DNA Reactivity and in Vitro Cytotoxicity of the Novel Antitumor Agent 1,5,2,4-Dioxadithiepane-2,2,4,4-tetraoxide (NSC-348948) in Human Embryo Cells

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ABSTRACT

1,5,2,4-Dioxadithiepane-2,2,4,4-tetraoxide (cyclic-SoSo) is structurally a novel synthetic compound but may functionally act as an alkylating agent. The effects of cyclic-SoSo on DNA were studied in IMR-90 and VA-13 human embryonic lung cell lines. Cyclic-SoSo produced no DNA-DNA interstrand cross-links in either cell line, even at concentrations which produced a greater than 3 log cell kill. At equimolar concentrations cyclic-SoSo induced DNA-protein cross-links in both cell lines to a similar extent. Frank DNA breaks and alkali-labile DNA lesions were detected in both cell lines. A greater quantity of strand breaks appeared in the IMR-90 than in the VA-13 cell line after exposure to cyclic-SoSo. However, cyclic-SoSo was more cytotoxic to the VA-13 cell line than to the IMR-90 cell line. Thus cyclic-SoSo may not be a typical bifunctional alkylating agent in that its mechanism of action does not appear to involve DNA interstrand cross-linking.

INTRODUCTION

Cyclic-SoSo is a novel type of antitumor agent whose structure suggests that it may act as an alkylating agent. Cyclic-SoSo was found to be active against the L1210 and P388 leukemias, M5076 sarcoma, and the human MX-1 mammary tumor xenograft in the National Cancer Institute screening program. Structural considerations suggest that cyclic-SoSo may react in a manner resembling two other classes of antitumor agents, the haloethanesulfonates and the dimethanesulfonic acid esters (1-3), both of which may produce bifunctional alkylations, including DNA cross-links which may be major factors responsible for cytotoxicity and antitumor activity (3, 4).

There are three major reasons for studying the biological effects of cyclic-SoSo. (a) It is expected that cyclic-SoSo will enter into phase-I clinical trials in the near future; (b) a cyclic structure like cyclic-SoSo would be expected to show the effects of steric factors, should they be important for this class of compound; and (c) the intermediate to be expected after the initial electrophilic attack would carry a negative charge. This compound thus appears unusual as few bisalkylating antitumor agents carry a negative charge after the first alkylation. The latter two reasons were the major stimulus for the synthesis of this structure.

We have recently shown that CIEtSoSo, a close chemical relative of cyclic-SoSo, is more cytotoxic to an SV40 transformed human embryonic lung cell line (VA-13) than to a normal human embryonic lung cell line (IMR-90). The increased toxicity of CIEtSoSo to the VA-13 cell line correlates with a higher level of DNA interstrand cross-linking observed after drug exposure (1).

In this study we have used these two cell lines to test the hypothesis, as suggested by the structural similarities to CIEtSoSo and the dimethanesulfonic acid esters, that cyclic-SoSo would act as an alkylating agent capable of cross-linking DNA. Unlike the majority of bisalkylating agents, cyclic-SoSo does not produce DNA-DNA interstrand cross-links in these two human cell lines. However, cyclic-SoSo selectively killed VA-13 cells in preference to IMR-90 cells. The mechanisms by which the various types of DNA damage occur and the reason for the differential cytotoxicity between the two cell lines are discussed. The differences between cyclic-SoSo and CIEtSoSo suggest that these compounds act by different mechanisms.

MATERIALS AND METHODS

Cell Culture. IMR-90, a human fibroblast cell line derived from embryonic lung was obtained from W. Nichols, Institute of Medical Research (Camden, NJ). The VA-13 cell line was derived by transformation of the normal human embryo cell strain WI-38 with SV40, and has been maintained in this laboratory for several years.

Stock cultures of IMR-90 and VA-13 cells were maintained by seeding cells at a density of 5 x 10⁴ cells/ml at 37°C, in Eagle's minimal essential medium (Dutchland Laboratories, Denver, PA) supplemented with the following components: 10% fetal bovine serum, gentamicin (0.05 mg/ml), glutamine (0.03 mg/ml), d-biotin (0.1 mg/ml), vitamin B₁₂ (1.36 µg/ml), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.02 M (2-hydroxyethyl)-1-piperazineethanesulfonic acid. For colony forming assays cells were seeded at 0.1, 0.3, 1, and 3 x 10⁴ cells per 25-cm² plastic flasks (Costar, Cambridge, MA) in Eagle's minimal essential medium. Cells were preincubated for 24 h to allow attachment to the surface of the dish. Cells were then exposed to various concentrations of cyclic-SoSo for 2 h at 37°C. After 10 days of incubation in fresh medium, the plates were rinsed with Hanks' balanced salt solution, fixed with methanol, and then stained with a solution containing 1 ml methylene blue, 1 ml 0.15 M Na₂HPO₄, and 1 ml 0.15 M KH₂PO₄ diluted to 50 ml with distilled water. Colonies were then counted and the observed control plating efficiencies were 23% for IMR-90 cells and 52% for VA-13 cells.

Drug Treatment. Cyclic-SoSo was obtained from the Drug Development Branch, National Cancer Institute, and was dissolved in sterile dimethyl sulfoxide immediately before treatment of cell cultures. The concentration of dimethyl sulfoxide in either treated or control cells was never greater than 2% (v/v). Treatments were terminated by aspiration of the drug-containing medium and replacement with fresh medium.

Alkaline Elution Experiments. For alkaline elution experiments 1.5-2.5 x 10⁵ cells were seeded into 25-cm² flasks in 10 ml medium which contained 0.02 µCi [³H]thymidine/ml (56 mCi/mmol; New England Nuclear, Boston, MA) and grown for 24 h. The radioactive medium was removed and the cultures were grown for an additional 18-24 h to allow for the maturation of labeled DNA into higher molecular weight DNA.

The alkaline elution analyses were carried out as previously described (5). For analyses of interstrand cross-links, strand breaks and alkali-labile lesions, cells were lysed on 0.8-µm-pore size polycarbonate (“Nuclepore”) filters with 2% sodium dodecyl sulfate, 0.025 M EDTA, pH 10.0, which was allowed to flow through the filter by gravity. Following lysis, 2 ml of 2% sodium dodecyl sulfate, 0.02 M EDTA, 0.1 M glycine (pH 10) containing 0.5 mg proteinase K/ml (EM Laboratories, Darmsstadt, West Germany) when necessary, were added to a reservoir over 1679

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the polycarbonate filters and pumped through the filter for approximately 1 h at 2 ml/h. DNA was eluted from the filter by pumping 0.02 M EDTA solution, adjusted to either pH 12.1 or 12.6 with tetrapropylammonium hydroxide, which contained 0.1% sodium dodecyl sulfate. For assay of interstrand cross-links, the cells cooled in 0.02 M EDTA solution, adjusted to either pH 12.1 or 12.6 with tetrapropylammonium hydroxide, which contained 0.1% sodium dodecyl sulfate. For assay of interstrand cross-links, the cells cooled in ice were irradiated with 3000 rads of 137Cs γ-rays. Internal standards were [3H]thymidine-labeled L1210 cells irradiated with 300 rads in the cold. Interstrand cross-link index is defined as

$$\left( \frac{1 - R_0}{1 - R} \right)^{1/2} - 1$$

where and $R_0$ and $R$, are relative retention of [14C]DNA at 25% retention of [3H]DNA (5); no corrections for strand breaks were applied.

For assay of DNA-protein cross-links, cells were irradiated with 300 rads 137Cs γ-rays in the cold. Cells were lysed on 2-μm-pore size polyvinylchloride filters with 2% sodium dodecyl sulfate, 0.025 M EDTA, pH 10.0. Elution was with tetrapropylammonium hydroxide-EDTA, pH 12.1. Internal standards were [3H]thymidine-labeled L1210 cells irradiated with 3000 rads. DNA-protein cross-link index is defined as

$$\left( 1 - r \right)^{1/2} - \left( 1 - r_0 \right)^{-1/2}$$

where $r$ and $r_0$ are the fraction of [14C]DNA or [3H]DNA, respectively, in the slow eluting component.

RESULTS

Differential Cytotoxicity. The transformed human embryo cell line, VA-13, was compared with the normal human embryo cell strain, IMR-90, to determine if any difference in the cell killing by cyclic-SoSo existed. Cytotoxicity assays based on reduced colony formation showed VA-13 cells to be more sensitive to the drug than were IMR-90 cells (Fig. 2). A 3-fold higher concentration of cyclic-SoSo was required to reduce the survival of IMR-90 cells to the same degree as a given drug concentration applied to VA-13 cells.

DNA Interstrand Cross-Linking. DNA interstrand cross-linking by cyclic-SoSo in IMR-90 and VA-13 cells was examined in a manner similar to that which had previously been used to study the effects of CIIeSoSo on these same cell types (1). Cyclic-SoSo produced no detectable interstrand cross-links in either cell line up to 18 h after drug treatment, even at concentrations which produced a greater than 3 log cell kill (Figs. 3 and 4). The increased rate of elution of the drug treated cells relative to the 300 rads control cells is due to DNA strand breaks produced at the same time after drug treatment (Fig. 4).

DNA-Protein Cross-Linking. Cyclic-SoSo produced DNA-protein cross-links equally in IMR-90 and VA-13 cells (Fig. 5). Immediately after the 2-h drug treatment (0 h in Fig. 5), DNA-protein cross-linking at corresponding drug concentrations was nearly the same in the two cell types. In VA-13 cells there appeared a slight increase in the DNA-protein cross-linking 6-12 h after drug exposure, followed by a decrease 18-24 h after drug removal (Fig. 5). The DNA-protein cross-linking in IMR-90 cells appeared to remain constant for the first 12 h after drug exposure, followed by a decrease 18-24 h after drug removal.

DNA Strand Breaks and Alkali-labile Lesions. Cyclic-SoSo produced an unusual pattern of formation and removal of DNA strand breaks. We have assayed both IMR-90 and VA-13 cells for the appearance and disappearance of frank DNA breaks and alkali-labile lesions. The frank DNA breaks and alkali-labile lesions can usually be distinguished by performing the alkaline elution assays at pH 12.1 and 12.6. An increase in the elution rates between the pH 12.6 assay and pH 12.1 assay strongly suggests the presence of alkali-labile lesions. The downward curvature of an elution profile at pH 12.1 may also be an indicator of alkali lability (6).

Assay to Detect Frank DNA Breaks. Cyclic-SoSo induced DNA strand breaks (assays performed at pH 12.1 indicating frank breaks) in both cell lines (Fig. 6). The formation of these breaks appeared to gradually increase during the 18 h post-drug treatment studied (Fig. 6). This profile was similar in both cell lines; however, cyclic-SoSo at equimolar concentrations induced a greater quantity of frank breaks in the IMR-90 cell line.

Assay to Detect Alkali-labile DNA Lesions. In the VA-13 cell line the strand breaks induced by cyclic-SoSo increase 6 h posttreatment, followed by a period of repair 12 h after drug removal with a further increase in the quantity of breaks by 18 h. In the IMR-90 cell line there is a large quantity of strand breaks followed by a period of repair 6-12 h after drug removal, with an additional increase in the quantity of breaks produced 18 h after drug treatment (Fig. 6). Again more breaks were observed in the IMR-90 cell line than in the VA-13 cell line.

To check whether the appearance of the frank DNA breaks was due to the destruction of a drug-induced DNA-protein
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Fig. 3. Alkaline elution profiles to show the lack of DNA interstrand cross-linking in VA-13 cells after a 2-h treatment with cyclic-SoSo and a variety of times after drug removal. The concentrations used are as indicated. These profiles are representative of at least three independent experiments.

Fig. 4. Alkaline elution profiles to show the lack of DNA interstrand cross-linking in IMR-90 cells after a 2-h treatment with cyclic-SoSo and a variety of times after drug removal. The concentrations used are as indicated. These profiles are representative of at least three independent experiments.

interaction, we compared the elution profiles obtained with or without the use of a proteinase K digestion step in the elution assay. No strand breaks were observed in either the VA-13 or IMR-90 cell line when proteinase K was deleted from the elution protocol. In contrast, the use of proteinase K resulted in a large increase in the elution rate of drug treated cells relative to the elution rate of control cells (Fig. 7). Thus it would appear that the breaks observed after cyclic-SoSo exposure are protein associated.

DISCUSSION

Cyclic-SoSo is a novel synthetic structure which was shown to possess significant antitumor activity in the National Cancer Institute screening system. It has close structural resemblances to two other types of antitumor agents, the haloethylsulfonates, and the dimethanesulfonates, both of which are thought to act by cross-linking DNA (1, 3, 4). We have previously shown that ClEtSoSo, a haloethylsulfonate, cross-links DNA presumably after an initial alkylation at the O6 position of guanine (1). In that study ClEtSoSo was more toxic to the Mer- cell line, VA-13, than the Mer+ IMR-90 cell line (1, 7). Thus a cell line which was incapable of repairing alkylations at the O6 position of guanine was found to be more sensitive to ClEtSoSo (1).

In close agreement with this increased sensitivity we observed that the Mer- cell line allowed a large quantity of DNA cross-links to form after ClEtSoSo exposure, whereas a Mer+ cell line did not. In this study, under identical conditions, we have found that a Mer- cell line (VA-13) is more sensitive to cyclic-SoSo than the Mer+ cell line (IMR-90). However, in contrast to ClEtSoSo, this cannot be accounted for by an increased level of DNA cross-linking in the Mer- cell line.

Our results are similar to the previous findings of Bedford and Fox (3) and Bedford et al. (4), who showed that ethylene dimethylsulfonate, the 2-carbon chain analogue of busulfan, produced no DNA interstrand cross-links in Yoshida lymphosarcoma cells, but the concentrations used produced little cytotoxicity. In contrast, busulfan-induced DNA interstrand cross-links in Yoshida cells at cytotoxic concentrations (3, 4). The inability of cyclic-SoSo to cross-link DNA at cytotoxic concentrations...
concentrations, however, may be due to factors such as steric hindrance, as the bulky sulfoxide groups may be repelled by the phosphate backbone of DNA. This may prevent cyclic-SoSo from alkylating specific sites in DNA which prevents the production of alkylated bases with the potential to form DNA interstrand cross-links. Another possible explanation for the failure of cyclic-SoSo to induce DNA interstrand cross-linking may be the fact that after an initial alkylation it results in an intermediate with a negative charge. This is unusual for a bisalkylating agent as ClEtSoSo and the dimethanesulfonic acid esters result in either a neutral or positively charged intermediate species. The negatively charged intermediate produced by cyclic-SoSo may preferentially react with the positively charged histones which surround the DNA helix and produce a DNA-protein cross-link in preference to a DNA interstrand cross-link. It does not appear, however, that DNA-protein cross-links are responsible for the differential cytotoxicity, since at equimolar concentrations there is an equivalent level of DNA-protein cross-links in both cell lines. The possibility always exists that cyclic-SoSo may form fewer, more selectively toxic, DNA interstrand cross-links than does ClEtSoSo, and that in this study the large quantity of strand breaks masked their detection by alkaline elution.

A complex pattern of DNA strand breaks was produced by cyclic-SoSo. Both frank DNA breaks and alkali-labile lesions were detected. The rate of formation and removal of these lesions complicates their interpretation. Lown and Singh (8) have suggested that there are at least two mechanisms by which DNA strand breaks may occur. Strand breaks may arise by phosphate alkylation which results in a rapid hydrolysis of the resultant phosphotriester under the alkaline conditions of their assay. The second mechanism of strand breakage consists of base alkylation, followed by depurination and depyrimidination and subsequent hydrolysis of the resulting base free site (8). DNA breaks induced by cyclic-SoSo in the VA-13 and IMR-90 cell lines may be due to either or both of these mechanisms. The formation and removal of cyclic-SoSo induced DNA breaks are different from those previously observed with ClEtSoSo (1). ClEtSoSo was found to produce minimal frank DNA breaks (pH 12.1 assay) and two components of delayed alkali-labile lesions (pH 12.6 assay), all of which appear to undergo repair (1). Evidence to suggest yet another alternative mechanism for DNA strand breakage came from the fact that cyclic-SoSo-induced DNA strand breaks, detected at pH 12.1, were found to be protein associated, in that they were not revealed unless proteinase K was used in the alkaline elution protocol.
A type of phenomenon has previously been observed with intercalating agents (9); however, the mechanisms responsible for such a phenomena are not clear. The importance of the breaks observed in this study in relation to cytotoxicity is also unclear. The greater quantity of DNA breaks observed in the IMR-90 cell line, the cell line which is less sensitive to cyclic-SoSo, suggests that DNA strand breaks are inversely correlated with cytotoxicity. It may be that these breaks reflect the ability of IMR-90 cells to repair cyclic-SoSo-induced DNA damage and this allows survival of these cells.

In conclusion, cyclic-SoSo does not appear to be a typical alkylating anti-tumor agent, in that its mechanism of action does not appear to be due to DNA-DNA interstrand cross-linking. More importantly, the differential cytotoxicity observed with cyclic-SoSo may be caused by a mechanism which is totally different to that previously suggested for chloroethylating agents such as CIEtSoSo. To this end we are currently trying to identify the mechanisms responsible for the variety of DNA strand breaks induced by cyclic-SoSo and to clarify their relationship to cytotoxicity.

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