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

Chun Jui Cheng, Shinji Fujimura, Dezider Grunberger, and I. Bernard Weinstein

Institute of Cancer Research and Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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 vitro 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. 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 glial 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/mmmole) or in the carbon atoms of the 2-chloroethyl moiety (ethylene-\( ^{14}C \)-labeled CCNU; 1.338 mCi/mmmole).

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.; *Escherichia coli* 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 T2 was from Sankyo Co., Tokyo, Japan; Pronase (B grade) was from Calbiochem, Los Angeles, Calif.; and poly-L-lysine, poly-L-arginine, poly U, poly A, poly (G1U3), poly G, and poly C were from Miles Laboratories, Inc., Elkhard, 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...
ethylene-14C-labeled CCNU (3 μCi) in 0.2 ml of dimethyl sulfoxide. The mice were sacrificed by decapitation 6 hr later, and brain, liver, and leukemia cells were quickly removed. The RNA, DNA, and cytoplasmic protein fractions were isolated by the method of Roberts and Warwick (18), with the following modifications. The RNA fraction (containing both tRNA and rRNA) was extracted with phenol, as described previously (6), and the crude DNA fraction was further purified by Pronase and RNase T2 digestion. Radioactivity in the isolated protein and nucleic acid fractions was measured in 10 ml of Bray’s solution with a Nuclear-Chicago Mark II liquid scintillation spectrometer.

**In Vitro Experiments with Cell Suspensions.** Leukemia cells were harvested as described above and were washed twice with ice-cold 0.9% NaCl solution. A 10% cell suspension was prepared in Krebs-Ringer phosphate buffer (pH 7.4) containing 0.1% glucose. To this was added either cyclohexyl-14C- or ethylene-14C-labeled CCNU, 50 μg/ml, and the suspension was incubated aerobically at 37°C, with gentle shaking.

After various incubation times, aliquots (50 μl) of cell suspension were pipetted onto filter paper discs (Whatman No. 3MM). The discs were washed 3 times with 5% cold trichloroacetic acid, ethanol, ethanol:ether (1:2, v/v), and ether. The acid-insoluble radioactivity remaining on the disc was counted as bound radioactivity, with 10 ml of toluene liquid scintillation solution.

The DNA, RNA, and protein fractions were isolated 3 hr after incubation, and the bound radioactivities were determined as described in the *in vivo* experiments.

**In Vitro Studies with Isolated Nucleic Acids and Proteins.** Twenty-five μg of either cyclohexyl-14C-labeled CCNU or ethylene-14C-labeled CCNU were mixed with 10 ml of Bray’s solution and counted as described above.

---

**Table 1**

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

**Table 2**

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

---

![Chart 1](image1.png)

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

---

![Chart 2](image2.png)

**Chart 2.** Time course of the binding of cyclohexyl-14C-labeled CCNU (A) and ethylene-14C-labeled CCNU (B) to the acid-insoluble fraction of a cell suspension of leukemia L1210.
ethylen-14C-labeled CCNU were incubated with 250 μg of either calf thymus DNA, E. coli tRNA, poly U, poly A, poly (G1U1), poly G, poly C, bovine albumin, bovine globulin, cytochrome c, histone, RNase A, or poly-L-lysine in 0.5 ml of 0.05 M cacodylate buffer (pH 7.2) at 37°C. After various incubation times, 50-μl aliquots were placed on filter paper discs, and the acid-insoluble radioactivity was determined, as described above.

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-14C 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-14C-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-14C to Nucleic Acids and Proteins in Leukemia Cell Suspensions. As shown in Chart 2, incubation of cyclohexyl-14C- or ethylene-14C-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

---

Table 3

<table>
<thead>
<tr>
<th>Radioactivity present (pmoles/mg) in</th>
<th>RNA</th>
<th>DNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexyl-14C-labeled CCNU</td>
<td>0</td>
<td>1.2</td>
<td>2006.7</td>
</tr>
<tr>
<td>Ethylene-14C-labeled CCNU</td>
<td>57.5</td>
<td>24.0</td>
<td>32.0</td>
</tr>
</tbody>
</table>

A 10% suspension of leukemia L1210 cells was incubated aerobically at 37°C 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-14C-labeled CCNU (A) and ethylene-14C-labeled CCNU (B).

---

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 1972 American Association for Cancer Research.
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-14C-labeled CCNU was bound to both nucleic acids and proteins. However, the level was considerably lower than that obtained with cyclohexyl-14C-labeled CCNU. With the ethylene-14C 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-14C-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-14C-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-14C-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 ε-amino group of lysine-forming nitrohomoarginine (13, 15, 21), one might expect CCNU to react with the ε-amino...
The modifications of proteins as well as of nucleic acids observed with ethylene-\textsuperscript{14}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-\textsuperscript{14}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).

ACKNOWLEDGMENTS

We thank Dr. Edgar M. Houspian for valuable support and suggestions. We are indebted to Dr. Steven K. Carter, Dr. Robert R. Engle, and the Cancer Chemotherapy National Service Center of the National Cancer Institute for making the labeled preparations of CCNU available to us.

REFERENCES


23. Wheeler, G. P., Bowdon, B. J., Adamson, D. J., and Vail, M. H. Effects of 1,3-Bis(2-chloroethyl)-1-nitrosourea and Some Chemically Related Compounds upon the Progression of Cultured...
Binding of CCNU to Nucleic Acids and Proteins


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

Chun Jui Cheng, Shinji Fujimura, Dezider Grunberger, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/32/1/22

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.