Carcinogenicity of dimethylarsinic acid in p53 heterozygous knockout and wild-type C57BL/6J mice

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Abbreviations: DMA, dimethylarsinic acid; IC, ion chromatography; ICP–MS, inductively coupled plasma mass spectrometry; LA–PCR, long and accurate polymerase chain reaction; MMA, monomethylarsonic acid; SSCP, single strand conformation polymorphism; TMAO, trimethylarsine oxide.

There is abundant epidemiological evidence that arsenic is an environmental carcinogen related to human cancers of the skin, lung, liver and urinary bladder, in particular. Dimethylarsinic acid (DMA) has also been reported to act as a carcinogen/or a promoter in rat models. To elucidate molecular mechanisms, we conducted an 18 month carcinogenicity study of DMA in p53 heterozygous (+/−) knockout mice, which are susceptible to early spontaneous development of various types of tumors, and wild-type (+/+) C57BL/6J mice. Totals of 88–90 males, 7–8 weeks of age, were divided into three groups each administered 0, 50 or 200 p.p.m. DMA in their drinking water for 18 months. Mice that were found moribund or died before the end of the study were autopsied to evaluate the tumor induction levels, as well as those killed at the end. Both p53+/− knockout and wild-type mice demonstrated spontaneous tumor development, but lesions were more prevalent in the knockout case. Carcinogenic effect of DMA was evident by significant early induction of tumors in both treated p53+/− knockout and wild-type mice, significant increase of the tumor multiplicity in 200 p.p.m.-treated p53+/− knockout mice, and by significant increase in the incidence and multiplicity of tumors (malignant lymphomas) in the treated wild-type mice. By the end of 80 weeks, tumor induction, particularly malignant lymphomas and sarcomas, were similar in treated and control p53+/− knockout mice. No evidence for organ-tumor specificity of DMA was obtained. Molecular analysis using PCR–SSCP techniques revealed no p53 mutations in lymphomas from either p53+/− knockout or wild-type mice. In conclusion, DMA primarily exerted its carcinogenic effect on spontaneous development of tumors with both of the animal genotypes investigated here.

Introduction

Arsenicals are widespread environmental and occupational carcinogens, dimethylarsinic acid (cacadyle acid, DMA) being a major methylated metabolite of inorganic forms which is also used as a silvicide, a non-selective herbicide and a cotton foliator and for various pharmaceutical and medicinal purposes (1). Recently, it was shown that human consumption of drinking water from high-arsenic artesian wells might result in an increased incidence of tumors of the urinary bladder, skin and respiratory tract, as well as possibly tumors of other internal organs (2). In experimental animals, administration of DMA in drinking water exerts potent promoting effects on the kidney, liver, thyroid gland and urinary bladder of rats initiated with genotoxic chemical carcinogens (3). Wanibuchi et al. (4,5) have demonstrated that DMA causes dose-dependent enhancement of urinary bladder and liver carcinogenesis in F344 rats. In another experiment in our laboratory, DMA was found to promote skin carcinogenesis in keratin (K6)/ODC transgenic mice (6). The first unequivocal evidence that DMA might be a complete carcinogen in animals was provided by Wei et al. (7), that administration of DMA at concentrations of 0, 12.5, 50 and 200 p.p.m. in the drinking water to F344 rats for 2 years inducing urinary bladder cancers, with 50 p.p.m. as the lowest effective dose. In another study, administration of 40 or 100 p.p.m. DMA to rats in the diet for 2 years (8), caused an increased incidence of urinary bladder tumors, females being more sensitive than males.

In the last few years, substantial scientific progress has been made in clarifying possible mechanisms underlying arsenic toxicity and carcinogenicity. In mammals, inorganic arsenics are metabolized to mono- and dimethylated species by methyltransferases enzymes in reactions that require S-adenosyl-methionine as a methyl-donating factor (9). Diversity in such activity may thus be responsible for the wide variability in human and animal sensitivity to arsenic adverse effects. Many reports have suggested that methylated [DMA and monomethylarsonic acid (MMA)] forms of the arsenic show different toxic potential than non-methylated inorganic (arsinite and arsenate) forms (10). For instance, DMA and MMA are known to cause DNA damage after interaction with ascorbic acid (11), and MMA appears to be the main causative agent for skin cancer in humans (12). Furthermore, studies have suggested that DMA is a potent clastogenic agent, causing gene amplification (13). Arsenicals may also interfere with DNA methyltransferases, resulting in inactivation of tumor suppressor genes through DNA hypermethylation (14).

The biological roles of p53 in human and experimental animal cancers have attracted a great deal of attention (15) as the encoding gene is one of the most commonly mutated tumor suppressor genes so far described. Cells lacking functional p53 are defective for entry into apoptosis following some, but not all, stimuli (16). In cellular in vitro systems, p53 is important for cell-cycle arrest at the end of G2 caused by DNA damage and other cellular stresses, leading to the suggestion that p53 occupies a central position in signaling pathways between DNA damage and cellular consequences (17). Recently, one focus has been the use of p53 transgenic mice as model animals to shorten the time to tumor development.
A dramatic demonstration of the role of p53 gene in carcinogenesis comes from studies with transgenic ‘knockout’ mice. Mice homozygous for a null p53 allele develop normally but show an early onset of spontaneous tumors, such as malignant lymphomas, soft tissue sarcomas and osteosarcomas at very high rates (18). Additionally, mice with a single heterozygote null p53 allele, p53<sup>+/−</sup> knockout mouse, show a latent period for spontaneous tumor development between that of the null and wild-type mice (19). p53<sup>+/−</sup> knockout mice are also sensitive to radiation (20), or chemical induction of tumors by agents such as the skin carcinogen 7,12-dimethylbenz(a)anthracene (21).

Identification of genetic changes in chemically induced neoplasms may help the understanding of the molecular pathogenesis of cancer, and allow comparison of mechanisms between experimental rodent models and humans. As noted above, a p53<sup>−/−</sup> knockout mouse model has been proposed for rapid identification of carcinogenic responses of mutagenic chemicals (18). Ozaki et al. (22) concluded that the p53<sup>−/−</sup> knockout mice are distinctly more sensitive to urinary bladder carcinogenesis than wild-type mice as evidenced by elevated DNA synthesis during carcinogen administration and an increased tumor yield. Therefore, to further characterize the carcinogenicity of DMA, we observed the effects of this chemical in the heterozygous p53<sup>+/−</sup> knockout mouse and compared the data with findings for wild-type mice administered similar doses of DMA in drinking water for the same period.

### Materials and methods

#### Chemical

DMA was purchased from Wako Pure Chemical Industries (Osaka, Japan). Its purity was 100% with no detectable inorganic arsenic when subjected to ion chromatography (IC) or inductive coupled plasma mass spectrometry (ICP-MS).

#### Animals

Eighty-eight male 6–7-week-old p53<sup>−/−</sup> knockout (genetic background is C57BL/6J), and 90 male 6–7-week-old wild-type littermates were purchased from Taconic, Germantown, NY, and maintained four or five to a plastic cage with wood chips for bedding in a room at 22 ± 2°C and 45 ± 5% relative humidity, with a 12-h light-dark cycle. The animals were fed a basal pellet diet (CE2, Clea Japan, Tokyo, Japan) and were administrated water ad libitum.

**Experimental protocol**

After 1 week of laboratory observation, at 7–8 weeks of age, mice were divided into six groups of 29 or 30 in each group (see Table II). Groups 1–3 (the p53<sup>−/−</sup> knockout mice) and 4–6 (the wild-type) were given DMA in the drinking water at 0, 50 and 200 p.p.m., respectively, and daily monitored until the final killing after 80 weeks. Body weights were measured weekly, and food consumption and water intake were calculated biweekly during the course of the experiment.

#### Histopathological examination of tumors

Most tumors were detected macroscopically by necropsy at the final killing. Animals becoming moribund during the course of the study, or when tumors appeared externally, were also killed for autopsy and microscopic examinations. The numbers of tumors were calculated and the latent periods calculated. On death, each tumor was divided into two halves; one was immersed in 10% phosphate-buffered formalin (pH 7.2) for histopathological examination, and the other was kept frozen at −80°C until applied for molecular analysis. Each observed tumor was subjected to histopathologic examination to determine its tissue of origin. After fixation in formalin for at least 48 h, parts of the tumors were prepared for routine histopathological examination of hematoxylin and eosin stained sections. A multi-organ killing protocol was followed, all body organs being excised, washed in saline, fixed and routinely prepared for histological examination. Because DMA was earlier found to be carcinogenic for the rat urinary bladder (7), particular attention was given to this organ regarding effects on the urothelium and urine characteristics.

### Table I. p53 primers used for the PCR–SSCP analysis

<table>
<thead>
<tr>
<th>PCR–SSCP</th>
<th>Exon 5</th>
<th>5′-TCTTTCCTCAATGACTCTCCTC-3′ (sense)</th>
<th>5′-GGAGGCCTTGAAGGCTTAC-3′ (antisense)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 6</td>
<td>5′-GCTTCTGACATTGTCG-3′ (sense)</td>
<td>5′-CAACCTGTCTCAGAACGAC-3′ (antisense)</td>
<td></td>
</tr>
<tr>
<td>Exon 7</td>
<td>5′-TCATTTGGATCCTGTGTCTT-3′ (sense)</td>
<td>5′-GAGCGTTAACCTAACCACCA-3′ (antisense)</td>
<td></td>
</tr>
<tr>
<td>Exon 8</td>
<td>5′-AATGAAGCTCAACAGGTCCTC-3′ (sense)</td>
<td>5′-TGAAGCTCAACAGGTCCTC-3′ (antisense)</td>
<td></td>
</tr>
</tbody>
</table>

#### Evaluation of urinary levels of electrolytes and biochemistry

Urine was collected by forced urination in the morning 2 weeks before final death (after 78 weeks) and the pH was immediately measured with a pH meter (Horiba, model F-15, Tokyo, Japan). Urinary samples were also stored at −80°C until assessed for sodium, chloride, potassium and calcium ions (Hitachi-710 Electrolyte Analyzer, Tokyo, Japan), and for levels of total protein and creatinine (Hitachi 736).

#### Concentration and speciation of arsenics in the urine after 78 weeks

A combination of IC, for separation (model IC 7000, Yokogawa Analytical Systems, Tokyo, Japan) and ICP-MS (model HP5000, DE) for element-selective detection, was used to analyze the arsenic species in urine samples. The levels of DMA, arsenobetaine (AsBe), arsenites (AsIII, AsV), MMA and trimethylarsine oxide (TMAO) were estimated after separation by IC under the conditions described previously (23).

#### Isolation of DNA

Twelve frozen tumors from p53<sup>−/−</sup> knockout mice, diagnosed as malignant lymphomas, 10 from enlarged lymph nodes found in the peritoneal cavity and two from spleens, were prepared for isolation of DNA. Another 12 frozen tumors from wild-type mice diagnosed as malignant lymphomas from enlarged lymph nodes found in the peritoneal cavity were also similarly treated. Homogenized cells from −0.5 cm<sup>3</sup> of each tumor were placed in sterilized disposable 1.5 ml tubes along with 150 µl of TE buffer (pH 9.0) with 0.25% NP-40 and 100 µl/ml protease K, and incubated at 55°C overnight. The reaction mixtures afterwards were purified with phenol–chloroform and the DNA pellets were precipitated with ethanol. The final concentration of DNA was adjusted to 50 ng/µl for PCR–SSCP analysis using a spectrophotometer (Ultraspex 3000, UV/Vis. Spectrophotometer; Pharmacia Biotech, Tokyo, Japan).

#### LA–PCR

To amplify the p53 exons 5–8 of normal alleles of the knockout mice, LA–PCR was carried out using a Takara LA-PCR kit (Takara SHUZO Co., Shiga, Japan). The pair of oligonucleotide primers used for LA–PCR were the sense strand in exon 5 and the antisense strand in exon 8 (Table I). After denaturation for 1 min at 94°C, 30 cycles of amplification each for 4 min at 68°C (annealing and extension), and denaturation for 20 s at 98°C were performed. After amplification, the mixtures were purified to obtain the target gene by low-melting temperature agarose gel electrophoresis, followed by phenol–chloroform extraction, and then ethanol precipitation. PCR products amplified from genomic DNA were subcloned using PCR-Script (SK+) cloning kit (Stratagene, La Jolla, CA), and five or more recombinant colonies were picked up and amplified in 3 ml LB culture medium. Double-strand plasmid DNA was extracted using Plasmid Mini Kit (QIAGEN, Chatsworth, CA) and was sequenced by a DNA sequencing system (Model 373A, Applied Biosystems, CA) using designed primers, T3 or T7 promoter primers, to confirm to be generated from the normal allele of the knockout mice. These plasmid DNAs were subjected to PCR–SSCP analysis as templates.

#### PCR–SSCP analysis

PCR–SSCP analysis was carried out to screen for mutations in exons 5–8 of the mouse p53 gene with four pairs of oligonucleotide primers designed for PCR. The primer sequences are presented in Table I. To screen the tumor samples for p53 gene mutations, PCR–SSCP analysis was performed using non-radioisotopic SSCP analysis as described previously (24). The primers were labeled at the 5′ terminus with rhodamine. Hot-start PCR was carried out in a 5 µl reaction volume using AmpliTaq Gold (Perkin-Elmer Cetus Instruments, Norwalk, CT) under the following conditions: initial preheating at 94°C for 9 min to achieve enzymatic activity followed by 38 cycles of denaturing (94°C) for 35 s, annealing (55°C) for 30 s and extension (72°C) for 60 s using GeneAmp PCR system 9700 (Perkin-Elmer Instruments). Five microliters of each PCR product were mixed with 145 µl of stop solution.
Carcinogenicity of DMA in p53<sup>+</sup>/– heterozygous knockout mouse

Table II. Average final body and relative organ weights of p53<sup>+</sup>/– knockout and wild-type C57BL/6J mice administered DMA in drinking water

<table>
<thead>
<tr>
<th>Group</th>
<th>DMA treatment (p.p.m.)</th>
<th>Initial no. of mice</th>
<th>No. of mice examined&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Final body weight</th>
<th>Relative liver wt (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Relative kidney wt (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Relative spleen wt (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>29</td>
<td>25</td>
<td>40.6 ± 4.9</td>
<td>7.6 ± 0.01</td>
<td>1.8 ± 0.004</td>
<td>0.5 ± 0.003</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>29</td>
<td>25</td>
<td>43.4 ± 6.1</td>
<td>7.5 ± 0.02</td>
<td>1.7 ± 0.003</td>
<td>0.6 ± 0.005</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>30</td>
<td>26</td>
<td>39.7 ± 5.5</td>
<td>7.0 ± 0.02</td>
<td>1.7 ± 0.003</td>
<td>0.4 ± 0.003</td>
</tr>
<tr>
<td>Wild</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>30</td>
<td>22</td>
<td>42.0 ± 4.4</td>
<td>5.0 ± 0.001</td>
<td>1.6 ± 0.004</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>30</td>
<td>22</td>
<td>54.0 ± 7.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.3 ± 0.006</td>
<td>1.2 ± 0.002</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>30</td>
<td>23</td>
<td>40.0 ± 4.3</td>
<td>5.0 ± 0.01</td>
<td>1.4 ± 0.005</td>
<td>0.3 ± 0.002</td>
</tr>
</tbody>
</table>

<sup>a</sup>No. of mice examined either when found moribund or at death. Data for body or organ weights of animals found dead at any time of the experiment were not included in this table.

<sup>b</sup>Relative organ weight = organ wt/body weight (%).

<sup>c</sup>Significantly different from other groups at P < 0.05.

Fig. 1. Kaplan–Meier survival curves.

(95% formamide, 20 mM EDTA), denatured for 3 min at 95°C and immediately put on ice for 10 min then loaded (2 µl/lane) onto 0.5× MDE gels either with or without glycerol. Gels were run at 40 W for 3–5 h or at 4 W for 16–18 h at room temperature. After electrophoresis, gels were visualized using an FMBIO II Multi-View fluorescent image analyzer (Takara SHUZO Co.).

The patterns of single-strand DNA bands were compared with those from normal control mouse DNA (from liver or kidney) to check for the presence of any mobility-shifted bands. Small areas of gels corresponding to the position of mobility shift bands were cut out, immersed in 70 µl distilled H2O and heated at 90°C for 5 min to elute the DNA from the gels. Ten microliters of each extracted DNA solution were amplified by PCR using the same primers used in PCR–SSCP, subcloned and sequenced as described above to detect mutations. Each PCR–SSCP reaction was repeated at least twice for confirmation.

Statistical analysis

Kaplan–Meier analysis was conducted to determine survival curves for p53<sup>+</sup>/– knockout and wild-type mice. The two-tailed Student’s t-test was employed to assess differences in mean values, and the χ<sup>2</sup> test for comparison of incidences of tumors. Logrank (Mantel-Cox) analysis was used to compare tumor latency (StatView ver. 4 E, Abacus Concepts, CA).

Results

General observations

DMA treatment did not cause any signs of toxicity or death in the early period of the experiment. Kaplan–Meier survival curves (Figure 1) showed survival rates to not differ significantly between DMA-treated and non-treated p53<sup>+</sup>/– knockout mice, or between DMA-treated wild-type mice and controls. The first mice died after 11 weeks in the 50 and 0 p.p.m.-treated groups for unknown reason, probably due to fighting. Also, one animal died in each of the three wild-type groups between weeks 18 and 20, also with causes unknown. Table II summarizes data for final body weights and relative organ weights. Wild-type mice treated with 50 p.p.m. DMA had significantly higher final body weights when compared with all other groups. Other mice treated with DMA had relatively higher body weights than non-treated mice during the course of the experiment. Relative liver, kidney and spleen weights did not differ among the groups. The average water intake was higher in p53<sup>+</sup>/– knockout mice treated with 50 p.p.m. DMA (P < 0.01) and the total DMA intake in general was related to the DMA dose, being 491.2 and 136.8 mg/mouse in 200 and 50 p.p.m. DMA-treated p53<sup>+</sup>/– knockout mice, and 454.2 and 125.4 mg/mouse in 200 and 50 p.p.m. DMA-treated wild-type mice, respectively. Average food intake was comparable among the groups (data not shown).

Tumor incidences and latency

The p53<sup>+</sup>/– knockout mice appeared more susceptible to the induction of different types of tumors than the wild-type mice. In p53<sup>+</sup>/– knockout mice, there was thus an increase in the incidence of tumors with 50 and 200 p.p.m.-treatment (62.1 and 63.3%, respectively) as compared with the non-treated controls (48.3%), albeit without statistical significance. Tumor multiplicity was significantly increased in 200 p.p.m. DMA (P < 0.05) and multiplicity of tumors with 50 and 200 p.p.m.-treatment (62.1 and 63.3%, respectively) as compared with the non-treated controls (48.3%), albeit without statistical significance. Tumor multiplicity was significantly increased in 200 p.p.m. DMA-treated p53<sup>+</sup>/– knockout mice (P < 0.02) as compared with non-treated controls. In the wild-type mice, DMA treatment significantly increased the incidence (P < 0.05) and multiplicity (P < 0.02) of tumors as compared with its non-treated controls (Table III). Figure 2 shows induction times for tumors in the experiment (tumor latency). Tumor latency curves in DMA-treated p53<sup>+</sup>/– knockout and wild-type mice showed a significant shift towards early induction (P < 0.03) as compared with their non-treated controls.
Table III. Incidence (%) and multiplicity of tumors

<table>
<thead>
<tr>
<th>DMA treatment</th>
<th>Heterozygotes</th>
<th>Wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 p.p.m.</td>
<td>50 p.p.m.</td>
</tr>
<tr>
<td>No. of tumor bearing mice (incidence %)(^a)</td>
<td>14 (48.3)</td>
<td>18 (62.1%)</td>
</tr>
<tr>
<td>No. of tumors/mouse in test group</td>
<td>23 (0.8)</td>
<td>33 (1.1)</td>
</tr>
<tr>
<td>No. of tumors/tumor bearing mouse</td>
<td>23 (1.6)</td>
<td>33 (1.8)</td>
</tr>
</tbody>
</table>

\(^a\)Numbers outside parentheses are for total no. of mice.
\(^b\)Significant versus the controls at \(P < 0.05\).
\(^c\)Significant versus the control at \(P < 0.02\).

Table IV. Distribution of tumors in different organs and tissues

<table>
<thead>
<tr>
<th>Site and type of tumor(^d)</th>
<th>DMA treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heterozygotes</td>
</tr>
<tr>
<td></td>
<td>0 p.p.m.</td>
</tr>
<tr>
<td>No. of mice</td>
<td>29</td>
</tr>
<tr>
<td>All sites</td>
<td></td>
</tr>
<tr>
<td>M.Lymphoma/Leukemia</td>
<td>8 (28)(^b)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Subcutis</td>
<td></td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>0</td>
</tr>
<tr>
<td>Lipoma</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Bones</td>
<td></td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>Adenoma</td>
<td>0</td>
</tr>
<tr>
<td>Metastatic tumors</td>
<td>0</td>
</tr>
<tr>
<td>Thyroid</td>
<td></td>
</tr>
<tr>
<td>Follicular cell carcinoma</td>
<td>0</td>
</tr>
<tr>
<td>Follicular cell adenoma</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Hemangiomat</td>
<td>0</td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td></td>
</tr>
<tr>
<td>Intestinal tubular adenocarcinoma</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^d\)Only organs with a neoplastic lesions are listed.
\(^b\)Number in parentheses represents % incidence.

Tumor spectrum and histopathology

Table IV shows the incidence and general distribution of tumors in treated and non-treated mice of both genotypes. Compilation of the results revealed differences in tumor spectrum between the p53\(^{+/-}\) knockout versus wild-type mice. The tumors induced in the p53\(^{+/-}\) knockout mice were mainly malignant lymphomas of B- and T-cell origin, soft tissue sarcomas and osteosarcomas. Lower incidences of other types of tumors such as hepatocellular carcinomas, thyroid follicular adenomas and one intestinal tubular adenocarcinoma. No soft tissue sarcomas or osteosarcomas were detected in wild-type mice. Extensive examination of the lower urinary tract in all treated and non-treated mice revealed no macroscopic or histological changes in the DMA-treated groups. Also, no urinary calculi were found formed in any DMA-treated mouse.

Urinary concentration of arsenic compounds

The urinary levels of arsenical compounds are given in Table V, dose-dependent increase being observed for all metabolites except AsBe. Non-changed DMA and TMAO were the major arsenic metabolites in the urine samples and lowest levels detected were for AsV and MMA. There was no trace of
Wild type

- Urine pH values did not differ among the p53 knockout and wild-type mice.
- Sodium and chloride levels were significantly increased in DMA-treated wild-type mice as compared with the 50 ppm-treated wild-type mice.
- DMA treatment significantly increased sodium and chloride levels at 78 weeks of the experiment.
- No significant differences were found in creatinine, total protein, or calcium levels between the groups.

### Urine chemistry

Figure 3 shows the data for urine total protein, creatinine, and ion concentrations of sodium, chloride, potassium, and calcium at 78 weeks of the experiment. No significant differences were observed in creatinine, total protein, or calcium levels between groups. However, DMA-treated wild-type mice (Groups 5 and 6) showed significant increase in sodium and chloride levels. Potassium levels of 50 p.p.m. DMA-treated knockout mice were significantly decreased below control levels (P < 0.05). Urine pH values did not differ among the p53 knockout and wild-type mice groups, but were significantly higher in the 200 p.p.m.-treated wild-type mice as compared with the 50 p.p.m.-treated group or the controls (Table V).

### Discussion

The present study was designed to provide additional information on the mechanisms underlying carcinogenicity of DMA. Long-term administration exerted carcinogenicity in both mice genotypes as indicated by: (i) earlier induction of tumors in the p53 knockout versus wild-type mice; (ii) multiple induction of tumors in more organs and tissue (tumor multiplicity) in DMA-treated p53 knockout mice than non-treated controls; (iii) significant induction of tumors in the treated wild-type mice. The non-specific organ carcinogenic effects of DMA as well as the no-p53 mutation effect in the p53 knockout versus wild-type mice are indicative that either a non-genotoxic carcinogenicity pathway for DMA effects occurs in p53 knockout and wild-type mice or that pathways other than p53 might account for the present findings.

A number of epidemiological and experimental studies have indicated that different chemical agents may act at different stages in the carcinogenic process, and several mechanisms may be involved (25). The IARC monographs focus on evaluating evidence of carcinogenicity of chemicals to experimental animals at any stage in the carcinogenic process, independently of the underlying mechanisms, although information on the latter may be used as an aide (26).
Practically, a positive relationship between DMA exposure and carcinogenicity was detected in the present experiment. Thus, the data of DMA here meet the IARC criteria established between 1976 and 1996 (27) for evaluation of the carcinogenic risk of different chemicals to humans. IARC concluded that the term ‘carcinogen’ should be used when the element is capable of increasing the incidence of malignant neoplasms; the induction of benign neoplasms may also in some circumstances contribute to a judgement of carcinogenic (25).

Although the mode of carcinogenic action of arsenic has been reviewed, the exact details remain (29). Accumulating evidence indicates that arsenic acts to a great extent at the level of tumor promotion by modulation of the signal transduction pathways that are responsible for cell growth or apoptosis (29). In our laboratory (39) we found DMA carcino- 

promotion effects at the molecular level.

The present PCR–SSCP analysis of DNA extracted from lymphomas found in p53+/− knockout and wild-type mice revealed a lack of mutational activity of DMA in the p53 gene (exons 5–8) in both cases, in line with the findings of Sukata et al. (35). Most genetic studies of arsenic have largely yielded no gene mutations but positive results for chromosomal aberrations (36), pointing to the existence of non-genotoxic pathway responsible for arsenic-induced cancer either through hypomethylation of DNA (13) or inhibition of DNA ligation (37). However, there has been one report (38) that arsenic is mutagenic to endogenous genes in mammalian cells, inducing mostly large multilocus deletions that are mediated by reactive oxygen species. In our laboratory (39) we found DMA carci-no- 

in the rat urinary bladder to feature only a low rate of H-ras mutations and no mutations in p53, K-ras or β-catenin genes. Furthermore, the study pointed to a contribution of mechanisms involving oxidative stress and/or alterations in cell-cycle regulatory proteins, p27kip1, and cyclin D1 and therefore cellular proliferation.

A number of reports have appeared in the p53+/− knockout mouse literature regarding spontaneous tumors or susceptibility to chemical carcinogenesis. Kemp et al. (20) found that loss of functional p53 has no effect on initiation but greatly enhances malignant promotion and progression in skin carcinogenesis. In another study (21), reduction in the p53 gene dose was not related to the increase of initiation but rather to enhanced promotion and malignant progression of chemically induced skin tumors. Tannet et al. (40) reported p53+/− knockout mice to be more susceptible to genotoxic than to non-genotoxic carcinogens. Therefore, the data support the conclusion that p53 genetic alterations are relatively infrequent in both chemically induced and spontaneous animal tumors in these mice (41–43).

Arsenic may either decrease p53 expression, or induce p53 phosphorylation and accumulation or, on the other hand, show completely no effect on p53 (34). In an early report a low dose of arsenite for 24 h increased p53 protein expression in normal non-mutated Hela cells (44). As2O3 (arsenic trioxide) up-regulate p53 expressions in the human B lymphoma cell line (45). In another study, a very low dose of arsenite induced DNA damage accompanied by phosphorylation and accumulation of p53 protein along with increase in p53 target proteins, including p21 and the human homolog of murine double minute-2 (MDM-2) (46). In contrast, low levels of arsenic acid (47) had no effect on p53-dependent transcription activity in p53 promoter-transfected JB6 cells over a wide range (12.5–200 mM). In human tumors, Boonchais et al. (48) found low levels of p53 expression in arsenic-related basal cell carcinomas and Castern et al. (49) could detect no p53 mutations in either benign or malignant skin tumors. However, in contrast, another study showed a high rate of p53 mutations (28.6–55.5%) in arsenic-related skin cancers (50). The present data for non- or relatively minor involvement of p53 in DMA carcinogenesis, and the available findings for both p53-dependent and independent pathways, indicate that the relationship of p53 and arsenic in carcinogenesis is probably cell and tissue type specific and may involve other molecular mechanisms. In support to it, is of particular interest to understand whether the reduction in p53 dose per se is sufficient to predispose to arsenic malignancy. Our recent work (unpublished data), shows that N,N-dibutylnitrosamine, a genotoxic carcinogen, when administered to p53+/− knockout and wild-type mice, induced high frequency of p53 mutations in the residual allele of p53+/− knockout mice, accompanied with significant reduction in the gene expression levels of the cyclin-dependent kinase inhibitor, p21WAF1/CIP1 in the esophagus and urinary bladder, all functioned together to predispose these tissues to a highly and accelerated rates of malignany as compared with the wild-type mice. Therefore, the influence of DMA and other organic arsenics on the p53 and p21WAF1/CIP1 gene expression levels per se within various animals and human tumors warrants further clarification. The carcinogenic action of arsenic could involve many molecular mechanisms (28,34) that are dependent on the length of arsenic exposure, dose, and/or chemical species (i.e. DMA, MMA, arsenate or arsenite). Bode and Dong (34) concluded that the possibility exists that the key to arsenics action is the extent to which it disrupts the normal control of apoptosis, by influencing signaling pathways, including the mitogen-activated protein kinases that can lead to activation of AP-1 or NFκB.

Taking into account the reports of human arsenic-related urinary bladder tumors (51) and rat-induced bladder carcinogenesis (7), we extensively examined the histology of the urinary bladders of DMA-treated mice in both genotypes. However, no calculi or histological lesions were found. In a previous study (52) we found an elevated sensitivity to urinary bladder carcinogenesis in p53+/− knockout mice using genotoxic carcinogen. Popovicova et al. (53) showed that when p53+/− knockout mice were exposed for 26 weeks to various combinations of arsenite, a choline-deficient diet, and p-cresidine, a known bladder genotoxic carcinogen, treatment with 50 p.p.m. arsenite in drinking water alone did not cause mouse urinary bladder tumors. In p-cresidine-exposed mice, co-exposure to either arsenite or a choline-deficient diet enhanced the extent of bladder hyperplasia and the incidence of bladder carcinomas. In another experiment (54), mice given 10 mg/l sodium arsenite in drinking water for 26 weeks had
a significant increase in yield of tumors after exposure to ultraviolet radiation (UVR) compared with mice given UVR alone, whereas no tumors appeared in mice given arsenite alone. Thus, although arsenite did not appear to be carcinogenic alone, with concomitant exposure to p-cresidine, or UVR, it acted as a co-carcinogen in the two experiments.

There are many different arsenic metabolites in the environment, but DMA is the major metabolite in most mammals. In a previous study with NBR rats (30), which are resistant to induction to bladder carcinogenesis, promoting activity of DMA on urinary bladder carcinogenesis was associated with a high urinary concentration of DMA and other methylated arsenic metabolites. In the present study, ingested DMA proved to be excreted mostly unchanged in the urine, some being methylated to TMAO. Both are known to induce mitotic arrest and exert effects on cell division and mitosis in vitro (55), causing tetraploidy in Chinese hamster V79 cells. The authors postulated that the cytotoxicity of different arsenics in vitro vary in the order of AsIII > AsV > DMA > MMA > TMAO. However, it was found recently that methylated trivalent arsenic compounds, methyloxoarsine (MMAIII) and iododimethylarsine (DMAIII), are more potent than iAs(III) in terms of induction of DNA damage (56). Monomethylarsonic acid (MMA) is reduced to MMA(III) before further methylation by methyltransferases. The fact that a large amount of unidentified arsenics (peaks 1 and 2) was excreted in the urine might suggest that these play a particular role. Further study is needed to elucidate this possibility. Also, the significance of increased body weights and water intake in groups of mice treated with DMA deserve further investigation because a similar observation was noticed in our previous study on F344 rats treated with DMA (7,39).

In conclusion, in the present study administration of DMA in drinking water for 18 months, was carcinogenic as indicated by the significant increase in the total numbers of tumors in wild-type mice and significant earlier induction of tumors in more organs and tissues of both treated p53+/− knockout and wild-type mice. The lack of organ-specificity or mutations in either the residual allele or wild-type alleles in both genotypes are all indicative of non-genotoxic carcinogenicity of DMA, at least in p53+/− knockout and wild-type mice.

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References

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