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Caffeine-derived N-nitroso compounds. V. Carcinogenicity of mononitrosocaffeidine and dinitrosocaffeidine in bd-ix rats

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Mononitrosocaffeidine (MNC) and dinitrosocaffeidine (DNC) are new N-nitroso compounds obtained from in vitro nitration of caffeidine, a hydrolysis product of caffeine present in a typically made and widely consumed tea from Kashmir (India), a high incidence area of esophageal and stomach cancer. The chemical synthesis, in vitro metabolic studies and mutagenicity of the compounds has been previously reported. DNC, a nitrosamide is highly mutagenic both with and without metabolic activation whereas MNC, like several other aromatic asymmetric nitrosamines, does not exhibit genotoxic or mutagenic properties. We now report the results of the first carcinogenicity experiments on chronic oral administration of these compounds in BD-IX rats. The acute LD50 of MNC and DNC were about 1300 and 230 mg/kg b.w., respectively. Lung oedema and gastrointestinal haemorrhages were the first symptoms of intoxication observed after 2 days for both the compounds.

All three dose groups of MNC treated rats showed localization of tumours in nasal cavity (93.9–100% of all malignant tumours). The tumours were histologically diagnosed as neuroepitheliomas of the olfactory epithelium (neuroblastoma of the bulbus olfactorii) and squamous cell carcinoma of the nasal cavity in the ratio of 3:1. No tumours of the nasal cavity were observed in the untreated controls. DNC, in contrast, induced squamous cell carcinoma of forestomach in 100% animals at low and high doses, of which nearly half the tumours metastasized predominantly into the peritoneum. No forestomach tumours were seen in the untreated controls. The data presented here clearly show the potential for induction of malignant tumours and distinct organ-specificity by MNC and DNC in rats, and support the postulate that a chronic exposure to these compounds may provide a carcinogenic risk for high incidence of gastrointestinal cancers in Kashmir.

Introduction

A high incidence of both esophageal and stomach cancer has been reported from Kashmir province in India (1,2). Our analytical field studies in the area have sufficiently documented a chronic human exposure to dietary N-nitroso compounds and their precursors (3–5), and biological monitoring studies on healthy volunteers indicated a high potential for endogenous nitrosation in the local population (6,7). We have earlier shown that the method of preparation of a commonly consumed salted tea in Kashmir (boiling of green tea leaves in presence of sodium bicarbonate followed by the addition of sodium chloride) leads to the formation of caffeidine and caffeidine acid due to the alkaline hydrolysis of caffeine in tea. The study also showed that caffeidine under acid-catalysed in vitro nitrosation produces mononitrosocaffeidine (MNC*) and dinitrosocaffeidine (DNC) besides other minor products (8). MNC, an asymmetric N-nitrosamine and DNC, a nitrosamide (for formulae see Figure 1) are new compounds whose chemical synthesis, in vitro metabolic activation and mutagenic potential has been reported (8–10). While DNC was found to induce strong mutagenic response in different strains of Salmonella typhimurium (Ames test) and exhibited a high potential for inducing DNA single-strand breaks in primary rat hepatocytes, MNC failed to show mutagenicity or genotoxicity in either system (10). We now report the results on the carcinogenicity of these compounds on chronic oral administration in BD IX-rats using three dose levels for MNC and two for DNC.

Material and methods

Chemical synthesis of MNC and DNC

Since large amounts of the test compounds were needed for carcinogenicity testing, it was necessary to modify and optimize the method reported earlier for the synthesis of MNC and DNC (9). In the present method, caffeidine nitrate 2 was obtained after alkaline hydrolysis of caffeine 1 (Figure 1). The caffeidine nitrate 2 was directly used for the synthesis of MNC 3 without further purification. Nitrosation of MNC was performed with NaN3, acetic anhydride and acetic acid (12), instead of using nitrosotetrafluoroborate (9). No by-products, such as nitro derivatives were formed.

All reagents were of analytical grade. The synthesized compounds were checked by 1H NMR, 13C NMR, IR and mass spectrometry. All obtained analytical and spectroscopic values agreed with the prior published data (9).

Caffeidine nitrate 2 (N,1-dimethyl-4-(methylammonium-nitrate)-1H-imidazole-5-carboxamide). Solutions of sodium nitrite (110 g, 1.6 mol) in 160 ml of water and 100 ml of 18.5% hydrochloric acid were added simultaneously to a cooled suspension of caffeidine nitrate 2 (206 g, 5.15 mol) in water (2.6 l) was added to caffeine 1 (500 g, 2.58 mol; Merck, Darmstadt, Germany) The suspension was stirred for 48 h at room temperature resulting in colourless solution. Caffeidine nitrate 2 was obtained as precipitate by the addition of 560 ml of nitric acid (65%) to the cooled reaction mixture. After an additional stirring for an hour, the precipitate was obtained after suction and washed with a small amount of cold water. The product was dried for 2 days at 60°C in vacuo yielding 229 g (38.5%) of a white powder with decomposition at 165°C.

Mononitrosocaffeidine 3 (MNC) (N,1-dimethyl-4-(methylnitrosatoamino)-1H-imidazole-5-carboxamide). Solutions of sodium nitrite (110 g, 1.6 mol) in 160 ml of water and 100 ml of 18.5% hydrochloric acid were added simultaneously to a cooled suspension of caffeidine nitrate 2 (184.8 g, 0.8 mol) in 800 ml of water. A pH of 2–3 was maintained during the reaction. The reaction mixture was stirred for 10 min at 5°C after complete addition of sodium nitrite. This was followed by addition of 600 ml of dichloromethane to dissolve the formed MNC. Nitrosation was terminated by ammonium sulphamate (30 g) added in small portions. After the separation of the CH2Cl2 phase, the aqueous reaction mixture was extracted with CH2Cl2 (4×100 ml). An additional amount of MNC was obtained by extraction with CH2Cl2 (3×100 ml) after neutralization of the aqueous phase with sodium hydroxide (40 ml, 5 N). The concentration of the combined organic layers (to 400 ml)
Dinitrosocaffeidine 4 (DNC) (\textit{N},1-dimethyl-4-(methylnitrosoamino)-N-nitroso-1H-imidazole-5-carboxamide). MNC 3 (39.5 g, 0.2 mol) was added to the cooled solution (0°C) of acetic acid (100 ml) and acetic anhydride (500 ml) followed by the addition of sodium nitrite (138 g, 2 mol) in small portions during 5 h at 0°C. The temperature was then brought to room temperature and the mixture was stirred for an additional 15 h, when a viscous solution was obtained. To this was added a mixture of ice and water (600 ml) and the excess of sodium nitrite was destroyed by addition of ammonium sulphamate (30 g) in small portions. Extraction with diethylether (6 \times 200 ml) gave a mixture of DNC and acetic anhydride. After drying the organic layer over magnesium sulphate, the solution was evaporated in vacuo at 40°C. Titration of the resulting yellow oil with pentane (200 ml) gave a crude yellow powder. This was dissolved in hot di-isopropylether (600 ml) and insoluble solids were removed. On concentration of the solution to 150 ml and cooling at 0°C, intense yellow crystals of dinitrosocaffeidine 4 were obtained, yielding 33 g (73%).

**Determination of LD\textsubscript{50}**

All animal experiments were performed according to the recommendations of Good Laboratory Practice (GLP). Six male adult BD-IX rats (300 g, b.w.) were used for each dose group. The dose factor was two. All animals were starved overnight before gavage of single dose of test compounds. The signs of toxicity and death were recorded during an observation period of 2 weeks. The mean lethal dose (LD\textsubscript{50}) was calculated according to Burn (13).

**Carcinogenicity experiments**

One-hundred-day-old BD IX-rats (Tierzucht Hannover) were used for chronic administration of the two test compounds. Animals were separated by sex, and then randomly distributed into the experimental and control groups. All animals were kept under conventional conditions and received Altromin pellet food. The experimental design is summarized in Table I for MNC and DNC.

The required daily dose of MNC was dissolved in 25 ml drinking water for males and 20 ml for females, respectively. This volume was mostly consumed in the first half of the day; after ensuring that the vessels had been emptied, tap water was given until the evening. Animals were then kept without drinking water overnight. MNC was administered once daily for 5 days/week.

Dinitrosocaffeidine 4 was administered by gavage twice per week. The amount of dose was dissolved in a small volume of alcohol and then diluted with tap water to an alcohol concentration of 20% (v/v) to obtain a clear solution. All animals were starved overnight before gavage. Untreated controls similarly received 20% alcohol twice a week. Treated animals and untreated controls for DNC were observed for their life time. Moribund rats were killed by ether anaesthesia and carefully autopsyed, including brain and nervous system. Tumours and other macroscopically visible lesions were registered and examined histologically.

**Results**

The acute LD\textsubscript{50} of MNC and DNC were about 1300 mg/kg b.w. and 230 mg/kg b.w., respectively. Lung oedema and gastrointestinal haemorrhages were the first symptoms of intoxication observed after 2 days for both the compounds. The final cause of death, which occurred ~1 week after the treatment, in most cases was also lung oedema.

The detailed data from carcinogenicity experiments are given in Table I. The oral administration of MNC was well tolerated in all treated groups (3 mg, 5 mg and 15 mg/kg, five times per week), and no signs of acute or sub-acute toxicity were observed. The body weights in all three dose groups were similar to those in the controls until the onset of tumour formation. Similarly, the low dose group of DNC (6 mg/kg) did not show any decrease in body weights compared to controls indicating the absence of acute or sub-acute toxicity at this dose. In the high-dose groups (60 mg/kg; twice weekly), however, the treatment led to appreciable loss of weight and, therefore, the dose was reduced to 30 mg/kg, twice weekly after 2 weeks. The reduced dose was well tolerated and animals gained body weight comparable to that of the controls. The median administered total dose (D\textsubscript{50}) and the median survival time (T\textsubscript{50}) are shown in Table I.
Table I. Experimental details of carcinogenicity testing of MNC and DNC

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial no. of animals</th>
<th>Dose group</th>
<th>Treatment² (mg/kg b.w.)</th>
<th>Median total dose applied</th>
<th>Median survival time³ T50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononitrosocaffeidine (MNC)</td>
<td>37</td>
<td>Control</td>
<td>0</td>
<td>825</td>
<td>534 (162–500)</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>Low dose</td>
<td>3</td>
<td>1355</td>
<td>406 (188–553)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Median dose</td>
<td>5</td>
<td>3330</td>
<td>418 (307–532)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>High dose</td>
<td>15</td>
<td>1500</td>
<td>346 (269–454)</td>
</tr>
<tr>
<td>Dinitrosocaffeidine (DNC)</td>
<td>22</td>
<td>Control</td>
<td>0</td>
<td>825</td>
<td>683 (204–295)</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>Low dose</td>
<td>6</td>
<td>384</td>
<td>222 (222–327)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>High dose</td>
<td>60 → 30</td>
<td>1500</td>
<td>174 (133–196)</td>
</tr>
</tbody>
</table>

²For MNC: five times per week in drinking water. For DNC: twice a week by stomach tube. In the high dose group, the single dose of 60 mg/kg was reduced to 30 mg/kg after 4 weeks (see text).
³Number in parentheses: time of appearance of first and last tumour.

Table II. Carcinogenicity of mononitrosocaffeidine (MNC)

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Malignant tumours no. (%)</th>
<th>Nasal cavity tumours</th>
<th>Other tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>66</td>
<td>6 (9,1)</td>
<td></td>
</tr>
<tr>
<td>Low dose (3 mg/kg/day)</td>
<td>42</td>
<td>3 (78.4)</td>
<td>Malignant</td>
</tr>
<tr>
<td>Medium dose (5 mg/kg/day)</td>
<td>45</td>
<td>29 (64.4)</td>
<td>Adenocarcinoma of mamma, 2</td>
</tr>
<tr>
<td>High dose (15 mg/kg/day)</td>
<td>43</td>
<td>32 (74.4)</td>
<td>Sarcoma of uterus, 2</td>
</tr>
</tbody>
</table>

As shown in Tables II and III, both MNC and DNC induced malignant tumours in high to very high frequency. Since no differences were observed with respect to tumour induction between male and female animals, the data have been pooled. All the three dose groups of MNC treated rats showed localization of tumours in nasal cavity (93.9–100% of all malignant tumours) with the exception of one hepatocellular carcinoma in the low-dose MNC group. Histologically, a majority of tumours were of neurogenic origin and diagnosed as neuro-epitheliums of the olfactory epithelium (neuroblastoma of the bulbus olfactorii). Squamous cell carcinoma of the nasal cavity were less frequently seen. Interestingly, the ratio of neuro-epitheliums and squamous cell carcinomas was ~3:1 in all three dose groups. No tumours of the nasal cavity were observed in the untreated controls. The identified malignant (9%) and benign tumours in control groups were of known spontaneous pathology of the BD IX strain of rats, and were similar in both controls.

As shown in Table III, DNC induced squamous cell carcinoma of forestomach in 100% animals at both low and high doses, of which nearly half metastasized predominantly into the peritoneum. The severe displasia of the forestomach
observed in the first dead animal could be considered as representative of preneoplastic lesions. No forestomach tumours were seen in the untreated controls.

Discussion

Although the epidemiological data suggest a multifactorial aetiology of human cancers, a chronic exposure to exogenous and endogenously produced N-nitroso compounds has been strongly implicated in several cancers among high risk populations (14). There is now considerable evidence supporting the causal role of tobacco-specific (TSNA), and betel-nut specific nitroso compounds in lung, oral and upper respiratory tract cancers (15). While no firm evidence is yet available, an involvement of intragastrically formed direct alkylating N-nitrosamides is long postulated to be the causal risk factor for stomach cancer (16) and organ-specific, metabolic activation dependent asymmetric N-nitrosamines are believed to be involved in providing high risks for human esophageal cancer (17).

The clinical and epidemiological data from Kashmir shows a high incidence both for esophageal and stomach cancers (1,2). This prompted us to conduct an analytical-field study in the area (2–5). The study showed a widespread contamination of preserved and stored food items with preformed known volatile and non-volatile N-nitrosamines (3,4) besides a high potential for endogenous formation of N-nitroso compounds in the local inhabitants due to a liberal consumption (average: 4 cups/day/adult) of salted tea in Kashmir (5). Estimates based on in vitro analysis of caffeine show that the daily exposure of MNC and DNC to salted tea drinkers in Kashmir may be ~150 µg and 0.5 µg/adult (5,9). It therefore, became imperative to investigate the carcinogenic potential of these compounds in order to estimate risk extrapolation to humans. This study presents the first carcinogenicity data on chronic administration of mononitrosocaffeidine (MNC) and dinitrosocaffeidine (DNC) in rats.

As shown in Table II, with the exception of one hepatocellular carcinoma, all tumours in three different dose groups were localized in nasal cavity showing an extreme organ-specificity in its carcinogenic action in rat. The cause of nasal cavity being the preferred organ for carcinogenic action of MNC, though unclear at the moment, may be related to the presence of specific activating enzymes in the organ and high stability of the compound under physiological conditions. MNC has been shown to undergo metabolic de-methylation preferentially at methyl nitrosamine group leading to the putative formation of imidazole diazonium ion capable of reacting with cellular nucleophiles (11). A lack of any nasal cavity tumours in control group further confirms the carcinogenic potential and organ-specificity of MNC, and rules out its action in merely enhancing the incidence of spontaneously induced tumours. Primacy of nasal cavity tumours also indicate the systemic action of MNC and shows that it can cause tumours in organs other than those which directly come into contact or where it may be formed from caffeine as it may occur under human conditions. The target specificity of MNC may vary in different species of animals and in humans depending on the levels and specificity of enzymes responsible for its metabolic activation and clearance of activated carcinogen. Change in target specificity of various N-nitrosamines in different animal species is well documented (18). Although data on the median survival time show a dose dependency in its carcinogenic action, it appears that the lowest dose used in the study was close to the dose required to produce optimal induction of tumours. DNC, a highly mutagenic and genotoxic compound (10) produced 100% tumours (squamous cell carcinomas of the stomach) at the site of its first contact under experimental conditions, thereby showing exceptional organotropy in its

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### Table III. Carcinogenicity of dinitrosocaffeidine (DNC)

<table>
<thead>
<tr>
<th>Eff. animals no.</th>
<th>Malignant tumours no. (%)</th>
<th>Squamous cell carcinomas of the forestomach</th>
<th>Other tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43</td>
<td>5 (8.6)</td>
<td>–</td>
</tr>
<tr>
<td>Low dose (2 × 6 mg/kg/week)</td>
<td>42</td>
<td>42 (100)</td>
<td>42 (100)</td>
</tr>
<tr>
<td>High dose (2 × 30 mg/kg/week)</td>
<td>42</td>
<td>40 (92.5)</td>
<td>40 (10)</td>
</tr>
</tbody>
</table>

- **Malignant**: Adenocarcinoma of mamma, 3
- **Benign**: Fibroadenoma of mamma, 5

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carcinogenic action. The target specificity of DNC is in agreement with its direct methylating action and low stability of the compound at physiological pH (9). The data also show that the low dose regimen was in itself sufficient to produce maximum number of tumours and hence dose response is obscured (Table III). It is therefore necessary that future experiments focus on low doses of DNC in order to obtain a dose–response relationship. An appreciable frequency of metastases in peritoneum and liver shows high carcinogenic potential, and capability of inducing fast growing tumours by DNC in rats.

The results obtained in the study further support the possible role of caffeine-derived nitroso compounds in providing high risk of esophageal and stomach cancer in the local population of Kashmir, where potential formation of such compounds has been already contemplated due to the large consumption of salted alkaline tea. The caffeine-derived N-nitroso compounds (MNC and DNC), therefore, also acquire immense importance as environmental carcinogens in other regions of the world where alkaline conditions may be used for tea preparation intentionally or due to the high alkalinity of natural water.

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References