Notes

Synthesis and Antitumor Evaluation of a Highly Potent Cytotoxic DNA Cross-Linking Polyamine Analogue, 1,12-Diaziridinyl-4,9-diazadodecane

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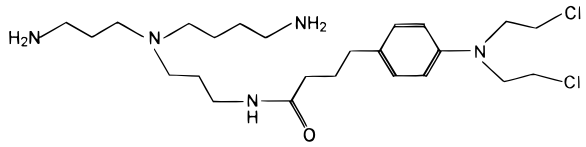
A diaziridinylspermine analogue, 1,12-diaziridinyl-4,9-diazadodecane (NSC-667005), was synthesized as a bisalkylating agent with a polyamine backbone. DNA cross-linking was detected in the reaction of linearized pBR322 DNA with 1,12-diaziridinyl-4,9-diazadodecane at concentrations comparable with that required for cross-linking by two nitrogen mustard drugs, mechlorethamine and melphalan. A significant increase in life span of female CD_2F_1 mice bearing L1210 murine leukemia was observed after intravenous administration of 1,12-diaziridinyl-4,9-diazadodecane in doses of less than 2.7 mg/kg, given on days 1, 5, and 9 of treatment.

Introduction

Polyamines are aliphatic polycations that appear to have a significant role in the regulation of normal and malignant cell proliferation.[1] Polyamine analogue development has become an attractive approach in drug design strategies for new cytotoxic or growth inhibitory compounds.[2] Many of these analogues may interact selectively with DNA because spermine and other polyamines bind avidly to DNA, and analogues bearing a polyamine backbone usually retain DNA-binding properties. Another feature of polyamine analogues useful in drug design is derived from the observation that a number of cancer cell lines possess polyamine uptake systems that selectively accumulate endogenous polyamines and structurally related compounds.[3] Polyamine uptake systems are more active in cells with high demands of polyamines, such as cancer cells,[4] suggesting that anticancer polyamines may show tumor selectivity.

A specific approach to the design of anticancer agents active through DNA-targeting mechanisms has been the combination of a polyamine structure with a bifunctional alkylating group. The polyamine portion provides a potential substrate for cellular polyamine uptake transporters, and the alkylating group imparts cytotoxic activity. As an example of this approach, Cohen *et al*. synthesized chlorambucil−spermidine conjugate **1** through an amide linkage of chlorambucil (4-[bis(2-chloroethyl)amino]benzenebutanoic acid) with a propylamino spacer attached at the secondary amine position of spermidine.[5] This combination is highly efficient at producing DNA cross-links compared with its parent compound, chlorambucil. On the other hand, the biological activity profile of the chlorambucil−spermidine conjugate suggests that this compound is not taken up well by cells because *in vivo* potency did not reach the level predicted from *in vitro* studies.[6] Stark *et al*. synthesized and evaluated spermidine analogue **2**, consisting of a nitrogen mustard functionality attached at the C-5 position of spermidine. The resulting compound was not more cytotoxic against B16-BL6 melanoma cells than was chlorambucil, and up-regulation of the polyamine transporter did not increase cytotoxicity.[7]



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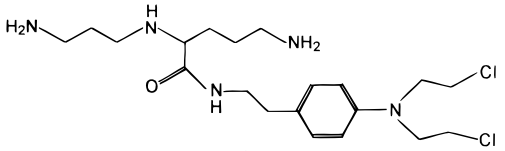

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Previously, we synthesized and evaluated two monoaziridinyl analogues of spermidine as irreversible cytotoxic agents *in vitro*.[8] N^1- And N^8-aziridinylspermidine [*N*-(3-aziridinylpropyl)-1,4-diaminobutane and *N*-(4-aziridinylbutyl)-1,3-diaminopropane, respectively] were found to be cytotoxic against a variety of cancer cell lines in submicromolar concentrations. Involvement of polyamine biochemistry in the action of these compounds was deduced from experiments that showed that depletion of cellular polyamines by pretreatment of L1210 cells with DFMO (2-(difluoromethyl)-DL-ornithine) enhanced the cytotoxicity of the aziridinylspermidine analogues, whereas co-incubation with spermidine pro-

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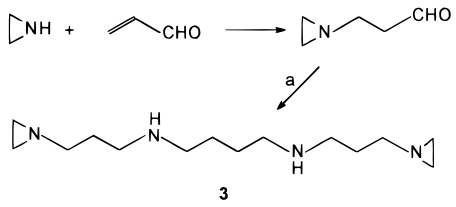
Scheme 1[a]



[a] Reagents: (a) 1,4-diaminobutane, $NaBH_4$.

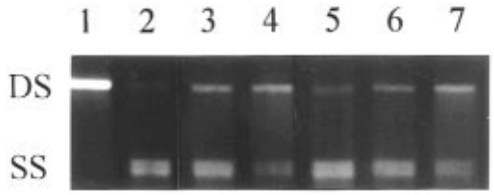


Figure 1. Agarose gel chromatography showing DNA cross-linking by 1,12-diaziridinyl-4,9-diazadodecane, mechlorethamine, and melphalan. Bands correspond to cross-linked or double-stranded (DS) and single-stranded (SS) DNA. Lane 1 (control nondenatured DNA); lane 2 (DNA denatured in the absence of test compound); lanes 3,4 (mechlorethamine, 1.0 and 2.0 μM, respectively); lane 5 (melphalan, 5.0 μM); lanes 6,7 (1,12-diaziridinyl-4,9-diazadodecane, 0.1 and 0.4 μM, respectively).

tected against cytotoxicity caused by the aziridinyl spermidine analogues. Utilization of a polyamine transporter was indicated by experiments that showed that L1210 cells accumulated N^1- and N^8-aziridinylspermidine.

Our current approach to aziridinylpolyamine analogue development consists of extension of the backbone to mimic the tetraamine, spermine, and the incorporation of two aziridine functionalities to provide the capability of cross-linking DNA without loss of polyamine-related properties. We now report the *in vivo* activity in a murine L1210 model and bifunctional alkylating properties of the spermine analogue, 1,12-diaziridinyl-4,9-diazadodecane (**3**, NSC-667005).

Results and Discussion

Reductive alkylation of both amino functionalities of 1,4-diaminobutane with 3-aziridinylpropanal in the presence of $NaBH_4$ afforded the spermine analogue, 1,-12-diaziridinyl-4,9-diazadodecane (Scheme 1).

Cross-linking resulting from the treatment of linearized pBR322 DNA with 1,12-diaziridinyl-4,9-diazadodecane in concentrations as low as 0.1 μM was easily detected (Figure 1). Linear, double-stranded, pBR322 DNA, consisting of approximately 4000 base pairs, was incubated with the bis(aziridinyl)spermine analogue and with two standard cross-linking drugs, mechlorethamine [2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine] and melphalan [4-[bis(2-chloroethyl)amino]-L-phenylalanine]. Interstrand cross-linking was detected as the presence of double-stranded DNA that could not be heat denatured to form single strands.

Incorporation of aziridine functionalities into the spermine nucleus provided a compound that was effective in forming interstrand cross-links in naked DNA. The concentrations needed for the observation of cross-links with this agent were equal to or less than the concentrations required to observe cross-links by two cross-linking, nitrogen mustard drugs (Figure 1). Competition studies carried out in the presence of spermine at a concentration of 1.0 μM indicated that spermine protects DNA from alkylation by 1,12-diaziridinyl-4,9-diazadodecane (data not shown).

Table 1. Effects of Treatment with 1,12-Diaziridinyl-4,9-diazadodecane on the Life Span of Mice Bearing L1210 Murine Leukemia

treatment[a] (mg/kg)	mean survival (days ± SD)	% ILS[b]	NLCK[c]
control (no treatment)	7 ± 0	0	
thiotepa[d]	15.3 ± 1.3	114	1
4.0 on days 1, 5, 9[e]	11.0 ± 5.4*	14	−7
2.7 on days 1, 5, 9	18.8 ± 1.1*	171	4
1.8 on days 1, 5, 9	17.4 ± 1.1*	143	2
4.0 on days 1, 8	17.2 ± 1.5*	157	4
2.7 on days 1, 8	15.2 ± 1.1*	129	2
1.8 on days 1, 8	12.6 ± 1.7*	86	−1
4.0 on days 1, 2[f]	5.4 ± 0.9	−29	−3
2.7 on days 1, 2	13.2 ± 3.0*	100	6
1.8 on days 1, 2	14.8 ± 1.1*	100	6
6.0 on day 1[g]	6.8 ± 1.3	−14	−1
4.0 on day 1	11.0 ± 1.9*	57	4
2.7 on day 1	10.4 ± 0.6*	42	3
1.8 on day 1	10.8 ± 0.5*	57	4

* Analysis of variance, $p \leq 0.05$; value different from the control, $p \leq 0.05$, using the Newman Keuls test for pairwise comparisons.[11] [a] Intravenous dose. [b] Percent increase in life span. [c] Net log cell kill. [d] As a standard, thiotepa was administered intraperitoneally at a dose of 10 mg/kg on days 1, 5, and 9. [e] LD20. [f] LD100. [g] LD50.

In vivo anticancer activity against implanted L1210 cells was determined (Table 1) with thiotepa as the positive control. Although 1,12-diaziridinyl-4,9-diazadodecane was highly toxic to mice with an estimated LD_{50} calculated to be 6 mg/kg after intravenous administration, three intravenous doses of 1.8 mg/kg spaced 4 days apart provided increased survival of mice implanted with 10^5 L1210 cells. With the likelihood that more frequent doses or continuous infusion would yield even greater activity, 1,12-diaziridinyl-4,9-diazadodecane joins the list of potent DNA cross-linking compounds with potential anticancer activity.

The apparent high level of cytotoxic activity shown by this aziridinyl polyamine in comparison with other aziridines suggests that the spermine backbone of the molecule may guide the molecule through cellular membranes to the proximity of binding sites on DNA. Proximity catalysis of electrophilic attack on DNA by the aziridino moieties is a likely explanation of the observed cross-linking of DNA. An alternative explanation based on high-affinity, noncovalent binding of the polyamine analogue with duplex DNA has not been ruled out by these experiments, although spermine alone at a concentration of 1.0 μM did not prevent denaturation of DNA.

On the basis of these preliminary findings, 1,12-diaziridinyl-4,9-diazadodecane is serving as a lead compound in the development of a new group of anticancer agents.

Experimental Section

CAUTION: acrolein and all aziridines should be treated as highly toxic and handled accordingly.

1,12-Diaziridinyl-4,9-diazadodecane (3). To a solution of aziridine[9] (3.72 g, 86.5 mmol) in diethyl ether (30 mL) was slowly added a solution of acrolein (4.60 g, 82.1 mmol) in diethyl ether (20 mL) cooled in an ice bath. After stirring for 1 h, the mixture was concentrated on a rotary evaporator to yield crude 3-aziridinylpropanal (7.28 g, 89%). Without further purification, 3-aziridinylpropanal (1.60 g, 16.2 mmol) in methanol (15 mL) was added dropwise to a solution of 1,4-diami-

nobutane (0.71 g, 8.1 mmol) in methanol (20 mL). After stirring for 10 minutes, $NaBH_4$ (1.3 g) was added in small portions. After 6 h, excess reducing agent was destroyed by adding 6 N methanolic HCl, and the reaction mixture was evaporated to near dryness. The residue was dissolved in water (10 mL) and adjusted to pH 12 with 40% NaOH, and the resulting solution was extracted with diethyl ether (3 × 30 mL). The combined organic extracts were dried (Na_2SO_4) and purified by fractional distillation to afford 1.07 g (52%) of **3** (bp 132−134 °C/0.15 mm): ^{1}H NMR ($CDCl_3$) δ 1.08 (m, 4 H, aziridine), 1.50 (m, 4 H), 1.70 (m, 4 H, aziridine), 1.74 (m, 4 H), 2.22 (t, 4 H, J = 7.4 Hz), 2.60 (m, 4 H), and 2.72 (t, 4 H, J = 7.5 Hz) ppm; mass spectrum (EI mode) (rel int), m/z (M^+, 0.2), 84 (100), 42 (aziridine, 51). Anal. ($C_{14}H_{30}N_4 \cdot 0.2H_2O$) C, H, N.

Reactivity with DNA. Determinations of DNA interstrand cross-linking were conducted following a modified procedure of Hartley *et al.*[10] Plasmid pBR322 DNA was linearized by digestion for 60 min at 37 °C with the restriction enzyme *HindIII* (Promega) at 1 unit/μg DNA. DNA (400 ng) was incubated with specified concentrations of test compounds in triethanolamine hydrochloride (25 mM) buffer, pH 7.4, containing EDTA (1 mM) at room temperature for 18 h. Alkylation reactions were terminated by the addition of an equal volume of a solution consisting of sodium acetate (0.6 M), EDTA (20 mM), and tRNA (100 μg/mL), and the DNA was immediately precipitated by the addition of 3 vol of anhydrous EtOH. Following centrifugation at 14000*g* for 15 min and removal of the supernatant fraction, the DNA pellet was dried by lyophilization. Experimental samples were dissolved in 10 μL of strand separation buffer [DMSO (30%), EDTA (1 mM), bromophenol blue (0.05%), and xylene cyanole (0.04%)], heated at 95 °C for 8 min, and chilled immediately in a CO_2/ethanol bath prior to loading onto agarose gels. Control, undenatured samples were dissolved in 10 μL of a sucrose (6%) solution containing bromophenol blue (0.04%) and loaded directly onto agarose gels. Agarose gels (1%, 10 cm) were made in TAE buffer [Tris (40 mM), acetic acid (20 mM), EDTA (2 mM), pH 8.1]. Electrophoresis was performed on submerged horizontal gels at 90 V for 1.5 h in TAE buffer. Gels were stained with ethidium bromide (0.5 μg/mL) and photographed under light at 302 nm.

***In Vivo* Antitumor Studies.** Virus-free, 12-week-old adult female CD_2F_1 mice were obtained from the animal program administered by the Animal Genetics and Production Branch of the National Cancer Institute. To minimize exogenous infection, mice were housed in microisolator caging and handled in accordance with the NIH guide for the care and use of laboratory animals (NIH No. 85-23, 1985). Mice were randomized to groups of five mice per group except titration groups which consisted of 10 mice and the untreated group which consisted of 20 mice. L1210 cells (G050141) were obtained from the Division of Cancer Treatment Tumor Repository at the National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD, and were passaged once ip in DBA/2 mice and stored in liquid N_2 and considered mouse passage 2. The viral profile of the cells was negative. Prior to implantation in CD_2F_1 mice, cells from liquid N_2 (mouse passage 2) were passaged ip in DBA/2 mice, the ascites cells were collected and washed with sterile media, and red cells were lysed. On day 0, mice were inoculated with L1210 cells (1×10^5 cells/mouse) intraperitoneally for treatment and control groups. Titration groups received 1×10^4 through 1×10^6 cells/mouse. Dosing solutions were freshly prepared just prior to treatment by dissolving 1,12-diaziridinyl-4,9-diazadodecane in 25 mM sodium phosphate buffer, pH 7.4, giving concentrations of 0.18−0.6 mg/mL. Beginning on day 1, dosing solutions were administered intravenously by tail vein at 0.01 mL/g of body weight. Doses of 1,12-diaziridinyl-4,9-diazadodecane were 6.0, 4.0, 2.7, and 1.8 mg/kg. Dosing schedules were as follows: a single dose on day 1, every 4 days beginning on day 1 for 3 doses, every 7 days for 2 doses beginning on day 1, or 2 doses, days 1 and 2.

Tumor doubling time was calculated from the median days of death for the titration groups and was found to be 0.3 days. Percent increase in lifespan was calculated using the formula, $(T - C)/C \times 100$, where T is the median day of death for the various treatment groups and C is the median day of death for the control group. Net log cell kill is an estimate of the number of cells that are killed by the drug at each specific dose and schedule and was calculated by the formula, $[[(T - C) - \text{duration of treatment}] \times 0.3]$/tumor doubling time.

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