Effects of Di-isononyl Phthalate, Di-2-ethylhexyl Phthalate, and Clofibrate in Cynomolgus Monkeys

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The effects of the peroxisome proliferators di-isononyl phthalate (DINP) and di-2-ethylhexyl phthalate (DEHP) were evaluated in young adult male cynomolgus monkeys after 14 days of treatment, with emphasis on detecting hepatic and other effects seen in rats and mice after treatment with high doses of phthalates. Groups of 4 monkeys received DINP (500 mg/kg/day), DEHP (500 mg/kg/day), or vehicle (0.5% methyl cellulose, 10 ml/kg) by intragastric intubation for 14 consecutive days. Clofibrate (250 mg/kg/day), a hypolipidemic drug used for cholesterol reduction in human patients was used as a reference substance. None of the test substances had any effect on body weight or liver weights. Histopathological examination of tissues from these animals revealed no distinctive treatment-related effects in the liver, kidney, or testes. There were also no changes in any of the hepatic markers for peroxisomal proliferation, including peroxisomal beta-oxidation (PBOX) or replicative DNA synthesis. Additionally, in situ dye transfer studies using fresh liver slices revealed that DINP, DEHP, and clofibrate had no effect on gap junctional intercellular communication (GJIC). None of the test substances produced any toxicologically important changes in urinalysis, hematology, or clinical chemistry; however, clofibrate produced some emesis, small increases in serum triglyceride, decreased calcium, and decreased weights of testes/epididymides and thyroid/parathyroid. The toxicological significance of these small changes is questionable. The absence of observable hepatic effects in monkeys at doses that produce hepatic effects in rodents suggests that DINP, DEHP, and clofibrate would also not elicit in primates other effects such as liver cancer. These data, along with results from in vitro hepatocyte studies, indicate that rodents are not good animal models for predicting the hepatic effects of phthalates in primates, including humans.

Key Words: tumor promotion; nongenotoxic carcinogens; phthalate esters; gap junctions; intercellular communication; hepatocytes; peroxisomes.

Peroxisomal proliferators are a chemically and structurally diverse class of chemicals that have been utilized in a wide spectrum of uses for a number of decades. This class of chemicals induces hepatic tumors in rats and mice and peroxisome proliferation in the liver (Ashby et al., 1994; IARC, 1995). While a mechanistic relationship between peroxisomal proliferation and hepatic cancer induction by this class of chemicals has not been confirmed, associated cellular changes, including inhibition of gap junctional intercellular communication, inhibition of apoptosis, and stimulation of cell proliferation by these agents appear to correlate with hepatic carcinogenicity. However, humans appear refractory to the induction of peroxisomal proliferation by chemicals that are peroxisome proliferators in rodents. Thus, the relevance of the various rodent liver effects, including hepatocellular carcinoma to humans, remains a topic of debate (Cattley et al., 1998; Doull et al., 1999). In the present investigation, the hepatic effects of two commercial phthalate plasticizers, di-2-ethylhexyl phthalate (DEHP) and di-isononyl-phthalate (DINP), were examined in male cynomolgus monkeys, since primates are a closer surrogate for predicting the human response than are rodents. The pharmaceutical agent clofibrate was included as a reference substance.

Clofibrate, a hypolipidemic drug developed and marketed in the early 1960s for the treatment of high cholesterol in humans (IARC, 1996; Tucker and Orton, 1993) is still used in the treatment of hypercholesterolemia. For therapeutic purposes, it is administered orally at a total daily dose of 2 g, or ~20–30 mg per kilogram body weight per day (mg/kg/day) (Brown and Goldstein, 1990; IARC, 1996). Clofibrate was used as a reference substance because it appears to be a more potent inducer of peroxisomal-proliferation-related hepatic effects in rats and mice than the phthalate esters (Ashby et al., 1994; Cohen and Grasso, 1981; Doull et al., 1999; Lake et al., 1984). Additionally, because there are nearly 40 years of human clinical experience with this drug (Brown and Goldstein, 1990; Tucker and Orton, 1993), the clofibrate data affords the opportunity to relate the primate data to humans.
IARC reviewed the comprehensive human data and found no evidence for cancer associated with the use of clofibrate when used by humans as a cholesterol-lowering drug (IARC, 1996). Likewise, numerous high-dose studies in rhesus monkeys and marmosets treated with clofibrate for up to 6.5 years at doses exceeding the therapeutic dose have shown no evidence of peroxisome proliferation, liver tumors, or related hepatic effects (Tucker and Orton, 1993). However, at a dietary dose of 5000 ppm (~250 mg/kg/day), clofibrate induced peroxisomes and produced liver tumors in 20 to 91% of chronically treated rats (Ashby et al., 1994; Doull et al., 1999; Hartig et al., 1982; Reddy and Qureshi, 1979; Svoboda and Azarnoff, 1979).

There are no comparable epidemiology data available for the phthalate esters, but there are extensive animal data. Chronic feeding of DEHP to male Fischer 344 rats produced an increased incidence of liver tumors (hepatocellular carcinoma or neoplastic nodules) at dietary doses of 12,000 ppm (~670 mg/kg/day) but not at 6000 ppm (~320 mg/kg/day) (Kluwe et al., 1982). More recent studies using lower doses of DEHP reported an increase in hepatocellular tumors in male F344 rats following treatment with 2500 ppm (estimated daily intake ~140 mg/kg/day), but not at 500 ppm (estimated as ~30 mg/kg/day) (David et al., 1999; Doull et al., 1999). DINP produced no increase in hepatic tumors in male F344 rats at lifetime dietary doses of 6000 ppm (>360 mg/kg/day) (Butala et al., 1996; Lington et al., 1997). However, the incidence of combined hepatic adenomas and carcinomas increased to 26% of rats on lifetime dietary doses of 12,000 ppm DINP (>730 mg/kg/day) (Butala et al., 1996). There are comparable results in chronic feeding studies in mice (Kluwe et al., 1982; Butala et al., 1997; David et al., 1999; Doull et al., 1999). Estimates of human exposure to phthalates range from approximately 10–50 μg/kg/day (ATSDR, 1993; Doull et al., 1999; Peijnenburg et al., 1991) but can be higher for some specific applications such as medical devices (Doull et al., 1999; Huber et al., 1996).

Previous studies of DINP and DEHP in non-human primates focused on hepatic and metabolic effects occurring within 14 to 21 days of treatment. Treatment of cynomolgus monkeys with up to 500 mg/kg/day of DEHP by oral gavage produced no histological (light and electron microscopy) or biochemical evidence of peroxisome proliferation as assessed by determination of peroxisomal beta-oxidation (PBOX) activity and lauric acid hydroxylation (Astill, 1989; Short et al., 1987). Likewise, marmosets treated with up to 200 mg/kg/day DEHP by oral gavage for 14 consecutive days did not exhibit morphological or biochemical changes in the liver (Rhodes et al., 1986). In several in vitro studies, rat and primate hepatocytes were exposed to the active monoester metabolite of DINP and/or DEHP. The dramatic differences in response provided additional evidence that primates, including humans, are insensitive to the hepatic effects of peroxisomal proliferators in contrast to rodents (Baker et al., 1996; Benford et al., 1986; Bichet et al., 1990; Dirven et al., 1993; Elcombe and Mitchell, 1986; Kamendulis et al., 1999 [unpublished]). In vitro studies with clofibrate, ciprofibrate, and derivatives show similar results (Bichet et al., 1990; Blauberger et al., 1990; Elcombe et al., 1997; Perrone et al., 1998).

Short-term (2–4 weeks) exposure of DINP and DEHP to rats and mice inhibits gap junctional intercellular communication (GJIC) and increases PBOX activity and replicative DNA synthesis (Isenberg et al., 1999; Smith et al., 2000). Compounds that block GJIC and increase replicative DNA synthesis appear to function at the tumor promotion phase of the chemical carcinogenesis process. Modulation of these endpoints has been implicated in peroxisome proliferator-induced hepatocarcinogenesis in rodents. The purpose of this study was to determine whether there were any effects on these more sensitive, additional hepatic endpoints, as well as other systemic effects, in cynomolgus monkeys treated with DINP and DEHP in comparison to those treated with clofibrate. The rationale was that observations in a non-human primate, such as the cynomolgus monkey, would provide more meaningful information on the human health response to phthalates and other peroxisome proliferators. The dose level of 500-mg/kg/day DINP and DEHP chosen for these studies produced hepatic effects in rats. Further, the administration of DEHP to cynomolgus monkeys at doses greater than 500 mg/kg/day will not necessarily increase the absorbed dose in this species (Short et al., 1987). Clofibrate was administered at a dose of 250 mg/kg/day, which is comparable to the dietary dose in rats that induces peroxisomes and produces liver tumors (5000 ppm, ~250 mg/kg/day) (Reddy and Qureshi, 1979).

**MATERIALS AND METHODS**

**Chemicals.** JAYFLEX® DINP Plasticizer (di-isononyl phthalate, DINP, >98% purity, CAS RN 68515-48-0) was obtained from Exxon Chemical Company (Houston, TX). Phthalates are produced by esterification of phthalic anhydride with various alcohols in a closed system. The alcohol used to make DINP is prepared by oligomization of propylene and mixed butenes resulting in a C9-rich mixture consisting of roughly equivalent amounts of 3,4-, 4,6-, 3,6-, 4,5-, and 5,6-dimethyl heptanol, with smaller amounts of methyl octanol, and iso-decanol. Di-2-ethylhexyl phthalate (DEHP, >98% purity, CAS RN 117-81-7) and phthalic acid (PA, 99+% purity) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Clofibrate (2-[4-chlorophenoxyl]-2-methylpropionic acid, ethyl ester, >99% purity) and methyl cellulose were purchased from Sigma Chemical Company, Inc. (St. Louis, MO). Mono-isononyl phthalate (MINP) was synthesized by Aldrich Chemical from the same type of alcohol feedstock used to make the DINP, and mono-2-ethylhexyl phthalate (MEHP) was a gift from Dr. Heindel (NIEHS, Research Triangle Park, NC).

The use of methylcellulose (0.5%) as the vehicle control was based on preliminary studies that indicated that the use of corn oil as a vehicle reduced appetite and produced gastrointestinal upset. Dosing solutions were prepared once weekly by dissolving the appropriate amounts of test articles in 0.5% methylcellulose to give the specified dose in a volume of 10 ml/kg.

**Animals and treatment.** Captive-bred, colony-raised, naive young adult (~2-year-old) male cynomolgus monkeys (Macaca fascicularis) were obtained from HRP-Texas Primate Center (Covance Research Products, Inc., Alice, TX) and acclimated for at least 30 days prior to treatment initiation. Monkeys were housed individually in stainless steel cages suspended over a flush pan in an isolated-temperature (66–78°F) and humidity-controlled (63–
EFFECTS OF DINP AND DEHP IN MONKEYS

RESULTS

Animal Observations

There were no overt changes in the general health or behavior of the monkeys following 14 days of dosing. With the possible exception of occasional emesis in two animals treated with clofibrate, following dosing, no adverse treatment-related effects were observed.

Body and Organ Weights

Treatment with DINP and DEHP for 14 days had no effect on body weights (Table 1), food consumption (data not shown), or relative weights of any organs assessed, including liver, kidney, thyroid/parathyroid, and testes/epididymides (Table 1). Statistically significant decreases in relative thyroid/parathyroid and testes/epididymides weights were observed in monkeys treated with clofibrate for 14 days (Table 1). Treatment with DINP, DEHP, and clofibrate for 14 days had no effect on absolute or relative weights of adrenals, brain, heart, lung, or spleen (data not shown).

Serum Chemistry and Urinalysis

Treatment of monkeys with 500 mg/kg/day DINP and DEHP for 14 days did not produce any changes in serum chemistry that were significantly different from control-treated monkeys. Statistically significant changes seen only in serum triglycerides and calcium levels were observed in monkeys treated with clofibrate when compared to controls (Table 2). Urinalysis was unremarkable (data not shown).

Hematology

There were no important changes in hematological parameters associated with the administration of DEHP, DINP, or clofibrate. Statistically significant increases in neutrophil count and decreases in lymphocyte count were observed in monkeys treated with DINP for 14 days (Table 3). These values were similarly affected in other treatment groups, but changes were not statistically significant.
Peroxisomal Beta-Oxidation (PBOX)

There were no statistically significant increases in PBOX activity in cynomolgus monkeys treated with DEHP, DINP, and clofibrate for 14 days (Table 4).

Gap Junction Intercellular Communication (GJIC). DEHP, DINP, and clofibrate had no effect on GJIC (Table 4). In the control group, the distance of dye transfer was 0.26 ± 0.03 mm. In the treated groups, the distance of dye transfer for DEHP, DINP and clofibrate was 0.28 ± 0.01 mm, 0.27 ± 0.02 mm, and 0.27 ± 0.03 mm, respectively.

Repetitive DNA Synthesis

There were no statistically significant changes in total hepatic DNA synthesis in animals treated with DEHP, DINP, or clofibrate (Table 4).

Histopathology

Microscopic evaluations were performed on tissues collected from the liver, kidney and testes of control and treated monkeys. Diffuse hepatocellular vacuolation was observed in one animal in the DEHP treatment group and in one animal in the clofibrate treatment group. There were no distinctive treatment-related effects observed in the kidneys or testes following DEHP, DINP, or clofibrate administration (Table 4). Furthermore, no signs of inflammation or necrosis were seen in any of the tissues evaluated.

Analysis of DINP, DEHP, and Metabolites in Liver and Serum

Low levels of DINP and DEHP were detected in the livers of monkeys treated with the methyl cellulose vehicle control. Due to the ubiquitous nature of the phthalate esters, the low level of phthalate esters measured in control samples may have resulted from leeching during storage of the samples. In the DINP and DEHP treatment groups, the parent diester, monoester metabolite, and phthalic acid (PA) were detected. DEHP (500 mg/kg/day) resulted in MEHP levels of 17.0 μmol/g compared with MINP levels of 2.2 μmol/g in monkeys treated with DINP (500 mg/kg/day). Higher levels of DEHP were found in the liver as compared to DINP; however, this finding was small and not statistically significant.

### Notes

- **Table 1** Effects of DEHP, DINP and Clofibrate on Body and Organ Weights in Cynomolgus Monkeys

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>Final bw (g)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Testes/epididymides</th>
<th>Thyroid/parathyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>2590 ± 138</td>
<td>1.915 ± 0.196</td>
<td>0.465 ± 0.063</td>
<td>0.083 ± 0.018</td>
<td>0.022 ± 0.006</td>
</tr>
<tr>
<td>DEHP</td>
<td>500</td>
<td>2378 ± 194</td>
<td>1.840 ± 0.188</td>
<td>0.473 ± 0.037</td>
<td>0.069 ± 0.005</td>
<td>0.018 ± 0.003</td>
</tr>
<tr>
<td>DINP</td>
<td>500</td>
<td>2458 ± 316</td>
<td>1.893 ± 0.145</td>
<td>0.476 ± 0.027</td>
<td>0.063 ± 0.010</td>
<td>0.016 ± 0.005</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>250</td>
<td>2482 ± 270</td>
<td>2.153 ± 0.110</td>
<td>0.484 ± 0.051</td>
<td>0.057 ± 0.013*</td>
<td>0.014 ± 0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant, p < 0.05.

- **Table 2** Effects of DEP, DINP, and Clofibrate on Serum Chemistry in Cynomolgus Monkeys

<table>
<thead>
<tr>
<th>Endpoint (units)</th>
<th>Control</th>
<th>DEHP 500 mg/kg/day</th>
<th>DINP 500 mg/kg/day</th>
<th>Clofibrate 250 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>669.8 ± 100.9</td>
<td>555.8 ± 132.4</td>
<td>545.4 ± 162.6</td>
<td>494.9 ± 103.4</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.9 ± 0.5</td>
<td>5.0 ± 0.3</td>
<td>4.9 ± 0.3</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>15.4 ± 3.5</td>
<td>15.4 ± 3.9</td>
<td>21.9 ± 5.0</td>
<td>20.8 ± 4.0</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>10.4 ± 0.1</td>
<td>10.5 ± 0.2</td>
<td>10.0 ± 0.2</td>
<td>9.9 ± 0.4*</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>140.6 ± 34.4</td>
<td>138.4 ± 21.9</td>
<td>140.9 ± 23.5</td>
<td>128.9 ± 18.0</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>108.9 ± 0.9</td>
<td>108.5 ± 2.7</td>
<td>110.1 ± 2.6</td>
<td>109.9 ± 1.3</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>2.8 ± 0.4</td>
<td>2.5 ± 0.3</td>
<td>2.4 ± 0.3</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Creatine (mg/dL)</td>
<td>0.77 ± 0.4</td>
<td>0.68 ± 0.1</td>
<td>0.99 ± 0.1</td>
<td>0.78 ± 0.2</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>4.84 ± 0.5</td>
<td>5.15 ± 0.7</td>
<td>5.10 ± 0.4</td>
<td>4.59 ± 0.6</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>36.8 ± 11.3</td>
<td>41.4 ± 11.4</td>
<td>52.4 ± 11.6</td>
<td>63.0 ± 13.6*</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.26 ± 0.04</td>
<td>0.31 ± 0.2</td>
<td>0.31 ± 0.1</td>
<td>0.23 ± 0.1</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>6.4 ± 0.4</td>
<td>5.6 ± 0.6</td>
<td>5.8 ± 1.3</td>
<td>6.0 ± 1.1</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>7.7 ± 0.3</td>
<td>7.5 ± 0.5</td>
<td>7.3 ± 0.4</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>

*Statistically significant, p < 0.05.
Effects of DEHP, DINP, and Clofibrate on Hematology in Cynomolgus Monkeys

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Control</th>
<th>DEHP 500 mg/kg/day</th>
<th>DINP 500 mg/kg/day</th>
<th>Clofibrate 250 g/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells (thousands/mm³)</td>
<td>14.8 ± 5.0</td>
<td>10.3 ± 2.3</td>
<td>9.5 ± 2.0</td>
<td>13.1 ± 3.4</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>25.0 ± 5.0</td>
<td>35.0 ± 16.0</td>
<td>51.0 ± 12.0</td>
<td>44.0 ± 12.0</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>69.0 ± 9.0</td>
<td>61.0 ± 17.0</td>
<td>44.0 ± 8.0*</td>
<td>55.0 ± 14.0</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>6.0 ± 5.0</td>
<td>3.0 ± 2.0</td>
<td>5.0 ± 7.0</td>
<td>1.0 ± 2.0</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.0 ± 1.0</td>
<td>2.0 ± 2.0</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Red blood cells (millions/mm³)</td>
<td>5.75 ± 0.4</td>
<td>5.86 ± 0.3</td>
<td>5.82 ± 0.5</td>
<td>5.46 ± 0.4</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.5 ± 0.5</td>
<td>13.3 ± 0.4</td>
<td>12.9 ± 0.4</td>
<td>12.5 ± 0.9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42.1 ± 2.4</td>
<td>42.3 ± 1.8</td>
<td>39.5 ± 2.2</td>
<td>39.6 ± 2.5</td>
</tr>
<tr>
<td>Mean corpuscular volume (µm³)</td>
<td>73.0 ± 5.0</td>
<td>72.0 ± 2.0</td>
<td>68.0 ± 2.0</td>
<td>73.0 ± 1.0</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (pg)</td>
<td>23.5 ± 1.5</td>
<td>22.7 ± 0.8</td>
<td>22.2 ± 1.3</td>
<td>22.8 ± 0.1</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (g/dL)</td>
<td>32.0 ± 0.7</td>
<td>31.5 ± 0.5</td>
<td>32.7 ± 0.9</td>
<td>31.5 ± 0.3</td>
</tr>
<tr>
<td>Platelet count (thousand/mm³)</td>
<td>390.0 ± 57.0</td>
<td>372.0 ± 131.0</td>
<td>427.0 ± 120.0</td>
<td>335.0 ± 144.0</td>
</tr>
</tbody>
</table>

Note. Values are mean ± SD; n = 4. *Statistically significant, p < 0.05.

PA were similar for monkeys treated with DEHP and DINP (Table 5).

DISCUSSION

The overall objective of this study was to assess the effects of DEHP, DINP, and clofibrate on peroxisomal proliferation in the cynomolgus monkey. The specific parameters measured included weights and histologic changes in target organs (liver, kidneys, and testes), induction of hepatic peroxisomal enzymes, hepatic replicative DNA synthesis, and hepatic gap junctional intracellular communication (GJIC). Recent evidence suggests that while induction of peroxisomal proliferation per se does not correlate with hepatic carcinogenesis, other cellular changes that are frequently seen with the peroxisome-proliferating compounds (blockage of GJIC, decrease apoptosis, increased cell proliferation) have been implicated in non-genotoxic/tumor-promotion aspects of the cancer process. Increased hepatic peroxisome proliferation is among the most prominent cellular effect observed with phthalate treatment in rodents. This change, along with inhibition of GJIC, is believed to be involved in rodent liver carcinogenesis. The absence of changes in the liver is in agreement with studies that have shown primates unresponsive to the hepatic effects of peroxisome proliferators (Foxworthy et al., 1990; Gray and De la Iglesia, 1984; Hall et al., 1999; Holloway, 1982; Kurata et al., 1997). On the other hand, these results differ significantly from rodent studies in which phthalates alter GJIC, PBOX and replicative DNA synthesis at doses similar to those used in the present study (Isenberg et al., 1999; Smith et al., 1999). The lack of modulation of these endpoints in primates by peroxisome proliferators provides additional evidence for species-specificity of phthalates in the induction of carcinogenesis.

Increases in liver and kidney weight have been shown to occur with peroxisomal proliferators. In the present study, all of the test compound studies failed to elicit an increase in liver or kidney weight. Relative weight of testes/epididymis was unaffected by DEHP or DINP treatment but was significantly decreased in clofibrate-treated animals. This finding was likely due to a decreased absolute weight in one of the animals (data not shown), and does not appear to be treatment-related.

Effects of DEHP, DINP and Clofibrate on Gap Junctional Intracellular Communication (GJIC) and Indicators of Peroxisomal Proliferation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GJIC (mm)</th>
<th>PBOX (fold increase)</th>
<th>DNA synthesis (fold increase)</th>
<th>Microscopic observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26 ± 0.03</td>
<td>—</td>
<td>—</td>
<td>Not remarkable</td>
</tr>
<tr>
<td>DEHP 500 mg/kg/day</td>
<td>0.28 ± 0.01</td>
<td>1.4 ± 0.47</td>
<td>1.07 ± 0.12</td>
<td>Diffuse hepatocellular vacuolation in one animal</td>
</tr>
<tr>
<td>DINP 500 mg/kg/day</td>
<td>0.27 ± 0.02</td>
<td>1.5 ± 0.40</td>
<td>0.95 ± 0.09</td>
<td>Not remarkable</td>
</tr>
<tr>
<td>Clofibrate 250 mg/kg/day</td>
<td>0.27 ± 0.03</td>
<td>1.4 ± 0.48</td>
<td>0.95 ± 0.16</td>
<td>Diffuse hepatocellular vacuolation in one animal</td>
</tr>
</tbody>
</table>

Note. Values are mean ± SD; n = 4.
have also observed that the relative thyroid/parathyroid weight was decreased in cynomolgus monkeys treated with clofibrate. Although this decrease was statistically significant, there were no obvious signs of toxicity associated with this change. In studies conducted in marmosets, a non-human primates species, Hall et al. (1999) demonstrated that neither DINP (2500 mg/kg/day) nor clofibrate had any effect on the relative liver weights over a 13-week treatment period. In similar studies, Kurata et al. (1998) showed that neither DEHP (2500 mg/kg/day) nor clofibrate (250 mg/kg/day) had any effect on relative liver, kidney, or testes weights.

There were no statistically significant changes in serum chemistry values in monkeys treated with DEHP or DINP in the present study. These results are in agreement with previous reports in which treatment with DEHP or DINP for 13 weeks in marmosets produced no effect on serum lipid measurements (Hall et al., 1999; Kurata et al., 1998). In the present study, clofibrate induced slight changes in blood triglyceride and calcium levels. No changes in cholesterol were observed following any of the treatments. The dose of clofibrate used in this study (250 mg/kg/day) exceeded the therapeutic dose for humans (20–30 mg/kg/day), (Brown and Goldstein, 1990; IARC, 1996). Clofibrate at 250 mg/kg/day produces marked effects on hepatic peroxisome proliferation in rodents (Ashby et al., 1994; Cohen and Grasso, 1981; Doull et al., 1999; Lake et al., 1984). It might therefore be expected that clofibrate treatment would alter the serum lipid balance in cynomolgus monkeys. Since the serum chemistry measurements in the present study examined total cholesterol and did not separate sub-fractions of cholesterol, clofibrate may have affected the overall lipid balance. Additionally, the efficacy of clofibrate on non hyperlipidemic rodents and/or primates is not known. These results are consistent with those reported previously for the marmoset that showed no change in cholesterol or triglyceride levels following clofibrate treatment for 13 weeks (Hall et al., 1999; Kurata et al., 1998).

The induction of peroxisome proliferation has been implicated as a mechanism in rodent hepatocarcinogenesis. It has been hypothesized that peroxisome proliferators increase the activity of peroxisomal peroxide-producing enzymes in the liver without elevating the levels of H2O2 scavenging enzymes (Rao and Reddy, 1991; Reddy and Rao, 1986). Following this line of reasoning, the level of oxidative stress to the cell would be expected to increase dramatically. The resulting oxidative stress could result in oxidative DNA adducts (OH8dG) and/or could modulate normal cellular processes. In the former, oxidative adducts may produce mutation, and/or modify gene expression resulting in increased cell proliferation. In the latter case, oxidative modification of cellular processes including GJIC, second messengers, gene transcription elements, and mitochondrial function may result in aberrant cell proliferation.

In the present studies, DEHP, DINP, and clofibrate showed no evidence for inducing PBOX activity in cynomolgus monkeys. Similar observations were made in marmosets treated for 90 days with DINP (2500 mg/kg/day) (Hall et al., 1999) and DEHP (2500 mg/kg/day) (Kurata et al., 1998).

In order to relate the results of the present work to humans, we chose clofibrate as a reference material, because its potency as a peroxisome proliferator is greater than the phthalates and also because it is used therapeutically for long periods of time by human patients with altered cholesterol metabolism. Liver biopsies from patients receiving hypolipidemic drugs such as clofibrate or gemfibrozil for long-term therapy did not reveal any evidence of increased peroxisomal enzyme activity (De La Iglesia et al., 1982; Hanefeld et al., 1980). Thus, the results of the primate studies of markers of peroxisome proliferation are consistent with human experience.

It has been hypothesized that elevated rates of cellular DNA synthesis represent an important mechanism by which DEHP and related phthalates induce tumors in rodents (Cattley et al., 1990; Chen et al., 1994; Roberts et al., 1995). In contrast DEHP, DINP, and clofibrate had no effect on DNA synthesis in cynomolgus monkeys. Similar to the species-specific response observed in the GJIC and PBOX assays, replicative DNA synthesis appears to be selectively modulated in rodents. Potent induction of replicative DNA synthesis has been reported in the rat following treatment with the hepatocarcinogen Wy-14,623 (Wada, 1992); however, only a slight increase was observed in hamsters (Durnford et al., 1995; Lake et al., 1993). Species differences have been reported in other studies where cipofibrate produced an acute increase in replicative DNA synthesis in rats, but resulted in a small decrease in DNA synthesis in human hepatocytes (Perrone et al., 1998).

There was no evidence of inhibition of GJIC in the primate studies. Similarly, in vitro studies using hepatocytes isolated from primates failed to demonstrate an inhibition of GJIC.

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**TABLE 5**

Analysis of DEHP, DINP and Metabolites in Cynomolgus Monkey Liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metabolite (µmoles/gram tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEHP</td>
</tr>
<tr>
<td>Control</td>
<td>3.3 ± 3.9</td>
</tr>
<tr>
<td>DEHP (500 mg/kg/day)</td>
<td>8.5 ± 11.2</td>
</tr>
<tr>
<td>DINP (500 mg/kg/day)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Note.* Values are mean ± SD; n = 4. ND = Not determined (value below detection limit); N/A = not applicable.
following exposure to peroxisome proliferators (Kamendulis et al., 1999, unpublished). The strongest evidence supporting a causal relationship between GJIC inhibition and cancer has been shown in rodents where GJIC is inhibited in vivo in rats and mice at tumorigenic doses of phthalates, but not at lower, non-tumorigenic doses (Isenberg et al., 1999; Smith et al., 1999). In studies using primary cultured rat and mouse hepatocytes, Kamendulis et al. (1999, unpublished) have shown that the major monoester metabolite of several phthalates inhibits GJIC; however no effects are observed in hepatocytes isolated from hamsters or humans. Based on these results, it appears that inhibition of GJIC by phthalates such as peroxisomal proliferation is species-specific and dose-dependent.

In an attempt to quantitate the actual levels of monoester metabolites present in the livers of monkeys, following DEHP and DINP treatment, HPLC analysis was performed on liver samples collected at the time of sacrifice. Consistent with published studies indicating that the monoester is the putative hepatotoxic metabolite, we observed significantly higher levels of MEHP in the liver compared to levels of the parent diester, DEHP. Interestingly, higher levels of MNP were not associated with DINP administration. This finding is possibly due to problems encountered in the extraction-recovery procedure rather than to incomplete hydrolysis of DINP to MNP. Following metabolism to the monoester metabolite, the phthalate monoesters are subsequently metabolized to phthalic acid. Similar levels of this metabolite were found in both DEHP- and DINP-treated animals and it does not appear to play a significant role in phthalate-induced liver toxicity. Although the monoester metabolites were detected in the livers of monkeys treated with DEHP and DINP, the levels of these metabolites were much lower than the levels reported in rodents (Isenberg et al., 2000; Smith et al., 1999). This finding may be attributed to the fact that the rate of DEHP hydrolysis in the gut of primates is much less than the rate observed in rats (Rhodes et al., 1986; Short et al., 1987).

In conclusion, the present findings showed primates to be unresponsive to the induction of DNA synthesis and peroxisomal β-oxidation, and to inhibition of GJIC following short-term treatment with these established rodent peroxisome proliferators and liver carcinogens. Furthermore, these data, in combination with previous research, lend additional support to the hypothesis that the hepatotoxicity of peroxisome proliferators is species-specific and that rodents may not be an appropriate model to predict the carcinogenic risk for peroxisome proliferating compounds in humans. Based on the present study and previous findings, phthalate esters do not appear to produce hepatic effects associated with peroxisome proliferation and hepatic carcinogenicity in humans.

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This manuscript is dedicated to the memory of our deceased colleague, Arthur W. Lington, for his enthusiasm and dedication to the mechanistic understanding of hepatocarcinogenesis induced by peroxisome proliferators. We thank Dr. Richard H. McKee for his critical review of and input on this manuscript.

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