Comparison of Biochemical and Biological Effects of Four Nitrosoureas with Differing Carbamoylating Activities

Herbert E. Kann, Jr.

Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

ABSTRACT

Four chloroethynitrosoureas with differing degrees of carbamoylating activity were compared for their effects on incorporation of radioactive precursors into macromolecules. The comparisons were made with concentrations that, for each drug, produced a defined biological effect, either an 0.5-log or a 2-log reduction in cloning efficiency from a 1-hr drug exposure. Dose-dependent inhibition of incorporation of labeled precursors into nucleic acids and proteins was observed during the 1-hr drug exposure with either of the two strongly carbamoylating nitrosoureas, 1-3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; the most extensive inhibition involved incorporation into DNA. No inhibitions were observed during exposure to a weakly carbamoylating nitrosourea (chlorozotocin) or during exposure to 1-(2-chloroethyl)-1-nitrosourea, a compound the carbamoylating activity of which is not agreed upon. By 4 hr after removal of 1-3-bis(2-chloroethyl)-1-nitrosourea or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea from the extracellular medium, the cells had partially recovered from the earlier inhibition of radioactive thymidine incorporation. This recovery was, however, dependent upon an undefined factor present in serum.

The inhibitions that were observed during the 1-hr drug exposure are clearly not essential to the cytotoxic effect of chloroethynitrosoureas since no inhibitions occurred with two of the four compounds studied.

INTRODUCTION

The chloroethynitrosoureas, compounds with clinically useful antineoplastic activity (for review see Ref. 29), have, as one of their well-recognized features, a marked instability under physiological conditions (26). On decomposition (see Chart 1), 2 products capable of alkylation are formed; these are chloroethyl diazohydroxide and a chloroethyl carbamion ion (4, 5, 23, 29). The other major product of decomposition is an organic isocyanate (23), the structure of which is determined by the N-3 substituent of the parent nitrosourea. Organic isocyanates undergo carbamoylation reactions and, depending on the extent of the carbamoylating activity of a given isocyanate decomposition product, its parent nitrosourea can be categorized as either weakly or strongly carbamoylating.

The 2 nitrosoureas in widest clinical use, BCNU and CCNU, are strongly carbamoylating (33). These 2 drugs have been used almost exclusively in previous studies of the biochemical effects of chloroethyl nitrosoureas. In those studies a variety of effects have been observed, including inhibition of macromolecular syntheses (11, 32), specific inhibition of DNA polymerase II (3), alteration in the pattern of de novo purine biosynthesis (12), selective modification of 1 class of histones (34), interference with processing of ribosomal precursor RNA and of nucleoplasmic RNA (1, 19), inhibition of repair of X-ray-induced strand breaks in DNA (17), production of strand breaks in DNA (8, 13), and cross-linking of DNA (20). Because several of these effects (3, 17, 19) can be produced by the strongly carbamoylating isocyanates formed on decomposition of BCNU (generates chloroethyl isocyanate) or CCNU (generates cyclohexyl isocyanate), it appears reasonable to attribute some of the biochemical effects seen with the parent compounds.

As was implied earlier, not all chloroethynitrosoureas form strongly carbamoylating isocyanates. Chlorozotocin is one example of a weakly carbamoylating nitrosourea with potent antitumor activity (2); CNU may be another (30). Since strong carbamoylation is not essential to antitumor activity, there is a question as to whether some of the reported biochemical effects, observed in the earlier studies involving nitrosourea compounds that happened to be strongly carbamoylating, may also be unrelated to cell killing. The present study comparing strongly and weakly carbamoylating nitrosoureas for their biochemical and biological effects is directed toward this question. The characteristics of the compounds studied are shown in Table 1.

MATERIALS AND METHODS

Cells. L1210 cells (24) in suspension culture were grown and maintained under conditions that have been described previously (17). Experiments were performed on cells in log-phase growth (doubling time, 12 to 14 hr).

Drugs. Drugs were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, stored at -20°, and freshly prepared for each experiment by dissolving in ethanol (chlorozotocin was dissolved in sterile 0.85% NaCl solution) within 5 min of the time of addition to culture. The relative volume of the ethanolic drug solution added, compared to the total volume of cell culture, was 0.001. Duration of exposure to drug was 1 hr; control cultures with solvent alone were included in each experiment.

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2 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CCNU, 1-(2-chloroethyl)-1-nitrosourea.
RESULTS

Drug Effects on Cell Viability. For each of the drugs studied, cell killing is dose dependent (see Chart 2). BCNU and CCNU are identical with regard to the amounts of lethality produced over the range of drug dosages examined. Both chlorozotocin and CNU are significantly more cytotoxic than BCNU or CCNU ($p < 0.01$ at concentrations of 10 to 30 $\mu M$). Based on the results of these cytotoxicity studies, 2 concentrations of each drug were selected for the biochemical studies: the lower concentration was the concentration that reduced cloning efficiency by 0.5 log; the higher concentration was the one that reduced cloning efficiency by 2 logs. The 2 concentrations used for each drug were: BCNU, 9 and 27 $\mu M$; CCNU, 9 and 27 $\mu M$; chlorozotocin, 7 and 21 $\mu M$; and CNU, 5 and 17 $\mu M$.

Incorporation of Radioactive Precursors during Drug Exposure. With chlorozotocin and with CNU, there was no inhibition of incorporation at either drug concentration during the 1-hr drug exposure (Chart 3, upper panel). With both BCNU and CCNU, however, there was dose-dependent inhibition of incorporation of radioactive precursors into DNA, RNA, and protein; the largest effect was on incorporation of radioactive thymidine. The inhibition of incorporation into DNA produced by low-dose BCNU or CCNU exceeded that produced by CNU even when the molar concentration of CNU was 12-fold higher (result not shown).

Incorporation of Radioactive Precursors 4 Hr after Drug Removal. With chlorozotocin and CNU, minimal late effects on incorporation are observed 4 hr after cell washing and resuspension in drug-free medium (Chart 3, lower panel). There is a small amount of inhibition of radioactive thymidine incorporation and, in the case of chlorozotocin, an equivocal effect on radioactive uridine incorporation. For BCNU and CCNU the finding of interest is a partial recovery from the early inhibition of radioactive thymidine incorporation; this recovery was evident at both low and high drug concentrations of each drug.

Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>NSC no.</th>
<th>Structure</th>
<th>Rate of decomposition ($t_{1/2}$ in min)</th>
<th>Alkylating activity ($A_{540}$)</th>
<th>Carbamoylating activity (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCNU</td>
<td>409962</td>
<td>$\text{ClCH}_2\text{CH}_2\text{-N-C-NH-R}$</td>
<td>43</td>
<td>1.382</td>
<td>28,716</td>
</tr>
<tr>
<td>CCNU</td>
<td>79037</td>
<td>$\text{ClCH}_2\text{CH}_2\text{-N-C-NH-R}$</td>
<td>52.5</td>
<td>0.520</td>
<td>42,000</td>
</tr>
<tr>
<td>Chlorozotocin</td>
<td>178248</td>
<td>$\text{ClCH}_2\text{CH}_2\text{-N-C-NH-R}$</td>
<td>21.1</td>
<td>2.350</td>
<td>824</td>
</tr>
<tr>
<td>CNU</td>
<td>47547</td>
<td>$\text{ClCH}_2\text{CH}_2\text{-N-C-NH-R}$</td>
<td>1.3</td>
<td>&gt;3.0</td>
<td>8,558</td>
</tr>
</tbody>
</table>

Chart 1. Abbreviated scheme for decomposition of CNU's, taken from Ref. 6.

Determination of Viable Cell Number. Following a 1-hr exposure to either drug or solvent, cells were washed twice to remove extracellular drug and resuspended in drug-free medium, and the number of viable cells was assessed from soft-agar cloning efficiency, determined by a standard technique (6).

Incorporation of Radioactive Precursors into DNA, RNA, or Protein. Relative rates of incorporation (drug-treated versus control) were determined over 30-min periods by standard techniques for which details have been given previously (21). Labeled precursors were [2-$^{14}$C]thymidine (55 mCi/mmol; 0.04 $\mu$Ci/ml cell suspension), [2-$^{14}$C]uridine (54 mCi/mmol; 0.04 $\mu$Ci/ml cell suspension), and L-$^{14}$[C]leucine (312 mCi/mmol; 0.4 $\mu$Ci/ml cell suspension).
Carbamoylation Effects of Nitrosoureas

93 -3
IO 2O 3O

Chart 2. Drug effects on cell survival. Duration of exposure, 1 hr. Each point represents the mean value from 5 or more independent experiments. ▲, BCNU; △, CCNU; ●, chlorozotocin; ○, CNU.

Chart 3. Drug effects on incorporation of radioactive precursors into macromolecules. The values represent the mean ± S.D. (bars). Dark gray boxes, incorporation into DNA; light gray boxes, incorporation into RNA; open boxes, incorporation into protein. For each drug the low dose is the concentration that during a 1-hr exposure reduces cell survival by 0.5 log; the high dose is the concentration that reduces cell survival by 2 logs. Top, 30-min incorporation (measured in the continuing presence of drug) following a 30-min drug preincubation; bottom, 30-min incorporation measured 4 hr after a 60-min period of drug exposure.

concentrations (p < 0.05 for 9 μM CCNU; otherwise, p < 0.01). This recovery, however, was not observed in a simultaneous set of BCNU experiments in which cells were suspended in medium supplemented with fetal calf serum from a different lot.

DISCUSSION

Many drugs with antitumor activity inhibit incorporation of radioactive precursors into macromolecules. These inhibitions are potentially lethal, and so the question arises as to whether these inhibitions may be primarily responsible for the cell-killing ability of the drug. This question was addressed, for chloroethylnitrosoureas, in this study. Under conditions of drug exposure that resulted in identical reductions in cell viability, different chloroethylnitrosoureas were found to produce strikingly different patterns of inhibition of radioactive precursor incorporation into macromolecules. With some chloroethylnitrosoureas (chlorozotocin and CNU), no prompt inhibitions occurred, and from this it was clear that the inhibitions that did occur with other chloroethylnitrosoureas (BCNU and CCNU) were not critically related to the cell-killing ability of this class of compounds.

The possibility that the prompt inhibition of radioactive precursor incorporation might be unrelated to the biological effect of chloroethylnitrosoureas came from consideration of strong carbamoylating activity, a feature that is not essential for antitumor activity, as a likely basis for inhibition of precursor incorporation. The results obtained with BCNU, CCNU, and chlorozotocin are in accord with this idea; the strong carbamoylators inhibit, whereas weakly carbamoylating chlorozotocin does not. However, interpretation of the result with CNU is not clear-cut, owing to the controversy regarding the carbamoylating activity of this particular compound (10, 31). CNU’s effects in this system are similar to those of chlorozotocin, the weak carbamylator. This similarity has been observed in several other biochemical systems as well (Res. 19; H. Kann, unpublished observations); without exception the biochemical effects of CNU have been seen to be those expected of a weak carbamylator. If the failure of CNU to exhibit characteristics of strongly carbamoylating nitrosoureas is to be explained on some basis other than a weak carbamoylating activity, an alternative explanation might be built around the idea that isocyanic acid, the carbamoylating decomposition product generated from CNU, lacks a bulky organic substituent that could, in theory, be required for production of the biochemical effects observed.

The late inhibition of incorporations into nucleic acids, which is seen in cells previously exposed to either chlorozotocin or CNU, is presumably not an effect of carbamoylation but may instead be the result of alkylation effects on DNA template activity. This possibility, proposed tentatively, arises in part from consideration of the chronology of events. With the chloroethylnitrosoureas, alkylation and cross-linking occurs slowly over several hr, and cross-linking progresses even after removal of the drug from the extracellular environment (20).

In the cells exposed to BCNU for 1 hr and incubated in drug-free medium for an additional 4 hr, there is a partial recovery from the early inhibition of radioactive thymidine incorporation into DNA. The basis for the recovery is not clear, beyond the fact that it is related to the presence or absence of an undefined serum component; reversibility was consistently observed in experiments performed with cells in medium supplemented with fetal calf serum from one lot, but it was consistently not observed in simultaneous experiments with fetal calf serum from a different lot.

In the comparison of drugs at equimolar concentrations for their effects on viability of L1210 cells in culture, CNU and chlorozotocin were found to be more cytotoxic than
REFERENCES


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