Inhibition of Rat Liver DNA Polymerase by Nitrosoureas and Isocyanates

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SUMMARY

The effects of 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and 1-(2-chloro-ethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea on two non-mitochondrial DNA polymerases (I and II) purified from rat liver and hepatoma were examined. The activity of DNA polymerase I was not altered by treatment with any of the nitrosoureas or the corresponding isocyanates, 2-chloroethyl isocyanate and cyclohexyl isocyanate. Incubation of DNA polymerase II with the nitrosoureas (1 mM) inhibited its enzymatic activity 30 to 45%. DNA polymerase II was inhibited 75 and 90% by 1 mM 2-chloroethyl isocyanate and cyclohexyl isocyanate, respectively.

The nitrosoureas appear to exert their inhibitory action on the enzyme (DNA polymerase II) rather than on the DNA template. Pretreatment of the enzyme increased the degree of inhibition by 1 mM nitrosourea (50 to 60% inhibition) or 2-chloroethyl isocyanate (>90% inhibition), whereas pretreatment of the DNA template did not enhance the inhibitory effect.

The three nitrosoureas are equally effective as inhibitors of DNA polymerase II. 2-Chloroethyl isocyanate and cyclohexyl isocyanate are better inhibitors than are the nitrosoureas. Since further decomposition products of the isocyanates, 2-chloroethylamine and cyclohexylamine, do not inhibit DNA polymerase II, we conclude that the isocyanates, which are decomposition products of the nitrosoureas, are the active inhibitors of the enzyme.

INTRODUCTION

The nitrosoureas BCNU, CCNU, and MeCCNU are effective antitumor agents when tested against a variety of experimental animal neoplasias as well as specific human neoplasias (7). The carcinostatic activity of the nitrosoureas has been recognized for some time, but the mechanism(s) by which the nitrosoureas act is not clear. Nitrosoureas are known to alkylate nucleic acids (9, 13, 19, 20, 28) and carbamoylate proteins (5, 9, 15). The carbamoylating activity of nitrosoureas does contribute to their carcinostatic effect, although alkylating activity probably plays a major role (27).

Although the nitrosoureas are not cell cycle phase-specific agents (3, 4, 26), they are known to inhibit DNA synthesis in cell-free extracts and in intact cells (10, 23, 24) as well as in brain tumors in vivo (17). Either alkylolation of DNA or carbamoylation of critical enzymes by the nitrosoureas could produce this inhibition of DNA synthesis.

DNA-dependent DNA polymerases have recently been purified from a variety of mammalian tissues (1, 2, 8, 16, 18, 22). Two DNA polymerases that are distinct from mitochondrial DNA polymerase have been purified from rat liver and hepatomas (1, 2, 11). The activity of one of these enzymes, designated DNA polymerase I, remains relatively constant in liver tissues of widely divergent growth rates (2). In contrast, the activity of the other enzyme, DNA polymerase II, shows a positive correlation with the growth rate of these same tissues (2). Although the functions of these 2 DNA polymerases are not known, this and other circumstantial evidence (P. Davis and E. F. Baril, manuscript in preparation) suggest that DNA polymerase II has some role in DNA replication.

Previous studies of nitrosourea action on DNA synthesis have been done with crude enzyme fractions, and it has not been possible to distinguish the effects of the compounds on different enzymes. We have examined the effects of BCNU, CCNU, and MeCCNU on the activities of purified DNA polymerases I and II from rat liver and hepatomas. Since the nitrosourea derivatives decompose under aqueous conditions (13), we have also examined the effects of some of the decomposition products on these 2 DNA polymerases.

MATERIALS AND METHODS

BCNU, CCNU, and MeCCNU were obtained from Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. CIC, CyIC, and cyclohexylamine were purchased from Eastman Chemical Co., Rochester, N. Y. TTP-3H and calf thymus DNA were obtained from Schwarz/Mann, Orangeburg, N. Y., and Worthington Biochemical Corp., Freehold, N. J., respectively. Stock solutions of the nitrosoureas and isocyanates were prepared in acetone.
DNA polymerases I and II were purified from homogenates of 30-hr regenerating rat liver and Novikoff tumor, as described previously by Baril et al. (1). Identical results were obtained with enzymes purified from either rat liver or Novikoff tumor. Most experiments were performed with enzymes purified through the phosphocellulose step, but identical results were obtained with enzymes purified only through the DEAE-cellulose step. DNA polymerases I and II were assayed as described previously (1), using activated calf thymus DNA as template. Activated DNA was prepared by the method of Oleson and Koerner (14). The standard incubation mixture (0.5 ml) contained: 20 μmoles of glycine-NaOH buffer (pH 8.0 for DNA polymerase II and pH 9.0 for DNA polymerase I); 8 μmoles of MgCl₂; 0.05 pmole each of dATP, dGTP, dCTP, and TTP-3H (25 mCi/m mole); 50 μg of bovine serum albumin; 50 to 150 μg of enzyme-containing protein; and 100 μg of activated calf thymus DNA. These conditions were previously determined to be optimal for assay of the 2 enzymes (1). The presence of bovine serum albumin at this concentration in the reaction mixture did not alter the effects of the nitrosoureas or the isocyanates on the DNA polymerases.

In some cases either the DNA template or the enzyme was preincubated with a nitrosourea or an isocyanate for 60 min at 25°C. DNA was usually preincubated with the agent in the absence of other components of the assay system. However, identical results were obtained if all components of the reaction mixture except enzyme were present during preincubation of the DNA with the agent. After the preincubation period, the DNA synthesis reaction was started by the addition of the remainder of the assay system. The enzyme was preincubated with the agent in the presence of all components of the reaction mixture except activated DNA. Enzyme activity was not stable at 25°C in the absence of those components. The incorporation reaction was started by the addition of activated DNA. In all cases incubation was at 37°C for 1 hr. The enzymes were stable under both preincubation and incubation conditions. In each experiment a control sample, which contained only 10 μl of acetone, was run in parallel with the experimental sample, which contained 10 μl of agent dissolved in acetone. After the incubation period the samples were processed as described previously (1). Radioactivity was determined in a Nuclear-Chicago Mark II liquid scintillation spectrometer in 2.5 ml of Omnifluor scintillator [3.92 g of PPO and 80 mg of p-bis(o-methylstyryl)benzene per liter of toluene].

RESULTS

The inhibitory effects of BCNU, CCNU, and MeCCNU on rat liver DNA polymerases I and II are shown in Table 1. The enzymes were preincubated with the nitrosoureas before the incubation period for the DNA-synthetic reaction. The experimental conditions are given in detail in "Materials and Methods." Under these conditions DNA polymerase I was not significantly inhibited by any of the nitrosoureas, whereas DNA polymerase II was inhibited 50 to 60% by all 3 nitrosourea derivatives.

Similarly, the isocyanates CIC and CyIC, which are produced upon the decomposition of BCNU and CCNU, respectively, in aqueous solutions, were tested with the 2 DNA polymerases. These results are also shown in Table 1. Both isocyanates inhibit DNA polymerase II virtually completely under these conditions. The isocyanates at 1 mM had no effect on DNA polymerase I. Even at 5 mM neither BCNU nor either isocyanate inhibited DNA polymerase I more than 20%. CCNU and MeCCNU were not tested at 5 mM because of their limited solubility in water.

Experiments were performed to determine whether the nitrosoureas and isocyanates inhibited the activity of DNA polymerase II by reaction with the enzyme or with the DNA template. The enzyme or activated DNA was preincubated with the reagent before the incubation for the DNA synthesis reaction. The preincubation conditions are detailed in "Materials and Methods." As shown in Table 2, when the enzyme was preincubated with the agent a greater degree of inhibition was observed than in the absence of preincubation. This was true with all 3 nitrosoureas and with CIC. However, CyIC inhibited the incorporation reaction maximally without preincubation with the enzyme. In contrast, pretreatment of the DNA template with the agents did not enhance the inhibition of the incorporation reaction and, in fact, decreased

### Table 1

<table>
<thead>
<tr>
<th>Agent</th>
<th>% inhibition by 1 mM agent</th>
</tr>
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<tbody>
<tr>
<td>BCNU</td>
<td>0 ± 5</td>
</tr>
<tr>
<td>CCNU</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>MeCCNU</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>CIC</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CyIC</td>
<td>0 ± 1</td>
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</table>

### Table 2

<table>
<thead>
<tr>
<th>Component preincubated</th>
<th>BCNU</th>
<th>CCNU</th>
<th>MeCCNU</th>
<th>CIC</th>
<th>CyIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28 ± 6</td>
<td>42 ± 12</td>
<td>45 ± 7</td>
<td>76 ± 6</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>DNA polymerase II</td>
<td>54 ± 5</td>
<td>60 ± 4</td>
<td>61 ± 7</td>
<td>93 ± 0</td>
<td>93 ± 1</td>
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<tr>
<td>DNA template</td>
<td>29 ± 11</td>
<td>36 ± 8</td>
<td>26 ± 5</td>
<td>0 ± 0</td>
<td>31 ± 11</td>
</tr>
</tbody>
</table>
it in the cases of the 2 isocyanates. Virtually identical results were obtained when nonsaturating concentrations (1 to 10 μg DNA per assay) of control or agent-pretreated DNA were used in the assays. Under these conditions decreased template activity as a result of nitrosourea or isocyanate treatment might be more apparent. However, inhibition of DNA synthesis was not enhanced under these conditions and was completely abolished after preincubation of 1 mM CIC with limiting DNA concentrations. These experiments suggest that the nitrosoureas and isocyanates are inhibiting the in vitro DNA-synthetic reaction as a result of reaction with the enzyme rather than with the DNA template.

The nitrosoureas progressively decompose under aqueous conditions (13). In order to determine whether the parent compound (nitrosourea) or one of its decomposition products (isocyanate or amine) is the actual inhibitor of DNA polymerase II, we have examined the effects of these compounds at various concentrations, and the results are shown in Chart 1. In each case the enzyme was preincubated with the agent under the conditions given in “Materials and Methods.” The concentration curves for the 3 nitrosoureas are very similar except at the higher concentrations. The semilogarithmic plot of the BCNU concentration curve is essentially linear, and at 5 mM inhibition of the enzyme approaches 100%. In contrast, the inhibition by CCNU and MeCCNU plateaus at approximately 65% inhibition (1.0 to 2.5 mM nitrosourea). CCNU and MeCCNU are not soluble at higher concentrations. The isocyanate concentration curves appear to be biphasic. At the higher concentrations (1 to 5 mM) the inhibition of DNA polymerase II approaches 100%. The isocyanates are more potent inhibitors of DNA polymerase II than are the nitrosoureas. The isocyanates produce 50% inhibition of the enzyme at concentrations 3 to 9-fold lower than the nitrosoureas. Neither 2-chloroethylamine (generated by reacting CIC with water) nor cyclohexylamine inhibits DNA polymerase II.

**DISCUSSION**

The results presented here show that the carcinostatic nitrosoureas, BCNU, CCNU, and MeCCNU inhibit only 1 of 2 nonmitochondrial DNA polymerases that were purified from rat liver and hepatoma. These carcinostatic compounds inhibit the DNA polymerase (DNA polymerase II) that may play a role in DNA replication in these tissues. The tentative assignment of this function to DNA polymerase II is based on the fact that the activity of DNA polymerase II is low in nonproliferating tissues and shows a positive correlation with cell proliferation and is believed to function primarily in a constitutive process such as DNA repair (2). This view is not in conflict with the recent report by Kann et al. (12) that CIC and, to a lesser extent, BCNU inhibit DNA repair in X-irradiated L1210 cells. DNA repair is undoubtedly dependent on many factors in addition to DNA polymerase.

It was previously observed that CIC, a decomposition product of BCNU, inhibited DNA synthesis in crude cell-free extracts (24). Similarly, we conclude that the isocyanates are the active inhibitors of DNA polymerase II. They are more potent inhibitors of this enzyme than either parent nitrosoureas or further decomposition products (Chart 1, chloroethylamine and cyclohexylamine).

CyIC, which is derived from CCNU, appears to be a significantly better inhibitor of DNA polymerase II than is CIC (derived from BCNU), since maximal inhibition by CyIC is obtained without preincubation with the enzyme (Table 2). Some of this difference between the isocyanates may reflect the chemical reactivities of the isocyanates toward DNA polymerase II. However, an equally important consideration is the stability of the inhibitor, and we have found that CyIC is substantially more stable under our conditions than is CIC. Under the conditions used to preincubate the enzyme, CyIC and CIC have half-lives of approximately 50 and 2 min, respectively. The half-lives were determined as loss of ability to inhibit DNA polymerase II after preincubation in aqueous solution in the absence of enzyme for various periods of time. The 3 nitrosoureas examined have similar stabilities. Under the preincubation conditions they have half-lives of 6 or more hr, as determined by loss of material absorbing at 230 nm. They are substantially more stable under these conditions (25°, glycine-NaOH buffer, pH 8.0) than reported previously (25, 27). Wheeler et al. (25, 27) found that these nitrosoureas have half-lives of less than 1 hr at 37° in phosphate buffer, pH 7.4. Phosphate has been found to catalyze the decomposition of the nitrosoureas (13); therefore, both the use of phosphate buffer and the higher temperatures would have contributed to the shorter half-lives observed previously (25, 27).

The observation that maximal inhibition of DNA polymerase II requires preincubation with the nitrosoureas (Table 2) probably reflects the time needed to generate the isocyanates. On the other hand, CIC is more stable at 25° than at 37° so...
that greater inhibition of the enzyme is observed after preincubation with CIC at 25°.

The detailed mechanism by which the isocyanates inhibit DNA polymerase II in vitro is not known at the present time. However, it is probable that the significant reaction in vitro is with the purified enzyme rather than with the DNA template (Table 2). Similarly, Wheeler and Bowdon (24) found that treatment of calf thymus DNA with BCNU did not alter its priming activity with a cell-free DNA-synthetic system.

It seems plausible that the isocyanates inhibit DNA polymerase II by reacting with critical amino acid moieties at or near the active site of the enzyme. Isocyanates are known to form stable carbamoyl derivatives with a number of amino acid moieties in proteins. Reactions have been demonstrated with lysine ε-amino (5, 9, 15), serine hydroxyl (6), and cysteine sulfhydryl groups (21). DNA polymerase II is inhibited by thiol-blocking agents (2) and the inhibition of the enzyme by the isocyanates might be due to carbamoylation of a critical cysteine moiety. However, it seems unlikely that this is the sole mechanism of inhibition since a carbamoyl derivative of cysteine has been shown to be unstable at the pH used for the enzyme assay (21). Experiments are in progress to determine directly what amino acid(s) in DNA polymerase II is carbamoylated by these compounds.

It was previously observed that bovine serum albumin is carbamoylated by CCNU (9). However, we do not detect any competition for the nitrosoureas or isocyanates when small amounts of bovine serum albumin are included in the DNA polymerase assays. The lack of competition may reflect the relative reactivities of DNA polymerase II and bovine serum albumin with the isocyanates. However, at a dose of 1 mM, where most of this work was done, the drug was probably in vast molar excess to the reactive sites in DNA polymerase II.

The nitrosourea concentration curves are all linear up to 1 mM (Chart 1). Above that concentration the solubilities of CCNU and MeCCNU become limiting and the curves plateau. In contrast, the isocyanate concentration curves appear biphasic. Studies are in progress to determine the basis for the biphasic nature of these curves.

Whether the nitrosoureas will similarly inhibit DNA polymerase II in cells in culture or in tissues in vivo is not yet known. Wheeler and Bowdon (24) found that administration of BCNU to intact L1210 cells reduced the total DNA polymerase activity in cell-free extracts of these cells. However, administration of BCNU to mice bearing L1210 ascites or solid tumors did not alter the total DNA polymerase activity in cell-free extracts from either form of L1210 cell (24).

Studies are in progress to determine the effects of CCNU and related compounds on DNA polymerase II in cells in culture (HeLa cells). Enzymes homologous to rat liver DNA polymerases I and II have been isolated from these cells (22) and we have determined that the 2 purified HeLa DNA polymerases are also differentially inactivated by the isocyanates.

Future studies must determine to what extent inhibition of DNA polymerase II accounts for the observed carcinostatic activity of the nitrosoureas. It must also be determined whether inhibition of DNA polymerase II is a general phenomenon in eukaryotic cells or is tissue specific. The results of such studies may provide insights into the carcinostatic actions of these nitrosoureas.

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Inhibition of DNA Polymerase by Nitrosoureas

Inhibition of Rat Liver DNA Polymerase by Nitrosoureas and Isocyanates


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