Original

Concordance between Results of Medium-term Liver Carcinogenesis Bioassays and Long-term Findings for Carcinogenic 2-Nitropropane and Non-carcinogenic 1-Nitropropane in F344 Rats

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Abstract: This study was conducted to determine the concordance of results for a pair of structural isomers, 2-nitropropane (2-NP) and 1-nitropropane (1-NP), using the rat medium-term liver carcinogenesis bioassay (Ito test) and previously published long-term carcinogenicity tests. Male F344 rats were given a single intraperitoneal injection of DEN (200 mg/kg b.w.) to initiate hepatocarcinogenesis. After 2 weeks, they received per os 0, 0.8, 4 or 20 mg/kg/day of 2-NP or 1-NP six times a week and were subjected to two-thirds partial hepatectomy at week 3. Non-initiated groups receiving 0 or 20 mg/kg/day were also included. The animals were sacrificed for quantitative analysis of GST-P-positive foci at week 8. With the highest dose of 2-NP, significantly increased numbers and areas of GST-P-positive foci were demonstrated as compared with the respective control but were not noted with 1-NP. In the non-DEN-initiated groups, many small GST-P-positive foci of less than 0.2 mm in diameter were also induced in the rats treated with 2-NP at 20 mg/kg/day but were lacking with 1-NP. These results strongly support that 2-NP is a complete hepatocarcinogen with a potent initiation activity, whereas 1-NP is not. (DOI: 10.1293/tox.24.207; J Toxicol Pathol 2011; 24: 207–213)

Key words: the rat medium-term liver carcinogenesis bioassay, Ito test, 1-nitropropane, 2-nitropropane

Introduction

2-Nitropropane (2-NP) was once widely used as a chemical intermediate and as a solvent component of paints, inks and varnishes[1,2]. It has also been detected in cigarette smoke in significant quantities[3]. Results of *in vivo* long-term bioassays in rats given 2-NP via gavage or inhalation in fact showed the compound to be a potent liver carcinogen[4,5], and the overall evaluation is category 2B in the IARC Monographs from positive animal data[2]. In contrast, 1-nitropropane (1-NP), a structural isomer utilized as a propellant fuel, gasoline additive and in chemical syntheses[1], was not found to be a hepatocarcinogen in a series of experiments[5–7]. Furthermore, while 2-NP proved to be mutagenic in a variety of short-term mutagenesis assays, including the Ames/Salmonella[8–10], *in vitro* sister chromatid exchange (SCE) and chromosome aberration[11], V79/HGPRT forward mutation and *in vitro* and *in vivo* unscheduled DNA synthesis (UDS) assays[12,13], positive data for 1-NP are limited to *in vitro* V79/HGRPT cells[12]. Neither was found to be mutagenic in micronucleus tests with polychromatic erythrocytes[14,15]. 2-NP does not appear to resemble any of the known classes of chemical carcinogens. Regarding the mechanisms of its carcinogenicity, 2-NP causes oxidative DNA and RNA damage in the rat liver resulting from intracellular generation of reactive forms of oxygen and/or 8-hydroxyguanine and 8-hydroxy-2'-deoxyguanosine[16,17]. Sodum *et al.* proposed from *in vivo* and *in vitro* experimental evidence that activation to an aminating species by rat liver aryl sulfotransferase is involved[18,19]. In addition, it was suggested that 2-NP

and its nitronate, an NO species, may mediate or contribute to genotoxicity[20]. A positive association between increased cell proliferation as assessed by incorporation of bromo-deoxyuridine and hepatocarcinogenesis has been reported for 2-NP, but not 1-NP[21]. Preneoplastic lesions, γ-glutamyl transpeptidase (γ-GT) and glutathione S-transferase placental form (GST-P)-positive foci, were also produced in Wistar rats injected intraperitoneally with 2-NP in the initiation stage[22].

The structures of 1-NP and 2-NP differ by only a little, but they differ greatly in their carcinogenic activity in the liver. In the present experiment, we investigated whether the two isomers promote development of GST-P-positive foci, as end-point lesions, in a medium-term liver carcinogenesis bioassay[23–25] to determine their sensitivity and specificity for distinguishing carcinogenic from non-carcinogenic chemicals[25–31].

Materials and Methods

All experimental procedures were performed in accordance with the in-house guidelines for the Care and Use of Laboratory Animals at DIMS Institute of Medical Science, Inc.

Test chemicals and initiator

2-nitropropane (2-NP) and 1-nitropropane (1-NP) were purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Diethylnitrosamine (DEN) was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan and used as an initiator of liver carcinogenesis. Regarding the reason for the determined dose level of test chemicals, a daily oral dose of 20 mg/kg/day, which did not show hepatocellular injury and increase in cell proliferation in a 2-week short-term oral administration study of 2-NP[21], was selected as the highest dose level in the present experiment. The lower levels were set at 4 and 0.8 mg/kg/day using a proportional factor of 5. The 1-NP dosage was the same as that for 2-NP to enable comparison.

Animals and maintenance

Male F344/DuCrj and F344/DuCrlCrlj rats, 5 weeks of age, were purchased from Charles River Laboratories Japan, Inc., Atsugi, Japan, and housed two or three to a polycarbonate cage with hardwood Beta chips (Northeastern Products Co., Warrensburg, NY, USA) for bedding. The animals were supplied with food (Oriental MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*, and the room temperature and relative humidity were controlled at 20–24 °C and 48–59%, respectively. Fluorescent lighting provided a 12-hr light/dark cycle. After a one-week quarantine and acclimation period, during which body weight and health conditions were monitored, a total of 159 rats were used for two experiments at 6 weeks of age.

Experimental procedure and autopsy

In experiment 1, from a total 78 male F344/DuCrj rats, 72 were allocated to 6 groups (15 rats each for groups 1 to 4 and 6 rats each for groups 5 and 6) using a computerized stratified body weight technique. At 6 weeks of age, animals of groups 1 to 4 were initially given a single intraperitoneal injection of DEN at a dose of 200 mg/kg b.w. dissolved in saline to initiate hepatocarcinogenesis[32]. Rats of groups 5 and 6 received the saline vehicle instead. After 2 weeks, animals of groups 1 to 4 were given 0, 0.8, 4 or 20 mg/kg/day per os of 2-NP, dissolved in corn oil, while non-DEN-initiated groups 5 and 6 received 0 and 20 mg/kg/day of 2-NP, respectively, for 6 weeks 6 times per week, with no dosing on Sunday. All animals were subjected to two-thirds partial hepatectomy (PH) at the end of week 3. The animals were observed daily for abnormalities, and body weights and food consumption were measured once a week.

In experiment 2, from a total 97 male F344/DuCrlCrlj rats, 87 were allocated to 7 groups (15 rats each for groups 1 to 5 and 6 rats each for groups 6 and 7) using the same technique as detailed above. At 6 weeks of age, animals of groups 1 to 4 were used under the same experimental regime, given 0, 0.8, 4 or 20 mg/kg/day of 1-NP dissolved in corn oil in place of 2-NP as the test compound, with an additional group of 15 rats receiving 2-NP at 20 mg/kg/day as a positive control. Non-DEN-initiated groups 6 and 7 received 0 and 20 mg/kg/day of 1-NP, respectively. Two-thirds PH was also conducted at the end of week 3. The animals were observed daily for abnormalities, and body weights and food consumption were measured once a week. Surviving rats in each group were sacrificed at the end of experimental week 8. At sacrifice in both experiments, livers were immediately excised and weighed to allow calculation of the liver-to-body weight ratio. A total of three 4 to 5 mm-thick slices from the cranial and caudal parts of the right lateral lobes, and the cranial part of the caudate lobe were cut with a razor blade and fixed in 10% buffered formalin for immunohistochemical demonstration of glutathione S-transferase placental form (GST-P)-positive foci.

Immunohistochemical staining and measurement of GST-P-positive foci

The avidin-biotin-peroxidase complex (ABC) method was used to stain GST-P-positive foci. After deparaffinization, liver sections were treated sequentially with normal goat serum, anti-rabbit GST-P antibody (Medical Biological Laboratories Co., Ltd., Nagoya, Japan; 1:2000) and biotin-labeled goat anti-rabbit IgG (1:200) for 1 hr using an Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The sites of peroxidase binding were visualized with diaminobenzidine tetrahydrochloride, and the nuclei were counterstained with hematoxylin. All GST-P-positive foci larger than 0.2 mm in diameter (the lowest limit for reliable evaluation) were measured using a color video image processor (IPAP-WIN, Sumika Technos, Osaka, Japan), and the numbers and areas of foci/square centimeter (cm^2) of liver sections were calculated.

Table 1. Final Body and Liver Weights of Male F344/DuCrj Rats Initiated with or without DEN Followed by 2-Nitropropane (2-NP) Administration and Partial Hepatectomy in Experiment 1

Group	Treatment		Effective No. of rats	Final body weight (g)[a]	Liver weight [a]	
	DEN	2-NP (mg/kg/day)			Absolute (g)	Relative (g/100 g body weight)
1	+	0	15	240.5 ± 9.8	5.63 ± 0.35	2.34 ± 0.07
2	+	0.8	13	244.5 ± 13.0	5.93 ± 0.36*	2.42 ± 0.07**
3	+	4.0	15	237.1 ± 12.2	5.61 ± 0.43	2.36 ± 0.08
4	+	20.0	15	239.7 ± 8.5	6.31 ± 0.36**	2.63 ± 0.10**
5	−	0	6	243.0 ± 14.7	5.85 ± 0.31	2.41 ± 0.10
6	−	20.0	6	253.0 ± 11.0	6.68 ± 0.34##	2.64 ± 0.07##

*, ** Significantly different from the control group (group 1) at *P*<0.05 and 0.01, respectively. ## Significantly different from the control group (group 5) at *P*<0.01. [a] Values indicate the mean ± SD.

Table 2. Final Body and Liver Weights of Male F344/DuCrlCrlj Rats Initiated with or without DEN Followed by 1-Nitropropane (1-NP) Administration and Partial Hepatectomy in Experiment 2

Group	Treatment		Effective No. of rats	Final body weight (g)[b]	Liver weight [b]	
	DEN	1-NP (mg/kg/day)			Absolute (g)	Relative (g/100 g body weight)
1	+	0	14	233.2 ± 12.9	5.58 ± 0.44	2.39 ± 0.09
2	+	0.8	14	234.5 ± 13.3	5.61 ± 0.43	2.39 ± 0.07
3	+	4.0	15	233.5 ± 8.8	5.68 ± 0.32	2.43 ± 0.11
4	+	20.0	14	221.2 ± 9.0**	5.55 ± 0.34	2.51 ± 0.14**
5[a]	+	20.0	15	231.7 ± 12.6	6.31 ± 0.48**	2.72 ± 0.10**
6	−	0	6	245.2 ± 16.5	5.91 ± 0.50	2.41 ± 0.13
7	−	20.0	6	243.0 ± 13.8	6.15 ± 0.61	2.52 ± 0.12

** Significantly different from the control group (group 1) at *P*<0.01. [a] 2-Nitropropane was used as a positive substance. [b] Values indicate the mean ± SD.

Small GST-P-positive foci of less than 0.2 mm in diameter were found in the non-DEN-initiated rats. The sizes of these GST-P-positive foci were evaluated under a microscope as follows: single cell, 2 to 4 cells, 5 to 10 cells or 11 cells or more.

Statistical analysis

The significance of differences for each parameter (excluding general conditions and food consumption) was analyzed and evaluated at *P*<0.05 or 0.01. Statistical comparisons between group 1 and groups 2 to 4 for numerical data were conducted using the Bartlett's test (evaluated at *P*<0.05). If homogeneous, the data were analyzed with the Dunnett's multiple comparison test (one sided), and if not, they were analyzed with the Steel's test (one sided)[33,34]. Statistical comparisons between groups 5 and 6 (experiment 1) and between groups 1 and 5 and groups 6 and 7 (experiment 2) for numerical data were conducted using the *F* test. If homogeneous, the data were analyzed with the Student's *t*-test (two sided), and if not, they were analyzed with the Welch test.

Results

Neither mortality nor clinical changes related to the test compound treatment were apparent in any of the groups. Average body weight values for animals exposed to DEN were lower than those of rats without DEN initiation at week 1 and thereafter continued to be depressed until termination

at week 8. Throughout the period of test material treatment (weeks 3 to 8), the mean body weight values of rats given 2-NP or 1-NP were comparable to the corresponding control values, although the values for rats treated with 20 mg/kg of 1-NP with DEN initiation were significantly lower than the control values in experiment 2 (data not shown). Food consumption by 2-NP- or 1-NP-treated animals was comparable to the corresponding control values throughout the experiment (data not shown).

Final average body weights for rats given 20 mg/kg/day of 1-NP with DEN initiation were significantly lower than those of control group 1. Those of the other groups were comparable to control values (Tables 1 and 2). Absolute and relative liver weights of rats treated with 0.8 and 20 mg/kg/day of 2-NP with DEN initiation were significantly higher than those of control group 1, and those of the rats treated with 20 mg/kg/day of 2-NP without DEN initiation were significantly higher as compared with control group 5 (Table 1). The relative liver weights of rats treated with 20 mg/kg/day of 1-NP with DEN initiation were also significantly higher than those of control group 1 (Table 2). The absolute and relative liver weights of rats treated with 20 mg/kg/day of 2-NP with DEN initiation, reference group 5, were significantly higher than those of control group 1 (Table 2). As macroscopic findings, pale color and several whitish/yellowish points in the liver were observed in rats treated with 20 mg/kg/day of 2-NP with DEN initiation (data not shown).

The numbers and areas of GST-P-positive foci for rats treated 20 mg/kg/day of 2-NP with DEN initiation were

Table 3. Numbers and Areas of GST-P-positive Foci (0.2 mm or More in Diameter) in Male F344/DuCrj Rats Initiated with or without DEN Followed by 2-Nitropropane (2-NP) Administration and Partial Hepatectomy in Experiment 1

Group	Treatment		Effective No. of rats	GST-P-positive foci [a]	
	DEN	2-NP (mg/kg/day)		Number (No./cm²)	Area (mm²/cm²)
1	+	0	15	3.39 ± 2.20	0.25 ± 0.17
2	+	0.8	13	3.50 ± 2.44	0.24 ± 0.18
3	+	4.0	15	4.60 ± 1.94	0.33 ± 0.18
4	+	20.0	15	10.83 ± 3.36**	0.88 ± 0.31**
5	-	0	6	0.00 ± 0.00	0.00 ± 0.00
6	-	20.0	6	0.00 ± 0.00	0.00 ± 0.00

** Significantly different from the control group (group 1) at $P<0.01$. [a] Values indicate the mean±SD.

Table 4. Numbers and Areas of GST-P-positive Foci (0.2 mm or More in Diameter) in Male F344/Du-CrlCrlj Rats Initiated with or without DEN Followed by 1-Nitropropane (1-NP) Administration and Partial Hepatectomy in Experiment 2

Group	Treatment		Effective No. of rats	GST-P-positive foci [b]	
	DEN	1-NP (mg/kg/day)		Number (No./cm²)	Area (mm²/cm²)
1	+	0	14	3.64 ± 1.01	0.31 ± 0.12
2	+	0.8	14	4.95 ± 1.99*	0.43 ± 0.26
3	+	4.0	15	3.91 ± 1.78	0.33 ± 0.18
4	+	20.0	14	4.38 ± 1.46	0.32 ± 0.11
5[a]	+	20.0	15	11.32 ± 1.54**	0.97 ± 0.17**
6	-	0	6	0.00 ± 0.00	0.00 ± 0.00
7	-	20.0	6	0.00 ± 0.00	0.00 ± 0.00

*, ** Significantly different from the control group (group 1) at $P<0.05$ and 0.01, respectively. [a] 2-nitropropane was used as a positive substance. [b] Values indicate the mean ± SD.

Table 5. Quantitative Analysis of GST-P-positive Foci of Less than 0.2 mm in Diameter in Male F344/DuCrj Rats Initiated without DEN Followed by 2-Nitropropane (2-NP) Administration and Partial Hepatectomy in Experiment 1

Group	Treatment		Effective No. of rats	Size distribution of GST-P-positive foci (No./cm²)[a]				
	DEN	2-NP (mg/kg/day)		Single cell	2-4 Cells	5-10 Cells	>11 Cells	Total[b]
5	–	0	6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.16	0.07 ± 0.16
6	–	20.0	6	2.80 ± 1.17**	4.76 ± 1.67**	3.09 ± 1.11**	0.63 ± 0.66*	8.48 ± 2.45**

*, ** Significantly different from the control group (group 5) at $P<0.05$ and 0.01, respectively. [a] Values indicate the mean ± SD. [b] Single GST-P-positive cells were excluded.

Table 6. Quantitative Analysis of GST-P-positive Foci of Less than 0.2 mm in Diameter in Male F344/DuCrlCrlj Rats Initiated without DEN Followed by 1-Nitropropane (1-NP) Administration and Partial Hepatectomy in Experiment 2

Group	Treatment		Effective No. of rats	Size distribution of GST-P-positive foci (No./cm²)[a]				
	DEN	1-NP (mg/kg/day)		Single cell	2-4 Cells	5-10 Cells	>11 Cells	Total[b]
6	–	0	6	0.42 ± 0.37	0.07 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.17
7	–	20.0	6	0.00 ± 0.00*	0.00 ± 0.00	0.07 ± 0.17	0.13 ± 0.20	0.20 ± 0.22

* Significantly different from the control group (group 6) at $P<0.05$. [a] Values indicate the mean ± SD. [b] Single GST-P-positive cells were excluded.

significantly higher than those of control group 1 in experiment 1 (Table 3). On the other hand, 1-NP did not promote the development of GST-P-positive foci in experiment 2, although a slight significant increase in number was evident in rats given 0.8 mg/kg/day of 1-NP with DEN initiation, with no dose dependence (Table 4). In the non-DEN-initiated groups, a number of small GST-P-positive foci of less than 0.2 mm in diameter developed in the rats given 20 mg/kg/day of 2-NP, and this was significantly higher than the control value (group 5) in experiment 1 (Table 5). However, no such findings were evident for 1-NP (group 6) in experiment 2 (Table 6).

Discussion

In the present investigation, the liver carcinogen 2-NP gave an unequivocally positive result in the *in vivo* medium-term liver carcinogenesis bioassay, whereas 1-NP proved negative. Preneoplastic GST-P-positive foci was significantly increased in the rats treated with 20 mg/kg/day of 2-NP with DEN initiation, but not 4 mg/kg/day of 2-NP or less (experiment 1), pointing to a dose threshold.

Most importantly, the results of the present carcinogenesis bioassay were in accordance with the earlier finding that 2-NP is a potent rat liver carcinogen according to long-term *in vivo* carcinogenicity tests with exposure by inhalation (207 ppm) and per os (1 mmol/kg)[4,5]. No toxicological effects were observed in the rats given 1-NP of 20 mg/kg/day except for the retardation of body weight. In contrast, increased liver weights were shown in the rats treated with 2-NP of 20 mg/kg/day. Moreover, the number of small GST-P-positive foci of less than 0.2 mm in diameter was significantly increased in the rats treated with 2-NP of 20 mg/kg/day without DEN initiation plus PH but not in the rats treated with 1-NP. Therefore, it was suggested that 2-NP is a complete hepatocarcinogen, displaying both promoting and initiating activities, in accordance with the results of a previous initiation bioassay focusing on induction of γ-GT-positive and GST-P-positive foci[22]. 2-NP, categorized as complete hepatocarcinogen, indicates increase of GST-P-positive foci in both with and without DEN initiation treatment. 1-NP, as non-hepatocarcinogen, demonstrates no increase in GST-P-positive foci even after DEN initiation treatment.

With regard to the mechanism of mutagenicity, oxidative damage to DNA and RNA was demonstrated for 2-NP, eliciting 8-hydroxyguanine and 8-hydroxy-2'-deoxyguanosine, products of hydroxyl radical attack that can cause DNA misreplication[16,17]. It is therefore very likely that a modification of DNA and/or RNA and hepatocellular proliferation are involved in 2-NP carcinogenicity[21]. Another mechanism might be related to NH_2^+, an aminating species, activated by aryl sulfotransferase, resulting in 8-aminoguanine products. In contrast, the isomer 1-NP appears not to be a substrate for aryl sulfotransferase[18,19].

With another pair of structural isomers, 2,4-diaminotoluene (2,4-DAT), but apparently not 2,6-diaminotoluene (2,6-DAT), proved to be hepatocarcinogenic[35,36], although both were found to be genotoxic in the Ames/Salmonella assay, and the metabolism of both compounds is mediated in a similar manner[37,38]. Cunningham and Matthews have documented that the difference may depend upon induction of cell proliferation. In the toxicological profile of the liver, clinical chemistry does not appear to differ between 2-NP and 1-NP[21]. However, increased cell proliferation was limited to the case with 2-NP, which proved to be carcinogenic[39]. Hepatocellular proliferation was similarly displayed at extremely high levels in the rats treated with 2,4-DAT but was lacking with 2,6-DAT[40]. Cell proliferation, therefore, may be an essential correlate of carcinogenicity with both 2-NP and 2,4-DAT liver carcinogens. Thus, after initiated cells are generated in the liver in the two-step theory model with partial hepatectomy, whereby pluripotential cells in the intermediate cell population undergo replication, the probability that another genetic alteration mistake can occur that leads to the development of cancer is augmented. With repeated cell divisions, initiated populations colonally expand to produce identifiable specific intermediate lesions such as GST-P-positive foci, which can be quickly and easily assessed as evidence for carcinogenic or non-carcinogenic potential[41,42].

In conclusion, the present medium-term liver bioassay clearly demonstrated 2-NP, but not 1-NP, to be a hepatocarcinogen, in accordance with published results for *in vivo* long-term carcinogenesis. Moreover, it was concluded that 2-NP was a complete hepatocarcinogen with genotoxic activity, showing that it displayed GST-P-positive activity both with and without DEN initiation treatment in this bioassay system.

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