The Effects of Diethyldithiocarbamate on the Hepatotoxic Action and Antitumor Activity of N-Methylformamide in Mice

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Abstract—The oral administration of diethyldithiocarbamate (DTC) prevented hepatic necrosis induced by N-methylformamide (NMF) in ddY-strain mice, in more susceptible BALB/c mice and in diethylmaleate-treated mice in which NMF hepatotoxicity was potentiated, as evidenced by suppression of increases of plasma glutamic pyruvic transaminase activity and liver calcium content or by histological observations. Early depletion of liver glutathione following NMF administration was also prevented by DTC. DTC markedly delayed the in vivo metabolism of NMF as indicated by a prolonged retention of plasma and liver NMF levels and an enhancement of urinary excretion of NMF. These observations support a bioactivation mechanism for NMF hepatotoxicity, and the hepatoprotective action of DTC may be due to an inhibition of the metabolic activation of NMF. Hepatotoxic manifestations after repeated administration of NMF also tended to be ameliorated by simultaneous treatment with DTC. Cotreatment with DTC, however, decreased the antitumor activity of NMF against Ehrlich ascites tumors, and Sarcoma 180. This also implies the involvement of a bioactivation mechanism in the antitumor action of NMF, but further studies are necessary to confirm this point. The possible therapeutic value of DTC as a hepatoprotector may be diminished by the suppression of the antitumor activity of NMF.

The antitumor activity of N-methylformamide (NMF) was originally found by Clarke et al. (1) and by Furst et al. (2) in Sarcoma 180 and Ehrlich ascites tumors, respectively. They also showed that NMF, among a number of related compounds, was most active against these tumors. In subsequent clinical studies, however, Myers et al. (3) observed that NMF was hepatotoxic without showing antitumor activity, and subsequently, the interest in this drug appeared to subside.

Recently, the antitumor activity against other murine tumors (4) and human xenografts in mice (5) was demonstrated, and experimental and clinical studies on NMF have been revived (6).

The hepatotoxic mechanisms of NMF are being investigated by Gescher and his associates: NMF depleted liver-soluble non-protein thiols shortly after administration, and the depletion was partially inhibited by pretreatment with SKF-525A (4). Glutathione (GSH) depletion was confirmed in experiments using isolated hepatocytes (7). In addition, radio-labelled NMF was covalently bound to liver macromolecules in vivo, the binding being well correlated with the depletion of liver GSH and reduced by pretreatment with SKF-525A (8). These findings suggest that the NMF hepatotoxicity involves a bioactivation mechanism, even though the active metabolite has not been identified yet. Depression of the mitochondrial calcium pump by NMF was also reported by Whitby et al. (9).

It is as yet uncertain, however, whether or not the antitumor activity of NMF is mediated through an active metabolite.

On the other hand, we previously reported that diethyldithiocarbamate (DTC) and its breakdown product, carbon disulfide (CS₂),
prevented mice from liver injury induced by structurally quite different hepatotoxicants, probably through an inhibition of bioactivation of the toxicants by the microsomal cytochrome P-450-containing monoxygenase system since both agents suppressed various microsomal drug metabolizing enzyme activities (10). DTC and CS₂ may, therefore, modify the pharmacologic and toxic actions of a number of drugs that are metabolized by the monoxygenase system. Conversely, these agents may serve as a convenient tool in predicting the involvement of a microsomal bioactivation mechanism. 

Thus, it appeared interesting from a therapeutic as well as mechanistic point of view to study whether DTC could prevent hepatotoxicity or not and how this agent could affect the antitumor activity of NMF.

Materials and Methods

Chemicals: The purity and sources of the test compounds were as follows: NMF (99%, GC) and diethylmaleate (DEM, 99%, GC) from Nakarai Chemicals Ltd., Japan; sodium diethyldithiocarbamate trihydrate (DTC, 99.5%, by nonaqueous titration) and carbon disulfide (CS₂, >99%) from Wako Pure Chemicals, Japan. All other chemicals were of the reagent grade commercially available.

Animals and treatments: SPF-grade male mice (ddY strain unless specified otherwise), 5 weeks old, were used, with food and water given ad libitum throughout the experimental period.

NMF dissolved in isotonic saline or distilled water was given i.p. in the acute hepatotoxicity tests and p.o. in the antitumor experiments, respectively. DTC and CS₂, freshly dissolved in distilled water and olive oil, respectively, were given p.o. 1 hr prior to the administration of NMF. Control groups received the vehicles alone. Details of the animal treatments are described under each experiment.

Urine was collected at intervals of 12 and 24 hr after administration of NMF by using a metabolic cage.

Evaluation of acute hepatotoxicity: Animals were killed by exsanguination, and the blood was collected in a heparinized syringe. After centrifugation of the blood, plasma glutamic pyruvic transaminase (GPT) activity was assayed according to the method of Reitman and Frankel (11). As direct proof of liver cell necrosis (10), liver calcium contents were determined in the same animals as follows: a portion of the liver was sonicated in 8% trichloroacetic acid (TCA), and calcium content in an aliquot of the TCA supernatant was determined by the calcein-fluorometric method of Von Hattingberg et al. (12). Liver GSH levels were estimated by determining the nonprotein sulfhydryl content according to the method of Sedlack and Rindsay (13). For microscopic observations, a portion of the median lobe of the liver was fixed in 10% neutralized formalin, embedded in paraffin, cut at 4 μm and stained with hematoxylin-eosin.

NMF determination: A portion of plasma, liver homogenate (40%) or urine was mixed with an equal volume of acetone and centrifuged. An aliquot (5 μl) of the supernatant was injected into a Shimadzu Gas Chromatograph (GC-4CMPF) equipped with a flame ionization detector and a glass column (1.5 m in length, 3 mm in diameter) packed with 100–200 mesh Chromosorb W (AW-DMCS) coated with 8% polyethylene glycol 20M and 2% KOH (Shimadzu Column Packing) under the following conditions: Temperature: column, 140°C; injector and detector 200°C; Gas flow: N₂, 40 ml/min; H₂, 60 ml/min; and air, 0.8 l/min.

Antitumor experiments: A suspension of Ehrlich ascites tumor cells, 10⁸ cells/ml in phosphate balanced saline, was inoculated i.p. at a volume of 0.1 ml/mouse. NMF was given p.o. in order to avoid direct contact with the tumor cells in the intraperitoneal cavity. The maximum dose of NMF was set at 200 mg/kg, since 400 mg/kg was too toxic to cause a marked loss of body weight in the preliminary experiment. DTC (100 mg/kg, p.o.) was given 1 hr before the NMF administration. Both treatments were started on the second day after the tumor cell inoculation and continued until the animals died. Body weights and the number of deaths were recorded every day. In the experiment with Sarcoma 180, animals were inoculated s.c. with 2×10⁶ cells (ascites type) and treated similarly with NMF and DTC; on the 17th
day, the solid tumors were cut out and weighed.

Subacute hepatotoxicity test: NMF (100 and 200 mg/kg, p.o.) was given every day for 38 days singly or in combination with DTC (100 mg/kg, p.o., 1 hr before the NMF administration). The animals were killed 24 hr after the last administration and examined for plasma GPT activity, liver GSH content and some microsomal drug metabolizing enzyme activities. The preparation of microsomes and assays for drug metabolizing enzyme activities have been described elsewhere (10). Microsomal glucose 6-phosphatase (G-6-Pase) activity was measured according to the method of Hübscher and West (14).

Statistics: Statistical analysis was performed by the method of Bonferoni as described by Wallenstein et al. (15) after the one-way analysis of variance for comparison of three or more groups, and P<0.05 was considered significant.

Results

Prevention of liver damage: NMF, at a dose of 400 mg/kg, i.p., produced a variable degree of liver damage in ddY mice as evidenced by increases in plasma GPT activity and liver calcium contents, with rather large standard deviations. The variation persisted at 600 and 800 mg/kg of NMF, whereas 200 mg/kg produced no increase in these parameters. Pretreatment with DTC (10–100 mg/kg, p.o.) and nearly equimolar doses of CS2 (3–30 mg/kg, p.o.) suppressed the hepatic damage induced by 400 mg/kg of NMF (Fig. 1).

Langdon et al. (16) reported a strain difference in NMF toxicity in mice, e.g., LD10 in BDF1 mice was about three times the value obtained in BALB/c mice. Therefore, the hepatotoxic potential of NMF in various strains of mice was examined. As shown in Table 1, BALB/c mice were the most susceptible to NMF, exhibiting marked increases in GPT activity and liver calcium content at 200 mg/kg of this agent. The preventive action of DTC was confirmed biochemically in BALB/c mice at 24 and 48 hr (Fig. 2). Histologically, NMF produced a marked centrilobular necrosis, which was prevented by pretreatment with DTC (data not shown). In subsequent experiments, effects of DTC were studied using male ddY mice.

DEM-pretreatment (500 mg/kg, i.p., 1 hr before NMF) potentiated hepatotoxicity induced by 300 mg/kg of NMF. This dose of DEM decreased liver GSH levels to approximately 40% of their original values by

![Fig. 1](image-url) Prevention of NMF-induced hepatotoxicity by pretreatment with DTC or CS2 in ddY-strain mice. DTC or CS2 was given p.o. 1 hr before NMF administration (i.p.). The animals were killed at 24 hr. Each bar represents X±S.D. (n=6 to 12). **Significantly higher than the other groups at *P<0.01 and **P<0.05, respectively.
the time of NMF administration, while DTC had no effect on the GSH levels of control and DEM-treated mice (data not shown). DTC again prevented this DEM-enhanced NMF hepatotoxicity (Fig. 3).

### Table 1. Acute hepatotoxic potential of NMF in various strains of mice

| Strain  | Dose of NMF (mg/kg) | Plasma GPT activity (KU) | Liver Ca content (μmol/g liver) |
|---------|---------------------|--------------------------|-------------------------------|
| BALB/c  | 0                   | 42±0                     | 0.75±0.30                    |
|         | 200                 | 2640±1410                | 4.70±2.46                    |
|         | 400                 | 8440±6250                | 5.49±2.25                    |
| ICR     | 0                   | 40±3                     | 0.70±0.03                    |
|         | 400                 | 170±150                  | 0.91±0.16                    |
|         | 800                 | 1670±1940                | 1.82±2.20                    |
| DBA/2   | 0                   | 30±10                    | 0.68±0.20                    |
|         | 400                 | 50±2                     | 0.88±0.06                    |
|         | 800                 | 2430±2100                | 1.71±0.91                    |
| C57BL/6 | 0                   | 27±0                     | 0.70±0.10                    |
|         | 400                 | 100±35                   | 0.81±0.06                    |
|         | 800                 | 4890±5470                | 1.02±0.49                    |
| C3H/He  | 0                   | 37±3                     | 0.76±0.30                    |
|         | 400                 | 600±460                  | 1.82±0.78                    |
|         | 800                 | 9160±3290                | 4.88±0.78                    |

Animals were killed 24 hr after administration of NMF (i.p.). Each value represents X±S.D. (n=5–6 for NMF-dosed groups and n=3–4 for controls).

**Fig. 2.** Prevention of NMF-induced hepatotoxicity by DTC in BALB/c mice. DTC was given p.o. 1 hr before administration of NMF (i.p.). The animals were killed at 24 and 48 hr. Each bar represents X±S.D. (n=6 to 10). *Significantly lower than the corresponding NMF-alone group at P<0.01.

**Fig. 3.** Enhancement of NMF-hepatotoxicity by diethylmaleate (DEM) in ddY-strain mice and its prevention by DTC. DEM and DTC were given i.p. and p.o., respectively, 1 hr before NMF administration (i.p.). The animals were killed at 24 hr. Each bar represents X±S.D. (n=10, but two mice died in the (DEM+NMF)-group.). **Significantly higher than the other groups at *P<0.01, **P<0.05, respectively.

Other pretreatments such as phenobarbital (0.1% in drinking water for 5 days), 3-
methylcholanthrene (40 mg/kg, i.p., for 4 days), β-naphthoflavone (80 mg/kg, i.p., 48 hr before) or fasting (48 hr) did not significantly enhance the hepatotoxicity induced by 300 mg/kg of NMF (data not shown).

**Effects on liver GSH levels:** As shown in Fig. 4, following the administration of 400 mg/kg of NMF, liver GSH levels decreased over 1 to 6 hr and then gradually recovered thereafter. Pretreatment with 100 mg/kg of DTC significantly suppressed this NMF-induced loss of GSH, whereas DTC alone had no significant effect on liver GSH levels throughout the experimental period.

**Effects on NMF metabolism in vivo:** In this experiment, the dose of NMF was lowered to 200 mg/kg, i.p., so as to be absorbed rapidly. Plasma NMF concentrations in the control group decreased exponentially from 1 to 12 hr, with a half life of about 2.5 hr. Pretreatment with DTC (100 mg/kg) markedly delayed the onset of the disappearance of the plasma NMF (Fig. 5A). In parallel with this, liver NMF concentrations remained high for a much longer period in the DTC-pretreated group (Fig. 5B). Urinary excretion of NMF, which occurred for the most part within 12 hr, was increased to about twice the control by DTC-pretreatment during a 24-hr period (Fig. 5C).

**Effects on antitumor activity of NMF:** As shown in Fig. 6, the control mice inoculated with Ehrlich ascites tumor cells survived an average of 18.5 days, on which treatment with DTC alone had no effect. NMF, 100 and 200 mg/kg, p.o., increased the survival days dose-dependently, i.e., averages of 34.4 and 40 days, respectively. These values, however, were shortened to 28.8 and 37.7 days, respectively by simultaneous treatment with 100 mg/kg of DTC. Dose-dependency of the DTC action was confirmed in a separate experiment, in which the mean survival days in each group were as follows: control (15.5), 200 mg/kg NMF, alone (38.2), 100 mg/kg DTC plus NMF (34.3), 300 mg/kg DTC plus NMF (28.4) and 300 mg/kg DTC, alone (15.0). With a high toxic dose of NMF (400 mg/kg, 2 weeks), DTC cotreatment (300 mg/kg) also shortened the mean survival days from 54 (NMF-alone group) to 30 days.

The antitumor activity of NMF against solid tumor growth of Sarcoma 180 was also
suppressed by cotreatment with DTC (Table 2). In these experiments, it is unlikely that DTC inhibited the absorption of NMF given orally, since DTC-pretreatment (300 mg/kg, p.o., 1 hr before NMF) did not affect plasma NMF concentrations early after adminis-

Fig. 5. Effects of DTC on A) plasma NMF disappearance, B) liver NMF levels and C) its urinary excretion in ddY mice. Open circles and columns: NMF 200 mg/kg, i.p. Closed circles and dotted columns: DTC (100 mg/kg, p.o.) was given 1 hr before administration of NMF. Each point represents X±S.D. (n=5 in A and B, n=6 in C). **Significantly higher than the NMF-alone group at *P<0.01 and **P<0.05, respectively.

Fig. 6. Effects of DTC on the NMF-antitumor activity against Ehrlich ascites tumor in ddY mice. Ehrlich ascites tumor cells were inoculated at day 1 and treatment with DTC and NMF (both given p.o.) was started on day 2 and continued until the animals died. DTC was given 1 hr prior to NMF administration. Experimental details were given in the Methods section. Arrows indicate the average survival days.
Table 2. Effects of DTC on the antitumor activity of NMF against Sarcoma 180

| Group                          | Tumor weight (g) |
|-------------------------------|------------------|
| Control                       | 0.88±0.29        |
| DTC, 100 mg/kg                | 0.98±0.42        |
| DTC, 300 mg/kg                | 1.03±0.34        |
| NMF, 200 mg/kg                | 0.19±0.07*       |
| DTC, 100 mg/kg+NMF, 200 mg/kg | 0.35±0.10        |
| DTC, 300 mg/kg+NMF, 200 mg/kg | 0.59±0.24*       |

Treatment with both agents (p.o.) was started on the second day after tumor inoculation and continued for 16 days. DTC was given 1 hr prior to NMF administration. Each value represents X±S.D. (n=10). *Significantly lower than the control group at P<0.01. **Significantly higher than the group given NMF alone at P<0.01.

Table 3. Effects of DTC-pretreatment on the hepatotoxicity induced by repeated administration of NMF

| Group                          | Body weight gain (g) | Plasma GPT activity (KU) | Liver GSH (mg/g liver) | G-6-Pase (μmol Pi mg prot. hr) | Aminopyrine demethylase (μmol HCHO mg prot. hr) | Aniline hydroxylase (nmol p-AP mg prot. hr) |
|-------------------------------|----------------------|--------------------------|------------------------|--------------------------------|-----------------------------------------------|------------------------------------------|
| Control                       | 12.2±1.2             | 31±10                    | 3.1±0.2                | 8.8±1.1                       | 0.40±0.03                                     | 68.6±2.5                                |
| DTC, 100 mg/kg                | 11.5±1.7             | 14±8                     | 3.2±0.2                | 9.4±1.4                       | 0.36±0.02                                     | 63.0±3.8                                |
| NMF, 100 mg/kg                | 11.5±1.7             | 28±7                     | 2.8±0.4                | 6.4±0.8*                      | 0.29±0.04*                                    | 60.6±9.2                                |
| NMF, 200 mg/kg                | 9.6±2.7              | 62±26*                   | 3.7±0.5                | 4.6±0.5*                      | 0.23±0.06*                                    | 55.1±2.7**                               |
| DTC, 100 mg/kg + NMF, 100 mg/kg | 10.4±1.8            | 29±8                     | 2.7±0.4                | 6.1±0.7                       | 0.33±0.03                                     | 69.7±10.4                               |
| DTC, 100 mg/kg + NMF, 200 mg/kg | 10.3±0.9            | 30±12*                   | 3.0±0.6                | 5.6±0.8                       | 0.30±0.03**                                   | 76.1±6.7*                                |

DTC and NMF were given p.o. everyday for 38 days. DTC was given 1 hr prior to NMF administration. Animals were killed 24 hr after the last treatment. Each value represents X±S.D. (n=5). *#Significantly different from the controls at P<0.01, ##P<0.05, respectively. *,**Significantly different from the corresponding NMF-alone group at *P<0.01, **P<0.05, respectively.
turation of NMF (200 mg/kg, p.o.) and markedly delayed its disappearance in a pattern similar to that shown in Fig. 5A, and this action of DTC was still evident even after 4 weeks-repeated administration of NMF and DTC (data not shown).

Effects of subacute toxicity of NMF:
Normal mice were treated with NMF and DTC for 38 days and killed 24 hr after the last administration. As shown in Table 3, with 200 mg/kg of NMF, some decrease in body weight gain and a slight, but significant, increase in plasma GPT activity were observed. Activities of liver microsomal enzymes such as G-6-Pase, aminopyrine N-demethylase and aniline hydroxylase were more evidently decreased by NMF-treatment. Cotreatment with DTC more or less ameliorated these NMF-induced changes. Liver GSH contents were not significantly different among the groups. The gross appearance of the liver was almost normal in all groups.

Discussion

Recent studies by Gescher et al. (4) and Pearson et al. (8) strongly suggest that NMF hepatotoxicity is caused by an active metabolite of NMF.

In the present study, NMF-induced hepatic necrosis in male ddY- and BALB/c-mice, the latter strain being most susceptible to NMF among various strains of mice, was prevented by pretreatment with DTC. On the other hand, DTC markedly delayed the plasma disappearance of NMF accompanied by a high and prolonged retention of unchanged NMF in the liver, while its urinary excretion was increased. Since we have already observed that DTC suppressed various cytochrome P-450-dependent drug metabolizing enzyme activities (10), the present observations suggest that NMF is metabolized by the liver monoxygenase system and that the hepatoprotective action of DTC, as in the case of other hepatotoxicants (10), may be due to an inhibition of the bioactivation of NMF.

Supporting this assumption are the facts that DTC prevented the early depletion of liver GSH after NMF administration, and DEM, a liver GSH depleter, potentiated the NMF hepatotoxicity, which was again prevented by DTC. These observations suggest a production of electrophilic metabolites, in agreement with the finding of Pearson et al. (8) that NMF covalently bound to liver macromolecules in vivo.

Pretreatment with cytochrome P-450 inducers such as phenobarbital, 3-methylcholanthrene and β-naphthoflavone did not significantly enhance NMF-hepatotoxicity. It is possible that detoxification of NMF or its metabolites could also be accelerated.

SKF-525A pretreatment is reported to partially reduce the NMF-induced loss of liver GSH (4) and the covalent binding of 14CH3-NMF in vivo (8); however, it does not influence the hepatotoxicity either (Gescher et al., personal communication).

Formaldehyde has been proposed as an active metabolite produced by oxidative N-demethylation of NMF, but this has not been proven yet (4). Nor could we detect formaldehyde production in the usual microsomal drug metabolizing enzyme system in vitro under various concentrations of NMF, microsomes or NADPH. Further studies on the metabolic pathways of NMF and identification of an active metabolite are awaited. DTC may be useful in such investigations and strain differences of the NMF-hepatotoxicity might also provide some clues.

Another aim of this study is to determine whether DTC could modify the antitumor activity of NMF. In confirmation of earlier reports (1, 2), NMF prolonged the survival days of Ehrlich ascites tumor-bearing mice and suppressed solid tumor growth of Sarcoma 180. It was discouraging, however, to find that DTC tended to reduce the antitumor activity of NMF in both types of tumors, even if it ameliorated hepatotoxic manifestations after repeated administration of NMF. The suppression of NMF-antitumor activity by DTC may partly be due to an enhancement of urinary excretion of NMF, but the facts, shown in Fig. 5, that the amount of the increased NMF excretion was not so much, accounting for approximately 10% of the dose administered, and nevertheless the plasma and liver NMF levels were maintained at a high level for a much longer period by DTC pretreatment may suggest another possibility, i.e., that the NMF-antitumor activity may also
be mediated by a reactive metabolite. In an attempt to examine this point, Ehrlich ascites tumor cell suspensions (10^7 cells/ml) were incubated for 2 hr in the presence of 10 mM NMF, liver microsomes (4 mg protein/ml) isolated from normal or DTC-treated (100 mg/kg, p.o., 1 hr) mice and an NADPH (1 mM)-generating system, and after washing, inoculated to mice. The survival days of both groups, however, were not different from those of the control group inoculated with the unincubated cells.

Cooksey et al. (17) studied the cytotoxicity and antitumor activity of N-hydroxymethylformamide, a putative metabolite of NMF, and concluded that this compound may not be responsible for depletion of liver GSH and in vivo antitumor activity. Thus, more experiments are necessary to prove whether or not a bioactivation mechanism is indeed involved in the antitumor activity of NMF.

The action of DTC, particularly when given orally, may be mediated by CS₂, since DTC easily decomposes to produce CS₂ under acidic conditions such as in the stomach. CS₂ is reported to be activated by the microsomal monoxygenase system to the active sulfur atom, which covalently binds to microsomal macromolecules, causing a suicidal destruction of cytochrome P-450 (18-20). Further studies on the effects of CS₂ on the microsomal drug metabolizing enzyme system are being conducted in our laboratory.

Finally, the suppression of the NMF antitumor activity by DTC may reduce the therapeutic value of DTC in preventing hepatic damage.

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