Biological and Biochemical Properties of the 2-Hydroxyl Metabolites of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea

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ABSTRACT

The lethal and bone marrow toxicity and antitumor activity of the cis- and trans-2-hydroxylated metabolites of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) have been correlated with their relative in vitro alkylating and carbamoylating activities. Both the isomers have considerably greater alkylating activity and shorter chemical half-lives than the parent compound and on a molar basis have greater antitumor activity against L1210 leukemia. However, in terms of molar doses resulting in the death of 10% of normal mice, the cis- and trans-2 isomers were 2- and 3-fold more toxic than was CCNU in normal mice. In comparing the antitumor activity produced by a maximum nonlethal dose for each compound, we found that the trans isomer had activity identical to that of CCNU (413 and 410% increased life span compared to control), and the cis isomer had considerably less (152%). Like chlorozotocin, both isomers possess low carbamoylating activity and increased water solubility, two features that have been considered possible contributors to the bone marrow-sparing character of chlorozotocin. However, in the murine model the human bone marrow colony formation (CFU-C) assay neither hydroxylated metabolite of CCNU was associated with reduced myelotoxicity.

INTRODUCTION

CCNU, a cyclohexyl chloroethylnitrosourea, is a lipid-soluble antitumor agent that has demonstrated activity in animal models and a wide variety of histological types of human cancer (4). Unfortunately, its major toxicity is cumulative bone marrow suppression, which may give rise to a steady state of chronic hypoplasia and significantly limit its clinical usefulness (7, 16).

Hansch et al. (6) predicted that nitrosoureas with greater water solubility might possess greater antitumor activity and reduced toxicity as measured by lethality in mice. Attachment of the cytotoxic group to a sugar moiety, as in the water-soluble nitrosoureas chlorozotocin and 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea has been demonstrated to reduce bone marrow toxicity at LD10 without impairing its antitumor effect for the murine L1210 leukemia (1, 5, 12). In rats and humans, CCNU is rapidly metabolized in vivo to polar monohydroxylated products by liver microsomes (10, 25), which retain both the cyclohexyl ring structure and the cytotoxic nitrosoureido moiety (15). The hydroxy group confers the property of increased solubility in water compared to CCNU. It has been proposed that the antitumor activity of CCNU is mediated by these hydroxylated metabolites (10).

Wheeler et al. (27) have studied 6 hydroxylated isomers of CCNU and found them all, on a molar basis, to be more active against L1210 leukemia than was CCNU, the parent compound. In addition, all the hydroxylated derivatives had better therapeutic indices (dose causing 50% of mice to survive at least 45 days after i.p. implantation of 10^5 L1210 cells/LD10) than either CCNU or chlorozotocin. The trans-2-isomer appeared the most promising, with antitumor activity similar to chlorozotocin but with a higher therapeutic index. The carbamoylating activity of this compound was also significantly lower than CCNU, a parameter that has previously been correlated with nitrosourea toxicity in 1 study (26) but not in other analyses (8, 19). Because of these features and the relatively increased water solubility conveyed by the hydroxy groups, it was suggested that the metabolites might have advantages over CCNU for clinical use. As yet, no data are available regarding their relative bone marrow toxicity. In this paper we correlate the lethal and bone marrow toxicity and antitumor activity of the cis- and trans-2-hydroxylated isomers of CCNU with their relative alkylating and carbamoylating activity.

MATERIALS AND METHODS

Animal Studies. Male BALB/c × DBA/2 F1, mice (hereafter called CD2F1), weighing 17 to 25 g and maintained on Lablox laboratory chow pellets and water ad libitum, were used throughout. Each group consisted of 10 mice.

The following drugs were investigated: CCNU (NSC 79037), trans-2-OH-CCNU (NSC 253947), cis-2-OH-CCNU (NSC 253946), and 2-[3-{2-chloroethyl)-3-nitrosoureido]-D-glucopyranose (chlorozotocin) (NSC 178248). CCNU and chlorozotocin were kindly supplied by Dr. Harry Wood, Drug Development Branch, National Cancer Institute, Bethesda, Md. The hydroxylated CCNU derivatives were synthesized by Dr. George S. McCaleb and kindly provided by Dr. Thomas P. Johnston of the Southern Research Institute, Birmingham, Ala.

CCNU was suspended in an aqueous solution of polyethoxylated vegetable oil, ethanol, and citrate buffer (5:5:90). The hydroxylated derivatives were dissolved in 5.0% alcohol solution in 0.01 M citrate buffer, pH 4.0, and chlorozotocin was dissolved in citrate buffer alone. All drugs were given i.p. as a single dose in a volume of 0.1 ml/10 g body weight.

The murine L1210 system was used to assess antitumor...
activity. Treatment was administered as a single LD_{50} on the second day after i.p. implantation of \(1 \times 10^5\) L1210 leukemia cells. The percentage of increased life span was calculated from the survival of control tumor-bearing animals that received appropriate volumes of the respective diluents (21). Groups of 10 normal CDF1 mice received a single i.p. dose of drug. Serial WBC were performed over a 28-day period; a 20-µl sample of retro-orbital sinus blood was placed in 9.98 ml of Isoton (Curtin-Matheson, Beltsville, Md.) and counted in a Model ZBI Coulter counter after lysis of RBC with Zap-oglobin (Curtin-Matheson). Differential WBC were performed on Wright-stained blood smears, taken on the third day following treatment. This time was chosen because it corresponded with the nadir of the WBC after nitrosourea treatment in this species (1). The absolute neutrophil counts are presented as a percentage of control values.

Biochemical Studies

Alkylating Activity. Each nitrosourea, 0.25 to 2.0 µmol, was dissolved in acetone, reacted with 4-(p-nitrobenzyl)-pyridine, and the absorbance was determined at 540 nm as described by Wheeler et al. (26). The micromoles of drug giving an absorbance of 0.5 were taken as the alkylating activity, and this was expressed as a percentage of the value for CCNU, the compound with the lowest alkylating activity. As a result, the more potent alkylators have higher percentage figures.

Carbamoylating Activity. In vitro carbamoylating activity was measured following a 6-hr incubation of 4.2 µmol of test nitrosourea with 4.2 µmol of [14C]lysine in 0.1 M sodium phosphate buffer, pH 7.4, at 37°C. After electrophoresis, the peak of radioactivity not associated with the parent [14C]lysine was regarded as the carbamoylated products. These counts, calculated as a percentage of the total radioactivity, were termed the carbamoylating activity of the compound (20) and were then expressed as a percentage of CCNU. Values obtained on separate occasions gave a S.E. of 2%.

Chemical Halflife. The half-life of the compounds, in min, was measured by following the change in absorbance at 230 nm during incubation at 37°C. Solutions of the soluble drugs were made in 0.1 M sodium phosphate buffer to give an initial absorbance between 0.5 and 1.0 at 230 nm. CCNU was first dissolved in absolute ethanol and then diluted 20-fold with the sodium phosphate buffer, pH 7.4.

Human Hematopoietic Cell (CFU-C) Assay. The washed buffy coat of human bone marrow aspirated from the posterior iliac crest of normal volunteers (age, 21 to 35 years) was suspended in McCoy's Medium 5A (NIH media; NIH, Bethesda, Md.) at a final concentration of 2.5 to 3.0 × 10^6 nucleated cells/ml (3). Chlorozotocin was prepared directly in McCoy's medium. CCNU and the cis and trans isomers were first dissolved in absolute ethanol and then diluted 20-fold with the sodium phosphate buffer. The results were expressed as a percentage of control values.

RESULTS

Biochemical Activity. The in vitro alkylating activity of the cis and trans isomer in Table 1 is 2 and 4 times that of an equimolar concentration of the parent compound, but neither drug is as active an alkylator as chlorozotocin. This increased alkylating ability of the CCNU metabolites is associated with an increase in their rate of decomposition, which is faster for the trans-2 isomer, the stronger alkylator. Hydroxylation of the cyclohexyl group of CCNU at position 2 results in an approximate 90% reduction in carbamoylating activity. However, both cis and trans isomers still retain 2 and 3 times as much in vitro carbamoylating activity when compared to chlorozotocin (Table 1).

Lethal Toxicity. The maximum nonlethal dose of CCNU, 128 µmol/kg, is twice that of the cis-2-OH isomer and about 3 times that of the more toxic compounds, the trans-2 isomer and chlorozotocin. The same relationship holds for LD_{50} (Table 2).

Murine Bone Marrow Toxicity. At the highest nonlethal dose of CCNU, the nadir absolute neutrophil count was 22% of control, which was not statistically different from 23 and 32% for the corresponding LD_{50} of the cis and trans isomers, respectively (p > 0.05). At 80 µmol/kg the trans-2 isomer is more myelosuppressive than was an equimolar dose of the cis form. Chlorozotocin, given at both a maxi-

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<td>CCNU</td>
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<td>Cis-2-OH</td>
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<td>4</td>
<td>663</td>
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* Measured in 0.1 M sodium phosphate buffer, pH 7.4, at 37°C.

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Table 2

Biological toxicity in normal CD2F1 Mice

<table>
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<tr>
<th>Drug</th>
<th>Dose (µmol/kg)</th>
<th>Lethality (% in normal mice)</th>
<th>% control</th>
<th>Nadir WBC</th>
<th>Nadir neutrophil count</th>
<th>Nadir lymphocyte count</th>
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<td>0</td>
<td>39</td>
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<td>54</td>
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<td>24</td>
<td>39</td>
<td>11</td>
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<tr>
<td>a</td>
<td>Peripheral WBC on Day 3 after single i.p. injection. Each group consisted of 10 mice.</td>
<td></td>
<td></td>
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<tr>
<td>b</td>
<td>Not statistically different from control (p &gt; 0.05).</td>
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</table>

Discussion

In vitro, under physiological conditions, the nitrosoureas degrade chemically to yield reactive intermediates. Of these the chloroethyl carbonium ions or diazonium hydroxide precursors are the principal alkylating products (2, 17). In addition organic isocyanates are generated that carbamylate the lysine residues of proteins (23). Despite the long chemical half-life of CCNU in 0.1 M sodium phosphate buffer, pH 7.4, at 37°, the biological half-life is considerably faster in both rat and human plasma (18, 24). A biphasic pattern is seen having an initial clearance with a half-life of 5 to 6 min and a second much longer component with a half-life of 47 min.

Several authors (9, 10, 15, 22) have demonstrated rapid enzymatic hydroxylation of the cyclohexyl moiety of CCNU by NADPH-dependent mixed-function oxidases. Five polar metabolic products of CCNU have been isolated and identified by mass spectrometry (10, 22). The chloroethyl, cyclohexyl, and nitroso groups of these metabolites are intact (15). After a 5-min incubation of CCNU with rat liver microsomes, only 2% of the parent compound remains, while 85% is in identifiable hydroxylated form, predominantly as the cis and trans-4 and trans-3 isomers (10). In rats an
equally rapid metabolism occurs in vivo; 96% of an i.v. dose of CCNU (30 mg/kg) is hydroxylated within 20 min (10). Less information is available in humans. However, in 4 patients studied immediately after an i.v. infusion of CCNU, 75% of the plasma drug was already in the hydroxylated form (11, 25). These observations lend support to the concept that much of the antitumor activity of lipid-soluble CCNU may be due to its more polar metabolites.

The 2-hydroxylated isomers were studied because they had been reported to have the highest therapeutic ratio, and their low carbamoylating activity allowed the assessment of carbamoylation as an independent variable. Our data confirm many of the findings of Wheeler et al. (27). Monohydroxylation of the cyclohexyl group at position 2 produces a less stable molecule in vitro than does CCNU. This is demonstrated by a shortened half-life of both isomers in phosphate buffer, pH 7.4, which is associated with a markedly increased alkylation activity. This inverse correlation between the t1/2 and the alkylation activity has been previously noted (20, 26).

Compounds with a high alkylation ability are usually more toxic to animals when assessed by lethality, but alkylation has also been reported to play a major role in determining antitumor activity (26). The cis-2 isomer has twice the alkylation activity and is 1.5 times as toxic as CCNU on a molar basis. The trans-2 isomer has 4 times the alkylation activity but is 3 times as toxic on a molar basis as is CCNU. At equimolar doses both metabolites are more effective antitumor agents than CCNU against i.p. L1210 leukemia in mice; however, because of the greater toxicity of the isomers, less drug is tolerated. At a dose producing comparable lethality in normal mice, LD(30), the trans isomer has antitumor activity equal to that of CCNU while the cis isomer is less effective.

While in vitro studies have correlated carbamoylating activity with an alteration of cellular chemistry, its role in the biological activity of the nitrosoureas is questionable (20). Both hydroxylated products have a significantly lower carbamoylating activity in vitro than does CCNU. The mechanism may be similar to that proposed for the reduced carbamoylating activity of streptozotocin and chlorozotocin, i.e., internal carbamoylation with the hydroxyl group on the cyclohexyl ring (19). Unlike chlorozotocin, which is non-myelosuppressive, both 2-hydroxylated isomers significantly depressed the absolute neutrophil count at their respective LD(30)'s. This confirms the lack of correlation between in vitro carbamoylating activity and neutrophil suppression (5), which was previously considered an important parameter contributing to toxicity (26). The greater water solubility of the metabolites similarly did not result in reduced bone marrow toxicity.

The dose-response curve for lethality is steeper for both 2-hydroxylated isomers than for CCNU or chlorozotocin, and the margin of safety is therefore narrower. There is a difference of only 10 mg/kg (40 μmol/kg) between the LD(30) (maximum nonlethal dose) and LD(100) (dose resulting in the death of 100% of normal mice) for the trans isomer and 20 mg/kg (80 μmol/kg) for the cis-hydroxylated derivative. These figures can be compared with 35 mg/kg (112 μmol/kg) for chlorozotocin and for the fact that 20 mg/kg (86 μmol/kg) for CCNU raises the lethal dose only from LD(30) to LD(90) (dose resulting in death of 30% of normal mice) (Chart 3).

The in vitro CFU-C assay measures human stem cells that are committed to granulocyte-macrophage differentiation and has been used to study the relative myelosuppressive
activity of chemotherapeutic agents (14). In this system the cis and trans-2 isomers produced a decrease in colony formation equal to that of CCNU at all 3 concentrations. Chlorozotocin had significantly less inhibition at each dose level (p < 0.01).

Apart from the greater solubility and therefore ease of handling of the 2 metabolic products, their lower carbamylation and higher alkylating activity is not translated into any clear advantage in either the mouse model or human CFU-C system. They do not have the potential marrow-sparing property of the water-soluble chlorozotocin, and the narrow margin of safety in mice may limit their usefulness as clinical agents.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Joan Bull for assistance in performing the CFU-C assay.

REFERENCES

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