Interaction of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037) with Nucleic Acids and Proteins in Vivo and in Vitro

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SUMMARY

The macromolecular binding of radioactivity from 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, labeled with \(^{14}C\) in either the cyclohexyl moiety or the ethylene residue, was studied in the L1210 leukemia-bearing mice, in a suspension of L1210 leukemia cells, and during in vivo incubation with isolated nucleic acids and proteins. In all three systems, radioactivity from cyclohexyl-\(^{14}C\)-labeled 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea was extensively bound to proteins, and there was negligible binding to nucleic acids. Radioactivity from the ethylene-\(^{14}C\)-labeled drug was bound to both nucleic acids and proteins, but the binding was only a fraction of the observed protein binding of the cyclohexyl label. Of the various macromolecules examined for in vitro binding, poly-L-lysine and albumin were the most active in binding the cyclohexyl-labeled material; polyguanylic acid, polycytidylic acid, and tRNA were the most active in binding the ethylene-\(^{14}C\)-labeled material.

The possibility that the carcinostatic activity of this drug reflects a dual capacity, namely, modification of cellular proteins via cyclohexylcarbamoylation and of nucleic acids via alkylation, is discussed.

INTRODUCTION

The nitrosoureas offer a relatively new and promising class of antineoplastic agents. Of particular interest is the compound CCNU.\(^3\) Because of its high lipid solubility and permeability through the blood brain barrier (16), CCNU might be particularly effective in the treatment of certain neoplasms of the central nervous system. Indeed, there is evidence that systemically administered CCNU and BCNU are effective in the treatment of meningeal leukemia in the mouse (11, 19) and in children who have acute leukemia with central nervous system involvement (9). In addition, preliminary studies indicate that CCNU and BCNU might exert a beneficial effect in patients with malignant gliar tumors (8, 22, 25).

Despite their clinical interest, the mechanism of action of CCNU and related nitrosoureas such as BCNU is not known. The following facts suggest that they do not function as conventional alkylating agents. (a) In human Hodgkin's disease, development of resistance to conventional alkylating agents is not associated with cross-resistance to BCNU (10). (b) CCNU and BCNU appear to act at phases of the cell cycle different than those of the known alkylating agents (2, 20, 23). (c) Although the alkylating potency of CCNU is lower than that of BCNU (24), it is somewhat more effective in the treatment of leukemia L1210 (4). For these reasons, we undertook a study of the possible binding of 2 different moieties of CCNU, the \(^{14}C\)-labeled cyclohexyl residue and the \(^{14}C\)-labeled ethylene residue, to cellular DNA, RNA, and protein, both in vivo and in vitro.

MATERIALS AND METHODS

Radioactive CCNU was kindly supplied by the Cancer Chemotherapy National Service Center, Bethesda, Md., with \(^{14}C\) label in 2 separate positions of the molecule (Chart 1), uniformly throughout the carbon atoms of the cyclohexyl ring (cyclohexyl-\(^{14}C\)-labeled CCNU; 2.498 mCi/m mole) or in the carbon atoms of the 2-chloroethyl moiety (ethylene-\(^{14}C\)-labeled CCNU; 1.338 mCi/m mole).

Bovine albumin Fraction V powder (unesterified, fatty acid-poor form), bovine globulin, 40% \(\beta\), 30% \(\gamma\) (Cohn Fraction II, III), and horse heart cytochrome \(c\) were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio; calf thymus histone (HLY, regular) and calf thymus DNA were from Worthington Biochemicals Corp., Freehold, N. J.; \(E_{scherichia\ colon} \) tRNA was from General Biochemicals, Inc., Chagrin Falls, Ohio; bovine pancreas RNase A (type 1-A) was from Sigma Chemical Co., St. Louis, Mo.; RNase T\(_2\) was from Sanyo Co., Tokyo, Japan; Pronase (B grade) was from Calbiochem, Los Angeles, Calif.; and poly-L-lysine, poly-L-arginine, poly U, poly A, poly (G\(_1\)U\(_3\)), poly G, and poly C were from Miles Laboratories, Inc., Elkhart, Ind.

In Vivo Experiments. DBF male mice, weighing around 20 g, were obtained from Breeding Laboratory, New York, N. Y. On the 7th day after i.p. inoculation of 2 \(\times\) 10\(^6\) cells of leukemia L1210, groups of 3 mice received i.p. injections of 0.5 mg of either cyclohexyl-\(^{14}C\)-labeled CCNU (5 \(\mu\)Ci) or...
Table 1

Binding of cyclohexyl-\(^{14}\)C-labeled CCNU to nucleic acids and proteins in vivo

<table>
<thead>
<tr>
<th></th>
<th>Radioactivity present (pmoles/mg) in</th>
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<tbody>
<tr>
<td></td>
<td>RNA</td>
<td>DNA</td>
<td>Protein</td>
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<tr>
<td>Brain</td>
<td>2.2</td>
<td>88.7</td>
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<tr>
<td>Liver</td>
<td>0.6</td>
<td>1.1</td>
<td>125.4</td>
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</tr>
<tr>
<td>Leukemia cells</td>
<td>1.1</td>
<td>9.4</td>
<td>431.6</td>
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</tr>
</tbody>
</table>

Table 2

Binding of ethylene-\(^{14}\)C-labeled CCNU to nucleic acids and proteins in vivo

<table>
<thead>
<tr>
<th></th>
<th>Radioactivity present (pmoles/mg) in</th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA</td>
<td>DNA</td>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>17.4</td>
<td></td>
<td>10.4</td>
<td></td>
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<tr>
<td>Liver</td>
<td>18.5</td>
<td>20.6</td>
<td>17.5</td>
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<tr>
<td>Leukemia cells</td>
<td>26.3</td>
<td>26.0</td>
<td>18.5</td>
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</table>

Chart 1. Molecular structure of cyclohexyl-\(^{14}\)C-labeled CCNU (top) and ethylene-\(^{14}\)C-labeled CCNU (bottom). *, labeled position.

Chart 2. Time course of the binding of cyclohexyl-\(^{14}\)C-labeled CCNU (A) and ethylene-\(^{14}\)C-labeled CCNU (B) to the acid-insoluble fraction of a cell suspension of leukemia L1210.
Radioactivity present (pmoles/mg) in RNA, DNA, and proteins follows.

Protein Cyclohexyl-\(^{14}\)C-labeled CCNU 0, 1.2, 0.7, 2.0
Ethylene-\(^{14}\)C-labeled CCNU 0, 24.0, 32.0

For the assay of radioactivity bound to poly-L-lysine, 5% trichloroacetic acid containing 0.25% sodium tungstate (pH 2.0) was used instead of 5% trichloroacetic acid (7).

Chemical Determinations. DNA, RNA, and protein were determined by the methods previously described by Burton (3), Dische (5), and Lowry et al. (12), respectively.

RESULTS

Binding of CCNU-\(^{14}\)C to Nucleic Acid and Proteins in Vivo. The radioactivity of the cyclohexyl moiety of CCNU was almost exclusively bound to cellular proteins, rather than to nucleic acids (Table 1). The specific activity of the protein fraction from leukemia cells was considerably greater than that of the protein fractions obtained from liver or brain. In view of subsequent results obtained with purified nucleic acids and proteins (see below), it seems likely that the small amount of radioactivity from the cyclohexyl-\(^{14}\)C-labeled CCNU found in DNA and RNA is actually due to contaminating protein. In contrast to these results, the radioactivity of the ethylene moiety was bound to both nucleic acids and proteins of brain, liver, and leukemia cells to about the same level (Table 2). In all 3 tissues, the binding of the cyclohexyl moiety of CCNU to protein fractions was 5- to 20-fold greater than the binding of the ethylene moiety to either protein or nucleic acids (compare Tables 1 and 2).

Binding of CCNU-\(^{14}\)C to Nucleic Acids and Proteins in Leukemia Cell Suspensions. As shown in Chart 2, incubation of cyclohexyl-\(^{14}\)C- or ethylene-\(^{14}\)C-labeled CCNU with a suspension of leukemia L1210 cells led to the binding of both radioactivities to acid-insoluble material, which increased progressively with time for at least 4 hr. However, the binding of radioactivity to acid-insoluble material was much lower for the ethylene moiety than for the cyclohexyl moiety (Compare Charts 2 and 3).

<table>
<thead>
<tr>
<th>Protein Fraction</th>
<th>Radioactivity present (pmoles/mg)</th>
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<tr>
<td>Cyclohexyl-(^{14})C-labeled CCNU</td>
<td>0, 1.2, 0.7</td>
</tr>
<tr>
<td>Ethylene-(^{14})C-labeled CCNU</td>
<td>57.5, 24.0, 32.0</td>
</tr>
</tbody>
</table>

Table 3

Binding of CCNU-\(^{14}\)C to nucleic acids and proteins of leukemia L1210 cells incubated in vitro

A 10% suspension of leukemia L1210 cells was incubated aerobically at 37° for 3 hr with 0.05 mg CCNU/mL. Nucleic acids and proteins were then isolated and assayed for radioactivity, as described in "Materials and Methods."

Chart 3. Time course of the binding of radioactivity to isolated nucleic acids and proteins, with cyclohexyl-\(^{14}\)C-labeled CCNU (A) and ethylene-\(^{14}\)C-labeled CCNU (B).
Binding of CCNU to Nucleic Acids and Proteins

A, or globulin. There was no detectable binding to purified calf thymus DNA, to E. coli tRNA (Chart 3A), or to poly-L-arginine (not shown here).

Chart 3B shows that, as in the in vivo and cell suspension studies, the ethylene-\(^{14}\)C-labeled CCNU was bound to both nucleic acids and proteins. However, the level was considerably lower than that obtained with cyclohexyl-\(^{14}\)C-labeled CCNU. With the ethylene-\(^{14}\)C label, the radioactivity bound to tRNA was approximately 5-fold higher than the binding to DNA or albumin. For determination of the effect of base composition, the binding of ethylene-\(^{14}\)C-labeled CCNU to synthetic polynucleotides was also studied (Chart 4). There was extensive binding to poly C and poly G, lesser binding to a random copolymer of G and U, in which the ratio of G to U was 1:3, and a low level of binding to poly A and poly U.

**DISCUSSION**

Alkylation has been suggested as the major mode of action of CCNU and related nitrosoureas (4, 19, 24), but the precise mechanism of action is still unknown (see “Introduction”). The present study clearly indicates that radioactivity from cyclohexyl-\(^{14}\)C-labeled CCNU is extensively bound to proteins and is negligibly bound to nucleic acids, both in vivo and in vitro. On the other hand, radioactivity from ethylene-\(^{14}\)C-labeled CCNU was bound at a low level to both proteins and nucleic acids.

In the present study, the in vivo labeling of nucleic acids and proteins in leukemic cells, which was greater than that with similar components in liver or brain, could simply reflect direct contact with the drug following i.p. injection and/or preferential uptake or metabolism by the leukemic cells. With respect to the therapy of brain tumors, there was significant labeling of brain nucleic acids and proteins. This is consistent with the previous data of Oliverio et al. (16), indicating that radioactivity from cyclohexyl- and ethylene-labeled CCNU does appear at appreciable concentrations in both the cerebrospinal fluid and brain tissue, although these authors presumably measured both free and bound material.

The binding of radioactivity from labeled CCNU to cellular macromolecules in a form that is trichloroacetic acid-insoluble and not extractable by ethanol or ether suggests covalent attachment of the cyclohexyl moiety to amino acid residues in proteins and of the ethylene moiety to both amino acids and base residues in nucleic acids. The precise identity of these derivatives remains to be determined. It seems likely that the cyclohexyl ring system remains intact during the protein binding and also retains an amino group, since cyclohexylamine and N,N′-dicyclohexylurea have been identified as major urinary metabolites (16). Our studies do not indicate whether the carbonyl moiety of CCNU is also part of the protein-bound adduct, since carbonyl-labeled material was not available at the time of these studies. We presume that this is the case, since cyclohexylisocyanate, but not cyclohexylamine, mimics certain biological effects of CCNU (2). By analogy with nitrosoguanidine, which substitutes on the e- amino group of lysine-forming nitrohomoarginine (13, 15, 21), one might expect CCNU to react with the e- amino group of lysine.
group of lysine to yield N<sup>6</sup>-cyclohexylcarbamoyllysine. Consistent with this is the extensive binding which we obtained with polylysine and cyclohexyl-<sup>14</sup>C-labeled CCNU. We are currently analyzing the hydrolysis products of this complex to definitively establish its structure.

The postulated lysine derivative is compatible with previous evidence that both BCNU and CCNU tend to decompose in aqueous solution to yield isocyanates which would be expected to be highly reactive with primary amino groups (14, 16). During the course of the present studies, Bowdon and Wheeler (1) reported the binding of BCNU to lysine residues of proteins, and therefore it is likely that this mechanism may apply to a variety of substituted nitrosoureas. The high reactivity of albumin noted in the present studies suggests that its lysine residues are more exposed than those in several other proteins which were examined.

The modifications of residues as well as of nucleic acids observed with ethylene-<sup>14</sup>C-labeled CCNU might be the result of alkylation of both classes of compounds. Further studies are required to determine whether the modified amino acid and base residues are similar to those found with more conventional alkylating agents (17). The present results with synthetic polynucleotides suggest that G and C residues in nucleic acids are more reactive than are A and U residues. On the other hand, the unusual reactivity of tRNA with ethylene-<sup>14</sup>C-labeled CCNU suggests that factors related to the secondary and tertiary structure of nucleic acids might also be important in exposing specific sites for modification.

In conclusion, the present experimental results suggest that CCNU chemically modifies proteins mainly via cyclohexylcarbamoylation of lysine residues and modifies nucleic acids via alkylation. The dual capacity of this compound may explain its broad cytotoxicity and its activity against tumors which are resistant to conventional alkylating agents (10).

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