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In vivo genotoxicity studies with 3-monochloropropan-1,2-diol

Stephen Robjohns[4], Richard Marshall[1], Michael Fellows[1,3] and George Kowalczyk[2]

Public Health Group, Department of Health, Skipton House, 80 London Road, London SE1 6LH, [1]Covance Laboratories, Harrogate HG3 1PY and [2]Directorate of Health and Social Care – North, Sunley Tower, Manchester M1 4BE, UK

[3]Present address: Genetic Toxicology, AstraZeneca, Mereside, Alderley Park, Macclesfield SK10 4TG, UK

3-Monochloropropan-1,2-diol (3-MCPD) is a contaminant of polyamine flocculants used in the production of drinking water, but more significantly for human exposure it can arise also in certain foodstuffs containing acid-hydrolysed vegetable protein. It is carcinogenic in the rat, producing tumours in males in the testes, mammary gland and the preputial gland and also kidney tumours in both sexes. It has given positive results in *in vitro* mutagenicity studies, but there have been no satisfactorily conducted *in vivo* studies in somatic cells published in the peer reviewed literature. As a result, and because of the absence of appropriate *in vivo* evidence, several international regulatory agencies had previously judged it prudent to assume that 3-MCPD possessed mutagenic activity *in vivo* and considered 3-MCPD to be a genotoxic carcinogen. We present in this paper results from two *in vivo* mutagenicity studies with 3-MCPD, namely a bone marrow micronucleus test in the rat and unscheduled DNA synthesis in the rat liver, both conducted in accordance with relevant OECD protocols. These studies show that 3-MCPD does not possess genotoxic activity *in vivo* in the tissues examined. On the basis of these findings, and along with evidence that tumours may be induced by mechanisms involving either hormonal disturbances or sustained cytotoxicity, we believe that 3-MCPD may now be considered to be carcinogenic to rodents via a non-genotoxic mechanism.

Introduction

3-Monochloropropan-1,2-diol (3-MCPD) is a member of a group of compounds known collectively as chloropropanols, a group that includes the genotoxic animal carcinogen 1,3-dichloropropan-2-ol (Olsen, 1993; Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment, 2001). 3-MCPD itself is an animal carcinogen producing tumours at various sites in male F344 rats (mammary tissue, testes and preputial gland) and renal tubular adenomas and carcinoma in both sexes of F344 rats (Sunahara *et al*., 1993; Lynch, 1998). It has given positive results in *in vitro* mutagenicity studies (Silhankova *et al*., 1982; Zeiger *et al*., 1988; Olsen, 1993; Lynch, 1998) but there have been no satisfactorily conducted *in vivo* studies in somatic cells published in peer reviewed journals.

As a result, and because of the absence of appropriate *in vivo* evidence, several international regulatory agencies had previously judged it prudent to assume that 3-MCPD possessed mutagenic activity *in vivo* and considered 3-MCPD to be a genotoxic carcinogen. Due to its mutagenic potential, practice in the UK had previously been to reduce exposure to 3-MCPD to as low a level as practicable. 3-MCPD has been detected as a contaminant of several foods and food ingredients, including acid-hydrolysed vegetable protein, malts and soy sauces (Food Standards Agency, 2001). The current approach in the UK is to ensure that levels of 3-MCPD in foods and food ingredients are <0.02 mg/kg based on 40% dry matter, the EU limit (European Commission, 2001). In respect of exposure through drinking water, the UK Drinking Water Inspectorate (1999) has set controls on impurity levels of 3-MCPD and on maximum usage rates of flocculant addition to water to achieve a maximum theoretical level in drinking water of 0.1 μg/l [note that this is identical to the level set in the EC Drinking Water Quality Directive (European Commission, 1998) for potentially genotoxic polymer-derived contaminants of drinking water such as acrylamide and epichlorohydrin].

The key missing evidence required to enable an adequate assessment of the health risks of 3-MCPD has been *in vivo* mutagenicity studies conducted to current standards. In this paper we present data on two new studies with 3-MCPD, namely a rat bone marrow erythrocyte micronucleus test and an assay of unscheduled DNA synthesis (UDS) in rat liver cells.

Materials and methods

The methods used for the micronucleus and UDS assays were based on current regulatory guidelines for these tests (OECD, 1997).

Reagents

3-MCPD (98.5% pure) was obtained from Sigma-Aldrich Chemical Co. (Gillingham, UK) and stored at room temperature in the dark. It was diluted in purified water prior to dosing. Negative control animals received purified water alone. 2-Acetamidofluorene (2-AAF) (Sigma Chemical Co., Poole, UK), suspended in corn oil, and dimethylnitrosamine (DMN) (Sigma Chemical Co.), dissolved in purified water, were used as positive controls for the UDS assay. Cyclophosphamide (CPA) (Aldrich Chemical Co., Gillingham, UK) was dissolved in saline to serve as the positive control for the micronucleus test.

Animals

Outbred Crl:HanWist (Glx:BRL) BR rats were obtained from Charles River UK Ltd (Margate, UK) or Harlan (UK) Ltd (Bicester, UK). The animals were housed in solid floored polypropylene cages (45 × 28 × 20 cm), with wood shavings for bedding. They were acclimatised for at least 6 days after arrival and were approximately 8 weeks of age (190–239 g) at the start of dosing. Animals were provided with a special-quality control (SQC) rat mouse maintenance diet [RM1 (E) SQC, Special Diets Services Limited, Witham, UK] *ad libitum*. All procedures carried out as part of this study were subject to the provisions of the UK Animals (Scientific Procedures) Act, 1986.

Dose selection

The doses of 3-MCPD selected for each assay were based on preliminary dose range finding experiments in which rats were given a single administration (UDS assay) or were dosed once daily for two consecutive days (micronucleus test) by oral gavage at a dose volume of 10 ml/kg. Signs of toxicity were then recorded. Maximum doses of 100 mg/kg and 60 mg/kg/day were selected for

[4]To whom correspondence should be addressed. Tel: +44 20 7972 1765; Fax: +44 20 7972 5156; Email: stephen.robjohns@doh.gsi.gov.uk

Table I. Micronucleus test data

Dose (mg/kg/day)	Treatment group	Animal ID number	PCE/NCE ratio		Micronucleated PCE per 1000 cells		
			Per animal	Group mean	Per animal	Group mean	SD
0	Vehicle	808	0.50	0.95	0.5	0.25	0.27
		778	0.48		0.0		
		810	0.99		0.0		
		799	1.26		0.5		
		811	1.38		0.0		
		780	1.09		0.5		
15	3-MCPD	787	1.35	0.92	0.5	0.33	0.41
		788	0.49		0.5		
		798	1.15		0.0		
		783	0.59		1.0		
		784	1.34		0.0		
		786	0.59		0.0		
30	3-MCPD	792	0.99	0.76	1.0	0.42	0.38
		797	0.78		0.5		
		802	0.47		0.5		
		804	0.44		0.0		
		807	0.60		0.0		
		793	1.26		0.5		
60	3-MCPD	794	0.97	0.54	0.0	0.25	0.27
		781	0.63		0.5		
		805	0.51		0.0		
		782	0.40		0.5		
		791	0.20		0.5		
		777	0.55		0.0		
40	CPA	809	0.21	0.40	4.0	2.83	2.42
		785	0.27		7.0		
		790	0.16		1.5		
		806	0.15		2.0		
		779	1.18		0.0		
		800	0.43		2.5		

PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes.

the UDS and micronucleus assays as being close to the maximum tolerated doses for single and double administration, respectively. Although signs of toxicity at these doses in the subsequent genotoxicity assays were limited, the results of the range finder demonstrated that it would not have been reasonable to administer appreciably higher doses. The UDS and micronucleus tests were performed using males only, as no substantial difference in toxicity was apparent between males and females.

Micronucleus test

Groups of six rats were dosed once daily by gavage for two consecutive days with the vehicle (water) or 3-MCPD at 15, 30 or 60 mg/kg/day. CPA was given as a single administration at 40 mg/kg, on the second day of dosing. All animals were killed and bone marrow sampled 24 h after the second 3-MCPD administration. Bone marrow smears were fixed in methanol and stained for 4 min in 12.5 μg/ml acridine orange made up in 0.1 M phosphate buffer, pH 7.4. Slides were rinsed in phosphate buffer and allowed to dry. Slides were randomised and 2000 polychromatic erythrocytes (PCE) scored for micronuclei per animal. The ratio of PCE to normochromatic erythrocytes (NCE) was determined based on a total of at least 1000 PCE + NCE.

UDS assay

Animals were dosed in groups of four with vehicle or 3-MCPD at 40 or 100 mg/kg via oral gavage. The assay was conducted as two experiments. In the first experiment, animals were sampled 2–4 h after administration using DMN as the concurrent positive control. In the second, rats were sampled 12–14 h after treatment using 2-AAF as the positive control. At the appropriate sampling time, each animal was maintained under deep anaesthesia with halothane to prevent recovery and the liver perfused with collagenase solution via the hepatic portal vein. The liver was cut free and the hepatocytes teased out into phosphate buffer. The separated hepatocytes were then resuspended in Williams E Medium–Complete and a sample of the suspension counted after staining with 0.4% (w/v) trypan blue. Each suspension was diluted to provide ~1.5×10^5 viable cells/ml and plated into 6-well multi-well plates (3 ml/well), each well containing a 25 mm round plastic Thermanox coverslip. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air for at least 90 min to allow the cells to attach. The medium was removed from the cells, the monolayers washed and medium containing 10 μCi/ml [^{3}H]thymidine added. After 4 h incubation the medium was removed, the cells washed with three changes of Williams E medium-Incomplete (WE-I) containing 0.25 mM cold thymidine and the cultures incubated overnight in the same medium.

To prepare the coverslips for autoradiography they were washed with phosphate-buffered saline and the cells fixed with three changes of acetic acid:ethanol (1:3 v/v). They were then washed with purified water, allowed to dry and mounted onto microscope slides, cells side up. The slides were dipped in Ilford K2 liquid emulsion, which was then allowed to gel. The slides were packed in light-tight boxes containing desiccant and refrigerated for 14 days. At the end of this time, the emulsion was developed in Kodak D19 developer and fixed using Ilford Hypam fixer. The cell nuclei and cytoplasm were stained with Meyers haemalum/eosin Y, dehydrated in ethanol, cleared in xylene and mounted with coverslips. Grain counting was performed using a microscope with a video camera connected to an image analysis system (Perceptive Instruments, Haverhill, UK).

Each slide was examined to ensure that the culture was viable. For each cell analysed, the number of grains over the nucleus was first counted. The field of view was moved and counts obtained for three separate adjacent areas of cytoplasm of equal size. Nuclear and mean cytoplasmic grain counts were then recorded and the net grains/nucleus (NNG) determined. One hundred cells were analysed per animal.

Data analysis

Micronucleus test. For each group, inter-individual variation in the numbers of micronucleated PCE was estimated by means of a heterogeneity χ^2 test (Lovell *et al*., 1989). The numbers of micronucleated PCE in each treated group were then compared with the numbers in vehicle control groups by using a 2×2 contingency table to determine χ^2 (Lovell *et al*., 1989) and also compared with the laboratory historical negative control range.

UDS assay. The population average NNG, the percentage of cells responding or in repair (i.e. % NNG) and the population average cytoplasmic and nuclear grain counts were calculated for each slide, animal and dose point (cells with 5 NNG or more were considered to be 'in repair'). Results for 3-MCPD-treated animals were compared with concurrent negative controls.

Table II. UDS assay (group mean data results)

Sampling time (h)	Dose (mg/kg)	Treatment group	Net grain count (NNG)		Cells in repair (%) (% NNG)		NNG of cells in repair	
			Mean	SD	Mean	SD	Mean	SD
2–4	0	Vehicle	–2.6	1.0	0.7	0.6	6.3	0.9
	40	3-MCPD	–2.4	0.2	–	–	–	–
	100	3-MCPD	–3.3	0.9	0.3	0.6	5.0	–
	10	DMN	11.3	1.4	74.3	6.4	14.8	1.2
12–14	0	Vehicle	–3.2	0.8	–	–	–	–
	40	3-MCPD	–3.0	0.7	0.3	0.6	7.7	–
	100	3-MCPD	–2.6	1.2	0.3	0.6	5.3	–
	75	2-AAF	19.8	4.0	96.7	3.5	20.2	3.6

Results

Micronucleus test

The results of the micronucleus test are shown in Table I. Rats treated with 3-MCPD had group mean ratios of PCE to NCE which were lower than that seen in the vehicle control group. This is considered to be a sign of toxicity and a clear demonstration of exposure of the target cells. Group mean frequencies of micronucleated PCE were similar and not significantly different from the value for the concurrent vehicle control group and fell within the laboratory historical negative control range.

UDS assay

Summary data for the UDS assay are shown in Table II. Treatment with 3-MCPD at all doses yielded NNG values <0, producing group mean NNG values at the two sampling times in the range –3.3 to –2.4, well below the threshold value of 0 NNG required for a positive response (Kennelly *et al*., 1993; OECD, 1997). No more than 0.3% cells were seen in repair at any dose of 3-MCPD.

Discussion

3-MCPD has a chemical structure which suggests that it may be metabolised to genotoxic intermediates, particularly glycidol. A number of studies have shown 3-MCPD to be mutagenic in the Ames assay both in the presence and absence of S9 metabolic activation (Stolzenberg and Hine, 1979, 1980; Silhankova *et al*., 1982; Majeska and Matheson, 1983; Zeiger *et al*., 1988; Ohkubo *et al*., 1995). 3-MCPD has been reported to be mutagenic in mammalian cells (Olsen, 1993; Lynch, 1998) and to yeast (Rossi *et al*., 1983). Malignant transformation of mouse M2 fibroblasts in culture has also been demonstrated (Piasecki *et al*., 1990). *In vivo* mutagenicity studies have shown 3-MCPD to be negative in the dominant lethal test in the mouse and rat (Jones *et al*., 1969; Epstein *et al*., 1972; Jones and Jackson, 1976). However, there are no data in the published literature relating to *in vivo* studies in somatic cells.

As 3-MCPD is clearly mutagenic *in vitro* in the *Salmonella* assay and in the mouse lymphoma assay in the absence of metabolic activation, it can be assumed that non-microsomal pathways can generate DNA-reactive species *in vitro*. The new *in vivo* data indicate that activity is not expressed in bone marrow or liver. Consideration of the metabolic profile of 3-MCPD indicates that, in rats, the predominant urinary metabolite following oral or i.p. doses of 3-MCPD was β-chlorolactic acid (Jones *et al*., 1978), a metabolite which is produced by an oxidative pathway not generating glycidol. Furthermore, a degradation product of β-chlorolactic acid, namely oxalic acid, has been documented to induce the nephrotoxic effect which is a key feature of 3-MCPD toxicity (Jones *et al*., 1979; Olsen, 1993). One study (Jones, 1975) has shown that 3-MCPD may also be metabolised to glycidol by a minor pathway, but this then undergoes conjugation with glutathione and ultimately forms a mercapturic acid [*N*-acetyl-*S*-(2,3-dihydroxypropyl)cysteine] in urine of rats. This suggests that glycidol may arise at low levels, but that it is subsequently inactivated. While it is appreciated that the metabolism of 3-MCPD in rats has not been fully examined, the available evidence supports the findings from the two new *in vivo* mutagenicity studies, that significant DNA-reactive metabolites are not produced in the whole animal.

The negative findings in the *in vivo* studies are also consistent with postulated non-genotoxic mechanisms for tumour formation in rats (Lynch *et al*., 1998). Tumours were reported in both sexes in the kidney and in males only at hormonally responsive sites (i.e. the testes, mammary gland and preputial gland) at dose levels which exceeded the maximum tolerated dose (Sunahara *et al*., 1993). In the kidney, tumours in both sexes were benign (renal tubular adenoma) and these were accompanied by chronic progressive nephropathy. As discussed above, metabolism to β-chlorolactic acid, and subsequently oxalate, is a major pathway in the rat. Oxalate is known to induce severe renal cytoxicity (Jones *et al*., 1979; Olsen, 1993) and this and other evidence, such as a study by Jones *et al*. (1978) in which crystals of oxalate were detected in the urine of rats treated with 3-MCPD (single dose of 100 mg/kg i.p.), supports a role for sustained cytotoxicity as a possible mechanism for the induction of kidney tumours in rats.

With regard to the sex-specific tumours in male rats (in the testes, mammary gland and preputial gland), it has been argued (Lynch *et al*., 1998) that the testicular tumours need to be viewed against the high spontaneous incidence of Leydig cell tumours common in the ageing F344 rat. Also, 3-MCPD has been shown to induce a prolonged increase in circulating hormone levels, a single i.p. dose of 80 mg/kg body wt causing increased serum levels of follicle stimulating hormone, luteinising hormone and prolactin (Morris and Jackson, 1978). It is possible that increases in the spontaneous rate of Leydig cell tumours may have been promoted by hormonal imbalance caused by 3-MCPD. Subsequently, the increase in tumours at other hormone-responsive sites (i.e. in the male mammary gland and the preputial gland) may be secondary to further hormonal disturbances, which are known to be induced by proliferating Leydig cells.

It is concluded that a non-genotoxic mechanism for the carcinogenicity of 3-MCPD in rodents is plausible and that these new negative *in vivo* mutagenicity studies suggest that reactive metabolites, if formed, do not produce genotoxicity in the bone marrow and liver.

The UK Department of Health referred these two *in vivo* studies to the Committtee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment and the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment and they also concluded that 3-MCPD could be regarded as having no significant genotoxic potential *in vivo* (Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, 2000; Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment, 2000).

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