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GENOTOXICITY

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Genotoxic effects of *N*-nitrosodicyclohexylamine in isolated human lymphocytes

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Abstract Dicyclohexylamine × nitrite is classified as an "experimental equivocal tumorigenic agent" by the National Toxicology Program. Since no genotoxic effects of the substance itself are known, the reported tumorigenic potential of dicyclohexylamine × nitrite could be due to generation of *N*-nitrosodicyclohexylamine (*N*-NO-DCHA), which occurs under conditions of use and can be detected in foils that contain dicyclohexylamine × nitrite. Therefore, we investigated possible mutagenic properties of *N*-NO-DCHA in the Ames test and the cytokinesis-block micronucleus assay with human lymphocytes. Since *N*-NO-DCHA is not commercially available, the substance was synthesized and purified by thin-layer chromatography. Identity was confirmed by gas chromatography/mass spectroscopy (GC/MS) and ^{1}H- and ^{13}C-NMR. More than 97% purity was achieved. Stability and availability in the solvent were checked by GC/MS. *N*-NO-DCHA induced micronuclei in isolated human lymphocytes at a dose range of 15–100 μg/ml (= 71.4–476.2 μM), exceeding the base rate significantly at one or two nontoxic concentrations in four out of six experiments. For the Ames test, arochlor-1254-, β-naphthoflavone/phenobarbital- and pyrazole-induced S9-fractions were used with *Salmonella typhimurium* TA100, TA1535, TA98 and TA104. No effects were seen in the Ames test, with the exception of microcolony induction at doses higher than 250 μg (= 1.2 mmol) *N*-NO-DCHA/plate using TA104 and 20% arochlor-1254 induced S9 at pH 6.5. In conclusion, *N*-NO-DCHA was negative in the Ames test using TA98, TA100 and TA1535, inconclusive using TA104, and weakly genotoxic in the in vitro micronucleus test with isolated human lymphocytes. With regard to the tumorigenicity of the majority of nitrosamines, our data underline the necessity of further studies on possible genotoxic effects of *N*-NO-DCHA.

Key words *N*-Nitrosodicyclohexylamine · Ames test · Cytokinesis-block micronucleus assay

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Introduction

Dicyclohexylamine × nitrite [CAS 3129-91-7] is used as an anticorrosive agent. According to the National Toxicology Program of the USA the substance is an "experimental equivocal tumorigenic agent" (National Toxicology Program 1991a). Since no data on the genotoxic effects of dicyclohexylamine × nitrite are available, the tumorigenic potential of dicyclohexylamine × nitrite might be linked to the generation of *N*-nitrosodicyclohexylamine (*N*-NO-DCHA), which is formed by the nitrosation of dicyclohexylamine (Fig. 1) (Kabacoff et al. 1984). Up to 250 mg/kg *N*-NO-DCHA can be detected in foils that contain dicyclohexylamine × nitrite (Reinhard et al. 2000). Since the vast majority of nitrosamines are carcinogenic (Hecht 1997), this could as well be the case for *N*-NO-DCHA.

Nitrosamines may show mutagenic effects in in vitro assays only under certain conditions. Therefore, we used three nitrosamines as positive controls (*N*-nitrosodiphenylamine, *N*-nitrosopyrrolidine and *N*-nitrosodiethylamine). The structurally closely related nitrosamine *N*-nitrosodiphenylamine [CAS 86-30-6] was shown to be carcinogenic (at 1,000 or 4,000 ppm) in both sexes of F344 rats, including induction of transitional-cell carcinomas of the urinary bladder, but was not carcinogenic in $B6C3F_1$ mice of either sex (National Toxicology Program 1990, 1991b). However, in vitro studies were inconclusive or negative (McGregor 1994) with the exception of one study where microcolony induction in *Salmonella typhimurium* TA104 was seen in the presence of arochlor-1254-induced S9 at pH 6.5 (Zielenska and Guttenplan 1988). *N*-Nitrosopyrrolidine is a potent experimental carcinogen (Berger et al. 1987) and can only

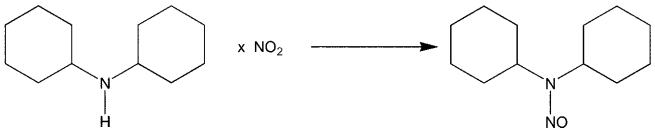

Fig. 1 Generation of *N*-nitrosodicyclohexylamine by the nitrosation of dicyclohexylamine

be effectively activated to Ames-test-positive metabolites by pyrazole-induced rat liver S9 (Burke et al. 1994). Pyrazole is known to induce CYP2E1 in the rat liver. *N*-Nitrosodiethylamine is one of the strongest known carcinogens (Lewis et al. 1997) and was proposed to be metabolized by CYP2E1 as well (Verna 1996). However, we could show that *N*-nitrosodiethylamine can be activated by β-naphthoflavone/phenobarbital (BNF/PB) - induced S9 to Ames-test-positive metabolites (Westphal et al. 2000). Mutagenic effects of these nitrosamines can only be detected using certain strains: *N*-nitrosopyrrolidine and *N*-nitrosodiethylamine were only mutagenic using TA1535 and *N*-nitrosodiphenylamine was only mutagenic in TA104.

The aim of this study was to evaluate possible mutagenic properties of *N*-NO-DCHA in the Ames test under conditions that have been reported to be effective for the above-mentioned nitrosamines. Additionally, we performed the in vitro cytochalasin the B-block micronucleus test.

Materials and methods

Chemicals

Synthesis and structure elucidation of N-nitrosodicyclohexylamine (N-NO-DCHA)

All chemicals used in the synthesis and the stability experiments were of analytical grade purity and were obtained from Sigma (Deisenhofen, Germany) or Sigma-Aldrich (Steinheim, Germany). Dichloromethane was a product of J.T. Baker (Griesheim, Germany). Preparative silica gel 60 F_{254} thin-layer chromatography (tlc) plates (20×20 cm, 0.25-mm layer thickness) were supplied by Merck (Darmstadt, Germany). Water was purified by passage through an Elix 3 and Milli-Q system (Millipore, Eschborn, Germany). This water was used for all aqueous solutions and buffers.

Mutagenicity testing

2-Aminofluorene [CAS 153-78-6], purity ≥99.0%, lot 9151564, 2-aminoanthracene [CAS 610–49–1], practical grade, lot 77H1867, *N*-nitrosodiethylamine [CAS 55-18-5], purity ≥99.0%, analysis number 194492/153097 and *N*-nitrosopyrrolidine [CAS 930-55-2], purity ≥99.0%, lot 70475-018 were obtained from Sigma-Aldrich (Steinheim, Germany). NADP, NAD, glucose 6-phosphate, mitomycin C and cytochalasin B were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany).

Other materials used were FICOLL, phosphate-buffered saline (PBS) and phytohemagglutinin (Biochrom KG, Berlin, Germany), RPMI 1640 (supplemented with 15% fetal calf serum, 1,5% glutamin, penicillin and streptomycin) and arochlor-1254-induced S9 (ICN, Eschwege, Germany), Trypan blue (Merck, Darmstadt, Germany) and Giemsa improved (Gurr/Promochem GmbH, Wesel, Germany).

Synthesis and purification of N-NO-DCHA

Synthesis was carried out according to a basic procedure for the formation of nitrosamines (Organikum 1986). Briefly, 48 ml of a 2.5 M sodium nitrite solution (0.12 mol $NaNO_2$) was added slowly to 20 ml dicyclohexylamine (0.1 mol DCHA) dissolved in 200 ml 10 M sulfuric acid under stirring (*Warning! nitrogen oxides may escape. The reaction should be conducted in a fume hood and with adequate skin and eye protection!*). The reaction mixture was continuously maintained below 5°C and stirred for 2 hs after the last addition of sodium nitrite solution. The reaction mixture was allowed to warm to room temperature when nitrogen oxides could escape from the reaction solution. Subsequently, the sulfuric acid was neutralized with a 10 M sodium hydroxide solution. Unreacted DCHA crystalized and was filtered from the neutralized solution. The product *N*-NO-DCHA was extracted from the aqueous solution using a total of 400 ml dichloromethane. The dried extract was concentrated to 10 ml in vacuo and 1 ml of this extract was applied to each preparative tlc plate. These were developed using dichloromethane as a solvent. A strong UV-absorbing band ($R_f = 0.32$) containing the desired product was scratched out from the glass plates. The product was eluted with dichloromethane from the combined silica fractions of ten plates. After filtration the extract was concentrated to dryness in vacuo, yielding a pale yellow residue. Overall yield was 221.8 mg *N*-NO-DCHA (1.06%) with a purity >97%, as judged by gas chromatography/mass spectroscopy (GC/MS).

Instrumental analysis

GC/MS analysis was carried out on a Hewlett-Packard GC 6890/MSD 5973 system (Agilent Technologies, Waldbronn, Germany) with splitless injection using a HP-5 MS capillary column (30 m×250 µm, layer thickness: 0.25 µm; Agilent Technologies, Waldbronn, Germany) and helium as the carrier gas (constant flow rate: 0.6 ml/min). A linear temperature gradient of 10°C/min from 50°C (1-min hold after injection) to 280°C (2-min hold until return to initial conditions) was applied. Injector and transfer line temperatures were kept at 250°C. The compound had a retention time of 16.1 min under these conditions. Mass spectra were acquired using electron impact (EI) ionization and the full-scan mode.

^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded in CD_2Cl_2 on a Varian VXR 200 instrument (Varian Inc., Darmstadt, Germany) at the Institute of Organic Chemistry, University of Göttingen.

Characterization of N-NO-DCHA

Mass spectrum (EI, 70 eV): *m/z* (relative abundances and assignments in parentheses) 210 (51) [M^+], 193 (9) [$M^+ - OH$], 129 (43) [$M^+ - C_6H_9$], 111 (12) [$M^+ - C_6H_{11}O$], 98 (33) [$M^+ - C_6H_{10}NO$], 83 (100) [C_6H_{11}], 67 (28) [C_5H_7], 55 (98) [C_4H_7].

^{1}H-NMR (200 MHz, CD_2Cl_2): δ = 1.5 (m, broad, 20 H, -C*H*$_2$), 4.15 (m, 1 H, -C*H*), 5.1 (m, 1 H, -C*H*).

^{13}C-NMR (50.3 MHz, CD_2Cl_2): δ = 27.6 (s, C-4′), 27.7 (s, C-4), 27.8 (s, C-3′), 28.3 (s, C-3), 31.6 (s, C-2′), 36.7 (s, C-2), 54.4 (s, C-1′), 60.6 (s, C-1).

^{13}C-NMR assignments of *N*-NO-DCHA are based on data of previously described ^{13}C-NMR spectra of analogous *N*-nitrosoamines (Kalinowski et al. 1984).

Stability of N-NO-DCHA in dimethyl sulfoxide (DMSO)

For the genotoxicity tests *N*-NO-DCHA was dissolved in DMSO (5 µg/µl). Ten microlitres of the freshly prepared solution was diluted with 990 µl water and extracted with 5 ml dichloromethane.

One microlitre of the dried dichloromethane extract was analysed by GC/MS. Only a single peak of *N*-NO-DCHA was detected. Subsequently, the DMSO solution of *N*-NO-DCHA (5 μg/μl) was stored for 24 h in a fridge at 4–8°C. Again, a 10-μl aliquot was taken, worked up as described above and analysed by GC/MS. No signs of decomposition of the synthesized compound upon storage in DMSO were detected.

Micronucleus test

The cytokinesis-block micronucleus assay was carried out according to Fenech (1993). Human blood was collected by venipuncture in heparinized tubes, diluted 1:1 in PBS and separated by a FICOLL gradient. 1.2×10^6 cells were seeded in 2.5 ml RPMI each. Cell viability was checked by the Trypan blue exclusion test. Lymphocyte stimulation was done by addition of 5 μg/ml phytohemagglutinin and cultivation at 37°C, 100% humidity and 5% CO_2 for 68 h. *N*-NO-DCHA (up to 25 μg/μl) was added as a solution in DMSO (in a volume of 20 μl/2.5 ml each) 24 h after starting the cultures. Cell division was blocked 44 h after starting the cultures by adding 4.5 μg/ml cytochalasin B. Mitomycin C was used as standard mutagen.

Cells were sampled 28 h after addition of cytochalasin B by centrifugation for 5 min at 175 *g* and 20°C. The cells were resuspended in 3 ml hypotonic saline (0.07 M KCl/0.15 M NaCl) and collected for 5 min at 175 *g* and 20°C. The pellet was resuspended in 3 ml ice-cold methanol/acetic acid (4:1). Finally, the cells were pelleted for 5 min at 175 *g* and 4°C, and transferred onto ice-cold degreased slides. The slides were coded and subsequently stained with 5% Giemsa solution for 15 min.

The maximum concentrations of the test article were determined by evaluation of the nuclear divison index (NDI) according to Eastmond and Tucker (1989). The cells were scored according to the criteria outlined by Fenech (1993). In addition, only cells with preserved cytoplasm containing not more than five micronuclei were included in the count. At least six concentrations were tested. Blood from three different donors was used. For each donor two independent experiments were performed. Acceptance criteria for a genotoxic effect were: NDI ≥1.1, a frequency of micronulei significantly exceeding the control, and stable pH. Samples were randomized prior to scoring.

Ames test

The Ames test was performed according to Maron and Ames (1983) using *S. typhimurium* TA100, TA98, TA1535 and TA104 with 30-min preincubation. Various S9 preparations were used. Pyrazole induction was performed according to Tu et al. (1981), and BNF/PB induction according to Ong et al. (1980). S9-mix was concentrated up to 20%, supplemented with up to 5 mM NADP and 25 mM glucose 6-phosphate. Arochlor 1254-induced S9 was additionally applied at pH 6.5.

N-NO-DCHA (50 μg/μl) dissolved in DMSO was added (maximal 20 μl DMSO per plate). The nitrosamine precipitates at concentrations above 500 μg/ml *N*-NO-DCHA. Therefore, in the absence of toxicity, a maximum of 2 mg *N*-NO-DCHA per plate was applied, which is at least twice the maximum solubility.

The compounds *N*-nitrosopyrrolidine, *N*-nitrosodiphenylamine, *N*-nitrosodiethylamine, 2-aminofluorene and methylmethanesulfonate served as positive controls. CYP2E1, 2A1, 1A1, 2B1 and 2B2 expression in the BNF/PB- and the pyrazole-induced S9-fractions, and the activity of the control mutagens *N*-nitrosopyrrolidine and *N*-nitrosodiethylamine are described elsewhere (Westphal et al. 2000). The acceptance criterion for a valid test was that solvent and positive controls were within the range of the historical controls. The positive controls for the *Salmonella* strains and the various S9 fractions are summarized in the Results section.

TA1535, TA98, TA100 and TA104 were kindly provided by the Bruce Ames Laboratory, Berkeley, Calif., USA.

Results

N-NO-DCHA was synthesized and characterized by GC/MS, ^{1}H- and ^{13}C-NMR. Probably because of the poor solubility in sulfuric acid of the starting compound DCHA, only a small yield of *N*-NO-DCHA (1.06%) could be recovered from the synthesis. Nevertheless, the product isolated by preparative tlc showed very high purity (>97% as judged by GC/MS). In addition, stability of the compound in DMSO tested over 24 h at 4–8°C proved to be excellent; thus fulfilling a prerequisite for valid genotoxicity testing.

Micronucleus test

N-NO-DCHA induced micronuclei in human lymphocytes at doses ranging from 5 to 100 μg/ml (=74.4–476 μM), exceeding the base rate significantly in four out of six experiments, apparent from the NDI at one or two non-toxic concentrations (Table 1). No pH deviations related to the test substance were observed. *N*-NO-DCHA was toxic above 100 μg/ml.

Ames test

In the absence of toxicity, the substance was tested up to 2 mg *N*-NO-DCHA per plate. This is at least twice the maximum soluble concentration, which was observed as a precipitation of the substance at concentrations above 1 mg/500 μl. In experiments involving TA98 and pyrazole-induced S9, 500 μg *N*-NO-DCHA per plate was slightly toxic. However, due to the limited solubility of the substance, pronounced bacterial toxicity could not be achieved.

With the exception of microcolony induction above 250 μg/plate (500 μl preincubation volume respectively) using TA104 in the presence of arochlor-1254-induced S9 at pH 6.5, *N*-NO-DCHA was not active in the Ames test (Table 2). The same effect was seen with *N*-nitrosodiphenylamine. However, owing to the small size of the colonies, reliable scoring was not possible. Additionally, the evaluation of this effect was impaired by pronounced base-rate deviations of TA104 at pH 6.5. Microcolony induction was not seen using TA98, TA100 or TA1535.

Discussion

No data on the genotoxic effects of dicyclohexylamine × nitrite are available. Dicyclohexylamine was intensively tested as a minor metabolite of cyclamate, without showing genotoxic effects (e.g. review by Bopp et al. 1986; Brusick et al. 1989). This was confirmed by our own preliminary experiments, in which we observed no mutagenic effects of dicyclohexylamine × nitrite (data

Table 1 Micronuclei (MN) per 1,000 binucleated cells and nuclear division index (NDI) induced by *N*-NO-DCHA in isolated lymphocytes of three different donors (two independent experiments each). Samples were randomized prior to scoring. Samples showing an NDI above 1.1 or a cell count below 1,000 valid cells were not included owing to toxicity (*tox*). Concentrations that were not examined are indicated as not determined (*nd*)

N-NO-DCHA (μg/ml)	Donor 1				Donor 2				Donor 3			
	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5		Experiment 6	
	MN	NDI	MN	NDI	MN	NDI	MN	NDI	MN	NDI	MN	NDI
0	3	1.6	8	1.5	2	1.6	4	1.6	7	1.4	5	1.5
5	nd	nd	8	1.2	4	1.4	1	1.4	nd	nd	nd	nd
10	5	1.4	8	1.2	3	1.5	3	1.5	5	1.5	9	1.3
15	nd	nd	11	1.4	8	1.5	7	1.5	20*	1.8	7	1.5
20	11*	1.5	28***	1.4	9*	1.5	5	1.5	5	1.3	11	1.4
30	6	1.7	tox	tox	2	1.7	2	1.5	24**	1.2	6	1.3
40	8	1.9	tox	tox	2	1.4	2	1.4	tox	tox	tox	tox
60	4	1.4	tox	tox	14**	1.3	4	1.4	tox	tox	tox	tox
80	9	1.4	tox	tox	nd	nd	nd	nd	tox	tox	tox	tox
100	21***	1.3	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox
0.15 μM Mitomycin C	14		88		27		23		151		162	

*$P < 0.05$, **$P < 0.005$, ***$P <= 0.0005$ (according to the chi-square test)

Table 2 Summary of the Ames test results. Various metabolic activation systems were used with four different strains. Each condition was tested at least twice

Activation	Strain	*N* -NO-DCHA	Positive control
Pyrazole S9	TA1535	Negative	*N*-Nitrosopyrrolidine
	TA100	Negative	2-Aminofluorene
	TA98	Negative	2-Aminofluorene
β-Naphthoflavone/ phenobarbital S9	TA100	Negative	2-Aminofluorene
	TA1535	Negative	*N*-Nitrosodiethylamine
Arochlor-1254 S9	TA100	Negative	2-Aminofluorene
	TA104	Microcolonies[a]	*N*-Nitrosodiphenylamine
Without activation	TA100	Negative	Methylmethanesulfonate
	TA104	Negative	Methylmethanesulfonate

[a]Microcolony induction was seen using arochlor-1254-induced S9 (ICN) at pH 6.5

not shown). Thus, we investigated whether *N*-NO-DCHA, which can originate from dicyclohexylamine × nitrite, has genotoxic properties.

Weak genotoxic effects of *N*-NO-DCHA were seen in the micronucleus test with human lymphocytes (Table 1). In consideration of the NDI (NDI > 1.1), this effect was seen at non-toxic concentrations. The dose response showed two maxima. The second maximum may be related to toxic effects, although the NDI was above 1.1. Reproducibility seems to be limited by the poor solubility of *N*-NO-DCHA and culture conditions. Nevertheless, a trend towards an elevation was seen in each valid experiment, significant in four out of six experiments. This meets the currently accepted requirements for a positive micronucleus test (Miller et al. 1997).

In the Ames test with TA104, *N*-NO-DCHA induced microcolonies above 250 μg per plate when arochlor-1254 at pH 6.5 was used. The same effect was observed with *N*-nitrosodiphenylamine. This probably represents an effect that has been described for *N*-nitrosodiphenylamine in an earlier report (Zielenska and Guttenplan 1988). Concurrent to the disappearance of 'normal-shaped' colonies, the background lawn differentiated to microcolonies. The evaluation was additionally complicated by strong base-rate deviations using TA104 at pH 6.5. Since isolated positive results with TA104 are not known (Gatehouse et al. 1994), with the exception of the findings with *N*-nitrosodiphenylamine (Zielenska and Guttenplan 1988), this effect might be pH-related rather than substance-related.

N-NO-DCHA could not be activated by pyrazole- or BNF/PB-induced S9, although we applied highly concentrated S9-mix. In the case of the positive control *N*-nitrosopyrrolidine, 4% pyrazole-induced S9 is sufficient to generate a clear mutagenic response, and 20% BNF/PB-induced S9 (5 mM NADP and 25 mM glucose 6-phosphate) is able to activate *N*-nitrosodiethylamine efficiently (Westphal et al. 2000). Thus, either the cyclohexyl residues of *N*-NO-DCHA seem to be extremely stable or else the decomposition products of *N*-NO-DCHA are not mutagenic.

By analogy to the mechanism discussed for *N*-nitrosodiphenylamine (McGregor 1994), the genotoxic effects of *N*-NO-DCHA in the in vitro micronucleus test may be due to transnitrosation reactions. In this case, *N*-NO-DCHA would act as a nitrite carrier. Lucas et al. (1999) demonstrated the formation of nitrite adducts following

incubation with purine nucleotides and 2′-deoxyguanosine with *N*-nitrosoindoles. However, we did not observe mutagenic effects in the Ames test without metabolic activation.

In conclusion, *N*-nitrosodicyclohexylamine is weakly genotoxic in the micronucleus test with human lymphocytes, negative in the Ames test with TA100, TA98 and TA1535, and inconclusive in the Ames test with TA104 with metabolic activation at pH 6.5.

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