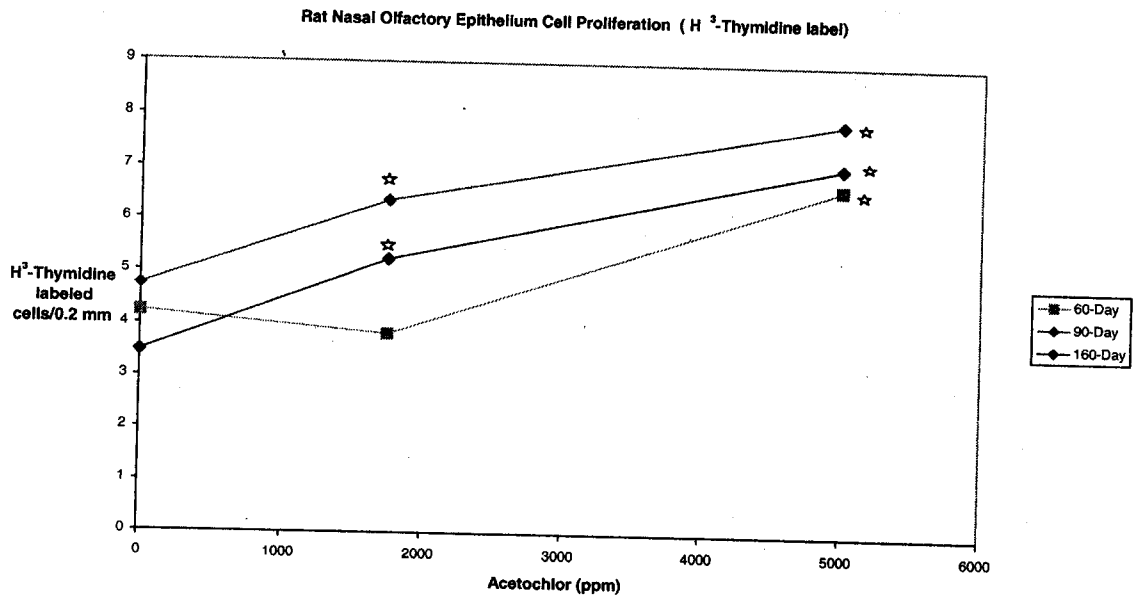


Figure 6. Cell proliferation dose response (H^3-Thymidine label) to various feed levels for 60, 90 or 160 days. Data from Table 5. * = $p \leq 0.05$

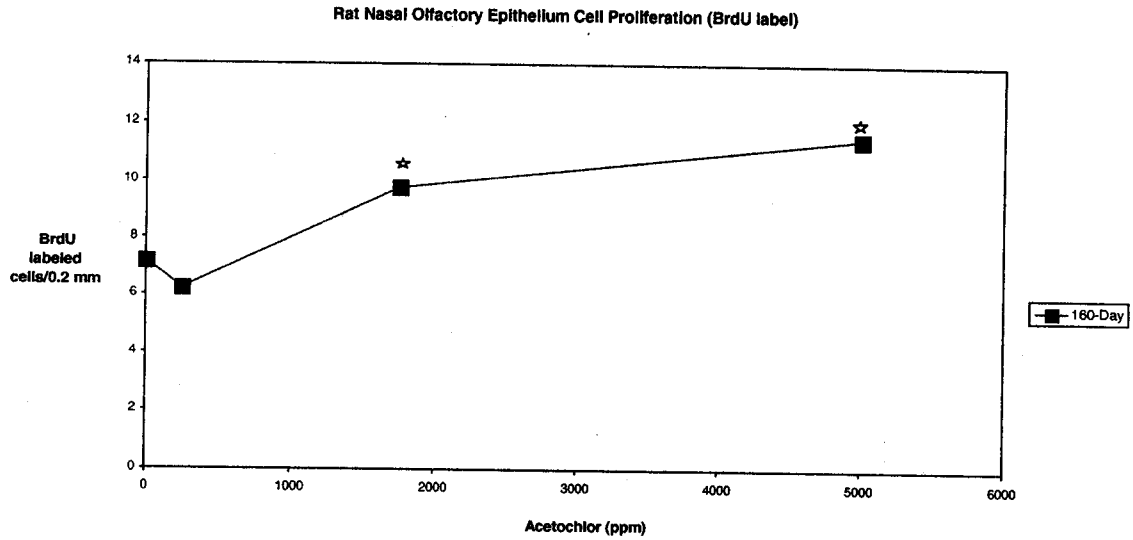


Figure 7. Cell proliferation dose response (BrdU label) to various feed levels for 160 days. Data from Table 5. * = $p \leq 0.05$

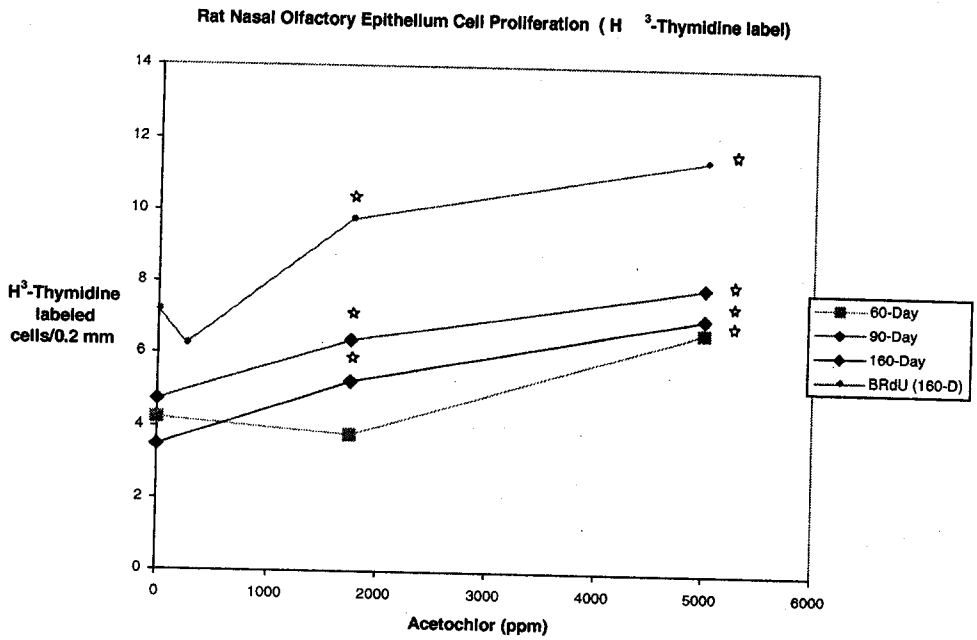


Figure 8. Same Figure 6, with the results of Figure 7, superimposed on it.
* = p≤ 0.05

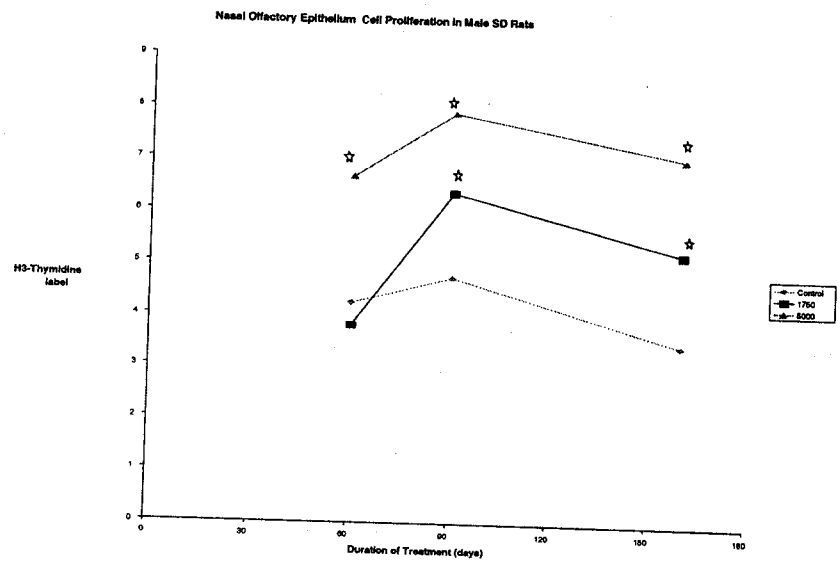


Figure 9. Effect of duration of treatment on cell proliferation. Values in Figure 6 were re-plotted using the duration of treatment in the x-axis. There is one curve for each dose level.

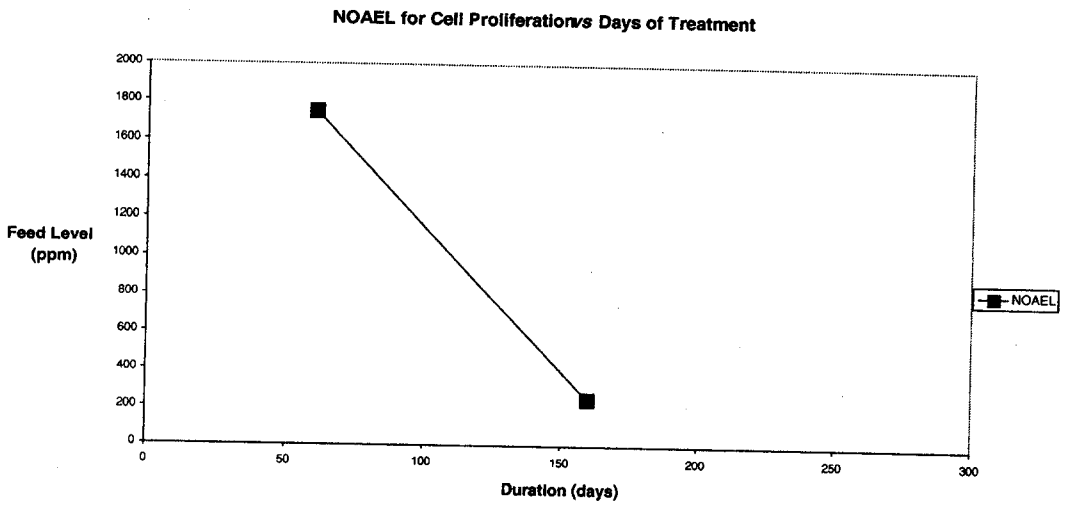


Figure 10a. NOAEL for cell proliferation vs days of treatment

5. Dose-response relationship

Several observations support a dose response relationship for the key events and the tumor endpoint.

a. Autoradiographic evidence indicates that when there is distribution to the nasal tissues and binding to the nasal turbinates (case of the rat), there are tumors. When there is no binding (presumably little or no dose of quinone imine, i.e. 0 dose), there are no tumors, such as in the mouse.

b. Chemical identification of bound residues indicates that the tumors are seen in species where the quinone imine is bound to proteins of nasal olfactory epithelium. When there is quinone imine binding, like in the rat, there are tumors. When there are undetectable levels of binding (i.e. no or undetectable levels of quinone imine) as in the mouse, there are no tumors.

c. Examination of Figures 6 and 7 reveals a dose-related increase in cell proliferation in the nasal olfactory epithelium of rats at doses coinciding with tumorigenic doses of acetochlor. In contrast, acetochlor does not produce cell proliferation in mice.

d. Examination of Table 14 indicates a dose-related increase in lipofucsin in the olfactory mucosa in both sexes. Lipofucsin formation is considered to be an indication of oxidative damage, which increases as a function of dose.

e. Using data from the analog alachlor (MRID 45852102), Table 8, it was shown that administration of alachlor in the diet for 60 days at tumorigenic doses resulted in significantly increased cell proliferation in the olfactory epithelium. Upon removal of the test material, cell proliferation had reverted to control levels after 60 days in basal diet.

Table 14: Histopathology findings in the nasal cavity [a]

Finding and severity	Sex	Dietary Concentration (ppm)							
		F0				F1			
		Control	200	600	1750	Control	200	600	175
Nasal Cavity Examined	M	26	26	26	25	26	26	26	26
No Abnormalities Detected		5	12	3	0	14	4	2	0
Nasal Cavity Examined	F	25	25	25	25	26	26	26	22
No Abnormalities Detected		10	4	0	0	15	7	0	0
Nasal cavity - Polypoid adenoma (Benign)	M	0	0	0	4	0	0	3	8 [c]
	F	0	0	0	6 [b]	0	0	1	17 [c]
Nasal cavity - Hyperplasia of the olfactory epithelium (Minimal to slight)	M	0	0	0	3	0	0	0	7

Nasal cavity - Hyperplasia of the respiratory epithelium (Minimal)	F	0	0	0	7	0	0	4	
	M	0	0	0	2	0	0	0	
Nasal cavity - Increased lipofuscin of the olfactory mucosa (Minimal to slight)	F	0	0	0	2	0	0	0	
	M	0	0	21	25	0	0	15	2
Nasal cavity - Chronic inflammation, nasolacrimal duct (Minimal to slight [e])	F	0	11	25	25	0	9	25	2
	M	12	13	8	8	10	17	14	1
Nasal cavity - Rhinitis (Minimal to slight)	F	14	18	9	9	10	15	20	1
	M	12	4	4	12	4	8	7	2
Nasal cavity - Hyperplasia, squamous epithelium (Minimal)	F	5	2	3	7	3	1	0	0
	M	0	0	0	0	0	0	0	0
Brain - Astrocytoma	F	0	0	0	1	0	0	0	0
	M	--	--	--	--	--	--	--	--
	F	--	--	--	--	--	--	--	1

a Data extracted from Tables 63 and 64 of the test report, **MRID 45357503,** (pages 223, 227, and 231).
b Includes 4 animals with single and 2 with multiple lesions.
c Includes 5 animals with single and 3 with multiple lesions.
d Includes 7 animals with single and 9 with multiple lesions.
e Minimal to moderate severity for F1 findings.

6. Temporal association

The database to assess the criterion of temporality is not complete. However, there are data to infer a sequence in time.

- The events associated with tissue distribution, binding, and genomic events are very early events. Radioautography of acetochlor-dosed rats shows that distribution and binding to nasal turbinates takes place after a single dose. If the binding is interpreted as due to the formation of a quinone imine, then one may say that the key event of quinone imine formation is taking place starting with the initial dose. In experiments with the analog alachlor, binding was seen to increase with time. It was found that nasal protein adducts (DEIQ-cysteine) increased with time in rats dosed for up to 2 weeks with C^{14}-labeled alachlor.

- Acetochlor produced significantly increased cell proliferation in cells of the nasal olfactory epithelium after 60 days (approximately 8 weeks) of treatment. There are no data to assess the earliest time of the proliferative response for acetochlor. However, Genter et al. (2000) reported no increase in cell proliferation after 1 month of dosing in rats treated with alachlor at a tumorigenic dose. Cell proliferation in the olfactory mucosa was seen by these authors at 6

months of alachlor exposure, at which time there were already nasal masses in 7 of 15 animals.

Although the time-frame for progression of acetochlor-induced olfactory mucosal tumors has not been studied, that of alachlor has been studied. Genter et al. (2002b) studied the progression of alachlor-induced olfactory mucosal tumors in rats. These authors did not observe histological changes after one month of treatment. At 3 months they reported respiratory metaplasia (i.e. replacement of olfactory mucosa by respiratory epithelium), increased cellularity, and epithelial disorganization. Alachlor-induced olfactory mucosal neoplasms were detected after 5 months of exposure.

The time-frame for tumor-progression for acetochlor-induced olfactory mucosal tumors is not known; however, short term data suggest that the time frame might not be too different from that of alachlor. In the case of acetochlor, polypoid adenomas of the olfactory epithelium have been observed in F0 and F1 animals in a rat multigeneration reproduction study with acetochlor. Additionally, preliminary data from a 1-year rat feeding study using the sulfoxide metabolite of acetochlor show a statistically significant increase in nasal polypoid adenomas in rats treated for 26 weeks (about 6.5 months).

Thus, one may conclude that there appears to be a time line for tumor formation with acetochlor that is consistent with the available data: early production and manifestation of the quinone imine and its effects, cell proliferation (60 days or less) that is reversible, and then tumor formation.

7. Strength, consistency and specificity of association of ultimate toxic effect with key events

a. There is consistency of formation of active species, tissue binding, and tumor formation site for acetochlor. The binding effect, like the tumors, is limited to the nasal olfactory epithelium.

b. The mouse, which does not bind the quinone imine to nasal olfactory epithelium, does not show nasal tumors in well conducted studies. In fact, there is binding of a mouse metabolite to nasal olfactory epithelium, but it is not the quinone imine.

c. Not only feeding of the parent compound produces nasal epithelium tumors, but also feeding of the sulfoxide metabolite of acetochlor, a proximate precursor of the quinone imine [Table 6 and Figure 10b].

d. Data for acetochlor are supported by data from the analog alachlor. Rats treated with alachlor for 1 month did not have detectable neoplasms when examined after a 5-month holding period in basal diet. No detectable olfactory mucosal lesions were observed in any of the "stop study" rats (Genter et al. 2002b).

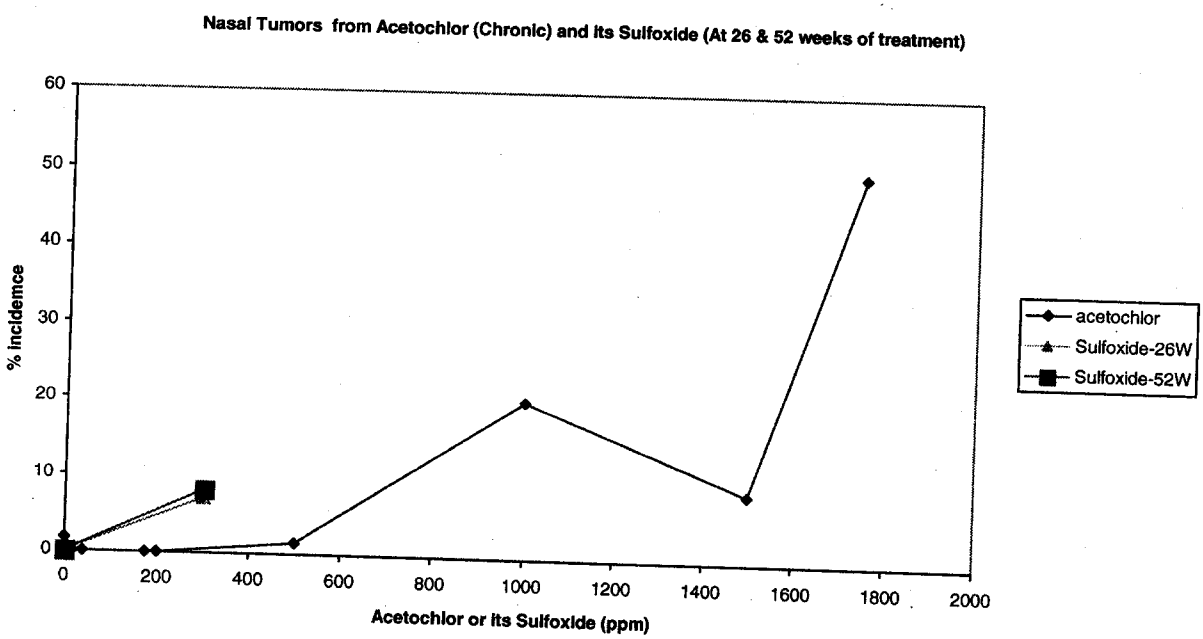

Nasal Tumors from Acetochlor (Chronic) and its Sulfoxide (At 26 & 52 weeks of treatment)
% incidemce
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Acetochlor or its Sulfoxide (ppm)
acetochlor
Sulfoxide-26W
Sulfoxide-52W

Figure 10b. Dose dependance of nasal adenoma formation in rats with increasing level of acetochlor or its sulfoxide metabolite in the feed. Acetochlor data from Table 2 and sulfoxide metabolite from Registrant's White Paper (MRID 46081801)

8. Biological plausibility and coherence

The data discussed above indicate that this mode of action is coherent and biologically plausible. As reviewed by the FIFRA Sap in 1997 (USEPA 1997, 2001), this mode of action is shared by the chloroacetanilides (alachlor and butachlor).

9. Assessment of postulated mode of action

There is considerable evidence in support of the non-genotoxic MOA discussed in this document. The evidence for the key events has been detailed above. This evidence is supported by the following:

a. The absence of demonstrated positive mutagenic effect of the chemical.

b. Acetochlor administration results in dose related increases in the binding of the putative active quinone imine metabolite in the target tissue.

c. Acetochlor administration results in dose related increases in cell proliferation in cells of the target tissue.

d. Absence of nasal epithelial tumors in mice correlated with their inability to form adducts of the quinone imine at the target site. This evidence was confirmed autoradiographically.

e. Rats administered the sulfoxide metabolite of acetochlor (a proximate precursor of the toxic metabolite, the quinone imine) show nasal olfactory mucosa adenomas after 26 weeks of treatment (MRID 46081801). It is noted these results on the sulfoxide metabolite were submitted to the Agency as a brief preliminary report, part of an ARP's position paper (MRID 46081801). The full report, presently in draft form, has not yet been submitted to the Agency to undergo a full review.

The data on acetochlor are supported by the entire database for the analog alachlor, in particular:

i. Reversibility of cell proliferation in rats treated with alachlor for 60 days at a tumorigenic dose, after placement in basal diet for 60 days (MRID 42852102)

ii. Rats treated with the analog alachlor for 1 month at a tumorigenic dose (126 mg/kg/day) did not have detectable neoplasms when examined after a 5-month holding period in basal diet. No detectable olfactory mucosal lesions were observed in any of the "stop study" rats (Genter et al. 2002b).

The weight of the evidence in support for the mode of action evaluated in this document is high. The evidence would have been strengthened if corroborative experiments, such as prevention or reversal of a precursor event (e.g. cell proliferation) by appropriate administration of a chemical (e.g. N-acetylcysteine) known to interfere with a key step (e.g. formation of quinone imine), had been available. Although dimethylaniline (DMA) and diethylaniline (DEA) [analogs of

ethylmethyl aniline (EMA)] have been found to form *in vivo* DNA adducts in rat nasal mucosa, concerns about a genotoxic mechanism for acetochlor are mitigated by several factors. These include absence of formation of DNA adducts in nasal mucosa in parallel experiments in rats using alachlor and the reversibility of cell proliferation of olfactory epithelium observed with the analog alachlor

10. Uncertainties, inconsistencies and data gaps

a. Two key observations in support of the MOA (reversibility of nasal mucosal cell proliferation and the "stop study") utilized the analog alachlor. Although it is plausible that the same results will apply with acetochlor, there is no similar data to confirm this.

b. Although there were direct data available to support "cytotoxicity" for acetochlor (respiratory metaplasia and lipofucsin pigment), additional information was inferred from data for the analog alachlor.

c. Although the Registrant measured glutathione (GSH) levels in nasal mucosal epithelium for alachlor and acetochlor, no changes in levels were observed. GSH decrease is a well recognized effect of quinone imine generation in tissues. Although no GSH decreases were seen, the structure of the nasal epithelial tissue protein adducts confirms that a quinone imine was formed. Decreases in GSH, however, were seen in the liver of rats gavaged with acetochlor (MRID 44863205). Recent work by Burman et al. (2003) with the analog alachlor seems to indicate that alachlor may induce oxidative stress in the nasal mucosal olfactory epithelium, which is consistent with the presence of an electrophilic metabolite.

d. Although the structure of the nasal protein adducts supports the formation of a quinone imine in nasal olfactory epithelium, it would have been optimal if direct confirmation of the quinone imine as a key step were available. It would have been very valuable if administration of an agent known to counteract the effect of the quinone imine (e.g. N-acetylcysteine) or an inhibitor of its formation could have been seen to prevent or reverse the key steps.

e. Dimethylaniline (DMA) and diethylaniline (DEA) [analogs of ethylmethyl aniline (EMA), an acetochlor metabolite] have been found to form *in vivo* DNA adducts in rat nasal mucosa. Concerns about a genotoxic mechanism for acetochlor nasal tumorigenesis are mitigated, however, by several factors. These factors include absence of formation of DNA adducts in nasal mucosa in parallel experiments in rats administered alachlor (an analog of acetochlor) and the reversibility of cell proliferation of olfactory epithelium (a key event) observed with the analog alachlor.

DMA has been found to be a nasal carcinogen in feeding studies with CR CD rats (NTP 1990, Technical Report TR-278). Recent work (Duan et al., 2004) indicates that DMA may form DNA adducts in F-344 rats. Nasal mucosal DNA extracted from rats administered DMA by gavage at 310 mg/kg/day for 7 days showed clear spots by postlabeling. In a parallel series of studies by the same authors (Duan et al., 2004), DEA, the putative precursor for the nasal tumor-causing quinone imine for alachlor was also found to show evidence of adduct formation in nasal mucosal

DNA. Nasal mucosal DNA extracted from rats administered DEA by gavage at 382 mg/kg/day for 7 days showed clear spots by postlabeling. Autoradiography of rats administered ^{14}C-DEA indicate binding of the compound to nasal turbinates.

On the other hand, as summarized below, although alachlor can form *in vitro* adducts with liver DNA or with deoxy-nucleosides and nucleotides, it appears not to be able to form *in vivo* DNA adducts with rat nasal mucosal DNA.

Brown et al. (1995) showed that alachlor and major metabolites, 2-chloro-N-(2,6-diethylphenyl)acetamide (CDEPA) as well as DEA, bind to mouse liver DNA and hemoglobin protein. Based on the *in vitro* reaction of alachlor and CDEPA with selected nucleosides and nucleotides, (thymidine 3'-monophosphate), Nesnow et al. (1995) demonstrated that both alachlor and CDEPA formed N-1 adducts with 2'-deoxyguanosine and N-3 adducts with thymidine as a result of chlorine displacement. Alachlor also formed N-7 adduct with 2'deoxyguanosine. In contrast to the *in vitro* work, recently Duan et al.(2004) administered alachlor to F344 rats to achieve doses of 126 mg/kg/day for 90 days. Analysis of extracted nasal mucosal DNA by postlabeling did not find evidence of DNA adduct formation, which is consistent with a non-genotoxic mode of action.

IV. CONCLUSIONS FOR MOA

The data supporting the mechanism of action for nasal olfactory epithelium tumors in rats by acetochlor have been evaluated by the CARC. It is concluded that the non-genotoxic MOA for nasal olfactory epithelium tumors in rats, discussed in this document, is supported by the data.

This evidence is supported by:

a. The absence of demonstrated positive mutagenic effect of the chemical.

b. Acetochlor administration results in dose related increases in the binding of the quinone imine metabolite to protein in the target tissue; this metabolite is considered to be the putative active species.

c. There is respiratory metaplasia of the nasal olfactory epithelium, an indication of cytotoxicity to the original olfactory tissue and its being replaced by respiratory epithelium, which originates from undifferentiated cells in the epithelium.

d. Lipofucsin granules are observed to increase in a dose related manner in the nasal olfactory epithelium of rats that show nasal olfactory tumors at the high dose. Lipofucsin granules are associated with oxidative damage to lipids and lipoproteins, which is consistent with the redox alterations known to be produced by quinones and quinone imines.

e. Acetochlor administration results in dose related increases in cell proliferation in cells of the target tissue.

f. Absence of nasal epithelial tumors in mice correlated with their inability to form adducts of the

quinone imine at the target site. This evidence of no quinone imine binding was confirmed autoradiographically.

g. Rats administered the sulfoxide metabolite of acetochlor (a proximate precursor of the toxic metabolite, the quinone imine) show nasal olfactory mucosa adenomas after 26 weeks of treatment (MRID 46081801).

The data on the non-genotoxic MOA for acetochlor are supported by the entire database for the analog alachlor, in particular:

i. Reversibility of cell proliferation in rats treated with alachlor for 60 days at a tumorigenic dose, after placement in basal diet for 60 days (MRID 42852102).

ii. Rats treated with the analog alachlor for 1 month at a tumorigenic dose (126 mg/kg/day) did not have detectable neoplasms when examined after a 5-month holding period in basal diet. No detectable olfactory mucosal lesions were observed in any of the "stop study" rats (Genter et al. 2002b).

The weight of the evidence in support for the mode of action evaluated in this document is high. The evidence would have been strengthened if corroborative experiments, such as prevention or reversal of a precursor event (e.g. cell proliferation) by appropriate administration of a chemical (e.g. N-acetylcysteine) known to interfere with a key step (e.g. formation of quinone imine), had been available. Although dimethylaniline (DMA) and diethylaniline (DEA) [analogs of ethylmethyl aniline (EMA)] have been found to form *in vivo* DNA adducts in rat nasal mucosa, concerns about a genotoxic mechanism for acetochlor are mitigated by several factors. These include absence of formation of DNA adducts in nasal mucosa in parallel experiments in rats using the analog alachlor and the reversibility of cell proliferation of olfactory epithelium observed with alachlor.

V. RELEVANCE TO HUMAN CANCER RISK ASSESSMENT

1. Data Presentation

This section covers the issue of relevance of the MOA for rat nasal turbinate tumors to human cancer risk assessment.

A. Introduction

In 1997, the SAP evaluated the weight-of-the-evidence for the biochemical transformation of **alachlor** to a reactive metabolite as the MOA for the induction of nasal olfactory tumors in the rat and also examined the relevance of this MOA for a human cancer risk assessment (USEPA 1996). Autoradiography data were presented showing localization of the alachlor metabolite only in the nasal tissues of the two rat strains and not in the mouse, hamster or squirrel monkey. These data were supported by *in vitro* metabolism results demonstrating a 30-fold increase in metabolism of **alachlor** in the rat compared to the mouse and a "several thousand-fold lower metabolism in the

human". Since the rat responds with nasal tumors and the mouse does not, it was reasoned that the difference in formation of the reactive product, which is retained by nasal tissue, was the "critical mechanism". It was further reasoned that because there was a 30-fold lower *in vitro* metabolism of **alachlor** by the mouse and no nasal tumors, by analogy, the "several thousand-fold lower metabolism in the human" would presumably not result in nasal tumor in humans. However, the SAP stated that the findings were only "suggestive of interspecies differences ... Thus, because bioactivation is thought to play a key role in the mechanism of nasal tumor formation, evidence that bioactivation in humans occurs at significantly lower rates should be compelling". Based on the registrants claim that a rate-limiting step in the metabolism of alachlor in the mouse was not the only factor for lack of a tumorigenic response in the mouse, the SAP concluded that "the limitation in the metabolism in the mouse may not be the real reason for the lack of a tumorigenic response in the mouse. If this is accurate, the argument that limitation in the metabolism of alachlor in the human precludes alachlor being considered as a human carcinogen can not be supported."

The following paragraphs analyze the significance of recently reviewed data on **acetochlor** and evaluate the relevance to human cancer of the postulated MOA for rats.

B. Metabolic differences between rats and other species

The ARP has presented several studies in support of qualitative and quantitative differences in the disposition of acetochlor between rats and other species.

i. Qualitative Differences in Metabolism in Rats and Mice

Several pieces of information supporting the position that marked differences in the metabolism of acetochlor exist between rats and mice include:

The hypothesized MOA for nasal carcinogenicity of alachlor and acetochlor is the bioactivation of these pesticides through several steps ultimately leading to the formation of the corresponding carcinogenic product, dialkylbenzoquinone imine (DABQI). Ashby et al. (1996) (MRID 44496215) and Green (1998) (MRID 44496203) and Green et al. (2000) showed that primary glucuronidation and oxidation of the ethoxymethyl side chain on acetochlor takes place in the rat liver followed by biliary excretion and enterohepatic recirculation causing the removal of the glucuronide and formaldehyde to yield the chloroacetylamide. The subsequent metabolism of the chloroacetylamide involves conjugation of the chloroacetyl group with GHS followed by a sequence of transformations leading to various sulfur-containing metabolites including secondary methyl sulfide. By contrast, the mouse forms a series of glucuronides of the ethoxymethyl side chain through glucuronidation and oxidation in the liver which are then excreted in the urine (Figure 11). Based on these findings, the Registrant concluded that the major route of metabolism of acetochlor in the mouse was glucuronide conjugation.

In another study, whole body autoradiography from four Sprague-Dawley rats administered oral

gavage doses of radiolabelled acetochlor sulfoxide metabolite (MRID 44496202) demonstrated that 8 hours after exposure, the highest concentration of radioactivity was found in the gastrointestinal tract and the nasal cavities. Five days postdosing, significant radioactivity was still found in the nasal passages. These findings are in agreement with the earlier whole body autoradiography of animals exposed to **alachlor** which indicated that the distribution to nasal turbinates is strain and species-specific to the rat and not observed in mice, hamsters or squirrel monkeys dosed with **alachlor** (MRID 43706001).

The absence of nasal cancer in mice administered acetochlor in the diet is attributed by the ARP (MRID 46081801) to differences in hepatic metabolism leading to a greatly reduced capacity to form DABQI. This decreased ability to form DABQI is manifested in mice, in addition to absence of tumors, as an absence of precursor events such as nasal tissue binding (MRID 44496211) and olfactory epithelium cell proliferation (MRID 44496209). It is not fully known if the qualitative differences between rats and mice exist between humans and rats.

ii. Quantitative differences in metabolism between rats, mice, monkeys and humans.

a. EMA hydroxylation (First reaction of Path A in Figure 3)

The ARP has studied *in vitro* quantitative differences in the rate of three reactions depicted in Figure 12 between rats, mice, and squirrel monkeys (MRID 44530002). The first reaction is the conjugation of acetochlor with GSH, the second reaction is the cleavage of secondary sulfide to EMA, and the third one is the p-hydroxylation of EMA to p-hydroxy-EMA (a precursor of the quinone imine). These three reactions are part of Path A, in Figure 3, one of the possible paths, probably a minor one for rats, leading to quinone imine. These reactions were studied using cytosolic and microsomal fractions from liver and nasal epithelium of the tested species. Olfactory and respiratory epithelium were separated for rats and mice, but not for the squirrel monkey.

Comparison of the initial reaction rates of CD rat vs. squirrel monkey tissue fractions indicated that the rates of all three reactions (glutathione conjugation of acetochlor, hydrolysis of the secondary sulfide metabolite of acetochlor to form EMA, and hydroxylation of EMA to pOH-EMA) in liver and nasal tissue fractions were higher in rats than monkeys. Reported reaction rates in the liver fractions ranged from 2.9-fold higher in rats (hydroxylation of EMA) to 10.3- and 10.9-fold for GSH conjugation of acetochlor and hydrolysis of the secondary sulfide of acetochlor to form EMA, respectively. All three reaction rates were significantly higher in the nasal olfactory tissue of the rat (26.2-fold, GSH conjugation of acetochlor, 86-fold, hydrolysis of secondary sulfide metabolite of acetochlor and 23.7-fold, hydroxylation of EMA), suggesting that the rate of formation of precursors to reactive metabolites (imino quinones) that are implicated in nasal tumor formation in rats may be greatest in the rat and much lower in monkeys or other primates (MRID 44530002).

Review of the data, however, indicated that **there is uncertainty** in these rat-to-monkey ratios of activities because olfactory and respiratory epithelium were not separately analyzed for the monkey and the respiratory epithelium in the monkey may be acting as an enzymatically inert

diluent for this *in vitro* assay. To evaluate the impact of this uncertainty, the ARP provided calculations (MRID 46081803) of the estimated rates (V_i) for p-hydroxylation of EMA assuming (1) a "conservative" estimate of monkey nasal tissue samples containing 10% olfactory tissue and (2) a "worst-case" estimate of samples containing 1% olfactory tissue. Based on these estimations, the presence of 10% olfactory tissue would give a primate $V_{olfactory}$ for p-hydroxylation of EMA that is 8.7-fold lower (not 23.7-fold lower, as reported initially) than that of rat olfactory tissue and 2.6-fold lower than rat respiratory tissues. If the samples contained only 1% olfactory tissue, the study author calculated that the primate $V_{olfactory}$ would be approximately 7.2-fold less than the rat olfactory tissue and 2.1-fold less than the respiratory tissues.

These results are summarized in Table 15. Examination of this table indicates that although-hydroxylation of EMA in the monkey is slower than in the rat, the rate is not so slow as to negate totally the possibility of oxidation of EMA in the monkey's olfactory epithelium, if EMA is available.

Table 15. Rat-to-monkey ratios of rate of p-hydroxylation of EMA to p-hydroxy-EMA using microsomal suspensions of rat olfactory epithelium and mixed monkey olfactory/respiratory epithelium (in unknown proportion). [From MRIDs 44530002 and 46081803]

Experimental ratio with no correction	Corrected ratio assuming 10% contamination with respiratory epithelium	Corrected ratio assuming 1% contamination with respiratory epithelium
23.7	8.7	7.2

b. Hydroxylation of Acetochlor sulfoxide metabolite (First reaction in Path B, Figure 3)

The ARP has studied *in vitro* the hydroxylation of the acetochlor sulfoxide metabolite in the rat mouse, monkey, and human liver and nasal tissues (MRID 46009402 and 46081802). Hydroxylation of the acetochlor sulfoxide metabolite is the first reaction in Path B, Figure 3]. Acetochlor sulfoxide is the major circulating acetochlor metabolite in plasma, which requires hydroxylation in the formation of DABQI (the quinone imine).

In this study, the rate of hydroxylation of radiolabeled acetochlor sulfoxide metabolite to p-hydroxy-acetochlor sulfoxide was compared using microsomal fractions derived from Sprague-Dawley rat and CD-1 mouse separated nasal olfactory and respiratory tissues and from squirrel monkeys and 33 human morphologically normal nasal tissue surgical explants (olfactory and respiratory tissues from primates and humans were combined and not separated). Reported results indicated that the highest rates of hydroxylation of acetochlor sulfoxide were observed in the olfactory tissue of the rat and the mouse with comparable activities in these species (6 to 7-fold higher than the activity in rat respiratory tissue, respectively). There was no detectable activity in the primate or human samples.

The investigators concluded, therefore, that acetochlor-induced nasal tumors in the rat are not relevant to humans because the quinone-imine metabolite derived from acetochlor sulfoxide and believed to be responsible for olfactory tumorigenesis would not be produced at sufficient levels in the human. Review of the study by the Agency indicated, however, that there is uncertainty in these data because olfactory and respiratory epithelium were not separately analyzed and the respiratory epithelium in the human and primate may be acting as an enzymatically inert diluent for this in vitro assay.

Following Agency review of the data, because of **concerns raised regarding the possible dilution effect of inactive respiratory epithelium**, the Registrant provided estimations of the activity that would be present if the sample contained only 10% or 1% olfactory tissue (MRID 46081803). Based on these estimates, hypothetical rates of hydroxylation of acetochlor sulfoxide in human or primate olfactory tissue would yield rates that were 132-fold or 88-fold lower than the activity in the rat olfactory tissue. These values for primates, although much smaller than those for rats, are still consisitent with a finite probability of bioactivation of acetochlor metabolites in the nasal mucosa. These ratios of activities are summarized in Table 16.

Table 16. Rat-to-monkey ratios of rate of p-hydroxylation of acetochlor sulfoxide metabolite using microsomal suspensions of rat olfactory epithelium and monkey or human unseparated olfactory/respiratory epithelium (in unknown proportion). (From MRIDs 44530002 and 46081803).

Experimental ratio with no correction	Corrected ratio assuming 10% contamination with respiratory epithelium	Corrected ratio assuming 1% contamination with respiratory epithelium
Extremely high (no activity for primates)	132	88

As part of this investigation, assays were performed with purified human cytochrome p-450 CYP2A6 enzyme (coumarin hydroxylase) (MRID 46081802). Results demonstrated that CYP2A6 did not cause hydroxlyation of acetochlor sulfoxide but rat olfactory microsomes hydroxylated both coumarin and acetochlor sulfoxide. These data suggest that the enzyme that hydroxylates acetochlor sulfoxide is not coumarin hydroxylase but may be related to the CYP2A subfamily. A likely candidate would be the cytochrome p-450 CYP2A3 enzyme, the predominant olfactory cytochrome found in rats but not humans (Fernandez-Salguero and Gonzalez, 1995). Genter et al. (2002a) proposed involvement of CYP2A3 in the final bioactivation of **alachlor** metabolites to the reactive quinone imine; however, the genomic analysis of **alachlor**-treated rat olfactory mucosa revealed that CYP2A3 was downregulated after 2 or 4 days or 1 month of **alachlor** treatment.

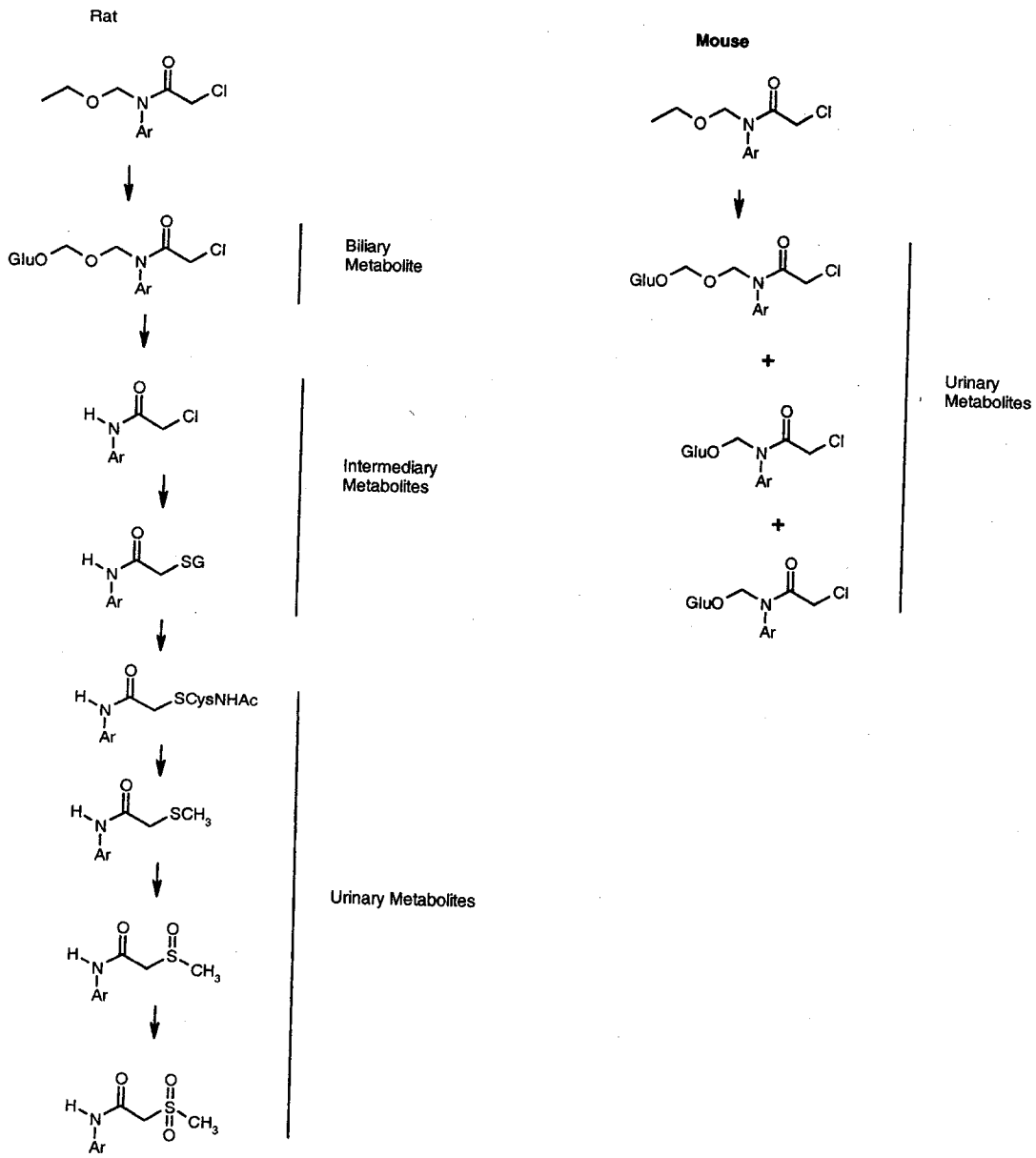
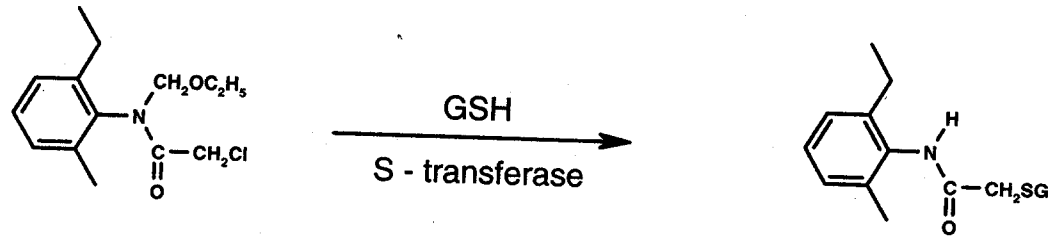
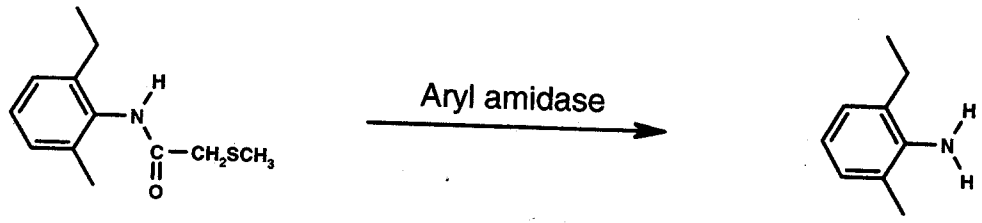

Rat
Mouse
Biliary Metabolite
Intermediary Metabolites
Urinary Metabolites
Urinary Metabolites

Figure 11. Biotransformation of acetochlor in the rat and mouse. (From MRID 44496203).

1) Acetochlor glutathione (GSH) conjugation



2) Secondary sulfide to 2-ethyl-6-methylaniline (EMA)



3) EMA to p-OH-EMA

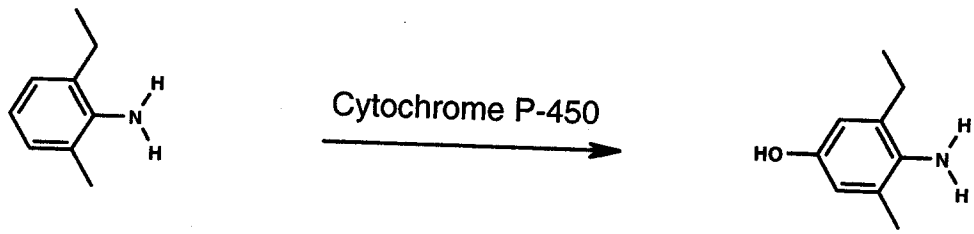


Figure 12. Metabolic reactions measured in rat, mouse, and primate liver and nasal tissues. (From MRID 44530002)

c. *In vitro* metabolism of acetochlor by rat and human liver

In contrast to the Registrant-sponsored studies indicating that acetochlor sulfoxide is not converted by humans to the quinone imine, an independent study by Coleman et al. (2000) demonstrated that human liver samples metabolized acetochlor to 2-chloro-N-(2-methyl-6-ethyl phenyl) acetamide (CMEPA, Figure 13), which is a step before transformation of acetochlor to the secondary methyl sulfide metabolite with subsequent bioactivation to the aniline substrate, 2-methyl-6-ethylaniline (EMA). Data presented further showed that commercially prepared liver microsomes from human males metabolized EMA at a 2-fold higher rate than liver microsomes from Long Evans rats (0.069 nmol/min/mg in human liver microsomes versus 0.035 nmol/min/mg in rat liver microsomes). Although human olfactory microsomes were not tested in this series of experiments, the potential for human liver microsomal fraction to produce the carcinogenic precursor EMA product is plausible. One may speculate that EMA will be carried in the blood to the nasal tissues and then could be further activated following Path A to produce the quinone imine. The related compound 2,6-dimethylaniline is a rat nasal carcinogen (NTP 1990, Technical Report TR-278) and 2,6-diethylaniline is the putative precursor to the alachlor rat nasal carcinogen.

Furthermore, Coleman et al. (2000) found that the cytochrome p-450 enzymes responsible for the human metabolism of acetochlor are CYP3A4 and CYP2B6.

2. Conclusion

On the basis of the *in vitro* metabolism data with microsomes derived from human livers showing the p-450 metabolism of acetocholor via the Scheme A pathway to the carcinogenic product, EMA, combined with the finding that the human microsomal fractions are about 2X more active than the rat in producing EMA (Figure 13), it is concluded that the Registrant argument that there is no relevance to humans can not be sustained.

This conclusion is supported by:

- The realization that production of a metabolite (EMA) with the capacity of undergoing transformation to a quinone imine is possible for humans (Coleman et al., 2000).

- *In vitro* studies of p-hydroxylation of EMA using olfactory epithelium enzymes indicate that rat-to-monkey ratios of activities (MRIDs 44530002 and 46081802) are not as large as 23.7 but could be as small as 7 or 8.

- In *in vitro* studies, the ratio of rat to monkey for p-hydroxylation of the sulfoxide metabolite of acetochlor may be not astronomically large, as initially postulated, but as small as 88 (MRID 46081802).

- Although nasal tissue was not included in the Coleman et al. (2000) study , the data indicate that human liver has the potential to produce EMA (Figure 13), a plausibly carcinogenic metabolite of acetochlor, which would then be available to all organs via the

circulatory system.

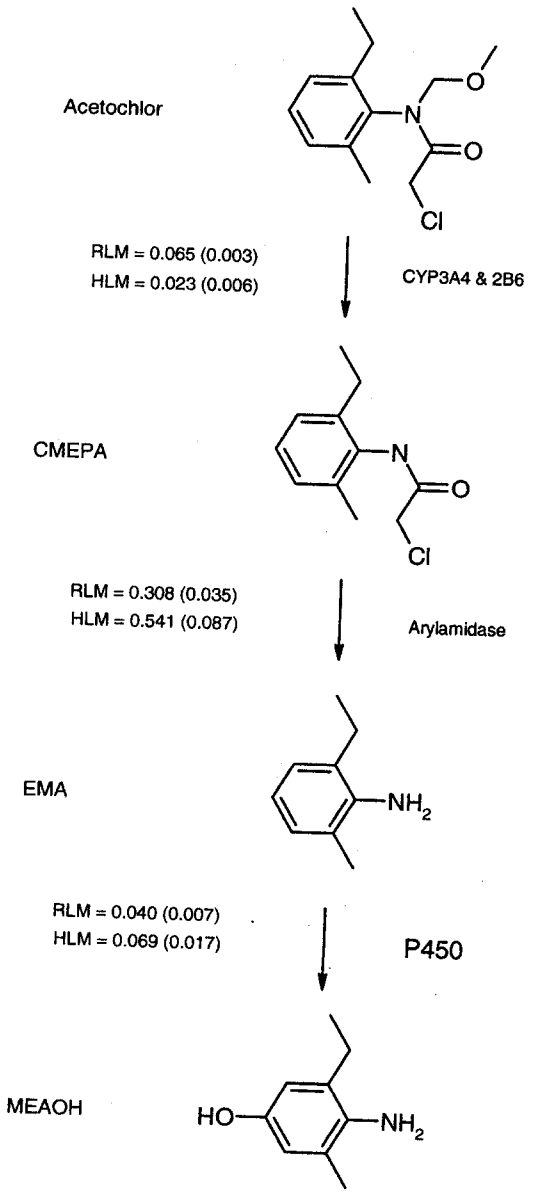


Figure 13. Comparison of the metabolism of acetochlor, CMEPA, and methyl ethyl aniline (EMA) between rat and human liver microsomes. HLM = Human liver microsomes, RLM = rat liver microsomes. Rates are nmol/min/mg ± (SEM). Adapted from Coleman et al. 2000).

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