2-Chloroethyl (Methylsulfonyl)methanesulfonate (NSC-338947), a More Selective DNA Alkylation Agent Than the ChloroethylNitrosoureas


ABSTRACT

The novel chloroethylating agent 2-chloroethyl (methylsulfonyl)methanesulfonate (CIEtSoSo) has been shown to act like the chloroethylnitrosoureas (CIEtNu's) in its DNA damaging and cytotoxic effects in human cell lines and has similar activity to the CIEtNu's in the National Cancer Institute antitumor screening tests. Its simpler chemistry, however, suggests that it may alkylate DNA more selectively than do the CIEtNu's. The DNA base adducts produced in calf thymus DNA by CIEtSoSo have been compared to a representative, non-carbamoylating CIEtNu, 1-(2-chloroethyl)-3-(cis-2-OH)cyclohexyl-1-nitrosourea, using high-pressure liquid chromatography. Two major modified base peaks were observed from the nitrosourea treated sample which have been subsequently identified by high-pressure liquid chromatography/mass spectrometric analysis to be 7-hydroxyethylguanine and 7-chloroethylguanine. In contrast only 7-chloroethylguanine was obtained from the CIEtSoSo treated DNA at equimolar doses. Thus CIEtSoSo was found to be more specific in its reaction with DNA in that it produced less variety of products than the nitrosourea, with no apparent generation of hydroxyethyl products, which are major side reactions of the CIEtNu's.

INTRODUCTION

A major goal in experimental chemotherapy is to identify as explicitly as possible the lesions that are responsible for the selective killing of tumor cells. The development of modified drugs that would produce mainly the type of reaction that selectively kills tumor cells with a minimum of side reactions would be distinctly advantageous to the host.

CIEtNu's are unstable compounds that decompose to a variety of reactive species which are capable of alkylating and carbamoylating nucleophilic targets (1, 2). The antitumor activity of CIEtNu's has been suggested to be a function of their alkylation activity, and two types of alkylation reactions have been demonstrated: chloroethylation and hydroxyethylation (2, 3). The important reaction of CIEtNu's with DNA appears to involve chloroethylation of the guanine-O6 position (4-6), followed by a slow series of reactions that lead to the delayed formation of interstrand cross-links over a period of several hours (5-8). Thus if the antitumor activity of CIEtNu's stems from a chloroethylation reaction, other side reactions such as hydroxyethylation could produce unnecessary toxicity and may even interfere with the antitumor potential of these drugs. Indeed hydroxyethylation does not contribute to antitumor activity, as compounds which produce solely this type of reaction lack antitumor activity. Hence a compound which retains chloroethylation potential but not hydroxyethylation activity would be preferred.

Shealy et al. (9, 10) at the Southern Research Institute investigated a series of chloroethylmethanesulfonates and identified some derivatives with moderately enhanced activities in the P388 murine leukemia system. In particular CIEtSoSo is highly effective against the murine P388 leukemia (9) and is as good as the CIEtNu's in the National Cancer Institute screening program. As a result this compound is being developed for clinical trial. We have investigated this compound previously for its DNA damaging and cytotoxic effects in human cell lines (11). We found that like the CIEtNu's it produced DNA interstrand cross-links after a delay of 6-12 h in the VA-13 (Mer-) but not in the IMR-90 (Mer+) cell lines, that it produced DNA-protein cross-links in both cell types without delay, and that it selectively killed the VA-13 cells. The results suggested that CIEtSoSo acts by chloroethylation guanine-O6 positions in DNA (11).

Another major side reaction of the CIEtNu's is their ability to undergo carbamoylation reactions. These reactions do not contribute to antitumor activity, but they do interfere with the selective action of CIEtNu's against human tumor cells, probably on the basis of an observed inhibition of the ligase reaction in DNA nucleotide excision repair (12, 13). In a rat mammary carcinoma cell line carbamoylation of glutathione reductase is thought to be important to the toxicity of CIEtNu's (14). From its chemistry, however, it appears that CIEtSoSo cannot carbamoylate nucleophilic sites; yet this compound retains antitumor activity. Thus CIEtSoSo lacks two prominent unnecessary side reactions of the CIEtNu's: hydroxyethylation and carbamoylation.

In the present study we have tested the concept that CIEtSoSo may react more selectively than the CIEtNu's by examining the DNA base adducts produced by CIEtSoSo as compared to a representative, low carbamoylating CIEtNu, cis-2-OH CCNU. The potential advantages of CIEtSoSo over CIEtNu's are discussed in light of the results.

MATERIALS AND METHODS

CIEtSoSo and cis-2-OH CCNU were obtained from the Drug Development Branch, National Cancer Institute. [ethyl-14C]CIEtSoSo (9.6 mCi/mmol) and [ethyl-14C]cis-2-OH CCNU (10.4 mCi/mmol) were obtained from the Research Triangle Institute. N,O-bis(trimethylsilyl)trifluoro-
acetamide was procured from Pierce Chemical Co., Rockford, IL. Poly(dG-dC)-poly(dG-dC) and poly(dA-dT)-poly(dA-dT) were purchased from P-L Biochemicals, Inc., Milwaukee, WI.

Drug Treatment. Calf Thymus DNA (Sigma Chemical Co., St. Louis, MO) at 5 mg/ml in 0.1 M NaCl:1 mM EDTA:1 mM NaPO₄, pH 7, was reacted with drug dissolved in dimethyl sulfoxide for 4 h at 37°C, conditions which produced maximum levels of covalent binding. The DNA was precipitated with 95% ethanol at −20°C overnight and recovered by centrifugation. Depurination was achieved by reaction with 0.15 M hydrochloric acid at 95°C for 30 min. Samples were neutralized with 0.3 M NaOH and filtered through 0.45-µm filters prior to analysis by HPLC.

High-Pressure Liquid Chromatography. Samples were analyzed by reverse-phase HPLC in 5-µm C18 Ultrasphere columns (Altex Ltd.) on a Beckman model 544 gradient liquid chromatograph. Analytical runs of approximately 1 mg hydrolyzed DNA were analyzed on a 4.6 mm x 25 cm column with a flow rate of 1 ml/min. The running buffer was 20 mM ammonium formate with a 1-10% acetonitrile linear gradient over 30 min; acetonitrile was then held at 10% for 10 min, followed by a linear gradient from 10–100% acetonitrile over 30 min. Preparative runs utilized a 10 mm x 25 cm column and a flow rate of 3 ml/min. The acetonitrile program was 1% for 15 min, 1–5% over 35 min, and 5–100% over 30 min. Approximately 10 times the sample could be accommodated in preparative compared to analytical runs. The preparative samples were reacted with a 1:10 ratio of labeled/unlabeled drug. One-min fractions were collected, 4 ml water and 10 ml Aquassure (New England Nuclear) scintillation fluid were added, and the samples were counted for radioactivity.

Preparation of Modified Base Standards. Standards were prepared for HPLC and mass spectral comparison with base adducts produced from alkylated DNA. O²-Hydroxyethylguanine was prepared by the method of Ashby et al. (15) by the reaction of 6-chloroguanine with sodium ethylene glycolate and purified by thin-layer chromatography. 7-Chloroethylguanine was prepared by the reaction of guanosine with 1-bromo-2-chloroethane according to the method of Tong and Ludlum (16). Unreacted guanosine was removed from the product by HPLC prior to hydrolysis to give the guanine product. 7-Hydroxyethylguanine was prepared by the method of Gombar et al. (3), by the reaction of guanosine with ethylene oxide in dimethyl sulfoxide. The product was purified by HPLC. Verification of the product preparation was by comparison of UV spectra with published data and by mass spectral analysis.

Gas Chromatography/Mass Spectrometry. Silylated derivatives of the synthetic modified guanines and samples collected from the HPLC were prepared by adding 50 µl each of acetonitrile and N,O-bis(trimethylsilyl)trifluoroacetamide to the sample and reacting for 15 min at 80°C. Mass spectral analysis was accomplished on a Ribermag R10-10C quadrupole mass spectrometer equipped with a series 32 Girdel gas chromatograph (Delsi-Nermag, Fairfield, NJ). The mass spectrometer was operated in the electron impact ionization mode at 70 eV. Separation of the compounds was achieved on a 15 m x 0.25 µm fused silica capillary column coated with SE-54 (0.25 µm thick) (Supelco, Inc. Bellefonte, PA). Helium gas at a linear velocity of 50 cm/s was used as the carrier gas. Injection of the sample onto the column was made through a split/splitless injector with a 10:1 split ratio. The injector and the transfer line temperatures were held at 270°C; the column temperature was held at 220°C.

RESULTS

Ludlum and his colleagues have shown that CIEtNu's produce a number of modified DNA base adducts in vitro; these include both hydroxethyl and chloroethyl purines, as well as some modified pyrimidines (2, 3, 16–18). In this study we have tested the concept that CIEtSoSo will produce a lesser variety of modified DNA base adducts, as suggested by its different chemistry to a CIEtNu. The structures of CIEtSoSo and cis-2-OH CCNU, a representative low carbamoylating nitrosourea, showing the positions of the 14C label are shown in Fig. 1. The two compounds were compared for their reaction products produced in calf thymus DNA. The compounds were reacted at 13 mm with calf thymus DNA which was then depurinated with 0.15 M HCl, and the radioactive products were separated using HPLC. Fig. 2 shows the analytical HPLC profile for cis-2-OH CCNU treated DNA showing 5 major peaks of radioactive eluting at 4, 9, 16, 21, and 27 min. Only 3 major peaks were found on analysis of the CIEtSoSo treated DNA (Fig. 3), eluting at 4, 9, and 26 min. The relative amounts of each peak in pmol/mg DNA/mM drug can be seen in Table 1. The peaks at 4 min (eluting close to the solvent front) and at 9 min were similarly observed from poly(dA·dT)·poly(dA·dT) and from poly(dG·dC)·poly(dG·dC) reacted with either CIEtSoSo (Fig. 4) or cis-2-OH CCNU (data not shown) and were thus assumed not to be modified base products. The products obtained upon a depurinating hydrolysis and eluting at 16, 21, and 26 or 27 min, however, were not observed...
CIEtSoSo, A MORE SELECTIVE ALKYLATING AGENT THAN CIEtNu's

Table 1
Relative amounts of the major alkylation products in calf thymus DNA following treatment with cis-2-OH CCNU and CIEtSoSo

<table>
<thead>
<tr>
<th>Peak retention time (min)</th>
<th>pmol product/mg DNA/mmol drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>94.6</td>
</tr>
<tr>
<td>9</td>
<td>234.6</td>
</tr>
<tr>
<td>16</td>
<td>99.2</td>
</tr>
<tr>
<td>21</td>
<td>26.9</td>
</tr>
<tr>
<td>25/27</td>
<td>53.4</td>
</tr>
<tr>
<td>cis-2-OH CCNU</td>
<td>23</td>
</tr>
<tr>
<td>CIEtSoSo</td>
<td>292.3</td>
</tr>
<tr>
<td>cis-2-OH CCNU</td>
<td>0</td>
</tr>
<tr>
<td>CIEtSoSo</td>
<td>44.2</td>
</tr>
</tbody>
</table>

Fig. 3. HPLC separation of alkylated products released from calf thymus DNA alkylated with CIEtSoSo using the analytical procedure described in "Materials and Methods." 

Table 2
Summary of the characteristics of synthesized standards and isolated peaks that coelute with standards under the high-pressure liquid chromatography and gas chromatography/mass spectrometry conditions

<table>
<thead>
<tr>
<th>HPLC retention times</th>
<th>Gas chromatography/mass spectrometry data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical (min)</td>
<td>Preparative (min)</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>s</td>
</tr>
<tr>
<td>7-Hydroxyethylguanine</td>
<td>16</td>
</tr>
<tr>
<td>cis-2-OH CCNU</td>
<td>16</td>
</tr>
<tr>
<td>cis-2-Hydroxyethylguanine</td>
<td>27</td>
</tr>
<tr>
<td>cis-2-OH CCNU</td>
<td>27</td>
</tr>
<tr>
<td>CIEtSoSo</td>
<td>26</td>
</tr>
<tr>
<td>O* -Hydroxyethylguanine</td>
<td>21</td>
</tr>
<tr>
<td>cis-2-OH CCNU</td>
<td>21</td>
</tr>
</tbody>
</table>

from the poly (dA-dT)-poly (dA-dT) treated sample, which thus suggests that they were modified guanine bases. The three standards 7-hydroxyethylguanine, O* -hydroxyethylguanine, and 7-chloroethylguanine were synthesized by methods described previously (3, 15, 16) and were found to elute at 16, 21, and 27 min, respectively, on the analytical column (see Table 2). It thus appeared from the retention times of the three standards that cis-2-OH CCNU produced both chloroethylguanine and hydroxyethylguanine products, whereas CIEtSoSo produced only 7-chloroethylguanine.

To verify this finding the radioactive peaks were collected and silylated, and electron impact mass spectra were obtained for comparison with those for the synthesized standards. To enable sufficient quantity of each peak to be collected, runs were performed on a preparative column from samples treated with a 1:10 ratio of labeled:unlabeled drug. In such runs 10 times the sample could be separated compared to analytical runs, and the acetonitrile gradient was changed to further separate the latter eluting peaks. Under the conditions used in the preparative HPLC runs, the three standards were found to elute at 23, 48, and 58 min, corresponding to 7-hydroxyethylguanine, O* -hydroxyethylguanine, and 7-chloroethylguanine, respectively. In this case cis-2-OH CCNU showed major radioactive peaks at 5, 12, 23, and 58 min but not at 48 min. The peaks at 23 and 58 min corre-
sponded to the 7-hydroxyethyl and 7-chloroethylguanine standards, but no radioactive peak was found to coelute with the O6-hydroxyethylguanine standard. In addition a number of minor peaks (less than 10 pmol/mg DNA/m/d drug) eluted at 7, 26, 28, 37, 40, 43, and 64 min. The identity of these peaks is at present not known. The CIEtSoSo profile again showed a reduction in the number of radioactive peaks, with 3 major peaks eluting at 5, 12, and 58 min, and some minor peaks accounting for less than 5% of the total radioactivity. The peaks at 23 and 58 min for cis-2-OH CCNU and at 58 min for CIEtSoSo were silylated and analyzed by gas chromatography/mass spectrometry. Since 7-hydroxyethylguanine and O6-hydroxyethylguanine have the same molecular ion but display different gas chromatographic retention times, this technique can distinguish between the two compounds without depending on differences in the electron impact fragmentation patterns (Table 2). A mass spectral comparison of the silyl derivative of authentic 7-hydroxyethylguanine and the silylated cis-2-OH CCNU 26 min HPLC peak is shown in Fig. 5. A molecular ion at m/z 411 and loss of CH3 radical from the molecular ion at m/z 396 were observed as listed in Table 2. Although the fragmentation patterns were identical for the standard and unknown samples, no attempt was made to identify the other ion fragments observed in the mass spectra. Fig. 6 shows the mass spectra of the disilyl derivative of authentic 7-chloroethylguanine, the cis-2-OH CCNU 27 min HPLC peak, and the CIEtSoSo 26 min HPLC peak. A molecular ion at m/z 357 and fragment ion due to the loss of a CH3 radical at m/z 342 were observed in all three spectra. The isotopic content of the ion clusters at these masses were consistent with a disilylated derivative of 7-chloroethylguanine. No attempt was made to identify the other ion fragments observed in the mass spectra. In addition to the mass spectral analysis identical gas chromatographic retention times were observed for the standard and unknown samples. A summary of the gas chromatography/mass spectrometry features is listed in Table 2.

The identity of the radioactive peak which coeluted with the O6-hydroxyethylguanine standard under the analytical HPLC conditions was not confirmed. Although the identity of this peak remains obscure, it is worthwhile noting three main points: (a) this radioactive peak was released from the DNA backbone under neutral thermal hydrolysis conditions as described by

Fig. 5. Mass spectrum of the trimethylsilyl derivatives of the 7-chloroethylguanine standard, the cis-2-OH CCNU 27-min peak, and the CIEtSoSo 26-min peak.

Beranek et al. (19), conditions which release 3-alkylated adenosines, cytidines and guanines, 7-alkylated adenosines and guanines, and O6-alkylated cytidines; (b) preparative HPLC analysis with a shallower acetonitrile gradient appeared to separate it into at least two components; and (c) no radioactive peak was obtained upon alkylation of poly (dA-dT)-poly (dA-dT).

This evidence suggests that the radioactive peak which elutes with our O6-hydroxyethylguanine standard under our analytical conditions may consist of at least two alkylated bases which at this time remain unknown.

In this study we found that cis-2-OH CCNU produced 99.2 and 53.4 pmol/mg DNA/m/d drug of 7-hydroxyethylguanine and 7-chloroethylguanine, respectively (Table 1), and that CIEtSoSo produced 44.2 pmol/mg DNA/m/d drug of 7-chloroethylguanine. These results are in close agreement with those of Tong et al. (2), who found that a variety of CIEtu's produced 80–150 pmol/mg DNA/m/d drug of 7-hydroxyethylguanine and 50–71 pmol/mg DNA/m/d drug of 7-chloroethylguanine.

In summary, these results indicate that CIEtSoSo is more specific in its reaction with calf thymus DNA in that it generates a reduced variety of products than does cis-2-OH CCNU. Both hydroxyethylguanine and chloroethylguanine products were observed with the nitrosourea, whereas only 7-chloroethylguanine was observed as a major product with CIEtSoSo at equimolar doses.

DISCUSSION

The usefulness of a variety of anticancer drugs is severely limited by non-selective toxicities produced in the host. An understanding of the mechanisms by which selective antitumor activity differs from the non-selective host toxicities may lead to the rational development of compounds with an improved therapeutic index. Dr. Shealy and his colleagues at the Southern Research Institute synthesized CIEtSoSo as a potential chloro-
ethylyating agent. The chemistry of CIEtSoSo suggests that it may be devoid of some unnecessary and complex reactive species produced upon CIEtNu decomposition. Hence the objective of this study was to compare the spectrum of alkylation products produced in DNA by CIEtSoSo and cis-2-OH CCNU.

CIEtSoSo was found, in the National Cancer Institute Screening Program, to be highly effective against the murine P388 leukemia (9) and against L1210 leukemia, B16 melanoma, and Lewis lung carcinoma.4 Thus CIEtSoSo has antitumor activity which appears analogous to that observed previously with the CIEtNu’s (9, 20). In addition we have shown recently that CIEtSoSo can selectively kill cells deficient in the repair of guanine O6-alkylations, in a manner similar to that reported for the CIEtNu’s (5, 11). Our interpretation of these results was that CIEtSoSo and CIEtNu’s have the same basic mechanism: an initial chloroethylation of DNA guanine-O6 position followed by a series of reactions that lead to a delayed production of interstrand cross-links.

The results obtained here suggest that the chemistry of alkylation by CIEtSoSo is in fact different from that of CIEtNu’s. CIEtSoSo produces a less heterogeneous mixture of modified DNA base adducts. In particular the CIEtNu’s are capable of both chloroethylating and hydroxyethylating DNA, whereas CIEtSoSo only chloroethylates DNA. The importance of chloroethylation of DNA to the selective antitumor effects of these agents has been discussed previously; however, hydroxyethylation of DNA is thought to contribute little to antitumor activity, as evidenced by a marked reduction in antitumor activity when ethylation of DNA is thought to contribute little to antitumor activity, and monoadduct repair in nitrosoureas-treated human tumor cells.4 This is due to the reduction in the cytotoxicity of the drug to the Mer cell line. Thus the elimination of hydroxyethylation of DNA by the CIEtSoSo structure without loss of antitumor activity suggests that this compound does not undergo some unnecessary side reactions that occur with the CIEtNu’s. It may be that this in turn will result in a reduction in the quantity of reactions that are responsible for the non-selective toxicities observed previously with the CIEtNu’s.

Another distinct advantage of CIEtSoSo is its lack of carbamylation activity. Again this type of reaction is not involved in the selective antitumor activity, as CIEtSoSo’s without carbamoylation activity retain activity against murine tumors in vivo (23). More importantly carbamylation has been shown to be a non-selective reaction in human cell strains that results in unwanted toxicity (12). CIEtSoSo produces a less complex number of reactive species than do the CIEtNu’s, yet it still retains antitumor activity. The lack of carbamoylation and hydroxyethylation activity of CIEtSoSo makes this more selective compound a suitable candidate for clinical trial in humans, as these reactions do not appear to be required for the therapeutic effect.

REFERENCES


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J. Plowman, personal communication.

Unpublished data.
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