Promotion by sodium barbital induces early development but does not increase the multiplicity of hereditary renal tumors in Eker rats


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Induced cell proliferation is important in the mode of action of many non-genotoxic renal carcinogens. Since Tsc2 mutant (Eker) rats are genetically predisposed to the development of renal cell tumors, they provide a useful animal model in which to study the action of renal carcinogens. Sodium barbital was used as a model non-genotoxic renal carcinogen to test whether a concentration that increased renal tubular proliferation without severe nephrotoxicity would enhance tumor induction in a hereditary tumor model. First, a subchronic concentration–response study was conducted in wild-type male Long-Evans rats to determine increased cell proliferation without severe nephrotoxicity. Rats were dosed with sodium barbital in the feed at 0, 50, 250, 500, 1000, 2000 or 4000 p.p.m. for 3 or 8 weeks. Cell proliferation within the cortex and nephrotoxicity were quantitated. Enhanced proliferation with minimal nephrotoxicity occurred at 500 p.p.m.. A second study was conducted in male Tsc2 mutant rats given sodium barbital in the feed at 0, 100 or 500 p.p.m. from 9 weeks of age to either 6 or 12 months of age. An additional group of rats was treated with sodium barbital for 6 months and then provided control feed until 12 months of age. Rats necropsied at 6 months of age had a concentration-dependent increase in preneoplastic and total renal lesions. Sodium barbital-treated rats necropsied at 12 months of age had numbers of lesions that were not different from controls. Total combined preneoplastic and neoplastic lesions in the 6 month, high dose group was the same as the 12 month control group. These data show that sodium barbital caused progression to the stage of spontaneous renal lesions in Tsc2 mutant rats but did not increase their overall number. These data suggest that enhanced cell proliferation without significant cytotoxicity exerted a promotional influence in this hereditary model.

Introduction

Among the many potential risk factors for developing renal cancer in human patients are gender, heredity and diseases that cause chronic nephritis and regeneration. The risk of kidney cancer after renal transplantation is 0.3% in men and 0.9% in women, compared with background renal adenocarcinoma rates of 0.01 and 0.004%, respectively (1,2). The risk of kidney cancer following chronic interstitial nephritis, analgesic nephritis or chronic pyelonephritis is higher than the risk following other kidney diseases (1). Taken together, these studies suggest an association of renal cancer with stimulation of cell proliferation alone or in diseases with persistent cytotoxicity and cell regeneration.

Every time DNA replicates it does so at less than 100% fidelity, which provides an opportunity for a mistake to be made and a somatic mutation to occur (3,4). Some non-genotoxic compounds can increase cancer incidence by increasing proliferation in transformed or intermediate cells. There is evidence that induction of increased cell proliferation can increase the risk of developing cancer (3,5,6). Increasing the proliferation rate of intermediate cells can increase the risk of cancer with time (7). Affecting the kinetics of intermediate cells is efficient in affecting tumor incidence because small changes in the rates of cell replication over cell death rates can lead to large changes in tumor incidence (7).

A xenobiotic can promote the development of tumors by several mechanisms. Tumor promoters may target initiated cells rather than non-initiated parenchymal cells for mitogenesis (4,8). The parenchymal cell may undergo hyperplasia as a reparative response to cytotoxicity (4). The initiated cells may have different growth control mechanisms than non-initiated cells and their response to mitogens or toxins may be different (4). Or, quite possibly, a particular promoter may work through more than one mechanism.

A rat model of hereditary renal cell cancer was first described by Eker (9). These hereditary renal tumors are due to a germline mutation in the tumor suppressor tuberous sclerosis 2 gene (Tsc2) (10,11). Tsc2 has been shown to be involved in the pathogenesis of renal cell carcinoma in humans and rats (12). The mutation in the Eker rat is an insertion of DNA into the 3’-portion of the Tsc2 gene. This insertional mutation results in premature stopping of transcription and elimination of the distal portion of the Tsc2 gene (13,14). Since this is a germline mutation, all the cells in the kidney are considered initiated in that they already have the first mutation in developing a renal cell tumor.

Sodium barbital (BB) has been used to promote intestinal, hepatic, thyroid, urinary bladder and renal tumor growth in rats alone or after treatment with various initiators (15–22). Male F344 rats promoted with 4000 p.p.m. BB in the diet developed renal carcinomas. The promoting effect was evident as a greater incidence of large renal tubular tumors after 52 weeks treatment. The incidences of dysplastic tubules and preneoplastic lesions were not different between treated and control animals, suggesting that the targets for the promoting activity of BB are the dysplastic lesions that arise from proximal tubule epithelium (23,24). Rats given BB had a positive correlation between increased cell proliferation in proximal tubules and the degree of nephropathy, with a 2-fold increase in proliferation over controls after 52 and 72 weeks treatment (23).

Abbreviations: BB, sodium barbital; BrdU, bromodeoxyuridine.
Prolonged estrogen treatment in Tsc2 mutant (Eker) rats enhanced the development of hereditary renal cell tumors and increased the severity of nephropathy, with a 2-fold greater number of poneoplastic and neoplastic renal lesions compared with untreated Eker rats (25). Ovariectomizing Eker rats resulted in 33% fewer renal lesions and was protective for nephropathic changes compared with the unmanipulated control group (25). These data illustrated an association between increased renal toxicity and hereditary renal tumor development. Treatment with a mutagenic chemical also increased the multiplicity of hereditary renal tumors in Eker rats (26). The present study was designed to identify a concentration of the nephrotoxicant and tumor promoter BB that increased proximal tubule cell proliferation with minimal nephrotoxicity and then to use that concentration to determine if enhanced cell proliferation in the absence of severe cytotoxicity is sufficient to increase tumor development in a genetically susceptible animal.

Materials and methods

Dose finding study

The purpose of this initial study was to identify the concentration of BB that induced no, limited or substantial increases in renal cortical epithelial cell proliferation in 6-week-old Tsc2 wild-type male Long-Evans rats. Dose groups were 0, 50, 250, 500, 1000, 2000 and 4000 p.p.m. BB (CAS no. 144-02-5; Sigma Chemical Co., St Louis, MO) in the feed (NIH-07 chow; Zeigler Bros, Gardener, PA) as the sole food source beginning at 6 weeks of age. Feed was analyzed after each batch was made (Table I). Feed consumption was determined twice a week.

Rats were randomly assigned to treatment groups by weight, six per treatment group, housed individually in plastic shoe box cages and uniquely identified. The rats were exposed to a 12 h light/dark cycle with a room temperature of 72 ± 2°F and humidity of 50 ± 5%. Water and feed were available ad libitum.

Osmotic minipumps (Alzet, Palo Alto, CA) containing a 20 mg/ml solution of bromodeoxyuridine (BrdU) (CAS no. 59-14-3; Sigma) in saline were implanted in the morning 3 days before euthanasia and necropsy to assess alterations in cell proliferation. After 3 or 8 weeks BB treatment, six rats from each dose group were euthanized by pentobarbital anesthesia, exanguinated and necropsied. Kidneys were removed, examined macroscopically, weighed and fixed in 10% neutral buffered formalin. Kidneys were cut mid-sagittally and placed in cassettes, each with a section of duodenum for confirmation that BrdU was present in the tissues. The kidneys were processed to paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. The kidneys were examined for poneoplastic and neoplastic microscopic renal lesions and severity of nephropathy as previously described (25). Macroscopic masses and microscopic lesions were counted and reported independently of each other.

Analyses for two types of responses were performed: whether or not there was any occurrence of each lesion and the number of lesions (multiplicity) that occurred. Fisher’s exact test was used for the analysis of presence of a lesion. If the overall test was significant, Fisher’s exact test was also used to test for significance of relevant treatment subsets. Lesion multiplicity data was first square root transformed and then analyzed by one-way ANOVA. If the overall F test for equal means was significant, the Tukey-Kramer test for all pairwise comparisons was applied. The significance level for all analyses was 0.05.

Results

Dose finding study

Male Long-Evans rats treated with BB had time- and concentration-dependent increases in labeling indices in the renal cortex after 3 and 8 weeks treatment. The kidneys from rats treated with 1000 p.p.m. BB or greater for 3 weeks and 500 p.p.m. or greater after 8 weeks treatment had a statistically significant 2-fold increase in labeling index compared with control rats (data not shown). The number of foci of regenerating proximal tubule epithelium as an indicator of nephrotoxicity was also increased in a concentration- and time-dependent manner at all concentrations. The number of foci of regeneration was significantly increased in rats given 500 p.p.m. BB or greater for 3 weeks and 250 p.p.m. or greater for 8 weeks (data not shown).
The liver also had moderate centrilobular hypertrophy in rats treated with 500 p.p.m. BB or greater in the feed for 3 and 8 weeks. These data, which were used to set doses, indicated that a feed concentration of 500 p.p.m. BB would provide increased cell proliferation in the proximal renal tubules with minimum nephrotoxicity.

**Tumor promotion study**

Untreated Eker rats had time-dependent significant increases in number and incidence of atypical hyperplasias and tumors and a significantly increased incidence in macroscopically visible masses (Tables II–V). These data confirm that spontaneous lesions develop early, grow over time and form tumors as previously reported. (27)

Rats treated for 4.5 months with BB had treatment-related but not concentration-dependent significant increases in incidence and number of atypical hyperplasias (Table III). There was also a significantly greater number of histologically identifiable lesions in rats treated with 500 p.p.m. BB compared with controls (Table III). Rats treated with 500 p.p.m. BB for 4.5 months had significantly more adenomas and more total tumors than those treated with 100 p.p.m. BB (Table III).

Rats treated for 10.5 months with 100 p.p.m. BB had significantly increased numbers of macroscopic masses and total tumors compared with those treated for 4.5 months (Tables II and IV). Rats treated with 500 p.p.m. BB for 10.5 months also had significantly increased numbers of macroscopic masses and significantly more carcinomas, but significantly fewer atypical tubules, compared with those treated for 4.5 months (Tables II and V).

A biologically significant finding in this study was that there was no statistically significant difference between the numbers of rats with lesions or the multiplicity of lesions in any category between any of the groups of animals necropsied at 12 months of age (Tables II–V). There was no statistically significant difference in any parameter measured between animals treated for 4.5 months with 500 p.p.m. BB and necropsied at 6 months of age and any of the groups necropsied at 12 months of age (Tables II–V). In addition, the recovery group, treated with 500 p.p.m. BB for 4.5 months and necropsied at 12 months of age, was not different from any of the other groups necropsied at 12 months of age (Tables IV and V).

The severity of nephropathy was slightly increased in the

### Table II. Mean number of lesions after 4.5 months treatment (6 months of age) with BB in the feed

<table>
<thead>
<tr>
<th>Treatment (p.p.m.)</th>
<th>Mean no. of masses ± SD (total number of masses)</th>
<th>Total number of masses according to size</th>
<th>Incidence of rats with macroscopic masses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of masses ± SD (total number of masses)</td>
<td>≤1 mm</td>
<td>&lt;1–5 mm</td>
</tr>
<tr>
<td>Control (15)</td>
<td>1.1 ± 1.1 (17)</td>
<td>5 (53%)b</td>
<td>7 (41%)</td>
</tr>
<tr>
<td>100 (18)</td>
<td>0.9 ± 1.1 (17)</td>
<td>8 (47%)</td>
<td>8 (47%)</td>
</tr>
<tr>
<td>500 (16)</td>
<td>1.3 ± 1.6 (20)</td>
<td>12 (60%)</td>
<td>8 (40%)</td>
</tr>
</tbody>
</table>

* n, total number of Tsc2 mutant rats.
* Total number of lesions counted per group.
* Percentage of all macroscopically visible masses.

### Table III. Mean number of lesions per rat ± SD and total number of lesions counted from one histological section from each kidney after 4.5 months treatment (6 months of age) with BB in the feed

<table>
<thead>
<tr>
<th>Treatment (p.p.m.)</th>
<th>Atypical tubule</th>
<th>Atypical hyperplasia</th>
<th>Adenoma</th>
<th>Carcinoma</th>
<th>Tumors</th>
<th>Sum of all lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (15)</td>
<td>6.9 ± 4.2 (103)a</td>
<td>0.7 ± 1.1 (11)</td>
<td>0.9 ± 1.0 (14)</td>
<td>0.2 ± 0.4 (3)</td>
<td>0.9 ± 0.8 (14)</td>
<td>8.7 ± 4.4 (130)</td>
</tr>
<tr>
<td>100 (18)</td>
<td>9.1 ± 5.1 (163)</td>
<td>2.6 ± 2.4 (47)</td>
<td>0.9 ± 1.1 (16)</td>
<td>0.1 ± 0.2 (1)</td>
<td>0.9 ± 1.1 (17)</td>
<td>12.6 ± 7.4 (227)</td>
</tr>
<tr>
<td>500 (16)</td>
<td>9.7 ± 3.6 (155)</td>
<td>3.6 ± 2.2 (58)b</td>
<td>2.6 ± 2.0 (41)c</td>
<td>0</td>
<td>2.6 ± 2.0 (41)</td>
<td>15.9 ± 5.3 (254)</td>
</tr>
</tbody>
</table>

* n, total number of Tsc2 mutant rats.
* Total number of lesions counted per group.
* Greater than (P < 0.05) concurrent control.
* Greater than (P < 0.05) the 6 months 100 p.p.m. group.

### Table IV. Mean number of lesions after 10.5 months treatment (12 months of age) with BB in the feed

<table>
<thead>
<tr>
<th>Treatment (p.p.m.)</th>
<th>Mean no. of masses ± SD (total number of masses)</th>
<th>Total number of masses according to size</th>
<th>Incidence of rats with macroscopic masses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of masses ± SD (total number of masses)</td>
<td>≤1 mm</td>
<td>&gt;1–5 mm</td>
</tr>
<tr>
<td>Control (13)</td>
<td>3.4 ± 3.1 (44)a</td>
<td>25 (57%)b</td>
<td>14 (32%)</td>
</tr>
<tr>
<td>100 (17)</td>
<td>3.2 ± 2.2 (54)c</td>
<td>36 (67%)</td>
<td>16 (30%)</td>
</tr>
<tr>
<td>500 (14)</td>
<td>4 ± 2.4 (56)d</td>
<td>31 (55%)</td>
<td>24 (43%)</td>
</tr>
<tr>
<td>500 stop (20)</td>
<td>4.3 ± 3.2 (87)</td>
<td>50 (58%)</td>
<td>34 (39%)</td>
</tr>
</tbody>
</table>

* n, total number of Tsc2 mutant rats.
* Total number of lesions counted for group.
* Percent of all macroscopically visible masses.
* Greater than (P < 0.05) the 6 months 100 p.p.m. group.
* Greater than (P < 0.05) the 6 months 500 p.p.m. group.

### Table V. Incidence of rats with macroscopic masses after 4.5 months treatment (6 months of age) with BB in the feed

<table>
<thead>
<tr>
<th>Treatment (p.p.m.)</th>
<th>Incidence of rats with macroscopic masses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (15)</td>
<td>10/15 (67%)</td>
</tr>
<tr>
<td>100 (18)</td>
<td>11/18 (65%)</td>
</tr>
<tr>
<td>500 (16)</td>
<td>10/16 (63%)</td>
</tr>
</tbody>
</table>

Rats treated for 10.5 months with 100 p.p.m. BB had significantly increased numbers of macroscopic masses and total tumors compared with those treated for 4.5 months (Tables II and IV). Rats treated with 500 p.p.m. BB for 10.5 months also had significantly increased numbers of macroscopic masses and significantly more carcinomas, but significantly fewer atypical tubules, compared with those treated for 4.5 months (Tables II and V).
Compounds such as BB, which are not directly mutagenic, may increase cancer incidence by increasing proliferation in the transformed or intermediate cell population. Merely increasing the number of proliferating cells may significantly increase the probability that cancer will arise in the target cell population (3,4,6,30,33). Even a doubling of the percentage of replicating cells may significantly increase the probability that cancer will arise in the target cell population (33). Subchronic exposure to BB at 500 p.p.m. in the feed resulted in a doubling of the percentage of cells in S phase in the target population of the kidney that develops renal tumors. While this exposure regimen did increase the number of renal tumors present after 4.5 months treatment, merely increasing the numbers of proliferating cells was not sufficient to increase the number of renal tumors after longer treatment. These data indicate that enhancing proliferation rates without additional cell stressors is not sufficient by itself to increase tumor numbers, even in a susceptible population.

Cancer may develop from chronic exposure to a xenobiotic that is not directly mutagenic by increasing the proliferation of normal or intermediate cells (4,6,7). This increased proliferation could increase transition rates from normal to intermediate or intermediate to malignant genotypes and phenotypes (7). Xenobiotics that increase the number of proliferating intermediate cells without affecting transition rates may increase cancer risk with time, and even small changes in the rate of cell proliferation over cell death rate can lead to large changes in tumor incidence (7). The Eker rat model of renal cancer has a mutation that allows increased susceptibility and sensitivity to renal carcinogenesis in all the cells, which means that all the cells in the kidney are intermediate cells because the rats are born with a proposed initiating mutation in the renal cancer process. The concentrations of BB used in the present study promoted spontaneous tumors, so that they reached a size described as adenoma earlier. Aiding from a 12% incidence in the labeling index in the renal cortex and outer medulla of male F344 rats (34). In all the studies using BB as a tumor promoter, there has not been a concentration-dependent response for tumor promotion in male rats treated with concentrations ranging from 500 to 4000 p.p.m. (34). All concentrations used appeared to have comparable promoting activity. Previous studies have shown concentration- and time-related BB-induced nephrotoxicity (35). BB given at 4000 p.p.m. in the feed resulted in a 62–70% incidence of dysplastic tubules and a 45% incidence in rats treated with 500 p.p.m. (35). BB-induced tumor incidence appeared to be time rather than concentration dependent (35). The present subchronic
study supports the previous work showing a concentration-dependent increase in nephrotoxicity in rats treated with BB and also indicates that 500 p.p.m. BB given in the feed is sufficient to act as a renal tumor promoter. In the Eker rat, the inherited mutation in the Tsc2 gene dramatically decreased the time to tumor. This model is useful in detecting renal carcinogens and renal tumor promoters. However, it is necessary to examine multiple time points to determine whether a particular xenobiotic is a carcinogen or tumor promoter.

While elevated cell proliferation does increase the likelihood of tumor formation, increased cell proliferation associated with mild toxicity was probably not sufficient to increase tumor formation in the present study. In some studies increased renal cell proliferation associated with mild nephrotoxicity was unrelated to tumor development (36,37). Others have shown that increased severity of nephropathy was associated with increased frequency of atypical hyperplasias and adenomas in the renal cortex (38). The presence of proliferative lesions was closely correlated with severity of nephropathy (38). Continual administration of cytotoxants can generate a hyperproliferative state characterized by persistent cell death and subsequent regeneration. This cytotoxic–proliferative response is an obligatory step in the cancer process such that concentrations that produced no cytotoxicity would not be associated with increased risk of cancer (39). In a previous study where female Eker rats were chronically treated with 17β-estradiol the treatment resulted in severe nephrotoxicity and increased numbers of renal tumors (25). In the same study there was a dramatic decrease in spontaneous nephropathy and an associated decrease in preneoplastic and neoplastic renal lesions when female Eker rats were ovariecotimized (25). The concentrations of BB given in the present study did not dramatically enhance nephrotoxicity and did not increase the multiplicity of preneoplastic and neoplastic renal lesions. Taken together, these studies indicate that for compounds not directly mutagenic there is a minimum level of ongoing cytotoxicity associated with regenerative proliferation to enhance the cancer process beyond promoting spontaneous lesions.

The results of the present study suggest that particular attention needs to be paid to the duration of treatment with respect to tumor latency when designing experiments using genetically susceptible or transgenic rodent models. If only the 6 month data were available in the present study, then the interpretation of those data would be that BB is a complete renal carcinogen that increases the multiplicity of both preneoplastic and neoplastic renal lesions. If only the 12 month data were available, then the interpretation would be that BB has no effect on renal carcinogenesis. This points out the necessity of designing studies appropriately when examining poorly characterized xenobiotics in genetically enhanced rodent models. The final interpretation of the potential hazard of a xenobiotic may be as much a function of the design of the study as of the properties of the compound.

In conclusion, the present study has shown that Eker rat hereditary renal tumors are sensitive to the promotional effects of BB. Without significant nephrotoxicity, there was no increase in the multiplicity of renal tumors. The interpretation of a study using genetically susceptible animals can be time dependent and this fact is very important when designing studies using transgenic or knockout animals. The present study and previous work have shown that the Eker rat model of hereditary renal cancer responds to mutagenic and non-mutagenic renal carcinogens and renal tumor promoters.

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