EFFECTS OF DI-ISONONYL PHTHALATE (DINP) ON PEROXISOMAL MARKERS IN THE MARMOSET - DINP IS NOT A PEROXISOME PROLIFERATOR

Michael HALL¹, Anne MATTHEWS¹, Lisa WEBLEY² and Robert HARLING²

¹Department of In Vitro Metabolism, ²Division of Toxicology,
Huntingdon Life Sciences Limited, England

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ABSTRACT — In the present study the systemic toxic potential of di-isononyl phthalate (DINP) was assessed in a 13-week study in marmosets. Particular attention was given to its potential for hepatic peroxisome proliferation. Three groups of four male and four female marmosets received DINP, by oral gavage administration, at dosages of 100, 500 or 2500 mg/kg/day for 13 weeks. A fourth group served as a concurrent Control group and received the vehicle (1% methylcellulose and 0.5% Tween) only. A fifth group received clofibrate at a dosage of 500 mg/kg/day to provide a positive Control for liver peroxisome activity.

At the end of the treatment period, the animals were killed and their livers were removed. 3000×g supernatant and microsomal subcellular fractions were prepared from homogenised liver by differential centrifugation. The peroxisomal marker enzyme activity, cyanide-insensitive palmitoyl CoA oxidase, was assayed in the former, while cytochrome P450 concentration and lauric acid 11- and 12-hydroxylase activities (selective for CYP2E1 and 4A, respectively) were assayed in the microsomes. No statistically significant changes were seen in any of these parameters measured following DINP treatment, compared with the Control. Clofibrate treatment resulted in an approximately 100% increase ($p < 0.01$) in both male and female marmoset cyanide-insensitive palmitoyl CoA oxidase activity and a similar increase ($p < 0.05$) in male (only) lauric acid 11-hydroxylase activity. No other changes were statistically significant at the 5% level. These data provide no evidence that DINP was acting as a peroxisome proliferator when administered to marmosets under the conditions of the study.

KEY WORDS: Cytochrome P450, DINP, Marmoset, Palmitoyl CoA oxidase, Peroxisome

INTRODUCTION

Certain hypolipidaemic drugs and phthalate esters, have been shown to produce liver enlargement, hepatic peroxisome proliferation, the induction of microsomal cytochrome P450 CYP4A and peroxisomal fatty acid oxidising enzyme activities in rats and mice (Cohen and Grasso, 1981; Gibson, 1993). Cyanide-insensitive palmitoyl CoA oxidase activity is located in the peroxisomes and thus can act as a marker for peroxisome proliferation that may result from administration of a test compound to experimental animals. Although well characterised in rodents, there appears to be equivocal

evidence for these compounds causing similar effects in non-human primates.

Di-isononyl phthalate (DINP) is being developed as an alternative to di(2-ethylhexyl)phthalate (DEHP) for use as a plasticiser (Fig.1). On administration to rats and mice, DEHP causes peroxisome proliferation and liver tumours, although it appears to be relatively weak compared to other compounds, such as clofibrate, which produce similar effects (reviewed by Huber *et al.*, 1996). In an evaluation of the effects of DEHP administration to primates, Kurata *et al.* (1998) dosed the compound to marmosets for 13 weeks. There was no evidence for an effect on peroxisomal enzymes of male

or female animals following treatment with DEHP. However, this compound did produce increases in both hepatic microsomal protein and cytochrome P450 concentrations.

In the present study, the effects of the oral administration to marmosets of DINP, at 3 concentrations for 13 weeks, on both peroxisomal and microsomal enzyme activities were examined and compared to those elicited by clofibrate.

MATERIALS AND METHODS

Animal management and experimental design

Kyowa Hakko Kogyo Co. Ltd (Tokyo, Japan) produced the test substance, DINP (Fig.1). Tokyo Kasai, Kogyo Co., Ltd (Tokyo, Japan) produced the clofibrate. Both substances were clear colourless liquids and were stored in a cool store (approximately 15°C).

Twenty male and twenty female purpose-bred common marmosets, *Callithrix jacchus*, approximately 16 to 25 months of age, were obtained from Harlan UK (Oxfordshire, England). Before treatment commenced the animals of each sex were ranked by weight and allocated to their groups using a random selection procedure ensuring, as far as possible, that all groups contained populations with similar mean and range of bodyweights. The animals were housed individually in aluminium cages with grid floors suspended above metal trays containing wood shavings. Environmental conditions were monitored daily; the target values were 24°C for temperature, 65% for relative humidity and at

least 10 air changes per hr. Lighting was controlled to allow a 14-hr light : 10-hr dark cycle. Treatment commenced after a three-week acclimatisation period. At commencement of treatment the animals weighed between 314 and 462 g.

Each animal was offered 10 g of malt loaf, 25 g of a commercially available pelleted New World primate diet (Mazuri, Special Diets Services Ltd., Essex, England) and 65 g of fresh fruit each day. The diet was moistened with 15 ml of water except for one day each week when it was moistened with 15 ml Cytac solution, a Vitamin B12 supplement (Duncan Flockhart and Company Ltd., Middlesex, England). On two days each week a further 20 g of forage feed (William Lillico and Son Ltd., Surrey, England) was provided. Diet and fruit was available at all times except when urine was being collected or overnight before blood sampling.

The DINP and clofibrate were prepared for administration weekly as an emulsion in 1% methylcellulose and 0.5% Tween. Animals were dosed daily by oral gavage at a volume dosage of 5 ml/kg bodyweight for 13 weeks.

Observations and laboratory investigations

Animals were examined daily, body weights were recorded weekly and food consumption was recorded daily throughout the study. Ophthalmoscopy examinations were performed before treatment commenced and during Week 12 of treatment. Blood samples were taken for haematology investigations before treatment commenced and during Weeks 6 and 13 of treatment,

Fig. 1. Chemical structure of DINP, DEHP and clofibrate.

and for blood chemistry analyses before treatment commenced and during Weeks 4 and 13. Further blood samples were obtained before commencement of treatment and during Week 12 for analysis of oestradiol and testosterone concentrations. Urine samples were collected for analysis before treatment commenced and during Weeks 5 and 12 of treatment.

Pathological examination

On completion of the 13-week treatment period, all animals were placed under intravenous sodium pentobarbitone anaesthesia and killed by rapid exsanguination. Each animal was examined externally and internally for macroscopic evidence of disease or adverse reactions to treatment. The main organs were weighed and a full spectrum of tissues retained in 10% neutral buffered formalin. Exceptionally, the eyes were preserved in Davidson's fluid and the testes and epididymides were retained in Bouin's fluid prior to fixing in 70% industrial methylated spirit. Immediately after removal and weighing of the liver, samples (minimum 5 g) were taken from each animal and processed as described under peroxisome proliferation. The tissues, taken from each animal, were embedded in paraffin wax, sectioned at approximately four to five micron thickness and stained with haematoxylin and eosin before microscopic examination.

Peroxisome proliferation

1. Chemicals

Bovine serum albumin (BSA), sodium dithionite, lauric acid, glucose 6-phosphate (disodium salt), NADP⁺ (sodium salt), glucose 6-phosphate dehydrogenase (type VII, from baker's yeast), palmitoyl CoA, NAD⁺ (sodium salt, grade VII, from yeast), coenzyme A (sodium salt), dithiothreitol and FAD (disodium salt) were all obtained from Sigma Chemical Co., Ltd. (Poole, Dorset, UK). [1-¹⁴C]Lauric acid (56 μ Ci/ μ mol, 50 μ Ci/ml) was obtained from Amersham International plc. (Amersham, Buckinghamshire, UK). Other chemicals were purchased either from Fisher Laboratory Supplies (Loughborough, Leicestershire, UK) from BDH Ltd. (Poole, Dorset, UK), or from Rathburn Chemicals (Walkerburn, Scotland).

2. Experimental design

Whole livers were removed from each marmoset at *post mortem* and weighed. Portions of the liver were then immediately frozen in liquid nitrogen and subsequently stored at *ca* -75°C pending processing. Before homogenisation, livers were thawed in ice-cold 50 mM

Tris-HCl buffer, pH 7.4 (at 25°C), containing 250 mM sucrose, on ice. A representative portion (*ca* 3 g) of each liver was homogenised in Tris-sucrose buffer, pH 7.4. 3000×g supernatant and microsomal subcellular fractions were prepared from each liver homogenate by differential centrifugation.

3. Liver enzyme assays

The protein concentration of each 3000×g supernatant and microsomal suspension was determined by the method of Lowry *et al.* (1951). Cyanide-insensitive palmitoyl CoA oxidase activity was measured in the 3000×g supernatant, by monitoring NADH formation at 340 nm using a UV/Vis spectrophotometer, essentially as described by Gray *et al.* (1983). Microsomal cytochrome P450 concentration was assayed by CO-difference spectrum, according to Rutten *et al.* (1987). Lauric acid 11- and 12-hydroxylase activities were determined in microsomes by a method based on that of Parker and Orton (1980). Carbon-14 labelled lauric acid and hydroxylated metabolites were separated by HPLC and quantified using an on-line radiodetector. All enzyme activities were assayed in duplicate, as a minimum. Results were expressed per milligram of protein in the appropriate subcellular fraction. Mean $\pm$ standard deviation was calculated for each group.

Statistical analyses

Data were analysed using the statistical tests listed below.

Students *t*-test using pooled error variance (Armitage, 1971) - haematology (except eosinophil, basophil and monocyte counts which are not normally distributed), blood chemistry and urinalysis (volume, specific gravity, pH and electrolytes only). Bartlett's test (Bartlett, 1937) for homogeneity and Behrens-Fischer/Dunnett's test (Cochran and Cox, 1957; Dunnett, 1955) - body-weight gain and organ weights. Fisher's Exact test (Fisher, 1973) - macroscopic pathology and histopathology. Levene's test for homogeneity of variance (Levene, 1960), both Williams' (Williams, 1971, 1972) and Dunnett's, (1955, 1964) tests to compare enzyme data in DINP treated groups with the Control group. A two-sided *t*-test (Snedecor and Cochran, 1989) was used to compare the clofibrate group with the Control group. Significance testing for liver assays was carried out at the 5% and 1% levels only.

RESULTS

Peroxisome proliferation

1. Liver weights

The weights of the livers removed from animals at *post mortem*, expressed both as absolute weights and relative to bodyweight, are shown in Table 1. These data do not provide any indication of a treatment-related or statistically significant effect of DINP or clofibrate administration.

2. Protein concentrations

Protein concentration of the 3000×g supernatant subcellular fraction was not affected by either DINP or

clofibrate treatment (Table 2).

Microsomal protein concentrations of both the male low and intermediate treatment groups showed a slight decrease (both 34%) following administration of DINP to marmosets (Table 3). However, these changes were not statistically significant at the 5% level. Clofibrate administration to female animals resulted in a statistically significant increase (of 69%) in microsomal protein concentration. A similar increase was seen in clofibrate-treated male marmosets, but this did not attain statistical significance at the 5% level.

3. Cyanide-insensitive palmitoyl CoA oxidase activity

There was no evidence for a dose-related or statis-

Table 1. Body weights and liver weights of marmosets following the oral administration of DINP and clofibrate for 13 weeks

Sex	Treatment	Dose (mg/kg/day)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
Male	Control	0	379±46	16.1± 5.4	4.19±0.95
	DINP	100	406±45	25.5±11.5	6.14±2.38
		500	404±30	20.1± 7.4	4.90±1.39
		2500	378±45	19.2± 7.5	5.01±1.65
	Clofibrate	500	375±31	18.5 ± 5.3	4.92±1.32
Female	Control	0	377±37	15.9± 3.5	4.20±0.69
	DINP	100	410±57	18.8± 5.3	4.53±0.84
		500	413±54	20.6± 7.1	4.94±1.52
		2500	384±53	16.3± 5.4	4.17±0.86
	Clofibrate	500	367±16	18.2± 4.7	4.96±1.20

Data are the mean of 4 animals per treatment ± standard deviation, except for the male clofibrate and female control groups, when n = 3.

Table 2. Marmoset hepatic 3000×g supernatant protein concentrations and cyanide-insensitive palmitoyl CoA oxidase activities

Sex	Treatment	Dose (mg/kg/day)	Protein concentration (mg/g liver)	Palmitoyl CoA oxidase activity (nmoles/min/mg protein)
Male	Control	0	112.3±18.2	2.43 ±0.43
	DINP	100	104.9±23.8	2.77 ±0.41
		500	112.5±10.9	2.65 ±0.23
		2500	113.0±11.7	2.72 ±0.39
	Clofibrate	500	124.9±26.0	4.79** ±0.30
Female	Control	0	99.8±12.6	2.58 ±0.38
	DINP	100	115.2±11.3	3.26 ±0.51
		500	103.9±21.2	2.85 ±0.50
		2500	119.1±19.0	2.63 ±0.40
	Clofibrate	500	116.5±14.1	4.91** ±0.55

Data are the mean of 4 animals per treatment ± standard deviation, except for the male clofibrate and female control groups, where n = 3. **p<0.01, compared with vehicle control group.

tically significant (at the 5% level) effect of DINP on cyanide-insensitive palmitoyl CoA oxidase activity, assayed in 3000×g supernatant prepared from liver homogenate (Table 2). However, clofibrate administration led to an increase of approximately 100% in this activity in both male and female marmosets ($p<0.01$, for both sexes).

4. Cytochrome P450 concentration and lauric acid hydroxylase activities

Several male marmosets had P450 concentrations that were below the limit of quantification of the assay. These included one animal from each of the 0 (Control) and 2500 mg/kg/day DINP groups and two from each of the 100 and 500 mg/kg/day DINP groups. The values below the limit of quantification were taken as zero in the calculations of mean $\pm$ standard deviation (Table

3). As a consequence of the number of observations below the limit of quantification, statistical analysis was not performed on the male P450 data. No statistically significant or treatment-related changes in cytochrome P450 concentration were seen following administration of either DINP or clofibrate to either sex of marmoset.

Lauric acid hydroxylase activity was measured by quantifying the formation of the two major products, viz. 11-hydroxylauric acid (ω -1-hydroxylation) and 12-hydroxylauric acid (ω -hydroxylation). There was only limited evidence for an effect of DINP on the formation of either product. Lauric acid 11-hydroxylase activity was increased by 27% and 19% following treatment of male and female marmosets, respectively, at 2500 mg/kg/day. Similarly, lauric acid 12-hydroxylase activity was increased by 29% and 35%, respectively, for these groups. However, none of these changes were

Table 3. Marmoset hepatic microsomal protein and cytochrome P450 concentrations and lauric acid 11- and 12-hydroxylase activities

Sex	Treatment	Dose mg/kg/day	Protein concentration (mg/g liver)	Cytochrome P450 concentration (nmoles /mg protein)	Lauric acid 11-hydroxylase activity (nmoles/min /mg protein)	Lauric acid 12-hydroxylase activity (nmoles/min /mg protein)
Male	Control	0	11.4 $\pm$ 6.6	0.126 $\pm$ 0.108	0.403 $\pm$ 0.279	0.093 $\pm$ 0.084
	DINP	100	7.5 $\pm$ 5.5	0.098 $\pm$ 0.122	0.305 $\pm$ 0.259	0.066 $\pm$ 0.064
		500	7.5 $\pm$ 4.7	0.103 $\pm$ 0.121	0.338 $\pm$ 0.221	0.068 $\pm$ 0.049
		2500	10.9 $\pm$ 5.9	0.160 $\pm$ 0.108	0.512 $\pm$ 0.242	0.120 $\pm$ 0.066
	Clofibrate	500	17.9 $\pm$ 5.8	0.268 $\pm$ 0.095	0.862* $\pm$ 0.175	0.118 $\pm$ 0.016
Female	Control	0	13.9 $\pm$ 2.5	0.223 $\pm$ 0.017	0.854 $\pm$ 0.053	0.170 $\pm$ 0.008
	DINP	100	15.8 $\pm$ 2.2	0.241 $\pm$ 0.060	0.876 $\pm$ 0.203	0.153 $\pm$ 0.035
		500	15.1 $\pm$ 1.5	0.276 $\pm$ 0.029	0.896 $\pm$ 0.107	0.219 $\pm$ 0.044
		2500	13.9 $\pm$ 2.3	0.256 $\pm$ 0.022	1.020 $\pm$ 0.123	0.230 $\pm$ 0.064
	Clofibrate	500	23.5** $\pm$ 4.7	0.301 $\pm$ 0.082	0.815 $\pm$ 0.225	0.143 $\pm$ 0.042

Data are the mean of 4 animals per treatment $\pm$ standard deviation, except for the male clofibrate and female control groups, where $n = 3$. * $p<0.05$, ** $p<0.01$, compared with the vehicle control group.

Table 4. Overall group mean bodyweight gain (Weeks 0 to 13)

Sex		Dosage group (mg/kg/day)				
		Control 0	Clofibrate 500	DINP 100	DINP 500	DINP 2500
Males	Body weight gain (g)	25	11	32	26	5
	as % of vehicle control	—	44	128	104	20
Females	Body weight gain (g)	35	10	37	30	16
	as % of vehicle control	—	29	106	86	46

statistically significant at the 5% level. Clofibrate treatment elicited an increase (of 114%) in male lauric acid 11-hydroxylase activity, which was significant ($p < 0.05$), but effects on female 11-hydroxylase activity and 12-hydroxylase activities of both sexes were not.

General toxicology

Clinical signs observed for males and females receiving DINP at 2500 mg/kg/day included ungroomed coat and slight or moderate reddening of the skin around the anus and back of the legs which became evident from Week 3 of treatment and persisted throughout the remainder of the study. The reddening was considered to be a superficial and localised reaction, possibly to the presence of the test substance in the faeces, whilst the ungroomed coat was considered to reflect the oily nature of the test substance. One male receiving the high dosage of DINP became underactive, and had a hunched posture during Week 13. It had been thin since Week 6 and had lost 13% of its bodyweight during the treatment period. Although this was the only animal with such findings it is considered that these may have been caused by treatment with DINP.

Salivation was regularly observed shortly after dosing for males and females receiving clofibrate. This sign became apparent immediately after dosing but was absent 1 to 2 hr later. During the first two weeks of treatment emesis was also occasionally observed for males and females receiving clofibrate but was rarely seen thereafter.

There were two deaths during Week 13 of treatment (one male receiving clofibrate and one Control female), but neither was related to treatment.

Suppression of body weight gain or body weight loss (see Table 4) was observed for the majority of animals receiving clofibrate (mean gains of 44% and 29% of the Controls for males and females, respectively). Body weight loss or body weight suppression was also observed for two males and one female receiving 2500 mg/kg/day DINP; the majority of animals were, however, apparently unaffected (mean gains of 20% and 46% of the Controls for males and females, respectively). Food consumption was unaffected by treatment with DINP or clofibrate.

There were no treatment-related ophthalmoscopic findings.

There were no treatment-related haematology changes for animals receiving DINP. Haematology investigations during week 13 of treatment revealed low packed cell volumes ($p < 0.05$), haemoglobin concentrations ($p < 0.05$) and erythrocyte counts ($p < 0.01$)

for all females receiving clofibrate when compared with the controls. Slightly high mean cell haemoglobin, mean cell volumes and platelet counts were also apparent for these animals. Similar changes were seen for the males although the inter-group differences were less marked than those for the females; only low erythrocyte counts and high mean cell volumes attained statistical significance ($p < 0.01$ and $p < 0.05$ respectively). Haematological changes during Week 6 of treatment were generally similar but less marked than those observed during Week 13 of treatment. Erythrocyte counts were low for animals receiving clofibrate when compared with the controls ($p < 0.05$ and $p < 0.01$ for males and females respectively). Slightly low packed cell volumes and haemoglobin concentrations were also apparent for these animals although the inter-group differences were minor and did not attain statistical significance. For the males, the inter-group difference was largely due to one animal.

There were no blood chemistry changes and no changes in testosterone or oestradiol concentrations that were clearly related to treatment with DINP or clofibrate.

The composition of the urine was unaffected by treatment with DINP. During week 12 of treatment, urinary sodium and chloride levels were slightly low for females receiving clofibrate when compared with the Controls and pretreatment values ($p < 0.05$ for Chloride). Chloride levels were also slightly low for these animals during week 5 of treatment when compared with the controls. The urine of males was unaffected by treatment with clofibrate.

Macroscopic findings and organ weight

There were no clear treatment-related organ-weight changes for males or females which received DINP. Slightly high absolute and bodyweight-relative kidney weights were evident for males and females which received clofibrate (approximate 30% increase). Absolute and bodyweight-relative salivary gland weights were also slightly high for females which received clofibrate (17% increase).

Thin build was apparent for one male and one female which received the high dosage of DINP; otherwise there were no treatment-related macroscopic findings for DINP or clofibrate.

Histopathology

There were no microscopic findings related to treatment with either DINP or clofibrate.

DISCUSSION

Several compounds, in particular certain phthalate ester plasticisers, such as DEHP, and hypolipidaemic drugs, such as clofibrate, have been shown to produce liver enlargement, hepatic peroxisome proliferation, the induction of CYP4A and peroxisomal fatty acid oxidising enzyme activities in rats and mice (Cohen and Grasso, 1981; Gibson, 1993; Huber *et al.*, 1996). Following prolonged administration to rodents, these compounds produce liver tumours, apparently by a non-genotoxic mechanism (Gibson, 1993; Ashby *et al.*, 1994). A number of non-rodent species, however, appear refractory to the effects of peroxisome proliferators. These include both humans and non-human primates (Ashby *et al.*, 1994; Woodyatt *et al.*, 1999). The administration of rodent peroxisome proliferators such as clobuzarit, for 15 days (Orton *et al.*, 1984), nafenopin, for 21 days (Lake *et al.*, 1989), ciprofibrate for up to 3 years (Makowska *et al.*, 1991; Graham *et al.*, 1994), and clofibrate and DEHP, both for 13 weeks (Kurata *et al.*, 1998), has been reported to produce little or no evidence of morphological changes in the liver or other organs of the marmoset. Small increases have been seen in palmitoyl CoA oxidase activity following treatment with nafenopin (< 200%) and ciprofibrate (up to approximately 350%), but not DEHP.

In the present study, clofibrate (500 mg/kg/day) administration to marmosets for 13 weeks resulted in statistically significant increases (of approximately 100%) in peroxisomal cyanide-insensitive palmitoyl CoA oxidase activity. These changes are similar to those reported by Kurata *et al.* (1998) but are much less than have been seen following clofibrate administration to rodents (Cohen and Grasso, 1981). There was no evidence for an effect of DINP on this activity following dosing at levels of up to 2500 mg/kg/day.

As well as inducing peroxisomal enzyme activities, the administration of clofibrate and other peroxisome proliferators to rats produces increases in the ω -(12)-hydroxylation of lauric acid (Parker and Orton, 1980). This activity is generally considered to be selectively catalysed by cytochrome P450 enzyme(s) of the 4A subfamily and the levels of these proteins are also increased in rats by peroxisome proliferators. It has been proposed that the increase in this activity and associated enzyme(s) forms an integral component of the mechanism of peroxisome proliferation (Gibson, 1993; Ashby *et al.*, 1994). There is no evidence to date for statistically significant induction of marmoset lauric acid 12-hydroxylase activity or CYP4A by peroxisome

proliferators (Lake *et al.*, 1989; Makowska *et al.*, 1991; Kurata *et al.*, 1998; results of the present study). Indeed, evidence for the presence of CYP4A protein in either treated or untreated marmoset liver remains ambiguous (Makowska *et al.*, 1991; Igarashi *et al.*, 1997). Interestingly, clofibrate induced lauric acid ω -1-(11)-hydroxylase activity, by >100% in male marmosets in the present study. A similar effect has been seen with nafenopin (Lake *et al.*, 1989), but not with ciprofibrate (Makowska *et al.*, 1991) or DINP (this study). This activity has been shown to serve as a marker for CYP2E1 in rat and human (Clarke *et al.*, 1994). The enzyme protein has been detected in marmoset liver (Schulz *et al.*, 1998).

In conclusion, oral administration of DINP to marmosets at dosages of up to 2500 mg/kg/day did not produce any evidence of significant toxicity. In particular, there was no indication that DINP acted as a peroxisome proliferator and there were no treatment-related histopathological changes. Minor changes were evident at 2500 mg/kg/day and the No-Observed-Effect Level (NOEL) was considered to be 500 mg/kg/day.

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