LACK OF MODIFYING EFFECTS OF 2, 2'-[(4-AMINOPHENYL) IMINO] BISETHANOL SULFATE ON INDUCTION OF PRENEOPLASTIC \( \gamma \)-GLUTAMYL TRANSPEPTIDASE-POSITIVE FOCI IN A MEDIUM-TERM BIOASSAY SYSTEM USING F344 RATS

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Abstract: The modifying effects of 2, 2'-[(4-aminophenyl) imino] bisethanol sulfate (4APE) on liver carcinogenesis were investigated in male F344/DuCrj rats initially treated with N-nitrosodiethylamine (DEN). Two weeks after a single dose of DEN (200 mg/kg, intraperitoneally), rats were given 4APE at dietary levels of 1,000, 330 and 110 parts per million (ppm), or 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) at 600 ppm as a positive control for 6 weeks. At week 3 following DEN administration, all animals were subjected to partial heptectomy. No adverse effects on survival and body weight were seen in rats treated with 4APE, even at the highest dietary levels. Marked growth retardation and significant increase of relative liver weight were found in animals treated with the known hepatocarcinogen 3'-Me-DAB, which demonstrated marked promoting activity as evidenced by significantly increased values for \( \gamma \)-glutamyl transpeptidase (\( \gamma \)-GT) positive foci as compared with the controls given DEN alone. In contrast, 4APE did not significantly increase the level of \( \gamma \)-GT positive foci over that induced by DEN initiation alone. From these results, it is concluded that 4APE does not possess promoting activity for liver carcinogenesis.

Key words: 2, 2'-[(4-aminophenyl) imino] bisethanol sulfate, tumor promotion, \( \gamma \)-glutamyl transpeptidase, medium-term bioassay, rat liver.

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— 77 —
INTRODUCTION

2, 2’-[(4-aminophenyl) imino] bisethanol sulphate (4APE) is extensively used in the United States as an ingredient in the "permanent type" hair dye which requires an oxidization procedure (Marzulli et al., 1978). It has been reported that 4APE does not possess any teratogenic activity or dominant lethal effects, which showing minimal toxicity as evidenced by slight growth retardation and darkened thyroid tissue without histopathological changes (Burnett et al., 1986). It was also noted that 4APE can induce a hypersensitivity skin reaction in guinea-pigs after multiple topical applications. Although toxicity studies of hair dyes, including 4APE, were performed, no adverse effects were found (Burnett et al., 1976) and to our knowledge, no other information concerning to the safety assessment of 4APE has been published.

Ito and his colleagues have developed an in vivo medium-term bioassay system for rapid and economical screening of environmental hepatocarcinogens, promoters and inhibitory agents (Ito et al., 1988; Tatematsu et al., 1977; Tsuda, et al., 1984) and using this system, have obtained highly positive results for all hepatocarcinogens tested. Moreover, they have demonstrated that the assay system dose not give any false-positive data.

The aim of the present study was to investigate the modifying effects of 4APE on liver carcinogenesis using the in vivo medium-term bioassay system for rapid prediction of hepatocarcinogenicity.

MATERIALS AND METHODS

Test chemical: 2, 2’-[(4-aminophenyl) imino] bisethanol sulphate (4APE)(Lot no. 3, purity: 92.9% normally or 98.7% after drying) manufactured by Lowenstein Dyes Cosmetics, Inc. (USA) was used in this study. The chemical formula is shown in Fig. 1.

Animals and maintenance: A total of 250 male F344/DuCrj rats were obtained from Charles River Japan, Inc., Kanagawa. The rats were about 6 weeks old at the commencement of the experiment and were housed five to a plastic cage with

![Chemical formula](attachment:image.png)

Fig. 1. Chemical formula of 2, 2’-[(4-aminophenyl) imino] bisethanol sulphate (4APE).
Lack of promoting activity of 4APE for liver carcinogenesis

hardwood chips for bedding. The animals were maintained at a 22±2°C of room temperature and a relative humidity at 60±10% with a 12-hr light/dark cycle. Positive air pressure was maintained with more than 15 air changes/hr.

Experimental procedure: The experimental design is shown in Fig. 2. Animals were divided into three groups, and groups 1 and 3 were subdivided into subgroups for treatment with test chemicals at three different dietary levels. Group 1 was given N-nitrosodiethylamine (DEN; Katayama Kagaku Kogyo Co. Ltd., Tokyo) at a dose of 200 mg/kg body weight intraperitoneally as an initiator (Shirai et al., 1985). Two weeks later, rats were given diet containing 4APE at 1,000, 330 and 110 parts per million (ppm) or 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB; Tokyo Kasei Kogyo Co. Ltd., Tokyo) at 600 ppm for 6 weeks. Group 2 was given DEN alone without secondary test chemical administration. Rats in group 3 were given saline intraperitoneally instead of DEN and then treated as for group 1. Three weeks after the beginning of the experiment, two-thirds partial hepatectomy was performed on all animals (Hasegawa et al., 1986; Higgins and Anderson, 1931). All survivors were sacrificed under ether anesthesia for examination at week 8.

Histopathological examination and quantitation of γ-GT positive foci: All survivors were autopsied, the liver and kidney weights measured and the organ weight to body weight ratios calculated. The livers were cut into 2-3 mm thick sections with a razor blade and fixed in ice-cold acetone for subsequent histochemical staining of γ-GT (Rutenberg et al., 1969; Tsuda et al., 1984) and routine hematoxylin and eosin staining. The numbers and areas of γ-GT positive foci were measured using a color video image processor (VIP-21C, Olympus-Ikegami Tsushin

![Graph showing experimental procedure](image)

Fig. 2. Assay method. Group 1. DEN+test chemical; Group 2. DEN alone; Group 3. test chemical alone. All rats were subjected to PH at week 3.
The results were assessed by comparing the values of γ-GT positive foci between group 1 (DEN+test chemical) and group 2 (DEN alone). Group 3 served for the estimation of carcinogenic potential. Other liver sections and the kidneys were fixed in 10% buffered formalin solution and preserved.

Statistical analysis: Numerical data obtained for liver weights and enzyme-altered foci were analysed using Student’s t-test.

RESULTS

Survivals, body weights, food consumption and chemical intake: Although several rats died after the surgical operation, no treatment-related deaths occurred. Final survival rates in each group were 92% or above. Marked growth retardation was evident in rats which received 3'-Me-DAB (groups 1-4 and 3-4), but not in rats exposed to 4APE at any of the dietary levels (groups 1-1~3) when compared to rats given DEN alone (group 2). Rats in group 1-4 consumed less food than group 2, but no adverse effects regarding food consumption data were found in groups 1-1~3. For the four treated subgroups (1-1~4), the intake of 4APE or 3'-Me-DAB, calculated from nominal dietary levels, the mean food consumption and the mean body weight for each subgroup, were 76, 24, 9 and 41 mg/kg body weight/day, respectively.

Gross findings: Enlargement and discolored area or nodules of liver were observed in rats exposed to 3'-Me-DAB (group 1-4 and 3-4). However, no treatment-related gross liver lesions were observed in rats receiving 4APE.

Organ weights: Relative organ weights for each group are shown in Table 1. Statistically significant increase was seen in the livers of rats treated with 3'-Me-DAB (group 1-4) when compared to group 2. The elevated relative kidney weight observed in group 1-4 seemed to be related to growth retardation. The reduced relative kidney weight found in group 1-2 did not seem to be due to 4APE treatment, since no dose-dependency was apparent.

Histochemical analysis: The number and area of γ-GT positive foci per cm² in animals exposed to 4APE (group 1-1~3) were not significantly different from the values for rats treated with DEN alone (group 2)(Table 2, Photo. 1 A and B). However, their development in rats given 3'-Me-DAB, as a positive control, was markedly increased as compared to group 2 (Photo. 1 C). Rats of group 3-4, subjected to the treatment with 3'-Me-DAB after saline injection, demonstrated marked development of γ-GT positive foci. In contrast, with exception of only a few γ-GT positive foci of very small size in animals of group 3-1, treated with 4APE at 1,000 ppm without initiation, no lesions were observed in the other groups without DEN initiation.

Histopathological examination of serial sections revealed that γ-GT positive foci coincided with clear cell type foci (areas)(Photo. 2 A~C).
Table 1. Final body weights and relative organ weights of rats initiated with DEN and subsequently treated with 4APE or 3’-Me-DAB.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Final body weight (g)</th>
<th>Relative organ weights (% b.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DEN</td>
<td>Chemicals (dose)</td>
<td>Initial</td>
</tr>
<tr>
<td>1-1</td>
<td>+ 4APE (1,000)</td>
<td>25</td>
<td>25</td>
<td>280±10b</td>
</tr>
<tr>
<td>2</td>
<td>+ 4APE (330)</td>
<td>25</td>
<td>23</td>
<td>282±12</td>
</tr>
<tr>
<td>3</td>
<td>+ 4APE (110)</td>
<td>25</td>
<td>24</td>
<td>284±12</td>
</tr>
<tr>
<td>4</td>
<td>+ 3’-Me-DAB (600)</td>
<td>25</td>
<td>23</td>
<td>227±14**</td>
</tr>
<tr>
<td>2</td>
<td>+ -</td>
<td>50</td>
<td>48</td>
<td>282±12</td>
</tr>
<tr>
<td>3-1</td>
<td>- 4APE (1,000)</td>
<td>25</td>
<td>23</td>
<td>306±11</td>
</tr>
<tr>
<td>2</td>
<td>- 4APE (330)</td>
<td>25</td>
<td>24</td>
<td>303±10</td>
</tr>
<tr>
<td>3</td>
<td>- 4APE (110)</td>
<td>25</td>
<td>25</td>
<td>300±12</td>
</tr>
<tr>
<td>4</td>
<td>- 3’-Me-DAB (600)</td>
<td>25</td>
<td>24</td>
<td>244± 9</td>
</tr>
</tbody>
</table>

a: Dietary levels at parts per million.
b: Values are means± S.D.
**: Significantly different from group 2 at P<0.01.
Table 2. Numbers and areas of $\gamma$-GT positive foci in the livers of rats initiated with DEN and subsequently treated with 4APE or 3'-Me-DAB.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>$\gamma$-GT positive foci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEN</td>
<td>Chemicals</td>
<td>(dose) $^a$</td>
</tr>
<tr>
<td>1-1</td>
<td>+</td>
<td>4APE (1,000)</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>4APE (330)</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>4APE (110)</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>3'-Me-DAB (600)</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>48</td>
</tr>
<tr>
<td>3-1</td>
<td>-</td>
<td>4APE (1,000)</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>4APE (330)</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>4APE (110)</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>3'-Me-DAB (600)</td>
<td>24</td>
</tr>
</tbody>
</table>

$^a$: Dietary levels at parts per million.
$^b$: Values are means±S.D.
$^{**}$: Significantly different from group 2 at P<0.01.

**DISCUSSION**

In the present investigation, no adverse effects on survival, body weights, and relative liver weights were found in rats treated DEN followed by 4APE as compared to rats given DEN alone. In addition, it was clearly shown that 4APE did not possess any modifying activity for liver carcinogenesis.

Ito et al. (1969) first reported that the substituted-benzenediamine $m$-toluylenediamine (2, 4-diaminotoluene) was a hepatocarcinogen in laboratory animals. Subsequently, a number of substituted-benzenediamine compounds were tested in the National Cancer Institute in large scale studies. Although Sontag (1981) and Weisburger et al. (1978) could not establish any clear relationships regarding structure-activity patterns, they did demonstrate that carcinogenic activity tended to decrease when the amino groups were para to one another. Since the 4APE, which is a common ingredient of permanent or oxidative type hair dyes, is a derivative of $p$-phenylenediamine ($p$-PD), it might be therefore be expected to have low if any carcinogenicity.

Tsuda et al. (1984) reported the results of thirty-one chemicals using the in vivo medium-term bioassay system, and showed that this was an useful model for prediction of hepatocarcinogenicity or promoting activity of test chemical with a high probability. Ito et al. (1988) also summarized the results for 112 chemicals in the same system using more advanced immunohistochemical staining with glu-
Lack of promoting activity of 4APE for liver carcinogenesis

**Photo. 1.** Specific Ψ-GT positive foci and nonspecific positive periportal cells in different groups. Ψ-GT staining, x40. A: DEN+4APE, 1,000 ppm, B: DEN alone, C: DEN+3'-Me-DAB, 600 ppm.
Photo 2. Serial sections to the preparations in Photo 1 showing γ-GT positive foci. Clear cell type foci are evident. H&E, x40. A: DEN+4APE, 1,000 ppm, B: DEN alone, C: DEN+ 3'-Me-DAB, 600 ppm.
Lack of promoting activity of 4APE for liver carcinogenesis
tathione S-transferase placental form, and found that the positive rates of known liver
carcinogens were 87.5%, while none of the non-liver carcinogens showed false-
positive. Furthermore, Ogiso et al. (1985) demonstrated that the dose-dependent
results obtained from this medium-term bioassay system using foci development as
the end-point clearly corresponded to those gained for carcinomas in long-term
carcinogenicity studies.

In conclusion, based on the evidence of the present investigation, it is strongly
suggested that 4APE is neither a hepatocarcinogen nor a hepatopromoter in rats.

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