Effect of the separate and combined administration of mestranol and phenobarbital on the development of altered hepatic foci expressing placental form of glutathione S-transferase in the rat

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Introduction

Epidemiological and experimental observations indicate that the development of cancer is multistage in nature, consisting of at least the stages of initiation, promotion and progression (1–3). Epidemiological data suggest that at least two rate-limiting events are required for the development of malignant neoplasia (4–6). In experimental systems, these two genetic changes occur to induce the initiation and progression stages of cancer development (3,7–9). In addition, a latency period between the first genetic event and the subsequent development of neoplasia has been observed in both experimental and human cancer development (cf. 10). The initiation–promotion–progression protocol of experimental carcinogenesis (3) mimics the stepwise development of cancer in humans (6,11,12) by coupling the two rate-limiting genetic events (initiation and progression) with the epigenetic process of promotion (3,6,10).

Use of the altered hepatic focus (AHF*) model of rat hepatocarcinogenesis (13), as analyzed by the technique of quantitative stereology, permits the classification of the stage at which compounds that pose a carcinogenic risk to humans are active (13,14). In addition, this experimental protocol provides a model system in which to determine the characteristics relevant to each stage in the cancer development process (15).

Tumor promotion is operationally defined as the reversible, altered phenotypic expression of protein markers and clonal expansion of single initiated cells (1,2,10). In the human, exogenous promoting agents can be encountered through lifestyle choices, in the diet, by workplace exposure and through the use of therapeutic agents (10,13). The dose–response characteristics of promoting agents differ from those of initiating agents in that promoting agents demonstrate a threshold dose below which they are ineffective in inducing a promotional response (3,10). The effects of promoting agents can be modulated by environmental factors (16), and the tumor promotion stage may be a useful target for chemoprevention strategies (17). While many properties of promoting agents have been described (1,2,10,13), several actions of carcinogenic agents of the promoter class need to be addressed prior to assuming that these agents are without risk under conditions of human exposure (18). Specifically, the issue of the additivity of promoting agents should be addressed before exposure to such carcinogenic agents is considered to be without risk, since the threshold dose may be altered by simultaneous exposure to several promoting agents.

Phenobarbital (PB) (9–24) and synthetic estrogens (25–32) such as mestranol (MS) are two well-studied promoting agents in the rat liver model of carcinogenesis (33,34). Both types of agents are capable of increasing the volume fraction of the liver occupied by AHF, as demonstrated in the studies cited. PB has previously been shown to exhibit a threshold dose for promotion in studies that examined the dose dependence of promotion of altered focus growth (35–40) and in studies that provided this compound intermittently (41). The dose–response characteristics for promotion with MS have been less well characterized, with one study demonstrating a promoting effect at the doses tested (28); however, a threshold dose has been demonstrated for ethinyl estradiol, the active promoting metabolite of MS (42). One mechanism postulated for the

Abbreviations: AHF, altered hepatic focus/foci; PB, phenobarbital; MS, mestranol; DEN, diethylnitrosamine; PGST, glutathione S-transferase, placental form; BrdU, bromodeoxyuridine; PBS, phosphate-buffered saline.
action of diverse promoting agents is the induction of a proliferative differential between the focal (presumptively initiated) and non-focal hepatocyte populations (23,43). This differential in proliferation may be partly due to the mito-inhibitory action of acute administration of certain promoting agents, including orotic acid (44) and PB (45–51). Numerous environmental compounds have estrogenic effects and/or induce P450s similar to those induced by PB. Since PB and MS are effective promoting agents for the rat liver, although with different mechanisms of action, we have examined the effect of MS and PB alone and in combination on promotion of diethylnitrosamine (DEN)-initiated AHF in the rat. Since the placental isozyme of glutathione S-transferase (PGST) has been reported to be the best single marker of rat hepatocarcino-genesis (52,53), these studies assessed the volume fraction of the liver occupied by AHF expressing PGST, as well as the focal and non-focal hepatocyte labeling index in initiated rats treated chronically with MS and/or PB.

Materials and methods

Animals and carcinogen administration

Animals were subjected to a neonatal initiation–promotion protocol as first described for rats by Persino et al. (54) and adapted by Dragan et al. (55) to assess the effect of the dose of DEN on initiation in this model. Late-gestation female rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and acclimatized for several days. DEN (Eastman Kodak, Rochester, NY) was dissolved in tricaprylin (Sigma Chemical Co., St. Louis, MO). At 5 days of age, the pups were injected i.p. with either the solvent tricaprylin or the initiating agent DEN (10 mg/kg body wt). Approximately 240 female rat pups were divided into experimental groups containing 7–12 rats each. At weaning (3 weeks of age), the female rat pups were housed in groups of 2–3 rats/cage and were permitted water and diet ad libitum. The basal diet was the purified, semi-synthetic AIN-76A diet (Teklad Test Diets, Madison, WI) in order to minimize exogenous promotion by factors other than the test compounds (56). Treatment groups were either initiated with DEN or treated with tricaprylin prior to administration of the basal diet, the basal diet containing PB (100 or 500 mg/kg diet PB as the free base), MS (0.02 or 0.2 mg/kg diet) or combinations of these concentrations of MS and PB. After 8 months of administration of the various promoting regimens the rats were killed. Bromodeoxyuridine (BrdU) was prepared as a 15 mg/ml stock in phosphate-buffered saline (PBS) and was administered to each animal at a dose of 100 mg/kg body wt BrdU by i.p. injection 2 and 10 h prior to sacrifice (57,58). At sacrifice, blood was collected and serum separated. PB levels were determined by Ms B.J.Basteyns at the State Laboratory of Wisconsin using a fluoroscent activated cell sorter kit (Toxi-Test, Chalmette, LA; fluoroscent activated cell sorter II assay; Abbott Labs). At sacrifice, 2 mm thick sections of the liver were fixed in ice-cold acetone for subsequent immunohistochemical analysis (59) of BrdU incorporation and PGST expression on the same section.

Immunohistochemical staining for BrdU incorporation and PGST expression

The liver samples placed in acetone at sacrifice were allowed to fix overnight at −20°C with one change to fresh acetone (59). The specimens were processed and embedded in low-melt paraffin. Sections (5 μm thick) were placed on poly-L-lysine (0.5% in water; Sigma Chemical Co., St. Louis MO)-coated slides, deparaffinized in xylene and rehydrated by graded alcohols to water. The tissues were rehydrated in PBS. Each section was then double labeled for the dual detection of PGST expression (rabbit polyclonal antibody generated by the protocol of Satoh et al; (60) and BrdU incorporation (mouse monoclonal antibody; DAKO, Carpinteria, CA) with the ABC method (61). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS for 2 min, while endogenous biotin was blocked with avidin (50 μg/ml). Non-specific protein binding was minimized by use of 5% normal goat serum (Zymed, South San Francisco, CA). The rabbit anti-rat PGST antibody was diluted in 1% bovine serum albumin and incubated with the section overnight at 4°C. Next, the sections were incubated with a biotinylated secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA). This was followed by incubation with streptavidin bound to β-galactosidase (Sigma Chemical Co.; 62) and subsequent chromagen development with 5-bromo-4-chloro-3-indolyl-β-galactopyranosidase (Boehringer Mannheim, Indianapolis, IN). Those cells which had incorporated BrdU in the hour prior to sacrifice were next assessed. The tissue section previously stained for PGST expression was briefly hydrolyzed with 0.1 M HCl, followed by several washes and application of the primary antibody. After addition of the biotinylated secondary antibody, streptavidin bound to peroxidase was used and chromagen development was with 0.5% diaminobenzidine and 0.015% hydrogen peroxide in 0.5 M Tris-HCl, pH 7.6. Nuclei were counterstained with Mayers hematoxylin and the sections were coverslipped with crystal mount (Biomeda, Foster City, CA). Background hepatic labeling index was determined as the number of hepatocyte nuclei with BrdU incorporation/1000 hepatocyte nuclei/rat (at least 2 sections/rat) for 5–12 rats/treatment group in hepatocytes not expressing PGST (58) Focal hepatic labeling index was determined for the PGST-expressing hepatocytes in 5–12 rats in treatment groups that had been initiated with DEN. Focal hepatic labeling indices were determined in sections double stained for the expression of PGST and BrdU. All nuclei within PGST-positive cells were scored (up to 1000/rat) or all observable PGST-positive hepatocytes/rat and the percentage of these nuclei that were also BrdU-positive was determined. The number was determined per rat and averaged for each treatment group. The focal and non-focal labeling indices were determined by multiplying the average number of BrdU-positive nuclei by the number of hepatic nuclei counted for each treatment group and multiplying by 100. The labeling index data are provided as the mean ± SEM.

Determination of volume fraction of liver as AHF

The method of stereological analysis as applied to hepatic focal analysis by Campbell et al. (63) was used to determine the volume fraction of the liver occupied by AHF. Briefly, each section was projected onto a Scannemicrograph digitizer connected with an HP9320 computer. The AHF were identified and quantitated on the magnified image of the tissue section. The outline of the tissue section and of each focal lesion was traced with a cursor. The location and area of each focal transection were recorded and, in combination with the liver weight, used to calculate the volume fraction of the liver occupied by AHF. The volume fraction of AHF was determined by multiplying the average number of BrdU-positive nuclei by 100. The labeling index data are provided as the mean ± SEM.

Results

The percentage or volume fraction of the liver occupied by AHF is considered the best single indicator of promoter action and has been used to assess the effectiveness of promotion by PB and MS both singly and in combination. Figure 1 addresses the effect of administration of PB alone (Figure 1A) and MS alone (Figure 1B) on the development of AHF. Figure 1A depicts the volume fraction of the liver occupied by AHF expressing PGST in DEN-initiated female rats as a function of the concentration of PB administered in the diet in the absence of DEN treatment. PB and MS treatment had no significant effect on the volume fraction parameter. At the doses tested, PB administration to DEN-initiated rats did not result in a statistically significant increase in the volume fraction parameter compared with the DEN-initiated rats fed only the basal diet. While a trend toward an increase in volume fraction was observed in DEN-initiated rats treated with 500 mg/kg diet PB compared with DEN-treated rats maintained on basal diet without PB, this trend was not significant (P < 0.05). In contrast, the DEN-initiated rats administered 500 mg/kg diet PB induced a significant enhancement of focal growth compared with the DEN-initiated rats fed the basal diet containing 10 mg/kg diet PB (P < 0.05). Analysis of serum levels of PB indicated that dietary administration resulted in a circulating level that was dose dependent. The serum PB level was <1 μg/ml for 0.001%, 6.9 ± 0.2 for 0.01% and 31.07 ± 0.9 for 0.05% PB in the diet.

Figure 1B depicts the volume fraction of liver in DEN-initiated female rats as a function of MS dose. In DEN-initiated rats, the lower dose of MS (0.02 mg/kg diet) was ineffective in increasing the volume fraction of liver occupied by foci above that detected in DEN-initiated rats maintained on a basal diet. Addition of 0.2 mg/kg diet MS, however,
resulted in an increased percentage of liver occupied by AHF expressing PGST (P < 0.05).

The incorporation of BrdU into focal (defined as those hepatocytes expressing PGST) and non-focal (defined as those hepatocytes not expressing PGST) hepatocytes is depicted as a function of PB and MS concentration for solvent-treated and DEN-initiated female rats (Table I). The non-focal labeling index was not altered by the lowest concentration of PB tested. Administration of the middle PB dose resulted in a trend toward a decrease in the non-focal hepatocyte labeling index. This trend toward a decreased non-focal hepatocyte labeling index with the middle PB dose was statistically significant for the DEN-initiated but not for the uninitiated rats (P < 0.05). The 500 mg/kg diet concentration of PB (Figure 1A) decreased the non-focal hepatic labeling index in DEN- and non-DEN-initiated rats compared with non-PB-treated rats or those administered the lower concentrations of PB (P < 0.05; Table I). The focal hepatic labeling index in DEN-initiated rats fed the basal diet alone was significantly different from the non-focal labeling index (P < 0.05). Administration of PB to DEN-initiated rats increased the focal hepatic labeling relative to the non-focal hepatocyte labeling index (P < 0.05). This focal to non-focal hepatic labeling index ratio was most marked at the highest PB dose (500 mg/kg diet), with the ratio being 1.6 in rats fed a basal diet and 4.0 in those administered the highest PB concentration.

Administration of the higher (but not the lower) concentration of MS significantly increased the non-focal hepatic labeling index in uninitiated rats compared with that observed in rats administered the basal diet only (P < 0.05; Table I). Similarly, MS administration to DEN-initiated rats resulted in a significant increase in the non-focal hepatocyte labeling index (P < 0.05). In DEN-initiated rats, the focal labeling index was enhanced by administration of both concentrations of MS compared with initiated rats that were fed only the basal diet (P < 0.05), but this effect was not dose dependent. The ratio of the focal to non-focal hepatic labeling index in DEN-initiated rats was increased (3-fold) with the higher dose of MS compared with the control or the lower MS dose.

Figure 2 depicts the volume fraction of liver occupied by AHF for the DEN-initiated rats administered several concentrations of PB in combination with 0.02 mg/kg diet MS. The volume fraction of the liver occupied by AHF was significantly increased by a combination of the lower MS dose with either the low (Figure 2A) or middle (Figure 2B) PB dose when compared with the DEN-initiated group not administered a promoting agent (Figure 1). In addition, administration of the combination of 0.02 mg/kg diet MS with 10 mg/kg diet PB (Figure 2A) or 0.02 mg/kg diet MS with 100 mg/kg diet PB (Figure 2B) to DEN-initiated rats markedly enhanced the volume fraction of the liver occupied by PGST-positive AHF compared with either MS or PB treatment alone (P < 0.05). This enhancement of the volume fraction of the liver occupied by AHF was greater than additive by χ² analysis. A trend toward an increase in the volume fraction was observed in DEN-initiated rats administered 0.02 mg/kg diet MS and 500 mg/kg diet PB (Figure 2C), but this trend was not significant.

Figure 3 depicts a comparison of the effect of the combination of MS (0.2 mg/kg diet) with PB (10, 100 and 500 mg/kg diet; Figure 3A–C respectively) on the volume fraction of the liver occupied by PGST-expressing AHF in DEN-initiated rats. Co-administration of 0.2 mg/kg diet MS with either the low or middle PB dose (Figure 3) significantly increased the volume fraction of liver occupied by AHF compared with DEN-initiated rats not administered a promoting agent (Figure 1; P < 0.05). Figure 3A demonstrates that co-administration of 0.2 mg/kg diet MS with 10 mg/kg diet PB increases the volume of liver occupied by altered foci compared with PB alone (P < 0.05), but not MS alone. In contrast, the combination of 100 mg/kg diet PB with 0.2 mg/kg diet MS resulted in a marked increase in the volume fraction parameter compared with promotion by either agent alone (P < 0.05). Promotion by the high dose of MS co-administered with the middle dose of PB did not, however, differ from that expected from addition of the separate effects of these agents. Promotion with 100 mg/kg diet PB co-administered with 0.2 mg/kg diet MS was more effective than the combination of 10 mg/kg diet PB and 0.2 mg/kg diet MS (P < 0.05). Further enhancement of promotion was not observed when 500 mg/kg diet PB and 0.2 mg/kg diet MS were combined compared with that observed with either agent alone.

Figure 4 provides the hepatic labeling indices for the combination of MS with PB to DEN-initiated rats. Figure 4A provides the non-focal hepatic labeling index. Administration of MS increased the non-focal hepatocyte labeling index compared with that observed in rats provided the basal diet (P < 0.05). Co-administration of PB at the concentrations tested (10, 100 or 500 mg/kg diet) with either of the two
concentrations of MS (0.02 and 0.2 mg/kg diet) resulted in a decrease in the non-focal labeling index compared with that observed for the corresponding concentration of MS alone (P < 0.05). Co-administration of 10 mg/kg diet PB with 0.02 mg/kg diet MS decreased the non-focal labeling index to the level observed in DEN-initiated but otherwise non-treated rats (P < 0.05). The non-focal labeling index observed for the combination of MS with the two highest concentrations of PB was increased compared with either the level in non-treated, DEN-initiated rats or that for the combination of MS with the lowest PB dose (P < 0.05). However, the non-focal labeling indices for the combinations of the middle and high dose of PB with the lower dose of MS did not differ. Combination of 0.2 mg/kg diet MS with any of the concentrations of PB tested resulted in a marked suppression of the non-focal labeling index compared with that observed with this concentration of MS alone (P < 0.05). Combination of the higher concentration of MS with PB resulted in a further suppression of the non-focal labeling index for the addition of 10 mg/kg diet PB (P < 0.05), but not for 100 or 500 mg/kg diet PB. Combination of the 100 mg/kg diet PB concentration with either concentration of MS decreased the non-focal labeling index relative to that observed with PB alone (P < 0.05). This decrease with the higher dose of MS and the middle dose of PB did not differ from that induced with the combination of the higher dose of MS with the lowest dose of PB. Co-administration of MS (at either dose) with the highest dose of PB did not result in a further suppression of the non-focal labeling index than was observed with this concentration of PB alone. Figure 4B provides the focal hepatic labeling index for administration of the various combinations of MS and PB in rats initiated with DEN. MS administration increased the focal hepatocyte labeling index compared with the control with a trend at the lower dose and a significant difference at the higher dose (P < 0.05). PB alone significantly increases the hepatocyte focal labeling index relative to control at the lower dose, but this trend is not significant at the two higher doses. In rats co-administered the lowest dose of PB with MS, a decrease in focal hepatocyte labeling index was observed with both MS doses compared with either MS or PB administered alone. With the higher dose of MS in rats co-administered PB, a significant decrease in focal hepatocyte labeling index was observed relative to the corresponding PB alone or MS alone groups (P < 0.05).

The effect of combining PB with MS on the focal (Figure 4B) to non-focal (Figure 4A) hepatocyte labeling index is provided in Figure 4. The focal (Figure 4B) to non-focal (Figure 4A) hepatocyte labeling index was significantly increased for the combination of the low dose of MS with either the middle or high dose of PB (P < 0.05). In addition, the focal compared with the non-focal hepatocyte labeling index was increased for the high, but not the low, MS dose combined with the low PB dose (P < 0.05). Combination of the high dose of MS with the high dose of PB suppressed the focal, relative to the non-focal, hepatocyte labeling index (P < 0.05). These data indicate that the labeling index cannot, of itself, explain the changes in volume fraction of the liver occupied by AHF (total number of cells) when PB and MS are given in combination.

Discussion

PB has been extensively studied as the prototype promoting agent for the liver since the demonstration of its ability to enhance the yield and decrease the latency of 2-acetylamino-fluorene-induced liver tumors (19). The promoting effect of PB has been extended to other classes of carcinogens, including polycyclic aromatic amines (67), polycyclic aromatic hydrocarbons (54) and nitrosamines (21,22). Tumor promotion by PB is organ specific (68,69) and dose dependent (35-40). In addition, a delay between administration of the initiating agent

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**Table 1. The effect of promotion with either PB or MS on hepatic incorporation of BrdU**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DEN</th>
<th>n</th>
<th>Labeling index (non-focal)</th>
<th>Labeling index (focal)</th>
<th>Labeling index ratio focal/non-focal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal diet</td>
<td>–</td>
<td>8</td>
<td>0.927 ± 0.109</td>
<td>1.35 ± 0.38c</td>
<td>1.6</td>
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<tr>
<td>Basal diet</td>
<td>+</td>
<td>9</td>
<td>0.845 ± 0.147</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>10 mg/kg diet</td>
<td>–</td>
<td>0.830 ± 0.160</td>
<td>2.93 ± 0.44ac</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12</td>
<td>0.986 ± 0.107</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>9</td>
<td>0.762 ± 0.14</td>
<td>2.10 ± 0.24ac</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10</td>
<td>0.706 ± 0.10</td>
<td>2.03 ± 0.22c</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>5</td>
<td>0.426 ± 0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10</td>
<td>0.594 ± 0.072</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mestranol</td>
<td>0.02 mg/kg diet</td>
<td>–</td>
<td>1.0 ± 0.13</td>
<td>2.07 ± 0.29b</td>
<td>1.2</td>
</tr>
<tr>
<td>0.2 mg/kg diet</td>
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<td>9</td>
<td>1.74 ± 0.209</td>
<td>2.93 ± 0.38</td>
<td>3.1</td>
</tr>
<tr>
<td>Mestranol</td>
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<td>1.68 ± 0.142b</td>
<td>5.35 ± 1.34bc</td>
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<tr>
<td>0.2 mg/kg diet</td>
<td>+</td>
<td>12</td>
<td>1.71 ± 0.142b</td>
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**Footnotes:**

1Female Sprague–Dawley rats were administered 10 mg/kg body wt DEN or the solvent tricaprilyn at 5 days of age. At weaning, those animals to be treated with promoting agents began their respective regimens. Rats were injected with BrdU at 2 and 10 h prior to sacrifice at 8 months of age. The non-focal labeling index was determined by assessing the number of hepatic nuclei containing BrdU in 1000 hepatocytes from two separate sections from each rat. PB and MS were admixed in the basal diet. At least 1000 focal cells or all of the focal cells per section were scored to determine focal labeling index. Data are presented as the mean ± SEM.

2Statistically different from the appropriate control rats administered only basal diet (P < 0.05).

3Statistically different from the focal and non-focal hepatocyte labeling index in DEN-initiated rats (P < 0.05).

4Statistically different from the hepatocyte labeling index of the next lower dose (P < 0.05).

5Statistically different from the lowest dose (P < 0.05).
and the start of PB administration has no effect on the subsequent yield of preneoplastic lesions (43,70). Although PB has many effects on the liver, including an increase in hyperplasia and hypertrophy (71) without increasing cell death, the mechanism underlying its promoting effects is unclear.

At least two general, but not mutually exclusive, theories concerning the mechanism of liver tumor promotion have been suggested. One mechanism involves the induction of adaptive growth in response to a chemical change, such as is observed with PB administration (33). The second mechanism involves the selective resistance of initiated cells to the mitoinhibitory effect of cytotoxic compounds (33,72–74). Several studies have demonstrated a correlation between the potency of P450 induction (specifically CYP1B1) and liver tumor promotion by the barbiturate class (75–77). Some researchers have suggested that alteration of an unknown transcription factor, as has been observed in PB-treated bacterial cells, may play some role in PB-induced alterations in mammals (78–84), but this possibility requires further examination. A receptor-based mechanism for PB has been postulated (85), but has not been demonstrated.

While PB is known to cause a transient increase in cell proliferation (24,48), this response is of insufficient magnitude and duration to account for promotion by PB (24). In addition, the spatial and temporal (86) effects of PB on hepatic proliferation are not coincident with hepatic focal development (49). Several studies indicate that PB administration results in a decreased responsiveness of hepatocytes to mitogenic signals (45,46,48,50,51), suggesting that chronic PB administration may cause a mitoinhibitory reaction in the liver. PB administration does not result in hepatic cytotoxicity, as has been observed with other agents that are mitoinhibitory (72–74). However, Barbason et al. (46) have demonstrated that sustained exposure to PB is mitoinhibitory. In addition, Tsai et al. (87) have demonstrated a decreased growth factor responsiveness of hepatocytes from rats treated chronically with PB. Several studies have intimated that promoting agents may selectively stimulate cell proliferation in the focal relative to the non-focal cells, but recent work from Jirtle et al. (49) indicates that an increased expression of TGFβ receptor ligands and the IGF2/mannose 6-phosphate receptor required for the activation of these ligands occurs with PB administration (47,48). The decrease in EGF receptor that occurs during chronic PB administration, such as in tumor promotion, may also contribute to this decreased responsiveness of tissue from PB-treated rats to mitogenic agents (88–90). The present study indicates that administration of PB alone results in a differential in the focal to non-focal hepatocyte labeling index that is greater at the
cells in the focus is intrinsically higher than that of the surrounding normal hepatocytes (33,94,95).

MS is a synthetic estrogen that is metabolized to the potent promoting agent ethinyl estradiol (96). This compound is extensively metabolized to hydroxylated products whose biological activity is less well characterized (97–102). Several studies have determined that MS and ethinyl estradiol are effective in promoting γ-glutamyl transpeptidase-positive focal growth (25–32). Yager et al. (28) have found a dose-dependent increase in focal hepatic lesions consistent with the no-effect result observed for our lower MS dose and the effective promotion with our higher MS concentration. Although effective promotion of the PGST-positive population was seen in this study with the 0.2 mg/kg diet concentration, a conflicting result was obtained in one study with a higher initiating dose of DEN (100 compared with 10 mg/kg body wt) and a higher promoting concentration (10 compared with 2 mg/kg diet) of ethinyl estradiol than in the present study (103). Our results are also consistent with the dose-dependent promotion observed by Campen et al. (42) in a study using a higher initiating dose of DEN and a different method of ethinyl estradiol administration. Liver tumor promotion by ethinyl estradiol is believed to be effected through interaction with the estrogen receptor, since promotion can be inhibited by concurrent administration of an anti-estrogen such as tamoxifen (29,104,105). While low doses of ethinyl estradiol cause hypertrophy coupled with a transient increase in cell proliferation, chronic administration of this estrogen can result in mitoinhibition (29,106–108) and enhanced cell proliferation (31,106), depending on the dose and duration of treatment. Mayol et al. (106) have demonstrated that chronic administration of a higher dose than employed in this study can result in cytotoxicity. While the mitotic index observed during the early transient phase of induced proliferation is of a greater magnitude than that observed after chronic administration, the proliferation with chronic administration is sustained to a greater extent (103). Ethinyl estradiol increases estrogen receptor levels and EGF receptors on hepatocytes (31,32). In addition, changes in hepatocyte ploidy are noted after ethinyl estradiol administration (31), which may be associated with the observed enhancement of cell proliferation. Thus, estrogenic compounds such as MS or ethinyl estradiol have many effects on target organs such as the liver and appear to be active at least in part through estrogen receptor-dependent mechanisms.

In DEN-initiated rats, combination of the 10, 100 or 500 mg/kg diet PB doses with an ineffective dose of the promoting agent MS (0.02 mg/kg diet) resulted in an increased promoting effect for the combination. However, combining PB (10, 100 or 500 mg/kg diet) with the higher dose of MS (0.2 mg/kg diet, a concentration that results in effective promotion) did not result in a consistent increase in promotion. At the middle PB dose, combination with MS caused at least an additive promotional effect. The observation of a lesser response with the other doses may reflect a balance between the increased conversion of MS to ethinyl estradiol and the increased clearance of ethinyl estradiol at these concentrations of PB (109–112). Although ethinyl estradiol and PB may induce promotion through different mechanisms, their interaction can result in an enhanced promotional activity at doses of each compound that are sufficiently low so as to be ineffective when administered singly. Both PB and estradiol induce a transient increase in hepatic cell proliferation (113), suggesting that liver tumor promotion by these agents may result from their

![Graph](image-url)

**Fig. 4.** The effect of co-administration of various concentrations of PB on the non-focal (A) and focal (B) hepatic labeling index induced by administration of MS to DEN-initiated rats. Female rats were administered the designated combinations of the two promoting agents for 8 months. The data are presented as the mean (bar) ± SEM (line) for each treatment. The open bar indicates 0 mg/kg diet MS, solid black bars indicate 0.02 mg/kg diet MS, and the stippled bars indicate 0.2 mg/kg diet MS. The non-focal hepatic labeling index was derived from 5–12 rats/treatment group, while the focal hepatic labeling index is from 5 rats/treatment group. Statistical differences from the appropriate control rats fed basal diet (P < 0.05). Statistical differences between the focal and non-focal hepatocyte labeling index for a given treatment (P < 0.05). Statistical differences between the hepatocyte labeling index of the next lower PB dose (P < 0.05). Statistical differences between the hepatocyte labeling index of the lowest and highest PB dose (P < 0.05). Statistical differences between the hepatocyte labeling index of DEN-initiated rats administered MS alone or in combination with PB (P < 0.05).
effects on cell proliferation. However, chronic administration of PB (50,51,87) or ethinyl estradiol (114) results in an inhibited cell growth with a compensatory increased growth rate of the initiated cell population. Concurrent PB and MS administration may alter the pharmacokinetics of MS (109,110,112), thus changing the in vivo effectiveness of this compound as a promoting agent. In fact, PB increases the conversion of MS to the promotionally active ethinyl estradiol (96). Additionally, PB is known to decrease the absorption of MS from the gastrointestinal tract and to promote its excretion (111). While at low doses of MS the PB effect might override the enhanced elimination of ethinyl estradiol observed at higher doses of PB, increasing the dose of MS by increasing bile flow and inducing hydroxylation pathways that mediate its elimination (98) may result in a decreased effectiveness when PB is co-administered. Although the promotion effects of the various combinations of PB and MS can be explained in part by alterations in the pharmacokinetics of MS wrought by concurrent PB administration, the depression of non-focal cell proliferation with co-administration of MS and PB compared with administration of only MS is not so readily explained and may have to do with the competing effects each of these agents has on signaling through the EGF receptor (31,90). Alterations in labeling index alone cannot explain the interactions of the two promoting agents at the doses examined in this study. The inhibition of apoptosis by some promoting agents, including PB (115–117), may provide one explanation for focal growth in the absence of increased cell proliferation.

Very few studies on the effect of mixtures of carcinogens have been undertaken, and the rat liver provides a useful model system in which to assess the effects of such combinations. Previous studies have determined that co-administration of genotoxic agents is at least additive (118–121). Combination of singly ineffective concentrations of promoting agents such as pesticides has been determined to be non-additive (122). However, in certain cases a greater than additive promoting action has been observed (123). Doses of promoting agents at or near the threshold for promoting response may, in combination with other promoting agents, result in at least an additive promoting response under some conditions of exposure, as demonstrated in the present study. These results indicate that the potential for additive or synergistic responses of promoting agents is probably dependent upon the dose, pharmacokinetics and pharmacodynamics of the individual compounds. Furthermore, the risk that exposure to agents with a promoting action poses for human cancer development requires a more complete understanding of their characteristics, including their ability to substitute for one another and the potential additivity of their actions (18).

Acknowledgements

The editorial comments of Dr Ilse Riegel are gratefully acknowledged. In addition, we wish to acknowledge the expert technical typing of Mrs Mary Jo Markham and Mrs Kristen Adler. The technical contributions of Mrs Jennifer Vaughan and the Histology Department at the McArdle Laboratory are gratefully acknowledged. This work was supported by grants CA-07175, CA-22484, CA-45700 and CA-57245 from the National Cancer Institute.

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Received on March 19, 1996; revised on May 24, 1996; accepted on May 24, 1996