

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

STATEMENT: GENOTOXICITY OF PARA-CHLOROANILINE. COM/09/S3

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. 4-CA is a potential human metabolite of the pesticide diflubenzuron. There is experimental evidence for urinary excretion of 4-CA in swine exposed to diflubenzuron, and for its presence as a metabolite in goats (liver) and in hens (liver and kidney). There is however no evidence for urinary excretion of 4-CA in rats, although it may be an intermediary metabolite in rats. 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 discussed the need for 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. 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 methaemoglobin (MetHb) 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 MetHb formation after 15-60 min and an oral dose of 40 mg/kg bw in rat induced significant levels of MetHb after 60-90 mins. Absorbed 4-CA is widely distributed with specific binding to erythrocytes reported. 4-CA 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 MetHb formation. Excretion as metabolites predominantly via the urine is rapid in rodents.

4. 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, MetHb formation and mild hepato- and renal toxicity. Following repeat dosing, the blood liver, spleen and kidneys are the predominant 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, and phaeochromocytoma of the adrenal gland). 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. It is notable that increased haemangiosarcomas were seen in both rats and mice (in spleen and/or liver).

Overview of genotoxicity data on Parachloroaniline

5. 4-CA in genotoxicity studies has been derived from a number of commercial sources. Where information is available purity is $\geq 97\%$. The COM noted that the lack of sulphotransferase activity in exogenous metabolic activation systems used in the available *in-vitro* mutagenicity tests systems may have limited the ability of the systems to convert the agent (an aromatic amine) to mutagenic species, thereby underestimating its *in vitro* mutagenic potential.

In vitro

Bacterial tests

6. 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 4-CA 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.typhimurium* strains may have been associated with use of lower dose levels and insufficient details were available to assess the conduct of all of these tests.¹¹⁻¹⁸ Evidence for genotoxicity was reported in a $\text{PolA}^+/\text{PolA}^-$ assay using *Escherichia coli*.¹⁸
7. Negative results were obtained in *umu* tests using *S.typhimurium* TA1535pSK1002 in the presence and absence of exogenous metabolic activation from phenobarbital/5,6-benzoflavone or oriental yeast.^{19,20}
8. The COM concluded that positive *in vitro* results had been seen in a number of test systems. This included the Ames test with

Salmonella typhimurium TA98 in the presence of exogenous metabolic activation where a small dose-response was consistently reported at doses levels of ≥ 1000 $\mu\text{g}/\text{plate}$.

Studies using fungi and yeast

9. Evidence for mutagenic activity was reported in *Aspergillus nidulans* (mei^- to mei^+) in a limited study using a single concentration of 4-CA.²¹
10. Negative results were reported for mitotic recombination in *Saccharomyces cerevisiae* D3 at one concentration of 4-CA in the 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
11. The COM agreed that these data were of very limited value in the genotoxicity assessment of 4-CA.

Studies using mammalian cells

12. 4CA induced chromosomal aberrations in CHO cells both in the presence and absence of exogenous metabolic activation using 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. No evidence for an increase in micronuclei was reported in one limited test using CHL cells in the presence and absence of exogenous metabolic activation from S-9 derived from phenobarbital/5,6-benzoflavone induced rats.²⁴
13. The COM concluded that clastogenicity in Chinese Hamster Ovary (CHO) cells had been reported both with and without exogenous metabolic activation but these results showed considerable inter study variation in the magnitude of response which was possibly influenced by cytotoxicity.
14. Mutagenic activity in mouse lymphoma L5178Y cells using the TK^{+/-} locus was 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 (RTG <10-20%) in some of these experiments.^{7,25-28}
15. The COM considered that the available mouse lymphoma studies were limited and were not adequate as assessed using the International Working Group on Genotoxicity Testing (IWGT) Global Evaluation Factor. The COM agreed a positive result was reported in mouse lymphoma L5178Y TK (+/-) cells without exogenous

metabolic activation at dose levels where cytotoxicity was reported.²⁸

16. 4-CA did not induce DNA strand breaks in mouse lymphoma LY5178 TK^{+/-} cells 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 activation²⁹
17. Positive results were reported in two UDS rat hepatocyte experiments at 10 µg/ml. A concentration of 50 µg/ml was toxic to hepatocytes in this study.³⁰ Negative results were reported in a separate study where a concentration of 12 µg/ml was reported to be toxic to hepatocytes.¹⁶

Conclusion *in vitro* studies

18. The COM noted that mutagenic effects in mammalian cells were reported, particularly after exposure to what appeared to be cytotoxic concentrations. Overall the COM concluded that 4-CA is an *in vitro* mutagen in bacteria and mammalian cells.

In vivo studies

19. Evidence for mutagenicity was reported in both repair proficient and deficient *Drosophila melanogaster*.³¹ The COM noted that the significance of this result for human health hazard assessment was unclear.

Studies in rodents

20. No evidence for hepatic DNA binding was reported (RAL <3.1 x 10⁻⁸).³² 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. Rat haemoglobin binding was reported to be 2386 ± 156 pmol/mg. 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. Positive results for DNA adduct formation were reported for concurrent studies using 4-aminobiphenyl.
21. The committee agreed that there was no clear evidence for DNA binding in the liver of rats given an oral dose of 64 mg/kg bw 4-CA. The study had limitations, such as the number of potential DNA adducts included in the analysis, and the chosen dose level may have been insufficient (a relatively low dose compared with the maximum tolerated dose in the rat).
22. Positive results for a comet assay were reported by Sasaki Y et al.³³ 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 was undertaken using isolated nuclei and comets assessed for 50 nuclei from each organ. Migration = Length-Diameter in μm . There was evidence for increased migration in stomach, bladder, lung and brain after 8h, and stomach, colon, liver, bladder, lung, brain after 24h.

23. The COM agreed there was evidence for a positive result in this comet assay in a number of tissues in mice given an oral dose of 200 mg/kg bw 4-CA. However, there were limitations in the conduct and reporting of this investigation, which reduced the weight that could be placed on this study.
24. Positive results have been reported in a bone marrow MN assay in mice.³⁴ Groups of 5 male B6C3F1 mice were dose orally with up to 400 mg/kg 4-CA (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 were 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 4-CA. The test material was administered as the hydrochloride salt in PBS and it considered likely that absorption from the gastrointestinal tract occurred.
27. The COM agreed that a positive response was also reported in an NTP oral bone marrow micronucleus (MN) assay in mice given three daily doses of 300 mg/kg bw/day with sampling 24 hours after the final dose. The assessment of toxicity in this study was inadequate (i.e. only up to 24 hours after the last dose) and it is possible that the 300 mg/kg bw dose level used may have induced significant toxicity. Members discussed the potential influence of methaemoglobin formation, which was likely to have occurred at the 300 mg/kg bw dose level used in this study, on the micronuclei

formation. It was noted that no change in the percentage of PCEs had been reported in the study and the investigators had used fluorescent staining which adequately identified micronuclei containing DNA. Thus, although a positive result was reported there is uncertainty over its biological significance.

28. The COM were aware of two abstracts that reported negative results for bone marrow MN studies in mice, but sufficient evaluation of these studies was not possible.

Conclusion *in vivo* studies

29. The COM concluded although that 4-CA was an *in vitro* mutagen, no definite conclusions on the *in vivo* mutagenicity could be drawn on the available information.

COM Discussion and consideration of further testing.

30. Regarding further testing for the assessment of potential *in vivo* genotoxicity of 4-CA, the committee agreed that as a first step a repeat MN test in mice conducted to internationally acceptable standards to include sampling of the bone marrow and peripheral blood for reticulocytes should be undertaken. Members noted that in previous studies of a substituted aniline, peripheral blood sampling had been more sensitive than bone marrow sampling^{35,36} and hence assessment of micronuclei in reticulocytes should be included in the repeat MN test to be undertaken with 4-CA. If this study was positive, then 4-CA should be regarded as an *in vivo* mutagen. If this study was negative or equivocal then a second *in vivo* study in rats should be undertaken. This second study should be a rat liver UDS assay with a concurrent rat comet assay to investigate DNA damage in the spleen, liver and other tissue (not considered to be a rat tumour target organ). The COM agreed that the proposed comet investigations would be adequate to assess for other tumour target organs in rats (e.g. adrenal) If this second study were positive, then 4-CA should be regarded as an *in vivo* mutagen. If this study was negative, then 4-CA would not be regarded as an *in vivo* mutagen. If the results were equivocal, then further consideration of testing would be required and further advice would be required.

COM conclusions

31. The COM concluded that 4-CA was an *in vitro* mutagen.
32. No definite conclusions on the *in vivo* mutagenicity could be drawn on the information reviewed.
33. A further *in vivo* genotoxicity testing strategy was agreed. This comprised two studies. Study ii) should be undertaken if the results of Study i) were negative or equivocal.

i) a repeat MN test in mice conducted to internationally acceptable standards to include sampling of the bone marrow and peripheral blood for reticulocytes

ii) The second study should be a rat liver UDS assay with a concurrent rat comet assay to investigate DNA damage in the spleen, liver and other tissue (not considered to be a rat tumour target organ).

December 2009

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