HEPATOTOXIC AND HEPATOCARCINOGENIC EFFECTS OF A SYDNONE DERIVATIVE, 3-BENZYLSYDNONE-4-ACETAMIDE, ON MICE: DIFFERENCES IN SUSCEPTIBILITY BY STRAIN AND SEX

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Abstract: Acute and chronic toxicity of a synthetic sydnone derivative, 3-benzylsydnone-4-acetamide (BSA), was examined. Hepatotoxic effect of BSA given in drinking water at concentrations up to 0.2% for 4 weeks varies considerably in differing strains of mice, DDD is the most susceptible followed by C57BL/6, DBA, BALB/c, and ICR. C3H mice were resistant at the tested doses. Female mice were more susceptible than the males in all strains. In DDD females liver cell necrosis developed in 2 weeks with 0.05 to 0.2% BSA in drinking water. With continuous administration of 0.05% BSA liver cell nuclei with polyploid DNA contents (8c, 16c, and 32c) increased in 2 weeks. Then hepatocytes having diploid nuclei predominated in 4 weeks. In DDD female mice hepatocellular carcinoma developed within 12 months with 0.01% and 0.02% of BSA in the drinking water. The incidence was 11.8 and 46.2%, respectively. By whole body autoradiography of female DDD mice given orally 10 µCi of [14C]-labeled BSA (about 60 mg/kg body weight) the radioactivity was shown to concentrate in the liver after 45 min and remained faintly but definitely up to 72 hrs, indicating the possible accumulation of the drug or its metabolite(s) in the liver. (J Toxicol Pathol 4: 45~54, 1991)

Key words: Sydnone derivative, Hepatotoxicity, Hepatocarcinogenesis, Whole body autoradiography, Mice

Introduction

One of the synthesized sydnone derivatives, 3-benzyl-sydnone-4-acetamide (BSA, Fig. 1), induced liver cirrhosis and hepatocellular carcinoma in rats1,2. Hepatotoxicity of BSA is more pronounced in male than in female rats1 and hepatocellular carcinomas4 were induced more frequently in male than in female rats (unpublished data). Many hepatotoxins show remarkable species3, strains4,5, and sex differences7~12 in their toxicity and carcinogenicity. To study the species difference in susceptibility of the toxin we performed acute and chronic toxicity test using several strains of mice of both sexes. A part of the results was reported in abstract form13.

Materials and Methods

Animals

Both sexes of mice of six different strains were used. DDD, C3H/He, and BALB/c mice were supplied from the Department of Pathology, the Institute of Medical Science, the University of Tokyo. DBA/2 and C57BL/6 were purchased from Shizuoka Agricultural Cooperation for Experimental Animals (Hamamatsu, Japan) and ICR/CL, from Clea Japan, Ltd. (Tokyo, Japan). They were all specific pathogen free, but maintained at the clean-conventional level before and during the experiment in an air-conditioned room. Usually 3 to 6 mice were housed in a plastic or aluminum cage, with free access to feed (CE-II, Clea Japan Ltd. or CRF1, Oriental Yeast Co.,
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Ltd., Tokyo, Japan) and running water.

3-Benzylsydnone-4-acetamide

3-Benzylsydnone-4-acetamide (Fig. 1, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was dissolved in drinking water at varied concentrations (ex. Table 1) and given to the mice ad libitum. It was soluble to water up to 0.4% and stable for at least 1 month at room temperature. Ingested amount of BSA was estimated by measuring the water consumption per cage.

\[^{14}C\]-BSA (Fig. 1) was synthesized by Fujisawa Pharmaceutical Co., Ltd.. The radioactive substance, with a specific activity of 6.6 μCi/mg, was dissolved in distilled water at 0.4%.

![Chemical structure of 3-benzylsydnone-4-acetamide. (*)]: where \(^{14}C\) was incorporated in radioactive BSA.

\[\text{Fig. 1. Chemical structure of 3-benzylsydnone-4-acetamide. (*)}: \text{where} \:^{14}\text{C} \text{was incorporated in radioactive BSA.}\]

\[\text{Acute and subacute toxicity tests}\]

Six-week-old animals received BSA in drinking water for up to 4 weeks (Table 1). At sacrifice by ether anesthesia blood was taken from the inferior caval vein for measurement of serum glutamate-pyruvate transaminase (s-GPT) activity by Reitman and Frankel method using a commercial kit (S.TA-Test Wako, Wako Junyaku Co., Ltd., Tokyo, Japan). The liver was weighed and fixed with 10% neutral-buffered formalin for histological examination. Paraffin-embedded sections were stained with hematoxylin and eosin and periodic acid–Schiff.

Histological changes after 4 weeks, similar to those of subacute liver necrosis, were evaluated in the order of none (0 in the tables), mild\(^{(5)}\), moderate\(^{(5)}\), and severe\(^{(5)}\), according to the criteria described below (Fig. 2). None: no lesion attributable to the toxin. Mild: centrilobular necrosis, presence of eosinophilic bodies, and pigment-laden Kupffer cells. Moderate: marked centrilobular necrosis and collapse with degenerative, swollen hepatocytes. Regenerative activity as well as bile ductular proliferation were also

\[\text{Table 1. Different Susceptibility of Varied Strains of Mice to the Hepatotoxic Effect of 3-benzylsydnone-4-acetamide Expressed by S-GPT Activity and Histological Changes of the Liver}\]

| Strains of mice | Male | | | Female | | |
|---|---|---|---|---|---|
| | S-GPT | | Histology | S-GPT | | Histology |
| | Cont | Exp | | | Cont | Exp |
| DDD (0.2%) | 27 | 27 | 0-1 | 35 | 226 | 2-3 |
| C57BL/6 | 29 | 30 | 0 | 44 | 49 | 1-2 |
| DBA/2 | 35 | 36 | 0 | 60 | 764 | 2 |
| Balb/c | 20 | 39 | 0 | 21 | 195 | 1-2 |
| ICR | 27 | 37 | 0 | 63 | 160 | 0 |
| C3H/He | 14 | 19 | 0 | 14 | 21 | 0 |
| DDD (0.1%) | 29 | 71 | 0 | 23 | 160 | 1-2 |
| (0.1%) | 215 | 2-3 | 630 | 2-3 | 82 | -1 |
| (0.02%) | | | | | | |
| DDD×ICR | | 43 | 0 | 315 | 3 |
| ICR×DDD | | 29 | 71 | 0 | 23 | 160 | 1-2 |
| DDD×C3H | | 24 | 0 | 255 | 3 |
| C3H×DDD | | 33 | 122 | -1 | 28 | 177 | 1-2 |

The drug was given in drinking water, 0.2% except for female DDD, for 4 weeks. S-GPT values were means of 6 mice in Kunkel unit. For histological grading see text and Fig. 2.
observed. Severe: in addition to the above, bridging necrosis and oval cell proliferation.

Long-term feeding test

In total 100 female and 30 male DDD mice of 6 weeks of age were used (Table 3). BSA was given in the drinking water at the concentrations of 0.002, 0.01 and 0.02%. Average water intake was about 2.3 to 3.3 ml/day for male and 2.2 to 2.4 ml/day for female mice. Because of the limited capacity of the animal facility, the number of control animals (9 male and 6 female) were limited, but in a simultaneously performed experiment14 22 non-treated female DDD mice showed no hepatic lesions after 12 or 15 months.

Three animals of each group were sacrificed at 3 months-intervals. All surviving animals were sacrificed after 12 months by excess ether anesthesia. At sacrifice blood was taken for s-GPT analysis as described above. The liver and grossly abnormal organs were histologically examined. The neoplastic lesions of the liver were classified histologically according to Frith and Ward15.

Quantitative cytophotometry of hepatocyte DNA by FACS-III

To determine the ploidy change of the hepatocyte nuclei during the early phase, liver cells were isolated by the method of Berry and Friend16 with some modifications17. The liver was perfused from the portal vein in situ with a recirculating dissociation medium containing 0.8% NaCl, 0.04% KCl, 0.2% glucose, and 0.05% collagenase at 37°C. The liver was removed and, after minced with a razor blade, incubated further 15 min in the same medium at 37°C under gentle stirring. Parenchymal cells were precipitated by centrifugation at 20 G for 5 min. The liver cells were resuspended in the solution containing 0.25 M sucrose, 0.2% nonidet P40, 0.05 M Tris–HCl, 25 mM KCl, 5 mM MgCl₂, and 1 mM CaCl₂ at pH 7.5. After homogenization the parenchymal nuclei were stained with CA3 solution (0.002% chromomycin A3 (Sigma), 0.3% MgCl₂ 6H₂O). The nuclei obtained here showed over 95% purity of parenchymal cells. Using FACS-III (Becton–Dickinson) DNA contents in each parenchymal nucleus was measured.
Fig. 3. Whole body autoradiography of DDD female mice administered with 10 μCi/animal or 60 mg/kg b.w. of [14C]-BSA. The autoradiographs are from the mice sacrificed after 15 (a), 45 min (b), 3 (c), 6 (d), 24 (e), and 72 hrs (f) of administration. Exposure time was 1 week except for the last two, in which it was 2.5 weeks. Radioactivity remains exclusively in the liver after 24 and 72 hrs.

by the fluorescence excited by laser beam at 458 nm.

Whole body autoradiography

To clarify if this drug accumulated in the liver, whole body autoradiographic study was performed. [14C]-BSA was administered to the 7-week-old animals, 10 μCi/mouse orally (about 60 mg/kg body weight). Each one mouse was sacrificed at 15, 30, 45 min, 1, 1.5, 2, 3, 6, 24, and 72 hrs after drug administration.

Whole-body autoradiography was performed by the technique of Ullberg18 with modification19. The anesthetized animals were frozen in acetone–dry ice bath at −50°C. The right paramedian plane was exposed and 40 μ thick sections were cut by a Leitz 1400 microtome at −20°C. The sections were placed on a Scotch tape (No. 810) and covered with mylar film for exposure using Fuji X-ray Film (Fuji Photo Film Inc., Tokyo, Japan). The extent and intensity of the radioactivity was assessed qualitatively and semi-quantitatively by comparing the blackening intensity of the films.

Operative methods and hormone treatment

Castration was performed at 4 weeks of age (2 weeks before BSA administration) under light ether anesthesia. In the groups of the male with or without castration betaestradiol, 1 mg/kg b.w., was given intraperitoneally 2 times/week during BSA administration.

Results

1. Strain- and sex-dependent differences of the acute toxicity of BSA (Table 1, Fig. 2)

To compare the susceptibility of different strains of mice to the drug, each 6 mice of both sexes of DDD, C57BL/6, DBA/2, BALB/c, ICR, and C3H/He were given 0.2% (for female DDD, 0.05%) of BSA in drinking water for 4 weeks. Ingested amount of BSA varied little when the body weight of the animals was considered.

In all of the experimental groups the rate of body weight gain was lower than in the controls; significantly lower (p<0.05 by Wilcoxon's rank sum test) in the female mice of DDD, DBA/2, and BALB/c. S-GPT activity was raised in the female mice of DDD, C57BL, DBA, and ICR. Histological changes of the liver were severe in DDD and C57BL females, moderate in DBA and BALB/c females, and mild in DDD males. C3H was resistant to this dose of BSA. In either strain of DDD, C57BL, DBA, and BALB/c the female mice were more susceptible than the male. Also
in ICR mice s-GPT activity showed the same tendency.

In the most susceptible strain, female DDD mice, as low dose as 0.02% or 1.4 mg/day/animal for 4 weeks caused definite liver injury as demonstrated by elevated s-GPT activity and histological changes. At the concentrations of 0.05% and higher, hepatic damage was severe with almost equal grade of hepatomegaly, elevated s-GPT activity and histological changes (Fig. 2d) of the liver.

Hepatotoxic effects of BSA on F₁ mice of DDD (the most susceptible strain) and ICR or C3H (the most resistant strains) were compared with those on the inbred strains. The same experiment designed as above showed that the male F₁ mice were as resistant, whereas female F₁ mice were as susceptible as their parent DDD. F₁ females were more susceptible when their mothers were DDD than when their fathers were DDD.

Fig. 4. Serum GPT activities were increasing from 2 to 5 weeks administration of BSA, indicating continuous degradation of hepatocytes.

Fig. 5. DNA contents distributions of the hepatocyte nuclei. Percentage of each ploidy class nuclei was calculated and plotted against experimental period. 4c or higher polyploid nuclei were increased in 2 to 5 weeks in the experimental group (right) compared to the control (left). Open circles are for 2c, closed circles, 4c, closed triangles, 8c, open triangles, 16c and open squares, 32c.
Table 2. Effect of Castration (TX) and/or Estradiol Administration (Est) on the Hepatotoxicity of 3-benzylsydnone-4-acetamide in DDD Male Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BSA Conc. (%)</th>
<th>Doses</th>
<th>S-GPT Mean (Range)</th>
<th>Liver damage (histology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>0.05</td>
<td>2.3</td>
<td>380 (270–730)</td>
<td>2</td>
</tr>
<tr>
<td>TX</td>
<td>0</td>
<td>0</td>
<td>26 (19–37)</td>
<td>0</td>
</tr>
<tr>
<td>TX + Est</td>
<td>0.05</td>
<td>3.9</td>
<td>510 (180–1020)</td>
<td>2</td>
</tr>
<tr>
<td>Est</td>
<td>0.05</td>
<td>3.5</td>
<td>190 (63–330)</td>
<td>1</td>
</tr>
</tbody>
</table>

BSA was administered for 4 weeks in drinking water at designated concentration or doses estimated from ingested amount of water (mg/day/animal). Castrated at 3 weeks of age. Beta-estradiol was injected intraperitoneally twice weekly throughout the experimental period.

2. Toxicological and pathological study in DDD mice

Distribution of the radioactivity of 14C-BSA in the body

The sequential distribution pattern of radioactivity after oral administration of the drug was studied by whole body autoradiography. After 15 min, the labeled material appeared diffusely in the body except for the brain (Fig. 3a). The urine in the renal pelvies and urinary bladder showed the remarkable radioactivity. After 45 min the radioactivity of the liver judged by the grade of blackening of the film was higher than other organs (Fig. 3b) and became more prominent thereafter (Figs. 3c and 3d). High radioactivity was also observed in the intestinal contents between 30 min and 6 hrs (Fig. 3b to 3d). After 24 and 72 hrs radioactivity was still discernible in the liver weakly but definitely (Figs. 3e and 3f). Preliminarily 69% of the administered radioactivity were recovered from the excretes including urine and feces within the first 24 hrs.

Nuclear DNA changes of hepatocytes

By flow cytophotometry of isolated liver cell nuclei DNA ploidy changes were examined at weekly intervals after BSA (0.05%) administration (Fig. 5). S-GPT levels rose gradually throughout the experiment (Fig. 4). One week after the beginning of BSA administration, the pattern was essentially unchanged from the control pattern. After 2 weeks 8c nuclei increased and even 16c and 32c nuclei appeared. At 3 weeks 32c nuclei increased and other polyploid nuclei decreased with preponderance of diploid nuclei. This tendency was further accentuated in the following week. In the control polyploid nuclei increased gradually, but no 16c or 32c nuclei were found as distinct peaks.

Table 3. Incidence of Hepatocellular Carcinomas in DDD Mice Administered 3-benzylsydnone-4-acetamide in Drinking Water for 12 Months

<table>
<thead>
<tr>
<th>Sex and doses</th>
<th>No. of mice</th>
<th>Hepato-cellular carcinomas</th>
<th>Hyperplastic nodules</th>
<th>Lung adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.02 (%)</td>
<td>16</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Female</td>
<td>0.02</td>
<td>25</td>
<td>13</td>
<td>6 (46.2%)</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>34</td>
<td>17</td>
<td>2 (11.8%)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>25</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Control (M and F)</td>
<td>15</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Each 3 mice of female groups were sacrificed at 3, 6, and 9 months and each 3 males at 3 and 6 months (See Fig. 6).
Effect of castration and estradiol treatment

To evaluate the effect of sex hormones on the toxicity of BSA, castration combined with estrogen administration was carried out. BSA was given to the castrated male DDD mice for 4 weeks (Table 2). Castration enhanced susceptibility of the male mice to BSA. Continual estradiol administration caused moderate enhancement of the BSA-induced liver damage in the male mice, but when combined with castration the damage was more severe (Table 2).

Long-term administration of BSA (Table 3, Fig. 6)

For chronic toxicity experiment BSA was given to DDD mice at the concentration of 0.02, 0.01, and 0.002% in drinking water for 12 months. There was no significant difference between the
mean body weight of the experimental and control groups. Spontaneous deaths in the 1 year experimental period occurred in 2 of 10 males and 3 of 16 females of 0.02%, 8 of 25 female mice of 0.01% of which 2 had leukemia, and in 2 of control females. All the deceased animals of the experimental groups had histologically chronic liver injury.

Sacrificed at 3, 6, and 9 months, s-GPT activity was elevated progressively in the female mice of 0.02% group (Fig. 6). In the females of 0.01% s-GPT level was elevated when the mice had distinct liver cell necrosis and hepatocellular carcinoma. At 9 months histological lesions of 0.02% females were of mild to moderate degree, but there was no hyperplastic areas or nodules, or small cell clusters except for one mouse which had hepatocellular carcinoma. Female mice of the 0.002% group and all male mice showed no hepatic injury at any points by enzyme assay as well as histological examination.

At 12 months the liver of all 13 female mice receiving 0.02% BSA showed severe prolonged liver cell injury but were featured by hyperplastic nodules. Hepatocellular carcinomas were found in 6 of them (Table 3) with significantly high incidence compared with no tumor occurrence in 0.002% group (p<0.05, chi-square test). All hepatocellular carcinomas were well differentiated showing trabecular pattern (Fig. 7). Two of 17 female mice receiving 0.01% BSA had well differentiated hepatocellular carcinoma. One of them had simultaneously a hemangiendothelioma with splenic and retroperitoneal lymph node metastases. Lung adenomas, usually single and up to milium-sized, were found in 7 mice (Table 3).

Discussion

Sydnone derivatives are well known mesoionic compounds. Acute toxicity of these chemicals are generally low and their carcinogenicity has never been known or tested so far. One of the synthetic sydnones, benzylsydnone-4-acetamide, has a potent hepatocarcinogenic effect on rats as well as hepatotoxic effect, causing acute necrosis, subacute atrophy, and liver cirrhosis. The present studies showed that BSA was hepatotoxic and carcinogenic also in mice. The confirmation of liver injury and carcinogenesis caused by BSA in rats and mice warns the possibility of such effects of other sydnone derivatives.

Acute toxicity of BSA is low; the oral LD50 of BSA have been estimated as 5,100 and 4,450 mg/kg b.w. for male and female rats and 1,920 mg/kg b. w. in male and 1,440 mg/kg in female ICR mice. A single administration of lethal dose of the drug, however, does not cause liver cell damage demonstrable by histological examination or by biochemical examination of the serum in rats and mice as well. In the mice drug-induced liver damage differed remarkably by strains; DDD, C57BL/6, DBA/2, BALB/c, ICR, and C3H in the descending order (Table 1). F1 hybrids of the most susceptible strain of DDD and the most resistant strains of C3H/He or ICR showed intermediate susceptibility (Table 1).

Furthermore, in each strain of mice females were more severely affected than males (Table 1), in contrast to rats in which the male are more susceptible than the female. This seems a rare characteristic of BSA, because many chemicals affecting the liver shows more severe toxic and carcinogenic effect on male than female mice. The effect of castration and sex hormone administration suggests the role of hormone dependent metabolic processes of BSA; androgen may play a suppressive role and estrogen seemed to work as an activator. High susceptibility of DDD female is possibly due to high concentration of BSA (or its active metabolites) in the liver, as shown by whole body autoradiography (Fig. 3) and in the case of luteoskyrin. It may be due to deficiency in so-called enzymatic detoxification. The trait enhanced by estrogen and suppressed by androgen seems to be dominantly inherited and expressed in females, judged from the hybridization studies. This emphasizes the importance of selection of animals, or even strains and sex of animals, in toxicity and carcinogenicity tests.

The metabolism of BSA has been scarcely known. When administered to rats for 1 to 2 weeks, BSA inhibited mixed function oxidases such as aminopyrin demethylase and aniline hydroxylase (Ueno, unpublished data), but these inhibitions may be secondary to hepatic necrosis.
and/or degeneration in the centrilobular area\textsuperscript{1}. A chemical analysis for BSA following single administration showed rapid disappearance of BSA from the liver and, with large doses, most of the drug was excreted unchanged in the urine (Yamashita, unpublished data). But the whole body autoradiographic study using [\textsuperscript{14}C]-BSA demonstrated that absorption of BSA was rapid; after 15 min the radioactivity was found diffusely in the body (Fig. 3a). Radioactivity remained in the liver after 72 hrs whereas all other tissues were negative after 24 hrs, indicating rapid excretion (Fig. 3a and 3f). Accumulation of the radioactivity in the liver indicates that the toxin may remain as a conjugate form in view of the rapid excretion of BSA as itself. This clearly explains the slowly developing liver injury. However, analysis of the radioactive substance(s) in the liver has not been performed. In vitro hydrolyzed substances, benzylhydrazine in acidic and N-benzyl-N-nitrosoaspartate in basic conditions were not elaborated by enzymatic hydrolyzation of BSA in the liver (Yamashita, unpublished data). Slowly progressive effect of BSA on the liver is also apparent from the sequential s-GPT examination and DNA analysis during the 2 to 5 weeks of administration. Enlarged parenchymal cells with polyploid (and probably heteroploid) nuclei appeared in 2 weeks (Fig. 5). Then, after 3 to 5 weeks of administration, small hepatocytes and oval cells with 2c nuclei increased, while hyperploid nuclei decreased relatively, reflecting an increasing proliferation of cells with 2c nuclei (Fig. 5). In all cases the liver was selectively damaged leaving other organs histologically unaffected. At least a part of administered BSA may be accumulated and activated slowly in the liver to cause slowly progressive liver damage in terms of weeks of consecutive administration.

The results presented here showed that BSA accumulated in the liver of mice to cause selective liver injury (necrosis) with low but divided doses; female mice are more susceptible than males, in contrast to rats, to toxic as well as carcinogenic effects of BSA; and hepatocellular carcinoma was induced following chronic liver damage and appearance of resistant cells. These characteristics of BSA are unique and it may be used as a tool to analyze the mechanism of drug metabolism, injury and carcinogenesis in the liver.

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References


