Enhancement of thyroid and hepatocarcinogenesis by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene in rats at doses that cause maximal induction of CYP2B

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To investigate the promoting effects of 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) on liver and thyroid carcinogenesis of rats at doses that cause maximal induction of hepatic CYP2B, 5-week-old male F344 rats were given either a single i.p. dose of 75 mg N-nitrosodiethylamine (NDEA)/kg body wt in saline or saline alone. After 2 weeks the rats were fed control diet or a diet containing 330 or 1000 p.p.m. TCPOBOP or 500 p.p.m. phenobarbital (PB; a positive control group). A total of four sequential sacrifices (9, 30, 52 and 79 weeks of age) was performed. At 30 weeks the mean volume (mm³) of hepatocellular foci in NDEA-initiated rats exposed to either dose of TCPOBOP or to PB was significantly increased as compared with rats exposed to NDEA followed by control diet (P < 0.05). In addition, the volume percentage of liver occupied by foci was significantly greater in NDEA-initiated/1000 p.p.m. TCPOBOP-promoted rats as compared with rats exposed to NDEA alone (P < 0.05, n = 6). At 52 weeks of age the incidences (and multiplicities, in units of tumors per tumor-bearing rat) of hepatocellular adenomas were 0, 83 (2.6 ± 1.3), 100 (3.4 ± 2.1) or 67% (2.5 ± 1.9) in rats exposed to NDEA alone or NDEA followed by 330 or 1000 p.p.m. TCPOBOP or 500 p.p.m. PB respectively (n = 12). Hepatocellular carcinomas were found only in rats given 1000 p.p.m. TCPOBOP (17% incidence) or PB (8% incidence) following NDEA initiation. The incidences of thyroid follicular cell adenomas were 0, 17, 33 or 8% in rats exposed to NDEA alone or NDEA followed by 330 or 1000 p.p.m. TCPOBOP or 500 p.p.m. PB respectively. Between 53 and 79 weeks of age 38% of rats treated with NDEA alone developed multiple (1.5 ± 0.8) hepatocellular adenomas. This incidence was enhanced to 100% in rats exposed to NDEA followed by either 330 or 1000 p.p.m. TCPOBOP. Multiplicities of hepatocellular adenomas were also increased significantly (10.5 ± 3.9, 10.4 ± 7.0 and 10.1 ± 6.7 respectively) in rats promoted with 330 or 1000 p.p.m. TCPOBOP or 500 p.p.m. PB. None of the rats exposed to NDEA alone developed hepatocellular carcinomas, while multiple hepatocellular carcinomas occurred in 38% of the rats exposed to 330 p.p.m. and 78% of the rats given 1000 p.p.m. TCPOBOP following NDEA initiation. Thyroid follicular cell tumours occurred at 79 weeks in more than 40 and 50% incidences in rats exposed to NDEA followed by 330 or 1000 p.p.m. TCPOBOP respectively. Also, a significant decrease in serum levels of triiodothyronine and thyroxine were observed in non-initiated 79-week-old rats fed 1000 p.p.m. TCPOBOP, compared with age-matched untreated controls (n = 6). Increases in hepatic CYP2B-mediated benzoyloxy-resorufin O-dealkylase activity detected in rats exposed to 330 and 1000 p.p.m. TCPOBOP for 2 or 23 weeks were similar in magnitude to those caused by 500 p.p.m. PB. Thus TCPOBOP at maximal CYP2B induction doses exhibits a strong promoting activity for both liver and thyroid of rats.

Introduction

The chlorinated hydrocarbon 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP*) was initially characterized by Poland and co-workers (1) as a relatively high affinity CYP2B induction agonist in the mouse. In a previous study (2) we found that TCPOBOP administered as multiple i.p. or oral doses at 3.0 mg/kg for 30 weeks was a complete carcinogen and an extremely potent promoter for mouse liver tumors initiated with N-nitrosodiethylamine (NDEA). In contrast, TCPOBOP was ineffective as a liver tumor promoter in F344 rats even at 10 times the dose (30 mg/kg) administered to mice. When tumor promoting activity and induction of cytochrome P450 subfamily 2B (CYP2B) were compared in the rat and mouse a strong association between these two parameters was observed (2). TCPOBOP was a potent CYP2B inducer and a tumor promoter in mice, but was negligibly effective as either an inducer or a promoter in F344 rats at a 10-fold higher dose (30 mg/kg). Thus our results were in agreement with those of Poland et al. (1,3) who first demonstrated that TCPOBOP is a potent inducer of CYP2B activity in mice, but is literally an order of magnitude less effective in F344 rats.

More recently (4) our group discovered that TCPOBOP displays maximal CYP2B induction in male rats [comparable with that caused by phenobarbital (PB)] when administered at higher dose levels. In that study, TCPOBOP at 1000 p.p.m. in the diet (14 days) appeared to be nearly as effective as 500 p.p.m. PB in this species. When extent of induction was related, on a molar basis, to serum total xenobiotic level, TCPOBOP appeared to be at least as potent as, if not more potent than, PB in the rat (4). In addition, this compound, like PB, induced other manifestations of the PB-type pleiotropic response, including CYP3A, epoxide hydrolase and glutathione S-transferase gene expression (4). Thus TCPOBOP would appear to be a complete agonist and not a partial agonist for PB-type response in the rat as suggested by the results of Poland et al. (1,3), Romano et al. (5) and our own laboratory (2).

Several PB-type hepatic microsomal enzyme inducers are known to cause thyroid follicular cell hyperplasia and promote thyroid carcinogenesis initiated by a variety of carcinogens in

Footnotes:

*Abbreviations: TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; NDEA, N-nitrosodiethylamine; PB, phenobarbital; T₃, triiodothyronine; T₄, thyroxine; TSH, thyroid-stimulating hormone.
rats (6–9). The hepatic PB-type pleiotropic response is thought to play an indirect role in the promotion process. The cumulative effect of PB-type inducers on various drug metabolizing activities and on liver mass leads to increased metabolic clearance of thyroid hormones, resulting in lowered circulating thyroxine (T4) and triiodothyronine (T3) levels. The subsequent chronic stimulation of thyroid follicular epithelium by thyroid stimulating hormone (TSH), produced in the pituitary in response to the abnormally low thyroid hormone levels, has been implicated as the primary cause of thyroid tumor promotion by PB-type inducers (8, 10, 11).

The present study was undertaken to re-evaluate the efficacy of TCPOBOP as a promoter of liver and thyroid carcinogenesis in male F344/NCr rats. The hypothesis to be tested was that dose levels of compounds that cause maximal CYP2B induction (i.e., equivalent to that caused by 500 p.p.m. PB) in the rat would be equally effective as liver and thyroid tumor promoters in this species.

Materials and methods

Chemicals

NDEA (purity 99%) was purchased from Eastman Chemicals (Rochester, NY). PB and triacryalin were obtained from Sigma Chemical Co. (St Louis, MO). NDEA was dissolved in saline at a concentration of 5 mg/ml Benzyloxyresorufin (BHRF2) was purchased from Molecular Probes Inc. (Eugene, OR). Dicumarol and resorufln were obtained from Aldrich Chemical Co. (Milwaukee, WI) and fluorescamine from Fluka Chemical Corp. (Ronkonkoma, NY). TCPOBOP was synthesized from 2,3,5-trichloropyridine as described by Polan et al. (1).

Animals

Male F344/NCr rats were obtained from the Animal Production Area, Frederick Cancer Research and Development Center (Frederick, MD). They were housed (three per cage) in polycarbonate cages on hardwood chip bedding and fed NIH-31 diet (Zeigler Brothers, Gardners, PA) and water ad libitum. The animals were housed at a temperature of 68–72°F and a relative humidity of 50 ± 5%, with a 12 h light/dark cycle. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, 1985).

Experimental design

Rats were randomized at 5 weeks of age into seven groups of 42 rats each. Rats in experimental groups 1–4 were given a single i.p. injection of 75 mg NDEA/kg body wt in sterile 0.85% NaCl. Controls (groups 5–7) received an i.p. injection of 0.85% NaCl alone. Starting 2 weeks later groups of rats were given diet containing either 500 p.p.m. PB (groups 4 and 6) or TCPOBOP (330 p.p.m. for group 2 and 1000 p.p.m. for groups 3 and 5). Dietary admixtures were prepared by combining the appropriate weight of TCPOBOP or PB with powdered diet in a W blender. Group 7 was an untreated control group. Six rats per group were killed at 9 and 30 weeks of age. 12 per group were killed at 52 weeks of age and the remainder were killed when moribund or at 79 weeks of age, when the experiment was terminated.

Pathology

All animals were carefully necropsied. The livers were removed in toto and weighed. At weeks 2 and 23 (9 and 30 weeks of age) a portion of the median lobe of liver from each rat was frozen at −70°C for enzyme analysis. At each time point (9, 30, 52 and 79 weeks of age) portions of liver lobes (two sections/lobe) and all liver lesions were fixed in 10% buffered formalin. All other internal organs were also observed. The lungs, nasal cavity, kidneys, thyroid gland, thymus and spleen, as well as other organs with gross lesions, were routinely removed and fixed in formalin. All visible lesions and representative samples from each major organ were embedded in paraffin, sectioned at 6 μm and routinely stained with hematoxylin and eosin for histological evaluation. Quantification of morphologically altered foci was performed using a video image analysis system (Bioquant System VI; R&M Biometrics, TN) and the stereology programs (12).

Serum thyroxine (T4) and triiodothyronine (T3) and TSH concentration

At terminal sacrifice blood was collected via retro-orbital sinus bleeding from six animals of groups 5, 6 and 7 (non-initiated rats fed 1000 p.p.m. TCPOBOP, 500 p.p.m. PB or control diet respectively). Blood samples were centrifuged (1000 g, 10 min) to obtain serum. Measurement of serum T3, T4 and TSH was conducted by Analytics Inc. (Gaithersburg, MD).

Biochemical studies

Liver samples of rats killed at 9 and 30 weeks of age were weighed and homogenized in 3 ml/g wet wt. 0.15 M KCl, 0.2 M sucrose (4°C). Postmitochondrial (S9) fractions were used for determination of benzyloxyresorufin-O-dealkylase activities as described earlier (9, 13, 14). Protein content in the S9 samples was determined using fluorescamine (15) with bovine serum albumin as the standard.

Statistical analysis

The significance of differences in the survival rate and the incidence of tumors among different groups was evaluated by Fisher’s exact test (one-tailed). Tumor multiplicity data, data on circulating levels of T3, T4, TSH and benzyloxyresorufin-O-dealkylase data were subjected to the Mann–Whitney U-test to determine statistical significance between the various promoted groups and the NDEA/control group. P ≤ 0.05 was considered to indicate a statistically significant difference.

Results

Survival and body weight gain

Survival was significantly affected in rats exposed to 1000 p.p.m. TCPOBOP following NDEA initiation. This was evident as early as 60 weeks of age and most (64%) of the rats in this group were killed in a moribund condition or died before 65 weeks of age (Figure 1). The major cause of death in these rats was the occurrence of multiple and malignant
Table I. Hepatocellular foci in 30-week-old rats

<table>
<thead>
<tr>
<th>Treatment (group)</th>
<th>No./cm³</th>
<th>No./liver</th>
<th>Mean volume (mm³)</th>
<th>Percent volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDEA/control (1)</td>
<td>27±11</td>
<td>390±143</td>
<td>0.018±0.009</td>
<td>0.045±0.025</td>
</tr>
<tr>
<td>NDEA/TCPOBOP 330 ppm (2)</td>
<td>22±11</td>
<td>457±230</td>
<td>0.040±0.007*</td>
<td>0.090±0.057</td>
</tr>
<tr>
<td>NDEA/TCPOBOP 1000 ppm (3)</td>
<td>23±13</td>
<td>531±304</td>
<td>0.058±0.007*</td>
<td>0.118±0.046*</td>
</tr>
<tr>
<td>NDEA/PB 500 ppm (4)</td>
<td>15±9</td>
<td>345±211</td>
<td>0.049±0.028*</td>
<td>0.087±0.049</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with NDEA/control group (n = 6).
No hepatocellular foci were observed in non-initiated groups at this time.

Table II. Hepatocellular and thyroid tumors in 52-week-old rats

<table>
<thead>
<tr>
<th>Treatment (group)</th>
<th>End point</th>
<th>Hepatocellular</th>
<th>Thyroid follicular cell adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adenomas</td>
<td>Carcinomas</td>
</tr>
<tr>
<td>NDEA/control (1)</td>
<td>Incidence</td>
<td>0/12 (0%)</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td></td>
<td>Multiplicity</td>
<td>(0)</td>
<td>(0)</td>
</tr>
<tr>
<td>NDEA/TCPOBOP 330 ppm (2)</td>
<td>Incidence</td>
<td>10/12 (83%)</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td></td>
<td>Multiplicity</td>
<td>(2.6±1.3)</td>
<td>(0)</td>
</tr>
<tr>
<td>NDEA/TCPOBOP 1000 ppm (3)</td>
<td>Incidence</td>
<td>12/12 (100%)</td>
<td>2/12 (17%)</td>
</tr>
<tr>
<td></td>
<td>Multiplicity</td>
<td>(3.4±2.1)</td>
<td>(1±0)</td>
</tr>
<tr>
<td>NDEA/PB 500 ppm (4)</td>
<td>Incidence</td>
<td>8/12 (67%)</td>
<td>1/12 (8%)</td>
</tr>
<tr>
<td></td>
<td>Multiplicity</td>
<td>(2.5±1.9)</td>
<td>(1±0)</td>
</tr>
</tbody>
</table>

Incidence = no. with tumors/no at risk (percent with lesions).
Multiplicity = no. of tumors/tumor-bearing rat (mean±SD).
P < 0.01, "P < 0.05, Fisher's exact test, compared with NDEA/control group.
No hepatocellular foci were observed in non-initiated groups at this time.

hepatocellular tumors. No significant differences were seen in survival rates between the NDEA group and the groups exposed to either the lower dose of TCPOBOP (330 ppm) or PB (Figure 1).

Body weight gains were not significantly different between different groups of rats. Although rats exposed to NDEA followed by 1000 ppm TCPOBOP showed significantly decreased survival, their body weight gain was not significantly affected as compared with rats in the group given NDEA only (Figure 2).

Hepatocellular foci
At 30 weeks of age no hepatocellular foci were found in any non-initiated rats that received either TCPOBOP (1000 ppm), PB (500 ppm) or control diets (groups 5–7). The number of foci/cm³ was not significantly different among different groups initiated with NDEA and promoted with either TCPOBOP or PB as compared with NDEA alone. However, the mean volume (mm³) of hepatocellular foci in rats exposed to either TCPOBOP or PB following NDEA initiation was significantly increased as compared with NDEA alone (Table I). In addition, the volume percentage of liver occupied by foci was significantly greater in NDEA-initiated and TCPOBOP (1000 ppm)-promoted rats as compared with NDEA alone. Hepatocellular foci in NDEA only rats were predominantly basophilic, while those in the promoted animals were mostly eosinophilic or of clear cell type. No hepatocellular tumors were found in rats of any group at this time.

Hepatocellular tumors
At 52 weeks of age hepatocellular foci in group 2 (NDEA followed by 330 ppm TCPOBOP), group 3 (NDEA followed by 1000 ppm TCPOBOP) and group 4 (NDEA followed by 500 ppm PB) were too numerous to count accurately. None of the rats treated with NDEA alone (group 1, Table II) developed any liver tumors at this time. On the other hand, 10/12 (83%) and 12/12 (100%) rats exposed to NDEA followed by 330 or 1000 ppm TCPOBOP respectively developed multiple hepatocellular adenomas. Although no hepatocellular carcinomas were seen in group 2 (NDEA followed by 330 ppm TCPOBOP), two rats (17%) exposed to the higher dose of TCPOBOP (group 3) following NDEA initiation did develop such malignant neoplasms. Eight of 12 rats (67%) exposed to PB following NDEA treatment had multiple hepatocellular adenomas, while only one such rat developed a solitary carcinoma. No metastases of liver tumors were observed in any rats at this time point. TCPOBOP (1000 ppm) or PB did not induce any liver neoplasms in non-initiated animals (data not shown).
Table III. Hepatocellular and thyroid tumor multiplicity in 53- to 79-week-old rats

<table>
<thead>
<tr>
<th>Treatment (group)</th>
<th>Hepatocellular Adenomas (mean±SD)</th>
<th>Carcinomas (mean±SD)</th>
<th>Thyroid follicular cell Adenomas (mean±SD)</th>
<th>Carcinomas (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDEA/control (1)</td>
<td>1.5±0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NDEA/TCPOBOP 330 p.p.m. (2)</td>
<td>10.5±3.9*</td>
<td>1.3±0.5</td>
<td>1.2±0.4</td>
<td>1±0</td>
</tr>
<tr>
<td>NDEA/TCPOBOP 1000 p.p.m. (3)</td>
<td>10.4±7.0*</td>
<td>3.0±1.9</td>
<td>1.3±0.5</td>
<td>1±0</td>
</tr>
<tr>
<td>NDEA/PB 500 p.p.m. (4)</td>
<td>10.1±6.7*</td>
<td>2.1±0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.001, compared with NDEA/control. 

Two rats exposed to 1000 p.p.m. TCPOBOP alone and one rat exposed to 500 p.p.m. PB alone developed a single hepatocellular adenoma each.

Figure 3 depicts the incidence of hepatocellular tumors in different groups of experimental rats observed between 53 and 79 weeks of age. The incidence of hepatocellular tumors was 38% (7/19) in rats treated with NDEA alone (group 1). This incidence was increased to 100% in rats exposed to either 330 or 1000 p.p.m. TCPOBOP (groups 2 and 3, P < 0.001 as compared with NDEA alone, Figure 3). A significant increase in the incidence of hepatocellular tumors was also observed in PB-promoted rats (Figure 3). None of the untreated control rats developed any liver tumors, while two rats exposed to 1000 p.p.m. TCPOBOP alone and one rat given PB alone had developed a single hepatocellular adenoma each.

Table III presents the data on the multiplicity of hepatocellular tumors in experimental groups. As shown in the table, the multiplicity of hepatocellular adenomas in the NDEA-only group (1.5 ± 0.8) was significantly increased by subsequently feeding either dose of TCPOBOP (groups 2 and 3) or PB (group 4). None of the rats treated with NDEA alone developed hepatocellular carcinomas. A significant (P < 0.02) dose-dependent promoting effect of TCPOBOP on the multiplicity of hepatocellular carcinomas was observed (Table III). Multiple hepatocellular tumors were also observed in NDEA-initiated and PB-promoted animals (group 4). Six rats in group 3 (NDEA followed by 1000 p.p.m. TCPOBOP) and four rats in group 4 (NDEA followed by PB) had carcinomas that metastasized to lungs.

Thyroid carcinogenesis

No thyroid follicular cell tumors were seen in any of the non-initiated groups at the interim sacrifice. Interestingly, thyroid follicular cell tumors developed in a dose-dependent manner in rats treated with NDEA followed by TCPOBOP following NDEA initiation. Thus, even at 52 weeks of age, 2/12 (17%; Table II) rats in group 2 (NDEA followed by 330 p.p.m. TCPOBOP) had thyroid follicular cell adenomas. This incidence almost doubled in rats that received 1000 p.p.m. TCPOBOP (group 3, P < 0.05 as compared to group 2). Between 53 and 79 weeks of age the incidence of thyroid follicular cell tumors increased to more than 50% in group 3 rats (NDEA followed by 1000 p.p.m. TCPOBOP) and to ~40% in rats of groups 2 and 4 (Figure 4). Multiple thyroid follicular cell adenomas (Figure 5) were observed in one rat of group 3 and in three rats of group 4. Two rats in group 3 and one rat in group 4 had invasive thyroid follicular cell carcinomas (Figure 6 and Table III). In addition, one non-initiated rat fed 1000 p.p.m. TCPOBOP and one non-initiated rat fed 500 p.p.m. PB developed a solitary thyroid follicular cell adenoma (data not shown).

Other neoplasms

Tumors other than hepatocellular and thyroid follicular cell epithelium that are related to NDEA treatment included nasal cavity and pulmonary alveolar tumors. Subsequent administration of either TCPOBOP or PB had no effect on the incidence or multiplicities of lung tumors. Although the incidences of nasal cavity tumors (adenomas and carcinomas) were slightly higher in TCPOBOP-treated rats (5/17 in group 2 and 7/18 in group 3), these were not significantly different from either the NDEA-alone group (4/19) or NDEA followed by PB group
Table IV. Effect of TCPOBOP (1000 p.p.m.) or PB (500 p.p.m.) on serum T₃, T₄ and TSH levels in non-initiated 79-week-old male rats

<table>
<thead>
<tr>
<th>Treatment (group)</th>
<th>T₃ (ng/dl)</th>
<th>T₄ (µg/dl)</th>
<th>TSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCPOBOP (5)</td>
<td>55.2±4.1</td>
<td>1.0±0.2</td>
<td>0.86±0.12</td>
</tr>
<tr>
<td>PB (6)</td>
<td>80.7±3.7</td>
<td>2.0±0.1</td>
<td>0.58±0.04</td>
</tr>
<tr>
<td>None (7)</td>
<td>83.2±6.2</td>
<td>2.8±0.1</td>
<td>0.67±0.09</td>
</tr>
</tbody>
</table>

bP < 0.002 as compared with untreated controls.
bP < 0.004 as compared with untreated controls.

(4/18). No nasal cavity tumors were found in any non-initiated animals. Testicular interstitial cell tumors occurred at a high incidence in all groups of rats killed at 79 weeks.

Serum T₃, T₄ and TSH levels

Serum T₃ and T₄ levels were decreased in both TCPOBOP- and PB-treated rats (Table IV). Both T₃ and T₄ levels in rats exposed to TCPOBOP were significantly lower than those in the untreated animals (P < 0.002). T₄ levels in PB-treated animals were also significantly decreased (P < 0.004), whereas no significant effect on T₃ level was observed. Serum TSH levels were not significantly different among different groups.

Biochemical studies

Rats fed PB or TCPOBOP for 2 or 23 weeks showed increased liver weight (not shown) and liver/body weight ratios (Table V) with respect to controls. This table shows a dose-dependent effect of TCPOBOP on the induction of cytochrome P450, as indicated by marked increases in the O-dealkylation of benzyloxyresorufin and testosterone 16β-hydroxylation. The ED₅₀ values (xenobiotic concentration in the diet associated with a half-maximal response) for CYP2B induction were found to be 300–400 p.p.m. dietary TCPOBOP. The maximal inductions (60–88%) of those resulting from exposure of the rats to 500 p.p.m. dietary PB) were observed in the rat when administered in the diet at 1000 p.p.m. However, based on the active site concentration–response curves generated by TCPOBOP and PB, it was clear that TCPOBOP was slightly more potent than the barbiturate in terms of CYP2B induction. This was reflected in lower EC₅₀ values (based upon total serum xenobiotic concentration) for TCPOBOP (2.4 µM) compared with PB (9 µM). On the other hand, total liver concentrations of PB and TCPOBOP were more similar. This increased potency of TCPOBOP relative to PB, which was indicated by the serum data, was confirmed by the experiment performed with primary hepatocyte cultures. The serum concentration–response curve for TCPOBOP in intact rats was remarkably similar to the curve generated with hepatocyte cultures. From these results we hypothesized that dose levels of TCPOBOP that cause maximal induction of CYP2B would be nearly as efficacious as PB in inducing most other elements of the hepatic PB-type response, including tumor promotion.

The results of the present study strongly support our hypothesis. Thus TCPOBOP given in the diet exerted a clear dose-dependent promoting effect on the number, size and progression of NDEA-initiated hepatocellular lesions. Like the classic liver tumor promoter PB, TCPOBOP given after NDEA significantly increased the size of preneoplastic hepatocellular foci at 30 weeks of age and dramatically increased the incidence and multiplicity of hepatocellular tumors at later time points. The majority of the foci and tumors promoted by TCPOBOP were of the eosinophilic or clear cell type. Also, a significant number of tumors in NDEA-initiated, TCPOBOP-promoted rats progressed to hepatocellular carcinomas. As shown in Figure 3, the results of the present study strongly support our hypothesis. Thus TCPOBOP given in the diet exerted a clear dose-dependent promoting effect on the number, size and progression of NDEA-initiated hepatocellular lesions. Like the classic liver tumor promoter PB, TCPOBOP given after NDEA significantly increased the size of preneoplastic hepatocellular foci at 30 weeks of age and dramatically increased the incidence and multiplicity of hepatocellular tumors at later time points. The majority of the foci and tumors promoted by TCPOBOP were of the eosinophilic or clear cell type. Also, a significant number of tumors in NDEA-initiated, TCPOBOP-promoted rats progressed to hepatocellular carcinomas. As shown in Figure 3.

Discussion

Poland and co-workers (1,3) were the first to report that TCPOBOP was a potent inducer of enzymatic activities associated with the CYP2B subfamily in mice, but not in rats. These results were later confirmed by Kelly et al. (16) and Pelkonen and co-workers (17). Studies by Dragani et al. (18) have shown TCPOBOP to be a potent promoter by the criterion of increased numbers of NDEA-initiated preneoplastic foci in mice. To investigate whether the relative tumor-promoting effects of TCPOBOP are associated with their ability to induce CYP2B, in our recent study (2) we compared the tumor promoting/carcinogenic effects of PB and TCPOBOP in two strains of mice (C57BL/6NCr and DBA/2NCr) that differ in their susceptibility to tumor promotion by PB, and in F344 rats. Our results indicated that TCPOBOP is an extraordinarily potent hepatocellular tumor promoter and carcinogen for mouse liver. In contrast, this compound was ineffective as a liver tumor promoter in rats, even at 10 times the dose administered to mice. PB induced CYP2B in the rats and in both strains of mice, whereas TCPOBOP was remarkably effective as an inducer in both strains of mice, but was negligibly effective in rats even at 10-fold higher doses. Thus our results give strong support to the hypothesis that the pleiotropic enzyme induction/gene derepression effect of these compounds is intimately associated with their promoting/carcinogenic activities in liver.

In a recent study (4) we examined the ability of TCPOBOP, administered for 14 days at dietary concentrations ranging from 12.3 to 1000 p.p.m., to induce CYP2B RNA, protein and associated activities in the male F344/NCr rat. The results clearly demonstrated concentration-dependent induction of hepatic CYP2B-mediated catalytic activities (benzoyloxy and pentoxyresorufin O-dealkylation and testosterone 16β-hydroxylation). The ED₅₀ values (xenobiotic concentration in the diet associated with a half-maximal response) for CYP2B induction were found to be 300–400 p.p.m. dietary TCPOBOP. The maximal inductions (60–88%) of those resulting from exposure of the rats to 500 p.p.m. dietary PB) were observed in the rat when administered in the diet at 1000 p.p.m. However, based on the active site concentration–response curves generated by TCPOBOP and PB, it was clear that TCPOBOP was slightly more potent than the barbiturate in terms of CYP2B induction. This was reflected in lower EC₅₀ values (based upon total serum xenobiotic concentration) for TCPOBOP (2.4 µM) compared with PB (9 µM). On the other hand, total liver concentrations of PB and TCPOBOP were more similar. This increased potency of TCPOBOP relative to PB, which was indicated by the serum data, was confirmed by the experiment performed with primary hepatocyte cultures. The serum concentration–response curve for TCPOBOP in intact rats was remarkably similar to the curve generated with hepatocyte cultures. From these results we hypothesized that dose levels of TCPOBOP that cause maximal induction of CYP2B would be nearly as efficacious as PB in inducing most other elements of the hepatic PB-type response, including tumor promotion.

The results of the present study strongly support our hypothesis. Thus TCPOBOP given in the diet exerted a clear dose-dependent promoting effect on the number, size and progression of NDEA-initiated hepatocellular lesions. Like the classic liver tumor promoter PB, TCPOBOP given after NDEA significantly increased the size of preneoplastic hepatocellular foci at 30 weeks of age and dramatically increased the incidence and multiplicity of hepatocellular tumors at later time points. The majority of the foci and tumors promoted by TCPOBOP were of the eosinophilic or clear cell type. Also, a significant number of tumors in NDEA-initiated, TCPOBOP-promoted rats progressed to hepatocellular carcinomas. As shown in Figure 3.
The subsequent chronic stimulation of thyroid follicular epithelium by TSH, produced in response to the abnormally low thyroid hormone levels, has been implicated as the primary cause of thyroid hormone promotion by PB-type compounds. Our results indicating dramatic decreases in serum thyroid hormone levels, particularly T₄, in TCPOBOP- and PB-treated rats are in complete agreement with those of earlier studies utilizing various other enzyme inducers (10,11,23,24). However, we found no direct correlation between decrease in thyroid hormone levels and compensatory increase in TSH levels in the present study. This is most likely a reflection of the small numbers of rats sampled, the use of retro-orbital blood for sample preparation and the fact that the samples were obtained from rats exposed to promoter for 72 weeks. After such prolonged duration of treatment compensatory mechanisms may come into play.

In our earlier study (2) we reported a small but significant promoting effect of TCPOBOP (30 mg/kg, i.p., twice per week for 71 weeks) on nasal cavity tumors induced by NDEA in male F344 rats. Although the incidence of nasal cavity tumors induced by NDEA (4/19, 21%) was increased by subsequent administration of TCPOBOP (5/17, 29% in group 2, 7/18, 38% in group 3) in the present study, the difference was not statistically significant. Thus the promoting activity of TCPOBOP on nasal cavity carcinogenesis is still questionable and needs further confirmation.

In summary, the results of the present study clearly indicated that, contrary to previous conclusions reached by many investigators, TCPOBOP at sufficiently high doses is a potent inducer of hepatic CYP2B activity in the rat. Thus at doses of 330 and 1000 p.p.m. in the diet this compound displayed maximal CYP2B induction (i.e. comparable with that caused by 500 p.p.m. PB). At maximal CYP2B induction doses TCPOBOP exhibited a strong liver tumor promoting effect in male F344 rats. Furthermore, a dose-dependent promoting effect of TCPOBOP was also observed in the thyroid glands of NDEA-initiated rats. Decreases in serum thyroid hormone levels clearly correlated with the promotion of thyroid carcinogenesis by this compound. Thus the present results give strong support to the hypothesis that the dose levels of compounds exhibiting maximal CYP2B induction would be equally effective as tumor promoters in the rat. Also, the mechanism by which TCPOBOP induces CYP2B and promotes liver and thyroid carcinogenesis appears to be similar to that caused by the prototype tumor promoter PB.

and Table IV, >60% of the rats in group 3 (NDEA followed by 1000 p.p.m. TCPOBOP) had multiple hepatocellular carcinomas, six of which metastasized to lungs. This compound thus exhibited a strong effect on progression of hepatocellular carcinogenesis. TCPOBOP alone, however, did not significantly increase the incidence of hepatocellular lesions in non-initiated rats. Thus, in contrast to its effects in mice, TCPOBOP does not appear to be a complete hepatocarcinogen in F344 rats.

In addition to its great ability to promote liver carcinogenesis in NDEA-initiated rats, a high incidence of thyroid follicular cell tumors was observed in rats exposed to TCPOBOP following NDEA initiation. The promoting effect of TCPOBOP on thyroid carcinogenesis was both time and dose dependent. Even at 52 weeks of age a significant number (4/12, 33%) of rats exposed to 1000 p.p.m. following NDEA initiation developed thyroid follicular adenomas. This incidence was increased to almost 50% by 79 weeks of age. Thus TCPOBOP, like PB, is a strong promoter of thyroid follicular cell carcinogenesis in rats. Although no thyroid tumors were observed in non-initiated rats, TCPOBOP alone induced follicular cell hyperplasia in several rats.

A striking correlation between the induction of CYP2B and liver tumor promotion by various compounds has been observed in rats by us (2,9,13,14,19) and several other investigators (20–22). Our present results are clearly in agreement with those of earlier findings. We have also confirmed our earlier findings (4) that given at high enough doses TCPOBOP is a potent inducer of CYP2B (benzoyloxysorufin O-dealkylase; Table V) activity in F344 rats. This effect was dose dependent and at 1000 p.p.m. TCPOBOP was slightly more effective than PB. Our earlier study has also shown that TCPOBOP induces other manifestations of the PB-type pleiotropic response, including CYP3A, epoxide hydrolase and glutathione S-transferase gene expression. Based upon our previous (4) and present results, TCPOBOP is as effective as PB for hepatic CYP2B induction, as well as liver tumor promotion, in the rat.

The hepatic PB-type pleiotropic response is thought to play an indirect role in the promotion of thyroid carcinogenesis (6,8,10,11). Several investigators have shown that microsomal enzyme inducers like PB cause a reduction in serum thyroid hormones. This reduction in thyroid hormone levels is the result of increased biotransformation and deactivation of T₄ by the microsomal enzyme UDP-glucuronosyltransferase, which catalyzes the formation of excretable T₄ glucuronide (23,24).
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References


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