

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT**DRAFT DISCUSSION PAPER: GENOTOXICITY OF PARA-CHLOROANILINE.****Referral**

1. The Advisory Committee on Pesticides have asked for a COM view on the available genotoxicity data on para-chloroaniline (4-chloroaniline, 4-CA).

Background

2. A copy of the referral letter is appended for members' information at Annex 1. In brief the 4-CA is a potential mammalian metabolite of the pesticide diflubenzuron. The ACP reviewed the available information from a WHO Concise International Chemical Assessment Document (No 48) on 4-CA published in 2003,¹ summary information on the metabolism of diflubenzuron (from the draft EU risk assessment document) and a published study on the metabolism of diflubenzuron in swine,² a draft risk assessment of the carcinogenicity of 4-CA in diflubenzuron produced by the EU Rapporteur Member State (Sweden) and a submission from the approval holder for diflubenzuron regarding the potential for metabolism of diflubenzuron to 4 CA. Based on the available data, the ACP requested additional studies to investigate the metabolism of diflubenzuron to 4-CA and *in vivo* comet assays to investigate potential genotoxicity in tumour target organs.
3. The ACP's request to COM concerns the available published scientific literature on the genotoxicity of 4-CA. This draft discussion paper is therefore made available on the COM internet site.

Introduction to current review

4. A literature search was undertaken using PUBMED and a total of 182 citations (all literature on 4-CA) were screened for potentially relevant references. The CICAD document was cross referenced for any additional publications. In addition to publications on genotoxicity, publication on the metabolism of 4CA and the acute toxicity of 4CA in rodents, in particular information on the mechanism of methaemoglobin (MtHb) formation induced by 4CA were noted.
5. An overview of the retrieved information is given below. Extracts from the CICAD document are given in Annex 2. A summary of the available genotoxicity data is given is appended at the end of this

draft discussion paper and extracts from relevant retrieved papers are given in Annex 3 (in alphabetical order).

Overview of toxicology data on 4CA.¹

6. In brief, 4CA is a crystalline aniline derivative. It is slightly soluble in water and soluble in a range of organic solvents. The n-octanol/water partition coefficient is reported to be 1.83 or 2.05. It is well absorbed orally or dermally and acute toxicity values and Mthb induction is similar following these routes of administration or after i.p. dosing. An oral dose of 76.5 mg/kg bw in rats induced significant Mthb formation after 15-60 min and an oral dose of 40 mg/kg bw in rat induced significant levels of Mthb after 60-90 mins. Absorbed 4CA is widely distributed with specific binding to erythrocytes reported. 4CA is rapidly metabolised with the predominant route in most mammalian species being hydroxylation at the *ortho*- position followed by conjugation with sulphate. A minor pathway involves N-hydroxylation to form 4-chloro-N-phenyl-hydroxylamine which can undergo oxidation to 4-chloronitrosobenzene in erythrocytes. Oxidation to 4-chloronitrosobenzene and its subsequent binding to oxy-haemoglobin are thought to be involved in Mthb formation. Excretion as metabolites predominantly via the urine is rapid in rodents.
7. The oral LD50 in rats is reported to be between 300-420 mg/kg bw and in mice 228-500 mg/kg bw. Signs include excitation, tremors, spasm, shortness of breath, cyanosis, Mthb formation and mild hepato- and renal toxicity. Following repeat dosing, the blood liver, spleen and kidneys are the predominate target organs. NTP bioassays for potential carcinogenicity have been undertaken in rats and mice. There was clear evidence of carcinogenicity in male rats (splenic sarcoma and osteosarcoma associated with fibrosis of the spleen). There was equivocal evidence for tumours of the spleen in female rats. There was some evidence for liver tumours in male mice and no evidence for carcinogenicity in female mice.

Overview of Genotoxicity data

8. 4CA in genotoxicity studies has been derived from a number of commercial sources. Where information is available purity is $\geq 97\%$.

In vitro

Bacterial tests

9. There are several bacterial reverse mutation assays in *Salmonella typhimurium* using plate incorporation approaches. There is consistent evidence from adequate studies for positive mutagenic activity of 4CA in *S.typhimurium* TA 98 in the presence of exogenous metabolic activation from Aroclor 1254 induced rats.³⁻¹⁰

Positive results were generally seen at dose levels of ≥ 1000 $\mu\text{g}/\text{plate}$. The available negative or equivocal results from other mutagenicity studies using *S.typhiumurium* strains may have been associated with use of lower dose levels or insufficient details to assess the conduct of the tests.¹¹⁻¹⁸ Evidence for genotoxicity was reported in a $\text{PolA}^+/\text{PolA}^-$ assay using *Escherichia coli*.¹⁸

10. Negative results were obtained in *umu* tests using *S.typhiumurium* TA1535pSK1002 in the presence and absence of exogenous metabolic activation from Phenobarbital/5,6-benzoflavone or oriental yeast.^{19,20}
11. What are members' views of the available tests using bacteria?

Studies using yeast

12. Evidence for mutagenic activity was reported in *Aspergillus nidulans* (mei^- to mei^+) in a limited study using a single concentration of 4CA.²¹
13. Negative results were reported for mitotic recombination in *Saccharomyces cerevisiae* D3 at one concentration of 4CA in presence and absence of exogenous metabolic activation using S-9 from Aroclor 1254 induced rats. It is noted that a range of other compounds requiring metabolic activation to form genotoxins were also negative in this assay.²²
14. What are members' views of the available tests in yeast?

Studies using mammalian cells

15. 4CA induced chromosomal aberrations in CHO cells both in the presence and absence of exogenous metabolic activation S-9 from Aroclor 1254 induced rats.^{7,23} Evidence for increased chromosomal aberrations was generally seen at relatively high doses. There were inconsistent results between the two participating laboratories regarding effect of metabolic activation.
16. Mutagenic activity was reported in mouse lymphoma assays using LY5178 $\text{TK}^{+/-}$ were reported in a number of laboratories both in the presence and absence of exogenous metabolic activation using S-9 mix from Aroclor 1254 induced rats. There was evidence of cytotoxicity ($\text{RTG} < 10\text{-}20\%$) in some of these experiments.^{7,24-27}
17. 4CA did not induced DNA strand breaks in mouse lymphoma cells LY5178 $\text{TK}^{+/-}$ in the absence of exogenous metabolic activation. Positive results were reported for a number of mutagens both in the presence and absence of exogenous metabolic activation²⁸
18. Positive results were reported in two UDS rat Hepatocyte experiments at 10 $\mu\text{g}/\text{ml}$. A concentration of 50 $\mu\text{g}/\text{ml}$ was toxic to

hepatocytes in this study.²⁹ Negative results were reported in a separate study where a concentration of 12 µg/ml was reported to be toxic to hepatocytes.¹⁶

19. What are members' views of the available mammalian cell genotoxicity tests?

Summary : *In vitro* mutagenicity studies.

20. 4CA is mutagenic in *S.typhimurium* TA98 in the presence of exogenous metabolic activation. 4CA is mutagenic in yeast in the absence of exogenous metabolic activation. 4CA induces chromosomal aberrations in CHO cells both in the presence and absence of exogenous metabolic activation. 4CA was mutagenic in the mouse lymphoma assay in LY5178 TK^{+/-} cells both in the presence and absence of exogenous metabolic activation. 4CA did not induce DNA strand breaks in mouse lymphoma LY5178 TK^{+/-} cells. 4CA induced UDS in primary rat hepatocytes.

In vivo studies

21. Evidence for mutagenicity was reported in both repair proficient and deficient *Drosophila melanogaster*.³⁰ The COM has previously attached little weight of evidence to *Drosophila* tests.

Studies in rodents

22. No evidence for hepatic DNA binding was reported (RAL <3.1 x 10⁻⁸).³¹ In this study two female Wistar rats were dosed (by gavage in 1,2 propanediol) with 0.5 mmol/kg (63.8 mg/kg) and sacrificed 24h post dose. Isolated liver DNA was sequentially digested to individual deoxynucleotides with DNase I, nuclease P₁, snake venom phosphodiesterase with alkaline phosphatase and analysed by HPLC/MS/MS. Rat haemoglobin binding reported to be 2386 ± 156 pmol/mg. Positive results for DNA adduct formation reported for concurrent studies using 4-aminobiphenyl.
23. Positive results for a comet assay were reported by Sasaki Y et al.³² A group of four mice were dosed with 0.5 of the oral LD50 (200 mg/kg bw) in olive oil, tissues samples (stomach, colon, liver, kidney, bladder, lung, brain and bone marrow) were obtained 3, 8, 24h post dose. Alkaline SCGE undertaken using isolated nuclei and comets assessed for 50 nuclei from each organ. Migration = Length-Diameter in µm. Evidence for increased migration in stomach, bladder, lung and brain after 8h, and stomach, colon, liver, bladder, lung, brain after 24h. The COM have previously considered results of comet screening assays published by Sasaki et al in 1999-2000 to be inadequately conducted for assessment purposes. (see extract from COM minutes February 2001 at the end of this draft discussion paper).

24. Positive results have been reported in a bone marrow MN assay in mice.³² Groups of 5 male B6C3F1 mice were dose orally with up to 400 mg/kg PCA (in phosphate buffered saline) on three consecutive days and bone marrow samples assessed 24 h post last dose. Two independent studies were undertaken. Doses in the first trial were 25, 50, 100, 200, 300, 400 mg/kg bw/day. In the second trial dose levels of 100, 200 and 300 mg/kg bw/day were used.
25. In the first trial a significant increase in mean MN-PCE/1000 PCE was recorded at 300 mg/kg bw/day (16.60 ± 2.75 , $P < 0.0001$). (Individual animal results 10.5, 26.5, 14.5, 13.5, 18). Slight increases noted at 25, 50, 200 mg/kg bw/day within control range. In second trial a significant increase in mean MN-PCE/1000 PCE was recorded at 300 mg/kg bw/day (5.60 ± 1.22 , $P < 0.0001$) (individual animal results (5, 10, 5.5, 2.5). Concurrent control 1.10 and 1.40 in the two trials. Positive control gave similar values in both trials (cyclophosphamide, 28.6 and 37).
26. A dose level of 400 mg/kg bw/day in the first study was reported by NTP to result in all animals being sacrificed due to excessive moribundity. A dose level of 300 mg/kg bw/day in the first trial did not result in any recorded signs of toxicity. In the second trial increased lethargy was reported at 300 mg/kg bw/day. No effects on %PCE were reported in either trial. The extent of toxicity reported by NTP is inconsistent with other acute toxicity data for 4CA. The test material was administered as the hydrochloride salt in PBS and it considered likely that absorption from the gastrointestinal tract occurred.
27. Negative results were reported in a separate mouse BM MN assay, but at the time of writing this draft discussion paper only limited details were available. A Single oral dose of 180 mg/kg bw (MTD) was administered to CFLP mice with sampling at 24-72h post dose.
28. In some earlier studies using azobenzene (metabolised to aniline) which is carcinogenic in rats but not mice and induces MN in rats but not mice. The authors report that MN induction was paralleled by increase in methaemoglobin levels. It was considered that accelerated erythropoieses (indicated by increase in PEs and reticulocytes count) was a contributory factor to MN induction in rats.³³ In contrast methaemoglobin induction by p-cresidine in p53 knockout mice was not associated with an increase in BM MN PCEs.³⁴
29. What are members' views of the available *in vivo* genotoxicity studies with 4CA.

Summary

30. There was no evidence for hepatic DNA binding in rats given an oral dose of 64 mg/kg bw 4CA. Evidence for a positive comet assay was reported in a range of organs in mice given an oral dose

of 200 mg/kg bw. The COM has previously considered the results of this study to be inadequate for assessment. A positive response was reported in one bone marrow oral MN assay in mice given three daily dose levels of 300 mg/kg bw. This dose level should have been consistent with severe toxicity, but no evidence of this was reported by the study investigators.

COM Discussion

31. The COM is asked to draw conclusions on the available genotoxicity data on 4CA. The COM has not been asked to formulate a mutagenicity testing strategy for 4CA.
32. What conclusions do members wish to reach regarding the *in vitro* genotoxicity data on 4CA?
33. What conclusions do members wish to reach regarding the *in vivo* studies on 4CA?

Secretariat August 2009

References.

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Summary of bacterial tests

A number of experiments using *Salmonella typhimurium* reported by Mortelmans 1986 were reproduced in the results reported by the NTP in the 1989 bioassay report and Zeiger et al 1990.

Reference, method	Results	Comments
Dunkel VC et al Environ Mut, 7 (suppl 5), 1-248, 1985. ³ Inter laboratory comparisons from 4 labs. Test material 99.4% pure, tested by plate incorporation in <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537, TA 1538 +/- S-9 from Aroclor 1254 induced F344 rats, B6C3F1 mice and Syrian hamsters. Tests also undertaken using <i>Escherichia coli</i> WP2 uvrA +/-S-9 mixes.	A positive result was reported for three out of four labs for TA98 +S-9 rat liver. A number of positive results were reported from some but not all labs in TA1538 using exogenous metabolic activation from rats, mice and hamsters.	Positive results generally seen at $\geq 1000 \mu\text{g}/\text{plate}$. The one negative result for TA98 +S-9 from rat liver used a highest dose levels of 333 $\mu\text{g}/\text{plate}$. Where positive responses were seen with TA1538, it was at doses of 1000 $\mu\text{g}/\text{plate}$.
Dunkel VC and Simmon, VF. IARC Sci Publ, 27, 283-302, 1980 ⁴ Results of plate incorporation test in <i>Salmonella typhimurium</i> TA 98 +/- S-9 from Aroclor 1254 induced F344 rats.	Limited data available. Positive result in TA 98 + S-9 from Aroclor 1254 induced F344 rats, showing dose-response.	Data suggest increased number of revertants from 666 $\mu\text{g}/\text{plate}$
Garner RC and Nutman CA, Mutat Res, 44, 9-19, 1977. ¹¹ Compound obtained from Aldrich. Plate incorporation study at dose levels of 50 or 100 $\mu\text{g}/\text{plate}$ tested <i>Salmonella typhimurium</i> TA 1538 +/- S-9 from Phenobarbital treated rats.	No evidence for mutagenic effect.	Part of study of a large number of structural analogs using fixed dose levels.
Gilbert, P et al, Arch Environ Contam Tox, 9, 533-541. ¹² Plate incorporation and fluctuation tests using <i>Salmonella typhimurium</i> TA 98, 100, 1530, 1535, 1537, 1538, 1532, 1950, 1975, 1978, G46 +/- S-9 from Aroclor 1254 induced Wistar rats. Very limited details of methods, no information on dose levels given. Compound obtained from Aldrich	Very limited details of results, reported as substantially negative.	Insufficient information to assess study.
McGregor D et al Env Mutagen, 6, 545-557, 1984. ⁵ Plate incorporation tests using <i>Salmonella typhimurium</i> TA 98 + S-9 from Aroclor 1254 induced F344 rats. (Microscope automated counting of microcolonies used to assess bacterial toxicity) Compound obtained from NIEHS repository.	Dose related increase in revertants reported in graphical presentation of data. Sharp decreases in bacterial cell survival were reported at doses where the number of mutants were decreased.	Peak response at 1000 $\mu\text{g}/\text{plate}$.
Mortelmans et al Env Mutagen,	Positive dose related responses	Data reproduced in NTP bioassay

8, (supp 7), 1-119, 1986. ⁶ Preincubation assay using <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537, TA 1538 +/- S-9 from Aroclor 1254 induced F344 rats, and Syrian hamsters. Test compound 99% pure. Two test laboratories.	reported in TA98 + S-9 from rat or hamster liver from 666 µg/plate from one testing laboratory. Negative results reported at 2 nd test laboratory but highest dose used was 333 µg/plate.	report.
NTP 1989. Tox and Carc studies of para-chloroniline. ⁷ Reproduces data from Mortelmans et al 1986.	Results of <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537, TA 1538 +/- S-9 from Aroclor 1254 induced F344 rats, and Syrian hamsters.	Tabulation of data clearer than in original publications.
Ono Y et al Wat Sci Tech, 26, 61-69, 1992. ¹⁹ Investigation of umuDC gene induction linked to lacZ, β-galactosidase in <i>Salmonella typhimurium</i> TA1535 pSK1002+/- S-9 from rats pretreated with Phenobarbital and 5,6-benzo-flavone.	Negative results reported for p-chloroaniline at one test concentration.	Study part of investigation of wide range of chloro aryl compounds.
Pai, V et al Mutation Research, 151, 201-207, 1985. ¹⁰ Fluctuation tests using <i>Escherichia coli</i> WP2 uvrA (-S-9 only) and plate incorporation tests using <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535 and <i>E.coli</i> WP2 uvrA (-S-9 only)	Positive response reported for TA 98 plate incorporation test	Data suggests equivocal response at 1000 µg/plate.
Rashid KA et al J Environ Sci health, B22, 721-729, 1987. ¹³ Compound obtained from Sigma. Plate incorporation tests using <i>Salmonella typhimurium</i> TA 98, TA 100 +/- S-9 from Aroclor 1254 induced rats.	No evidence for mutagenic effect.	Doses of up to 1000 µg/plate tests.
Rosenkranz, HS and Poirer, LA. J Natl Cancer Inst, 62, 873-892. ¹⁸ Plate incorporation assays using <i>Salmonella typhimurium</i> TA 1535, TA 1538 +/- S-9 from untreated rat liver. PolA ⁺ /PolA ⁻ assay using <i>Escherichai coli</i> . (Test materials tested in duplicate on three occasions).	Positive results reported for PolA ⁺ /PolA ⁻ assay at one tested dose level of 5 µg/ml both +/-S-9. Negative results reported in plate incorporation tests in TA1535 TA 1538.	Single dose level of 250 µg/plate used. Study included 99 chemcials.
Sakagami, Y et al Mutat Res, 209, 155-160, 1988. ²⁰ Results of umu test using <i>Salmonella typhimurium</i> TA1535 pSK1002+/- S-9 (Oriental yeast), Rec assay and Ames test. Very limited details of methods provided. Compound obtained from Wako Pure Chemical.	Very limited details of results provided. Para-chloroaniline reported to be negative in all tests.	Insufficient information to assess report.
Seuferer, SL et al Pesticide Biochem and Physiol, 10, 174-	Data for net mutants/plate reported at one test concentration	Equivocal result

180, 1979. ¹⁴ Plate incorporation tests using <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537, TA 1538. Paper unclear whether S-9 was used. Limited details of methods provided. 4CA obtained from Aldrich.	(1000 µg/plate). Authors report borderline positive result but do not specify which strain.	
Simmon, VF. J Natl Cancer Inst, 62, 893-899, 1979. ¹⁵ Plate incorporation tests using <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537, TA 1538 +/- S-9 from Aroclor 1254 induced rats. Compound supplied by NCI.	Negative results reported for the highest concentration tested (1000 µg/plate).	Limited details of methods and results presented. Study reported results for 101 compounds.
Thompson, CZ et al, Environ Mutagen, 5, 803-811, 1983. ¹⁶ Modified Ames gradient plate tests undertaken using <i>Salmonella typhimurium</i> G46, TA1535, TA100, C3076, TA1537, D3052, TA1538, TA98 and <i>Escherichia coli</i> WP2/WP2uvrA-. Test designed to give 10,000 fold concentration range both +/- S-9 from Aroclor 1254 induced rats.	Negative results reported. Limited details of results presented.	Positive results with Streptozotocin and 2-Acetylaminofluorene. Study reported results of 45 chemicals.
Van der Bijl et al J Dental Ass S Africa, 39, 535-537, 1984. ⁸ Spot and plate incorporation tests using <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537, TA 1538 +/- S-9 from Aroclor 1254 or phenobarbitone induced rats. Compound obtained from BDH, Poole, England.	Negative results were obtained in spot tests using up to 50 µg/ml (which was toxic to TA 100, TA 1537, TA 1538 – S-9, and TA 100 +S-9). In plate incorporation tests, positive results were obtained in TA98+S-9 from Aroclor 1254 treated male and female rats, with a greater effect from S-9 from male rats. No mutagenic effect was reported using S-9 from phenobarbitone-induced male rats	Data from three concentrations reported. No effect at 0.1 mg/plate, positive at 1 mg/plate (peak effect), reduced but still positive mutagenic response at 2.5 mg/plate.
Zeiger, E Env Mol Mut, 16 (suppl18), 32-54, 1990. ⁹ Reproduces data from Mortelmans 1986	Data from three laboratories. Positive in TA 98 +S-9 from rat liver	Tabulated data presents more details of effect of varying levels of S-9 mix added.
Zimmer, D et al Mutat Res, 77, 317-326, 1980. ¹⁷ Preincubation and plate incorporation tests using <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1537 +/- S-9 from Aroclor 1254 induced rats. Very limited details of methods provided. No details of concentrations tested. Compound obtained from Aldrich.	Very limited details of results presented. Compound reported to be negative in strains reported TA 98, TA 100, TA 1537	Insufficient information to assess report.

Summary of studies in Yeast

Reference, method	Results	Comments
Prasad, I. Can J Microbiol, 16, 369-72, 1970. ²¹ Mutation of <i>Aspergillus nidulans</i> strain requiring methionine and pyridoxine (<i>meth⁻</i> to <i>meth⁺</i>) was determined using a single concentration of parachloroaniline	Two morphological types of revertant colonies detected on selective media containing methionine (at 0.1 mg/ml). The total frequency of <i>meth₃</i> revertants was 28 in 4CA treatment compared to 3/10 viable spores in the control cultures	Concentration tested was 200 µg/ml Range of chloroaniline compounds tested.
Simmon VF J Natl Cancer Inst, 62, 901-909, 1979. ²² <i>Saccharomyces cerevisiae</i> D3 heterozygous for D3. Inresaed formation of homozygous mutants formed by mitotic recombination investigated. +/- S-9 from Aroclor 1254 induced rats.	Negative results were reported for one concentration of p-chloroaniline (0.2% in DMSO) +/- S-9.	Positive response reported for a range of alkylating agents. Negative response reported for B(a)P and 7,12 DMBA (tested + S-9). Authors concluded improved metabolic activation procedure was required.

Summary of Studies in Mammalian cells

Reference, method	Results	Comments
Chromosomal Aberrations		
Anderson, BE et al Env Mol Mut, 16 (suppl 18), 55-137, 1990. ²³ CHO cells were incubated with test material in DMSO for 8h with colcemid present for final 2h. test material was incubated for 2h in presence of S9 mix from Aroclor 1254 induced rats, and then for 8-10h in absence of S-9, with colcemid present for final 2h. Growth period was extended for 6-8h in some trials when cell cycle delay was reported)	Data from two independent laboratories reported. 100 cells per dose scored. In laboratory 1, toxicity was reported at 500 µg/ml in the absence of S-9. In the presence of S-9, increased incidence of CAs was reported at ≥900 µg/ml.(Delay cell cycle was reported). In laboratory 2, a positive result in the absence of S-9 at ≥500 µg/ml. Negative results were reported in the presence of S-9 at 600 µg/ml.	Results were repeated in one or more trials. Data for SCEs reported positive both in absence of S-9 (both labs) and presence of S-9 (one lab).
NTP 1989. Tox and Carc studies of para-chloroniline. ⁷ Reproduces data from Anderson et al 1990.	Same results	Clearer tabulation of data.
Mouse Lymphoma Assay		
Caspary WJ et al Env Mol Mutagen, 12 (suppl13), 195-229, 1988. ²⁴ Results of interlaboratory reproducibility study of 63 chemicals. Limited details of methods provided.	Para-chloroaniline was positive in both laboratories both in presence and absence of exogenous metabolic activation (S-9).	Most trials were positive. Between 2-4 trials undertaken for both + and – S-9 assays. Graphical presentation of data.
Mitchell, AD et al Env Mol Mut, 12 (suppl 13), 37-101, 1988. ²⁵ Mouse lymphoma L5178Y TK ^{+/-} assay +/- S-9 from Aroclor 1254 induced rats.	Positive results (two fold increase over solvent control) in two out of three trials in the absence of S-9 and positive in the two trials conducted in the presence of S-9.	In the absence of S-9 positive results reported at highest dose of 500 µg/ml at relative total growth of between 5-15%. Positive in the presence of S-9 at dose levels of 15.7 µg/ml in one trial and 24.6 µg/ml in a separate trial with some limited evidence for a dose-response in the second trial. RTG in these S-9 trials were around 30% in the first trial and 20%, 25% in the second trial.
Mhyr BC and Caspray, WJ. Env Mol Mut, 12 (suppl 13), 103-194, 1988. ²⁶ Mouse lymphoma L5178Y TK ^{+/-} assay +/- S-9 from Aroclor 1254 induced rats.	Positive results in four trials in the absence of S-9. Positive in three trials in the presence of S-9.	Positive results in the three non activation trials at around dose levels of ≥375 µg/ml (RTG between 3-30%) and in the fourth trial at 100 µg/ml (RTG 47-58%). In the presence of S-9 positive dose-related increase from 15. 6 µg/ml (RTG 48%, 63%) in one trial and positive results in two other trials at 75 µg/ml (RTG 30-39%) and 125 µg/ml (RTG 15-17%).
NTP 1989. Tox and Carc studies of para-chloroniline. ⁷	Same results	Clearer tabulation of data.

Reproduces data from Caspray 1988, Mitchell 1988 and Myhr 1988		
Wagenheim J and Bolcsfoldi G 1988. ²⁷ Mouse lymphoma L5178Y TK ⁺ assa. 4h exposure, 48 expression. No S-9 used.	Positive dose-related increase reported in absence of S-9	Significant increase in MF reported at 1.52 mM (at 19% RTG), 2.04 mM (at 15% RTG) and 2.54 mM (6% RTG).
<u>DNA Strand Breaks</u>		
Garberg P et al Mutat Res, 203, 155-176, 1988. ²⁸ Mouse lymphoma L5178Y TK ⁺ cells were incubated with ³ H-thymidine and then exposed to test compound in the absence of S-9 for 3h. Cells were treated with alkali, then neutralised, sonicated and treated with SDS and DNA separated on hydroxyapatite columns. Relative content of dsDNA and ssDNA was determined. Compound obtained from Janssen Chimica (Belgium).	Negative results reported. Positive results reported for assays with cyclohexamide (-S-9) and 7,12-DMBA (+S-9).	Slight increase in %ssDNA at highest concentration tested 3mM but within range of solvent control.
<u>Hepatocyte UDS Assays</u>		
Thompson CZ et al Env Mutagen, 5, 803-811, 1983. ¹⁶ Hepatocytes from F344 rats were exposed to concentrations (1,000-0.5 nmol/ml), and autoradiography performed and nuclei of 20 morphologically unaltered cells were counted. Each experiment was replicated. Nuclear Grain counts	Negative data for 50 nmol/ml (6 µg/ml)	100 nmol/ml (12 µg/ml) was toxic. The authors considered that the variance between these results and those of Williams et al 1982 may reflect a very limited dose-response curve for UDS with this compound.
Williams GM et al Mutat Res, 97, 359-370, 1982. ²⁹ Hepatocytes from F344 rats were exposed to 3 concentrations for 18-20h in the presence of ³ H-thymidine. Autoradiography was undertaken for 7d and NNG count undertaken for 20 nuclei. Two replicate experiments were undertaken.	Positive results were reported in two trials at 10 µg/ml.	Negative results were reported at 5 µg/ml and hepatotoxicity was reported at 50 µg/ml.

In vivo mutagenicity tests

Reference, methods	Results	Comments
<u>Drosophila tests</u>		
Graf, U et al Env Mol Mut, 16, 225-237, 1990. ³⁰ 50 to 100 Drosophila larvae (72h old, repair proficient females (<i>mei-9</i>) and repair deficient males) heterozygous for multiple wing hairs (<i>mwh</i>) and flare (<i>flr</i>) were allowed to feed on cellulose powder impregnated with 7.84 mM 4CA for 6h. Adult flies were collected and wings prepared for microscopic inspection of wing spots. Single spots indicating mutagenic activity and twin spots showing mitotic recombination were scored. (Compound gift from Dr Van Bilj S.Africa)	In both excision repair proficient and deficient larvae an increase in the number and frequency of single (small and large spots) and twin spots and total spots was reported. Both excision repair proficient and deficient larvae were considered to be equally sensitive.	2-aminofluorene was negative in excision defective larvae, but positive in repair proficient larvae where higher dose levels were used. Positive results also reported in this tests for 4,4'-methylene-bis-(2-chloroaniline).
<u>Mammalian DNA binding</u>		
Jones RJ and Sabbioni, G, Chem Res Tox, 16, 1251-1263, 2003. ³¹ Two female Wistar rats were dosed (by gavage in 1,2 propanediol) with 0.5 mmol/kg (63.8 mg/kg) and sacrificed 24h post dose. Isolated liver DNA was sequentially digested to individual deoxynucleotides with DNase I, nuclease P ₁ , snake venom phosphodiesterase with alkaline phosphatase and analysed by HPLC/MS/MS.	No evidence for hepatic DNA binding was reported (RAL <3.1 x 10 ⁻⁸)	Rat haemoglobin binding reported to be 2386 ± 156 pmol/mg. Positive results for DNA adduct formation reported for concurrent studies using 4-aminobiphenyl. Evidence for covalent protein binding in liver, kidney and blood was reported in an earlier study where male F344 rats were dosed with ¹⁴ C-4CA and TCA precipitation used to identify protein bound material. Subcellular fractionation indicated that radiolabel was widely distributed in all subcellular fractions (nuclear, mitochondrial, microsomal and cytosolic). ³⁵
<u>DNA Damage Assays</u>		
Sasaki YF et al, mutat Res, 440, 1-18, 1999. ³² A group of four mice were dosed with 0.5 of the oral LD50 (200 mg/kg bw) in olive oil, tissues samples (stomach, colon, liver, kidney, bladder, lung, brain and bone marrow) were obtained 3, 8, 24h post dose. Alkaline SCGE undertaken using isolated nuclei and comets assessed for 50 nuclei from each organ. Migration = Length-Diameter in µm.	Evidence for increased migration in stomach, bladder, lung and brain after 8h, and stomach, colon, liver, bladder, lung, brain after 24h.	COM have previously considered results of comet screening assays published by Sasaki et al in 1999-2000 to be inadequately conducted for assessment purposes. (see extract from COM minutes February 2001).
<u>Bone Marrow MN assays</u>		
NTP 1995. ³⁶ Groups of 5 male B6C3F1 mice were dose orally with up to 400 mg/kg PCA (in phosphate	In the first trial a significant increase in mean MN-PCE/1000 PCE was recorded at 300 mg/kg bw/day (16.60 ± 2.75, P<0.0001).	A dose level of 400 mg/kg bw/day in the first study was reported by NTP to result in all animals being sacrificed due to excessive

<p>buffered saline) on three consecutive days and bone marrow samples assessed 24 h post last dose. Two independent studies were undertaken. Doses in the first trial were 25, 50, 100, 200, 300, 400 mg/kg bw/day. In the second trial dose levels of 100, 200 and 300 mg/kg bw/day were used.</p>	<p>(Individual animal results 10.5, 26.5, 14.5, 13.5, 18). Slight increases noted at 25, 50, 200 mg/kg bw/day within control range.</p> <p>In second trial a significant increase in mean MN-PCE/1000 PCE was recorded at 300 mg/kg bw/day (5.60 ± 1.22, $P < 0.0001$) (individual animal results (5, 10, 5.5, 2.5)</p> <p>Concurrent control 1.10 and 1.40 in the two trials..</p> <p>Positive control gave similar values in both trials (cyclophosphamide, 28.6 and 37)</p>	<p>moribundity. A dose level of 300 mg/kg bw/day in the first trial did not result in any recorded signs of toxicity. In the second trial increased lethargy was reported at 300 mg/kg bw/day.</p> <p>No effects on %PCE were reported in either trial.</p>
<p>BUA 1995.</p> <p>Single oral dose of 180 mg/kg bw to CFLP mice with sampling at 24-72h post dose. Only summary of study available.</p>	<p>No increase in bone marrow MN</p>	<p>Suspension in gum tragacanth. MTD used.</p>

COM Minutes of consideration of Sasaki et al Critical Rev Toxicol, 30, 629-799, 2000.

ITEM 5: IN VIVO COMET ASSAY: DATA ON 208 CHEMICALS AND ADVICE ON DICHLORVOS (AND DIMETHOATE) (MUT/01/4) addendum to MUT/01/4 and MUT/01/10 (tabled)

14. The Committee considered a recent report on a substantial data set on the *in-vivo* comet assay (Sasaki Y F *et al*, 2000. Critical Rev.Toxicol. Vol 30, p 629-799). Comet assay results were given for 8 organs in the mouse with 208 chemicals for which carcinogenicity bioassay data were available. This assay is given specific consideration in the revised COM guidance, where it is stated that earlier problems regarding the distinction between cytotoxic and genotoxic chemicals have now largely been resolved. Overall the assay does appear to give a high correlation with *in-vitro* genotoxicity (i.e. Ames positives); but there are some results that give rise for concern. Twenty percent of rodent non-carcinogens were positive. There was poor correlation between compounds positive in the bone marrow in the Comet assay and positive in the bone marrow mouse micronucleus test (26 compounds positive in both, but 37 negative in comet and positive in micronucleus test, and 9 positive in the Comet assay and negative in the micronucleus test).

15. Members considered that the approach adopted by Sasaki *et al* to testing several hundreds of chemicals had a number of drawbacks, for example, limited reporting of signs of toxicity seen in animals. Members considered that the appropriateness of the isolated nuclei method used by Sasaki *et al* had not been established and noted that it was not possible to have concurrent evaluation of cytotoxicity using this method. Hence there would be difficulties in evaluating the significance of positive results. In this respect members highlighted the results obtained with a number of polycyclic aromatic hydrocarbons and commented that positive results had been reported in the new Comet assay data for some PAHs (eg pyrene) which were considered to be non-carcinogenic in rodents.

16. Members concluded that the new data did not affect the advice given in the COM Guidance document regarding the *in-vivo* Comet assay which should be regarded as a secondary test for use in specific situations not as a primary screen for mutagenic activity. It was considered useful for following up positive *in-vitro* mutagenicity tests, and to have the advantage of being able to look at every tissue *in-vivo* in one assay.