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p-Dichlorobenzene

Investigations on the subject of carcinogenicity

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1. SUMMARY

Studies on the putative mechanism of tumor formation in male rats and male and female mice were performed to evaluate their relevance to human carcinogenic risk. A pilot study in mice, as well as the longterm NTP study with this species indicate that p-DCB (as many chlorinated aliphatic or aromatic compounds) induces liver toxicity at relatively high doses probably as a rather unspecific side effect of overloading detoxifying microsomal enzyme systems. Therefore exposure to p-DCB in non-hepatotoxic doses or concentrations is unlikely to increase human cancer risk.

Short-term studies revealed that p-DCB induces a nephropathy in male Fischer 344 rats in the dose range of 75 to 600 mg/kg, which has the typical characteristics of the so-called light hydrocarbon or alpha$_{2\mu}$-globulin nephropathy. This type of nephropathy may be associated with the development of renal cortical tumors after long-term treatment. The nephrotoxicity (and presumably the associated carcinogenicity) depend on the presence of alpha$_{2\mu}$-globulin which has been found only in adult male rats, but not in female rats, mice, guineapigs, dogs, monkeys and humans. The literature on this subject generally regards these renal effects as not predictive for man.

The absence of genotoxic/mutagenic or cell transforming activity has been reconfirmed in CHO/HGPRT forward mutation assays, in micronucleus assays in mice and in vitro transformation of BALB/3T3 cells assays with p-DCB and its major metabolite 2,5-dichlorophenol. In addition, p-DCB did not induce gene mutation in V-79 Chinese hamster lung cells, or chromosome abberrations in human lymphocytes in vitro or unscheduled DNA synthesis in cultered HeLa cells. These data are in agreement with numerous literature references using other test systems.

The available data therefore do not indicate that p-DCB pose a carcinogenic risk to humans.

2. INTRODUCTION

p-dichlorobenzene (p-DCB), a registered pesticide is used as a space deodorant, for moth control and as an intermediate in organic synthesis. Previous in vitro and in vivo investigations have revealed the lack of any genotoxic potential for both p-DCB itself and its major metabolite 2,5-dichlorophenol (Connor et al., 1985; Haworth et al., 1983; Lawlor and Haworth, 1979; Löser and Litchfield, 1982; Myhr, 1973; NTP, 1986; Perocco et al., 1983; Rapson et al., 1980; Shimizu et al., 1983). Long-term inhalation studies in male and female rats and female mice exposed for 5 hours per day on 5 days per week to concentrations of 0 (controls), 75 or 500 ppm p-DCB for a total period of 76 weeks (rats) or 57 weeks (female mice) followed by 36 weeks (rats) or 19 weeks (female mice) post-exposure observation have failed to demonstrate carcinogenic activity (Löser and Litchfield, 1983).

Very recently an increased incidence of renal tubular adenocarcinomas was observed in the kidneys of male Fischer 344 rats treated with doses of 150 and 300 mg/kg/day once daily on 5 days per week over a period of 2 years by gavage (NTP, 1986). Although treated at higher doses (300 and 600 mg/kg/day), females were unaffected. Male and female B6C3F1 mice similarly treated with doses of 300 and 600 mg/kg/day had significantly more hepatocellular neoplasms than the control animals. Besides these tumor responses there were several morphological alterations in the affected organs. It is therefore reasonable to assume, that chronic organ damage may be a contributing factor.

In order to clarify the etiology of the increased kidney tumor incidence in male rats as well as the increased liver tumor incidence in male and female mice a number of studies have been performed. The results are briefly summarized and their relevance with respect to the observed carcinogenic effects are discussed.

3. METHODS AND RESULTS

3.1. Subacute gavage study on the subject of hepatotoxicity in mice

Male and female mice were treated with p-DCB dissolved in corn oil in doses of 0 (controls) 300, 600 and 900 mg/kg respectively once daily for 4 weeks by gavage (Bomhard and Luckhaus, 1986). The doses of 300 and 600 mg/kg corresponded to those used in the long term NTP-study.

Treatment did not affect general behaviour, food and water consumption, growth or mortality. A significant and dose dependent increase in plasma GPT activity was indicative of a hepatotoxic effect in males and females at 600 and 900 mg/kg/day. Cholesterol and bilirubin concentrations in plasma were increased after 900 mg/kg/day in both sexes. Liver weights were markedly and dose dependently increased in all treatment groups. In liver parenchyma there was a dose dependent fatty degeneration in all treated groups and centrilobular and mid-zonal hepatocytes were markedly enlarged at 600 and 900 mg/kg/day.

From this study it can be concluded, that p-DCB exerts hepatotoxicity in mice in the range of doses used for long-term treatment. Chronic liver damage in mice is regularly accompanied or followed by increased rates of hepatocellular tumors (Butler and Newberne, 1975; ECETOC, 1982; Grasso and Crampton, 1972; Schach von Wittenau and Estes, 1983; Tomatis et al., 1973). For agents acting by these so called non-genotoxic mechanisms which enhance liver tumor incidence only under conditions of high exposure and/or chronic toxicity, it is possible to identify convincing reasons why there should be no risk under conditions of low exposure.

3.2. Subchronic toxicological investigations in rats on the subject of nephrotoxicity

Male and female Fischer 344 rats were administered p-DCB in corn oil once daily (seven times per week) by gavage in the following doses: 0 (controls), 75, 150, 300 and 600 mg/kg respectively. Half of the animals were sacrificed after 4 weeks, and the remaining animals after 13 weeks of the study. A number of blood and urinary parameters were studied after 2 days, 1, 4 and 12 weeks of treatment. These and the histopathological and ultrastructural investigations served to clarify the time course of possible renal effects (Bomhard et al., 1987).

Increased urinary LDH and epithelial cell excretion as well as exacerbation of hyaline droplet accumulation in the cytoplasm of renal cortical cells were observed in male rats over the entire dose range investigated. Tubular cell necrosis and granular cast formation as well as tubular dilation of the outer zone of the medulla were evident in male rats after 4 and 13 weeks of treatment with doses of 150 to 600 mg/kg/day. Ultrastructurally there was a dose dependent increase in the accumulation of polymorph-hyaline bodies in the proximal tubular epithelial cells exclusively in males of all treated groups. At 150 mg/kg and above single cell necrosis and (at the higher dose levels) desquamation of longer parts of the tubuli were seen occasionally. At higher magnifications the polymorph-hyaline bodies represent crystalloid structures, which have no obvious effects on intracellular elements. Those structures were also found in male control rats (although to a much reduced extent) but not in females, which also showed none of the other pathomorphological alterations.

These changes in the kidney corresponded in all aspects to those which have been reported after subacute to chronic administration of a large number of aliphatic and cyclic hydrocarbons (McFarland, 1984; Phillips and Cockrell, 1984; Alden et al., 1984; Alden et al., 1985, Craig, 1986). Extensive investigations by these and other authors have demonstrated,

that nephrotoxic effects of these substances occur only in male, but not in female rats or in other species. The reasons for this exceptional response in the male rat have not yet been finally clarified. Numerous results point to a key role for the protein alpha$_{2\mu}$-globulin in the pathogenesis, which has been found only in post-pubertal male rats (Alden, 1986). It has been suggested that certain aliphatic or cyclic hydrocarbons may bind to alpha$_{2\mu}$-globulin to form a complex which is re-absorbed by the renal proximal tubulus and which than accumulates within the cells because of a defect in protein catabolism by renal lysosomes (Kloss and Bus, 1985). It is probable that the increase in renal cortical tumors in the range of nephrotoxic dosages (such as also occurred with the investigated aliphatic and cyclic hydrocarbons) is not indicative of a carcinogenic potential for man for these compounds (Alden, 1986).

3.3. Investigations on the genotoxicity and transforming activity of p-DCB

The effects on 6 test systems, namely V79 and CHO/HGPRT forward mutation assays, chromosome aberration test in human lymphocytes in vitro, micronucleus test, UDS test in cultured HeLa cells and in vitro transformation of BALB/3T3-cells assay were investigated.

3.3.1. CHO/HGPRT forward mutation assay

p-DCB was assayed for point mutagenic activity at the HGPRT locus in CHO cells from 80 µg/ml to 240 µg/ml without activation and from 70 µg/ml to 350 µg/ml with activation. Under both conditions a wide range of toxicity was induced. Without activation these treatments did not cause any statistically significant increases in mutant frequency and total mutant colonies. The test material was therefore considered to be inactive in the CHO/HGPRT forward mutation assay without activation (den Boer and Horn, 1986a). Two experiments were performed in the presence of metabolic activation systems. Small but statistically significant increases in mutant frequency in the first study could not be confirmed in the second study, where the treatment did not cause any consistent increases in mutant frequency, and no dose-response relationships were evident for either total mutant colonies or mutant frequencies. The test material was therefore also considered to be nonmutagenic in the CHO/HGPRT forward mutation assay with activation.

3.3.2. V79/HGPRT forward mutation assay

In concentrations of 1 to 100 µg/ml (without metabolic activation) and 1 to 200 µg/ml (with metabolic activation) p-DCB did not induce an increased frequency of gene mutations at the HGPRT locus of V79 Chinese hamster lung cells. The reference mutagens ethylmethane sulphonate (EMS) and dimethylnitrosamine (DMN) induced an evident increase of HGPRT frequency (Pirovano and Milone, 1986a).

3.3.3. Chromosome aberration test in human lymphocytes in vitro

Cultured human lymphocytes were exposed to p-DCB at concentrations of 1, 10, 100 and 500 µg/ml, both in the presence and in the absence of metabolic activation. By this test, damage to chromatids caused by a test substance can be identified as chromosome aberrations. Metaphase examination did not reveal a p-DCB induced increase of chromosome aberrations with and without metabolic activation (Pirovano and Milone, 1986b).

3.3.4. Micronucleus test

p-DCB was investigated in male and female mice for possible clastogenic effects on the chromosomes of bone marrow polychromatic erythrocytes. The clastogen and cytostatic agent cyclophosphamide served as a positive control. The animals received a single dose of 2500 mg p-DCB/kg body weight or 20 mg cyclophosphamide/kg by gavage. Femoral marrow from the p-DCB groups was prepared after 24, 48 and 72 hours (Herbold, 1986a).

All animals survived until the end of the test, the p-DCB treated animals showing signs of toxicity. Erythrocyte formation, as measured by the ratio of polychromatic to normochromatic erythrocytes was affected. No indications of a clastogenic effect of p-DCB were found, while cyclophosphamide had a clear clastogenic effect.

3.3.5. Unscheduled DNA synthesis in cultured HeLa cells

p-DCB was assayed in 2 separate experiments in 5 concentrations each ranging from 1 to 500 µg/ml and 0.5 to 100 µg/ml respectively without metabolic activation. The concentrations 1 to 500 µg/ml were also tested in the presence of S9 mix (Pirovano and Milone, 1986c).

Without metabolic activation p-DCB proved to be toxic at 50 or more µg/ml. The toxicity was significantly reduced in the presence of the metabolic activation system, the cell survival at the maximum concentration assayed (500 µg/ml) being 43 %.

p-DCB did not induce statistically significant increases in the incorporation of tritiated thymidine in cultured human cells (HeLa) in the dose levels examined both with and without metabolic activation.

3.3.6. In vitro transformation of BALB/3T3 cells assay

p-DCB did not induce a statistically significant increase in the number of transformed foci of BALB/3T3 cells over the concentration range of 60 to 140 µg/ml. This concentration range corresponded to a survival ranging from approximately 2 % to near 100 % as predicted from the preliminary cytotoxicity test and confirmed in the concurrent cytotoxicity test. Therefore, p-DCB is considered to be inactive in the BALB/3T3 in vitro transformation assay (den Boer and Hoorn, 1986b).

3.4. Investigations on the genotoxicity and transforming activity of 2,5-dichlorophenol, the major metabolite of p-DCB

3.4.1. CHO/HGPRT forward mutation assay

2,5-dichlorophenol was assayed in 5 concentrations ranging from 100 µg/ml to 250 µg/ml without S9 activation and 6 concentrations from 62.5 µg/ml to 200 µg/ml with S9 activation (den Boer and Hoorn, 1986c).

In both cases the test material showed dose-related toxicity as evidenced by decreasing relative survival and relative population growth with increasing test material concentrations. There were no dose related increases in the mutant frequency at the HGPRT locus in CHO cell cultures. The test material was therefore considered to be inactive in the CHO/HGPRT forward mutation assay.

3.4.2. Micronucleus test

2,5-dichlorophenol was investigated in male and female mice for a possible clastogenic effect on the chromosomes of bone marrow polychromatic erythrocytes using the micronucleus assay. The clastogen and cytostatic agent cyclophosphamide served as positive control (Herbold, 1986b).

The animals received a single administration of 1500 mg 2,5-dichlorophenol/kg body weight or 20 mg cyclophosphamide/ kg by gavage. Femoral marrow was prepared from the 2,5-dichlorophenol groups 24, 48 and 73 hours after administration. Dichlorophenol treated animals showed signs of toxicity including 6 intercurrent deaths.

Erythrocyte formation, as measured by the ratio of polychromatic to normochromatic erythrocytes was reduced in 2,5-dichlorophenol treated animals, but no indications of a clastogenic effect were found. Cyclophosphamide, the positive control, had a clear clastogenic effect, as can be seen from the biologically relevant increase in polychromatic erythrocytes with micronuclei.

3.4.3 In vitro transformation of BALB/3T3 cells assay

2,5-dichlorophenol was tested for the ability to transform BALB/3T3 cells in vitro. Concentrations of 20.0, 40.0, 50.0, 65.0 and 80.0 µg/ml were selected since higher concentrations proved to be very toxic to the 3T3 cells. 3-methylcholanthrene was used as a positive control (den Boer and Hoorn, 1985).

The concentration range of 2,5-dichlorophenol corresponded to approximately 90 % to 13 % survival as predicted in the preliminary cytotoxicity test and confirmed in the concurrent cytotoxicity test. The test compound did not induce a significant increase in the number of transformed foci. Therefore it is considered to be inactive in the BALB/3T3 in vitro transformation assay.
The sensitivity of the assay was shown by the significantly increased number of foci after 3-methylcholanthrene treatment.

4. DISCUSSION AND CONCLUSIONS

Long-term studies of the US National Toxicology Program (NTP), using high doses of p-DCB in corn oil via gavage, showed a higher incidence of renal adenocarcinoma in male rats and of hepatocellular tumors in male and female mice. The studies were interpreted as providing "clear evidence of carcinogenicity" in male rats and mice of both sexes. It is important to recognise, that the categories adopted in June 1983 for use in technical reports within NTP refer to the weight of experimental evidence from the studies considered and not to either potency or mechanism of carcigenicity.

In order to clarify the relevance of the increased tumor incidences for man a thorough analysis of the underlying mechanism of tumor formation seems to be mandatory. This is also necessary for any decision for inclusion or omission from lists of substances that pose a carcinogenic risk to humans.

The investigations summarized have mainly focussed on 3 aspects:

a) short-term effects on the liver of mice;
b) short-term effects on the kidney of rats;
c) genotoxicity and cell transforming ability of p-DCB and its major metabolite 2,5-dichlorophenol.

Subacute treatment of male and female mice with doses ranging from 300 to 900 mg p-DCB/kg body weight/day administered in corn oil by gavage resulted in a dose dependent liver toxicity at all doses examined. Non-neoplastic alterations in liver morphology were also seen in the majority of male and female mice within the long-term NTP study.

The mouse is extremely sensitive to chronic overloading of metabolic pathways of the liver, chronic induction of microsomal enzymes or direct liver damage. Increased incidences of hepatocellular tumors are regularly observed after respective long-term treatment.

The accumulated knowledge on the induction of tumors of this type in mice in general and after long-term treatment with chlorinated aromatic and aliphatic hydrocarbons in particular show that the mouse liver tumor data are not necessarily indicative of a carcinogenic risk for humans.

Treatment of Fischer 344 rats with p-DCB in the dose range of 75 to 600 mg/kg/day for up to 3 months provided clear evidence of the induction of the so-called light hydrocarbon or alpha$_{2\mu}$-globulin nephropathy in male animals only. A large number of studies with different aliphatic and cyclic hydrocarbons have shown identical toxic effects in the kidneys of male rats. Recent advances in the understanding of mechanisms involved indicate that induction of a nephropathy of this type is a pre-requisite for the tumor induction following long-term administration of these chemicals. Light hydrocarbon nephropathy has been shown to develop after oral and inhalation exposure to various hydrocarbons.

This nephropathy was not seen following long-term inhalation of p-DCB in rats in a previous study (Löser and Litchfield, 1983) probably because the exposure concentrations were below the threshold for the effect. However the study does show that tumor induction does not occur even at substantial concentrations in the absence of this type of nephropathy.

Numerous studies in rodents, such as those performed by NTP on p-DCB, point to the fact, that chronic damage of certain organs or tissues of rodents is an extremely confounding variable in chronic carcinogenicity studies.

The conclusion, that p-DCB is not a primary carcinogen, is supported by a great number of available in vitro and in vivo assays for genotoxicity/mutagenicity or cell transformation ability for both p-DCB itself and its major metabolite 2,5-dichlorophenol.

In summary, the reported investigations support the already available data showing p-DCB to be neither genotoxic nor a primary carcinogenic agent. Increased tumor incidences in certain chronically injured rodent organs may not necessarily pose a carcinogenic risk for humans.

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