Comparison of Mutagenicity, Antitumor Activity, and Chemical Properties of Selected Nitrosoureas and Nitrosoamides

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ABSTRACT

A number of nitrosoureas and nitrosoamides have been compared with respect to mutagenicity for Salmonella typhimurium, in vitro cytotoxicity, in vivo toxicity and antitumor activity against murine L1210 leukemia, and chemical properties. Despite chemical similarities between the nitrosoureas and nitrosoamides, they show important differences in biological activity. Some of the nitrosoureas are very active antitumor agents, and they are less mutagenic than are the corresponding nitrosoamides, which lack antitumor activity.

INTRODUCTION

The chloroethyl nitrosoureas BCNU, 5 CCNU, and N-(2-chloroethyl)-N'-(trans-4-methylcyclohexyl)-N-nitrosourea are established antitumor agents of significant clinical value in the treatment of a variety of human tumors (8, 14, 36). However, nitrosoureas and related nitro compound are known to be potent mutagens and carcinogens (26, 28, 31). An increasing number of clinical reports are establishing an occurrence of new cancers in patients treated with alkylating agents, and at least 2 such instances have been reported in patients treated with a nitrosourea (10). Since it is likely that the currently used nitrosoureas will prove to be carcinogenic in humans, it is important to attempt to dissociate the chemical alterations which enhance the antitumor effect from those modifications which increase the carcinogenic effect. Such a dissociation would not only allow the selection of improved antitumor agents but also lead to a better understanding of the molecular events responsible for antitumor selectivity, mutagenicity, and carcinogenicity. Such understanding would then provide further opportunity to improve the selectivity of antitumor agents and the definition of compounds with mutagenic and carcinogenic potential.

We have compared the antitumor and mutagenic effects of a series of nitrosourea and nitrosoamide derivatives.

REFERENCES

1. Recipient of National Cancer Institute Grants CA-16783 and CA-06973.
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3. Recipient of National Cancer Institute Grant CA-13837.
4. Recipient of National Institute of Allergy and Infectious Diseases Grant AI-01650.
5. The abbreviations used are: BCNU, N,N'-bis(2-chloroethyl)-N-nitrosourea; CCNU, N-(2-chloroethyl)N'-cyclohexyl-N-nitrosourea; CCNU, N-(2-chloroethyl)-N-nitrosourea; MNU, N-methyl-N-nitrosourea; MNA, N-methyl-N-nitroso-acetamide; CENA, N-(2-chloroethyl)-N-nitroso-acetamide; NMR, nuclear magnetic resonance; t, triplet; s, singlet; m, multiplet; PNA, N-nitroso-N-propylacetamide; NPU, N-nitros-N-propylurea; NPU, N-nitroso-N-propylurea.

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Differences in molecular structure lead to a significant degree of dissociation of antitumor and mutagenic effects.

MATERIALS AND METHODS

Source of Drugs. BCNU and CENU were kindly provided by Dr. Harry Wood of the Drug Development Branch of the National Cancer Institute. MNU was prepared following the directions of Arndt (2). MNA was prepared by nitrosating N-methylacetamide with sodium nitrite and formic acid and purified by chromatography (ether on silica gel) to give a yellow oil which crystallized at -10° [literature, -8.5° (13)].

CENA was prepared as follows. To a mixture of N-(2-chloroethyl)-acetamide (3) (2.44 g, 20 mmol) and anhydrous sodium acetate (12 g, 0.15 mol) in 15 ml of methylene chloride was added dropwise a solution of dinitrogen tetroxide (5.5 g, 60 mmol) (from NO2) in 20 ml of methylene chloride at -10°. After addition, the reaction was stirred for an additional 45 min at -10°. The mixture was poured onto ice water (100 ml), and the organic phase was washed with ice-cold 5% sodium bicarbonate (3 x 30 ml) and with ice water; the organic phase was separated and dried over sodium sulfate at 0° for 40 min. The solution was filtered and concentrated at reduced pressure to yield an orange oil (2.65 g, 17.6 mmol, 88%); 1H NMR (CDCl3, internal standard, tetramethylsilane), δ 4.10 (t, 2H), 3.40 (t, 2H) and 2.76 (s, 3H); IR (CH2Cl2), 1730, 1510, 1380, 1345, 1117, 640 cm⁻¹; UV (η = 0.1), 428 nm (ε 136), 409 (128), 393 (76).

PNA was prepared as follows. A solution of N-(isopropyl)-acetamide (20 g, 19.8 mmol) in methylene chloride (20 ml) was cooled to -15° in a flask equipped with a magnetic stirrer and bar, and sodium acetate (11.3 g, 0.14 mol) was added. Dinitrogen tetroxide (5.5 g, 60 mmol) in methylene chloride (20 ml) was then added dropwise over a period of 10 min. The mixture was stirred at -15° for 3 hr. The reaction mixture was poured onto ice water (100 ml), and the organic phase was washed with ice-cold 5% sodium bicarbonate (3 x 30 ml) and with ice water; the organic phase was separated and dried over sodium sulfate at 0° for 40 min. The solution was filtered and concentrated at reduced pressure to yield an orange oil (2.25 g, 16.6 mmol, 84%); 1H NMR (CDCl3, internal standard, tetramethylsilane), δ 4.10 (t, 2H), 3.40 (t, 2H) and 2.76 (s, 3H); IR (CH2Cl2), 1730, 1510, 1380, 1345, 1117, 640 cm⁻¹; UV (η = 0.1), 430 nm (ε 106), 410 (98), 394 (59).

N-(isopropyl)-N-nitroso-acetamide was prepared as follows. N-(isopropyl)-acetamide (2.0 g, 19.8 mmol) was nitrosated with dinitrogen tetroxide (12.5 ml, 19.0 g, 0.21 mol) in 30 ml of methylene chloride in the presence of sodium acetate (25 g, 0.31 mol) at -15° to 0° for 2 hr. The work-up with 5% sodium carbonate solution (2 x 100 ml), saturated sodium chloride solution (1 x 100 ml), and water (1 x 100 ml) and the subsequent removal of solvent by rotary evapo-
ration gave 1.9 g (15 mmol, 74%) of the nitrosoamide: IR (CHCl₃) 1727, 1510, 1390, 1370, 1090, 980 cm⁻¹; UV\textsubscript{max} \(430\) nm (ε 77), 410 (72), 394 (47).

PNU was prepared as follows. A mixture of n-propylurea (2.04 g, 0.02 mol), methylene chloride (50 ml), and anhydrous sodium acetate (16.4 g, 0.20 mol) was cooled to \(-10°\), and 7.24 g (5 ml, 79 mmol) of dinitrogen tetroxide were added in one portion. The resultant light blue solution was allowed to warm gradually to room temperature (at \(-5°\), foaming occurred and the blue color was discharged). The methylene chloride solution was washed with two 100-ml portions of ice-cold, 5% sodium carbonate followed by two 100-ml portions of ice-cold, saturated sodium chloride solution. The organic layer was separated, dried over sodium sulfate, and evaporated on a rotary evaporator at 0° to give 2.6 g (0.15 mol, 73%) of N-(2-chloroethyl)-N-nitrosobutyramide (3) and 32.8 g (0.40 mol) of anhydrous sodium sulfate, filtered, and concentrated under vacuum at \(-10°.\)

The course of nitrosation of the amides and ureas was best followed by the shift of the carbonyl IR frequencies and the development of the UV absorption at \(\approx 400\) nm. The nitroso compounds contained no unnitrosated starting amide or urea detectable by IR.

**In Vitro Cytotoxicity.** Murine L1210 leukemia cells were harvested from female C57BL/6 × DBA/2F₁ (hereafter called B6D2F₁) mice 4 days after inoculation i.p. with \(10^6\) cells (11). The cells (2 \(×\) \(10^6\) ml) were then incubated at 37° in RPMI Roswell Park Memorial Institute Medium 1630 (Grand Island Biological Co. Grand Island, N.Y.) buffered at pH 7.4 with 0.05 m phosphate for 30 min with the desired concentration of drug. At the end of the incubation, the cells were sedimented at 600 rpm, resuspended in Medium 1630, and injected into B6D2F₁ mice (0.5 ml, \(10^6\) cells/mouse, 5 mice/group). The mean survival time of these mice was compared to those of mice given injections of the same number of cells incubated with medium alone.

**In Vivo Antitumor Effect and Toxicity.** Female B6D2F₁ mice (5/group) were given i.p. injections of \(10^6\) L1210 leukemia cells. On Day 4, the mice were given injections i.p. of the desired amount of drug in 0.2 ml of ethanol: propylene glycol/water (30:30:40). (Compound XI, was given in dimethyl sulfoxide). The mean survival time of these mice was compared to that of tumor-bearing mice which had been given injections of solvent alone. Long-term survivors were counted as having died on Day 60.

**Decomposition Rates.** The nitrosoureas (0.1 mg/ml) or the nitrosoamides (0.3 mg/ml) were incubated at 37° in 0.1 m sodium phosphate buffer, pH 7.4, containing 1% ethanol. At intervals, aliquots were removed and analyzed for content of nitroso compounds by the Britton-Marshall technique (25).

**Decomposition Products.** The nitroso compound (0.02 mmol) was incubated in 0.1 m sodium phosphate buffer, pH 7.4, (2.5 ml) for 4 days at 37°. The solution was then analyzed by isothermal gas chromatography using a 10-foot glass column packed with Chromosorb 101 (Supelco, Inc., Bellefonte, Pa.) at 120° (12). No liquid phase was used.

**Salmonella Mutagenicity Screen.** Ames tester strains were plated by a standard procedure (1) and by a preincubation procedure described in the legend to Chart 1.

**RESULTS**

**Chemical Studies.** Previous studies (12, 33) have demonstrated that the chloroethynitrosoureas decompose in aqueous solutions at physiological pH to form chloroethanol, acetaldehyde, vinyl chloride, and dichloroethane. Evidence has been presented (6) that these products arise from the intermediate chloroethyldiazohydroxide or diazonium ion. The decomposition of the methyl and chloroethynitrosoureas and nitrosoamides (Table 1, Compounds I to IV), yielded the products shown in Table 2. These products are analogous to those from the nitroso compounds and are consistent with the hypothesis that the nitrosoamides, like the nitrosoureas, decompose in aqueous base to yield an alkyldiazonium intermediate.

**Antitumor Effect and Whole-Animal Toxicity.** The compounds tested were either nitrosoureas (III to V, VIII) or nitrosoamides (I, II, VI, VII, IX, X). Only the nitrosoureas III to V showed a significant antitumor effect (>20% increase in life span of treated animals (Table 1)) against L1210 murine leukemia. Further, the chloroethynitrosoureas were at least 20-fold more active than methyl nitrosourea on a
molar basis. As can be seen in Table 1, the chloroethylnitrosoureas were significantly more toxic to the whole animal than the other compounds, with the exception of Compound II. CENA exhibited whole-animal toxicity at levels similar to the chloroethylnitrosoureas but exhibited no antitumor activity.

Mutagenicity. Seven compounds were screened for relative mutagenic activity in a limited number of trials using the standard Ames procedure designated 'Ames' in Chart 1 and by a preincubation procedure described in the chart legend. In Chart 1, relative mutagenicity decreases progressively from the upper left to the lower right. Comparison of the paired data for MNA, CENU, and PNU indicates that the preincubation procedure enhanced mutation yield some 20-fold over the standard Ames test. A similar enhancement of mutagenicity was found for the 3 other compounds tested (data not shown). Relative mutagenicity decreased with increasing chain length, e.g., MNA compared to PNA, and it appeared that the slopes of the dose-response curves similarly decreased. In a similar test, N-isopropyl-N-nitrosoacetamide failed to exhibit mutagenic activity for strains TA1535 and TA100. In the 3 cases where pairs of analogs of equal chain length were tested (MNA and MNU, CENA and CENU, PNA and PNU), the nitrosourea was lower in mutagenic activity, indicating that most of the activity found for CENA operates other than by displacement of the chloride ion. Mutagenicity of none of the compounds was enhanced by the addition of S9 (microsomal) extract to the standard Ames test (1); in fact, mutagenic activity often was decreased slightly by S9 addition (data not shown). CENA failed to increase the mutation frequency in Ames frame-shift tester strains TA1537 and TA1538 (chart 1, bottom curve), indicating that this class of mutagens predominantly causes base substitution mutations and is ineffective in eliciting frame-shift mutations.

DISCUSSION

Recent reviews have summarized knowledge concerning the mutagenicity (28, 31) and carcinogenicity (26, 28) of some of the compounds surveyed here. Our observations on mutagenicity, which can be considered only as preliminary tests, showed a decrease in mutagenicity of nitrosoacetamides and nitrosoureas with an increase in N-alkyl chain length. This observation is in accord with the ability of Endo et al. (17) to nitrosate methylurea to a potent mutagen while butylurea failed to yield detectable mutagen for S. typhimurium strain TA1535. In addition, Lee et al. (24) found that MNU was a more potent mutagen for TA1535 than was N-ethyl-N-nitrosourea. This relationship in measured genetic activity is opposite that found in some other systems (20, 30, 31); for example, Heineman (19) found that the minimal concentrations (µg/ml) effective in induction of λ prophage of Escherichia coli were 25 for MNU, 10 for N-ethyl-N-nitrosourea, and 2 for CENU. Such differences in relative dose relationships with different test systems remain unexplained, but Neale (31) and Lee et al. (24) discuss a number of factors that could be involved. In our tests, there is little evident effect of the uvrB mutation or of the pKM101 plasmid, factors that enhance mutagenicity to other classes of compounds (1). On the other hand, the rfa mutation, which enhances permeability of larger molecules, may enhance mutagenicity by compounds with longer alkyl chain length.

The relative mutagenic activities of various homologs vary substantially; e.g., over a 10-fold difference in mutagenic activity exists between MNA and MNU and between MNA and PNA. Thus, this series of compounds could be
### Properties of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>ILS(^a) (%)</th>
<th>ED (mmol/kg)</th>
<th>(\text{in vivo} \text{ LD}) (mmol/kg)</th>
<th>(\text{in vitro} \text{ LD}) (μM)</th>
<th>(t_{1/2}) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>MNA</td>
<td>0</td>
<td>None (^c)</td>
<td>2.0</td>
<td>25</td>
</tr>
<tr>
<td>II</td>
<td>CENA</td>
<td>0</td>
<td>None</td>
<td>0.1</td>
<td>50</td>
</tr>
<tr>
<td>II</td>
<td>MNU</td>
<td>70</td>
<td>1.0</td>
<td>2.5</td>
<td>100</td>
</tr>
<tr>
<td>IV</td>
<td>CENU</td>
<td>500</td>
<td>(all indefinite survivors)</td>
<td>0.01</td>
<td>12</td>
</tr>
<tr>
<td>V</td>
<td>BCNU</td>
<td>500</td>
<td>(all indefinite survivors)</td>
<td>0.05</td>
<td>12</td>
</tr>
<tr>
<td>VI</td>
<td>PNA</td>
<td>0</td>
<td>Undetermined (^d)</td>
<td>(2.0)</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>iPNA</td>
<td>0</td>
<td>None</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>PNU</td>
<td>0</td>
<td>Undetermined (^d)</td>
<td>(2.0)</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>CNBA</td>
<td>0</td>
<td>None</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>CBA</td>
<td>0</td>
<td>Undetermined (^d)</td>
<td>(0.1)</td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>CNPA</td>
<td>0</td>
<td>None</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ILS, maximum increase in life span over tumor controls; ED, lowest dose giving >20% increase in life span over tumor controls; \(\text{in vivo} \text{ LD}\), lowest dose giving >20% decrease in life span compared to tumor controls; \(\text{in vitro} \text{ LD}\), lowest concentration killing all tumor cells during 30 min incubation; iPNA, N-(isopropyl)-N-nitrosoacetamide; CNBA, N-(2-chloroethyl)-N-nitroso-n-butyramide; CBA, N-(2-chloroethyl)-n-butyramide; CNPA, N-(2-chloroethyl)-N-nitrosopivalamide.

\(^b\) In 0.05 M sodium phosphate buffer, pH 7.4.

\(^c\) No effective dose found before lethal dose reached.

\(^d\) The highest dose tested (in parentheses) was neither effective nor lethal.
The 2 nitrosoureas have significant antitumor activity with exhibit interesting differences in their biological activities. The 2 methyl compounds (MNU and MNA) give methanol as the major product detected but not quantitated; —, product not present.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CICH2CH2OH</th>
<th>CH3OC-CH3</th>
<th>CICH2CH2Cl</th>
<th>CICH2CH2OC-CH3</th>
<th>CH3CH2OH</th>
<th>CH2OC-CH3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CENU</td>
<td>82</td>
<td>13</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENA</td>
<td>75</td>
<td>17</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MNU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>MNA</td>
<td></td>
<td></td>
<td>+</td>
<td>85</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mol percentage of nitroso compound reacted.

\(^b\) +, product detected but not quantitated; —, product not present.

valuable in assisting consideration of the predictive value of various short-term tests for genetic activity in relation to carcinogenic potency (cf. Ref. 27). An understanding of structure-activity relationships is important because of the striking organospecificity of carcinogenesis by nitrosoamides.

The results reported here are in agreement with the previous findings of the structure-function studies of Montgomery (29) and Hyde et al. (21). These studies indicated that certain structural features are essential for optimal antitumor activity of the molecule. In particular, the compound must be a nitrosourea, and the alkyl group must be a chloroethyl or fluoroethyl group.

Compounds I to IV are a series of chemically similar compounds. All the compounds are small, nonpolar molecules. The octanol:water partition coefficients of the nitrosoureas and nitrosoamides (log P of CENA = 1.2) are well within the range of nitrosamines with antitumor activity (29). The nitrosamines also have half-lives in pH 7.4 buffer similar to those of the antitumor nitrosoureas (Compound V). Both the nitrosoureas and the nitrosoamides are believed to decompose through a diazohydroxide intermediate (18). The similarity in the decomposition mechanisms is supported by the decomposition products found in this study. The 2 chloroethyl compounds, CENU and CENA, give chloroethanol and acetaldelyde as major products and 1,2-dichloroethane and vinyl chloride as minor products. The 2 methyl compounds (MNU and MNA) give methanol as the major product.

Despite these chemical similarities, the 4 compounds exhibit interesting differences in their biological activities. The 2 nitrosoureas have significant antitumor activity with the chloroethyl derivatives being more effective than the methyl, while neither of the nitrosoamides exhibits antitumor activity. All 4 compounds are, however, cytotoxic to tumor cells in vitro. All 4 are also toxic to the whole animal with the chloroethyl derivatives being more toxic than the methyl. Surprisingly, high in vitro cytotoxicity to tumor cells and low in vivo toxicity do not correlate with in vivo antitumor activity. For example, Compound IV (CENU), an effective antitumor agent producing indefinite survivors, has the lowest in vivo lethal dose to in vitro lethal dose ratio, while Compound I (MNA), which has the highest ratio, has no in vivo antitumor activity. All 4 compounds are mutagenic with the nitrosoamide being more mutagenic than the corresponding nitrosourea.

Thus, in this series there is a dissociation of biological effects. The nitrosoamides, which have no antitumor activity, are more mutagenic than the nitrosoureas, which are very active antitumor agents. While there are many factors involved in mutagenicity and antitumor activity, the chemical similarity of the nitrosoureas and nitrosoamides eliminates many of them. In particular, if both antitumor activity and mutagenicity are due to alkylation, the event responsible for this dissociation of biological effects must occur before or during decomposition to diazohydroxide since both the nitrosoureas and nitrosoamides appear to react via an identical diazohydroxide-alkylating intermediate. One possibility is that the decomposition occurs at different sites and the that diazohydroxide alkylates before it can diffuse away. The preference for different decomposition sites could be due to the fact that the decomposition of the nitrosoureas is initiated by proton extraction (40) while the decomposition of the nitrosoamides is initiated by the addition of a nucleophile (40). This idea is supported by the fact that the closely related nitrosocarbamates, which like the nitrosoamides decompose by the nucleophile addition mechanism, also are inactive as antitumor agents and are mutagens (22, 28).

A second possibility to explain the dissociation in activity is that the compounds are taken up differently by tumor cells, normal cells, and the bacteria that we have studied. This possibility is made less likely by the fact that the nitrosoureas (BCNU in particular) are not actively transported but enter cells by diffusion (5).

A third possibility must be considered since the nitrosoureas decompose not only to alkylating species but also to isocyanates with carbamoylating activity. Cheng et al. (9) demonstrated that the ethylene carbons from the nitrosocarbamates of CCNU bound to DNA, RNA, and proteins, while the carbons of the cyclohexyl portion of the molecule bound almost exclusively to proteins. Subsequently, these investigators demonstrated that the binding of the cyclohexyl group to proteins was due to the carbamoylation of lysine residues by cyclohexyl isocyanate (34). The carbamoylation of the terminal α-amino group of protein also occurs (39). The functional significance of the carbamoylation of proteins by isocyanates has been examined by several investigators. Wheeler and Bowdon reported that BCNU inhibited DNA polymerase activity in L1210 cells and demonstrated that the effect was mediated by chloroethyl isocyanate (37). Baril et al. (4) have subsequently demonstrated that DNA polymerase II, but not DNA polymerase I, is inhibited by the alkyl isocyanates produced from BCNU and CCNU. Kann et al. (22) have described the inhibition of the repair of irradiation-produced DNA strand breaks by BCNU and chloroethyl isocyanate. More recently,
Kann et al. have reported that the organic isocyanates generated from nitrosoureas inhibited RNA synthesis and processing in L1210 cells (23). The role of these in vitro carbamoylating effects of nitrosoureas in the antitumor effect and toxicities of nitrosoureas remains uncertain. However, Wheeler et al. (38) found that a measurement of alkylating activity (nitrobenzylpyridine reactivity) of a series of nitrosoureas was correlated with the antitumor activity of the compounds, while the ability to carbamoylate lysine was not. Also, Panasci et al. (32) in recent work have found that the carbamoylating activity of a series of nitrosoureas correlated with neither the toxicity nor the antitumor effect of the drugs. In this study, the alkylating activity of the compounds was correlated with the whole-animal toxicity but not with the antitumor effect.

The further elucidation of the biochemical basis for the dissociation of the antitumor and mutagenic effects of such similar compounds as CENA and CENU should lead to a better understanding of the chemical and biochemical determinants of antitumor effect versus mutagenicity (and presumably carcinogenicity) and be of major importance to the design of antitumor agents.

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