Cytotoxic Effects and Biological Activity of 2-Aza-8-germanspiro[4,5]-
decane-2-propanamine-8,8-diethyl-N,N-dimethyl Dichloride (NSC 192965; Spirogermanium) in Vitro

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ABSTRACT

Lethal and other biological effects of 2-aza-8-germanspiro-[4,5]decane-2-propanamine-8,8-diethyl-N,N-dimethyl dichloride (NSC 192965; spirogermanium), representing a new chemical class of compound exhibiting antitumor activity, have been studied in vitro. Survival curves for NIL 8 hamster cells were exponential with greater kill occurring with increasing drug concentrations and longer exposure times. Cytotoxicity was temperature dependent. "Quiescent" cultures were significantly less sensitive to spiroygermanium than were logarithmically growing cells. These lethal effects showed no phase specificity. There was no evidence of progression delay through the cycle following spiroygermanium treatment.

When spiroygermanium was tested against a range of human cell lines, the consistency of the values for the drug concentration required to reduce survival by 50% on the exponential part of the survival curve, derived from colony-forming assays, was most marked. The survival curves, characterized by an initial shoulder, were steep and exponential with measurements possible over only a narrow concentration range since complete cell lysis occurred at levels causing a >2-log kill. Cell membrane damage by spiroygermanium, as judged by dye exclusion, was most striking. The precise mode of action of spiroygermanium remains to be established, and these data further illustrate its apparent lack of specificity.

INTRODUCTION

Spirogermanium2 represents a new type of synthetic compound that incorporates germanium into a heterocyclic ring structure (10). Laboratory studies have shown cytotoxic activity against certain tumor cell lines both in vitro and in vivo (10, 11, 17), although its mechanism(s) of action remains unclear. The most striking feature of spiroygermanium in these animal studies was the lack of any evidence of tissue toxicity from relatively large and continuous dosing. Transient central nervous system toxicity, however, was experienced following high-dose, single-push injections, but this problem could be overcome by administration of the same or larger doses by slow infusion over 15 to 30 min (17). Phase I clinical studies have confirmed the absence of acute cumulative hematological, renal, or hepatic toxicities with spirogermanium (2, 10, 12, 17). Its limiting toxicity is neurological. However, infusions over 15 to 30 min have allowed the administration of repeated weekly doses of 80 and 120 mg/sq m without toxicity, and these dosages or higher have been recommended for Phase II studies. A recent Phase II study reporting the use of a dose of 50 mg/sq m twice weekly shows the value of spirogermanium as a palliative agent in advanced ovarian cancer (15). Objective partial remissions were obtained in 2 (11%) of the 18 patients treated, and an additional 4 (22%) patients had stable disease.

To provide more background information for ongoing clinical studies, we have determined the lethal and kinetic effects of spiroygermanium against a range of mammalian cell lines, including various human tumor lines, so as to identify any tumor type specificity, and we have attempted to establish the mode of action of the drug in vitro.

MATERIALS AND METHODS

Materials. Spirogermanium was provided for these studies by Unimed, Inc., Somerville, N. J. Low gelling temperature agarose was obtained from Unicience, Ltd., Cambridge, England. Mithramycin used for FMF was purchased from Pfizer Co., Ltd., Kent, England, as Mithracin, and fluorodeoxyuridine was from Sigma Chemical Co., St. Louis, Mo. Media and sera were supplied by Gibco-Biocult, Ltd., Croydonshire, Scotland. The radioisotopes (purchased from the Radiocchemical Centre, Amersham, Buckinghamshire, England) used in this study were [methyl-3H]thymidine (specific activity, 23 Ci/mmol), [5-3H]uridine (specific activity, 6 Ci/mmol), and L-[4,5-3H]leucine (65 Ci/mmol).

Cell Cultures. NIL 8 cells isolated from Syrian golden hamster ovary cell lines were grown in Eagle's medium plus 10% calf serum at 37° in a humidified atmosphere of 10% CO2 in air as described previously (5). Logarithmically growing cultures were obtained 24 hr after subculture while "quiescent" cultures were obtained from cells grown to confluency (5 days after plating without media changing) in which the cell count per dish had not increased over the previous 36-hr period. Cell counts and cell volumes were obtained using a Model ZBI Coulter Counter. Synchronous cultures were obtained by mitotic selection as described earlier (7).

COLO 205 cells, derived from a human colon carcinoma (14), were maintained in suspension culture in Roswell Park Memorial Institute Medium 1640 (Gibco-Biocult, Ltd.) supplemented with 10% fetal calf serum. Human neuroblastoma cells (CHP100) were maintained in monolayer culture in Eagle's medium plus 10% fetal calf serum. Mithracin, and fluorodeoxyuridine was from Sigma Chemical Co., St. Louis, Mo. Media and sera were supplied by Gibco-Biocult, Ltd., Croydonshire, Scotland. The radioisotopes (purchased from the Radiocchemical Centre, Amersham, Buckinghamshire, England) used in this study were [methyl-3H]thymidine (specific activity, 23 Ci/mmol), [5-3H]uridine (specific activity, 6 Ci/mmol), and L-[4,5-3H]leucine (65 Ci/mmol).

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Primary culture fetal rat hypothalamic neurons (REH99) were prepared as described previously (16) and maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, nonessential amino acids, and subtoxic levels of fluorodeoxyuridine (40 μM) to prevent overgrowth of nonneuronal cells.

All cultures were maintained at 37° in a humidified atmosphere of 10% CO2 in air. Only data obtained from Mycoplasma-free cultures are presented.

**Colony-forming Assays.** Cells in logarithmic growth were exposed to a selected range of drug concentrations for 1 or 24 hr. Cell survival was assessed by determining the ability of cells to form colonies. Monolayer cultures were washed with serum-free medium, trypsinized (0.25% trypsin as Trypsar from Armour Pharmaceuticals Co., Ltd., Eastbourne, England), and resuspended in fresh medium with serum. The appropriate number of cells to yield approximately 100 to 200 colonies per assay point were then either plated directly onto plastic in the case of NIL 8 and LOVO cells or, for the CHP100 and HN-1 cells, placed into Falcon 2051 plastic tubes to which were added 5 ml of agarose (final concentration, 0.17%) containing medium plus 10% fetal calf serum. For the HN-1 colony assays, “feeder” cells were provided by exposing cells to 14,000 rads and adding these to the agarose at a concentration of 105 cells/ml. The COLO 205 cells were removed from suspension, washed with serum-free medium, and resuspended in medium containing 10% serum prior to assessing their colony formation as described above for CHP100 cells. Under these conditions, the colony-forming efficiencies were: NIL 8 cells (logarithmically growing), 50 to 60%; NIL 8 cells (stationary phase), 35%; COLO 205, 30%, CHP100, 17%, HN-1 cells, 20%; and LOVO, 26%.

The mean and S.E. of the colony counts were expressed as a percentage of the control cultures, which were given the value of 100%. The curves were fitted by eye to the data points.

**Vital Dye Exclusion.** Logarithmically growing COLO 205 cells were exposed to a range of spirogermanium concentrations prior to monitoring at 0.5-hr intervals after drug addition for the ability of the cells to exclude trypan blue (Sigma). Cells in medium with serum were mixed with an equal volume of 0.2% trypan blue in phosphate-buffered saline, and the number of cells excluding the dye was scored using a Leitz microscope (x40).

**FMF Analyses.** The effects of spirogermanium on cell cycle progression of logarithmically growing NIL 8 and COLO 205 cells were analyzed by measurements of relative DNA content of individual cells. Individual cells were fixed with ethanol and stained with mithramycin (20 mg/ml in 20% ethanol and 300 mM MgCl2) and analyzed using a fluorescence-activated cell sorter (FACS-1, Falcon Plastics, Oxnard, Calif.) as described previously (5).

**Cellular Incorporation of Radioactively Labeled Precursors.** Logarithmically growing COLO 205 cells in medium containing 10% serum were treated with spirogermanium (0.5, 0.75, or 1.5 μg/ml). At 2, 6, and 24 hr after treatment, the cells were removed from suspension by centrifugation and washed with Hank’s balanced salt solution prior to incubation at 37° in fresh Hank’s solution with glucose containing the radiolabel of [3H]thymidine, [3H]uridine, or [3H]leucine at a final concentration of 1 μCi/ml for 1 hr. Triplicate samples of both drug-treated and control (untreated) cells were analyzed at each time point for radioactivity associated with trichloroacetic acid-insoluble material as described earlier (6).

After extraction by the procedure of Scott, the cellular DNA, RNA, and protein levels were determined using standard methods detailed previously (6).

**Effects of Temperature on the Cytotoxicity of Spirogermanium.** Serum-containing medium was precooled or preheated to the required temperatures using a water bath containing a Thermomix 1420 Braun Unit (F. T. Scientific Instruments, Breden, Gloucester, England), which controlled the temperature with an accuracy of ±0.02° (S.E.). The appropriate medium was then added to dishes containing monolayers of NIL 8 cells established at 37°. Some of the dishes were treated with spirogermanium, while others served as controls. All were then placed in boxes, gassed with 10% CO2 in air, sealed, and returned to the appropriate water baths for the incubations. Subsequently, the colony-forming abilities of the cells were measured as described above. Plating efficiencies in the range of 45 to 56% were not significantly influenced by temperature.

**RESULTS**

**Lethal Effects of Spirogermanium**

**Asynchronous Cells.** Chart 1 shows the effects of spirogermanium on the survival of NIL 8 cells after drug exposure for 1 hr (Chart 1A) or 24 hr (Chart 1B). The survival curves are of exponential type showing increasing cell kill with increasing drug concentration and with longer exposure time. These lethal effects of spirogermanium were temperature dependent, being reduced at temperatures below 37° and almost negligible at 4° but enhanced at the higher temperature of 40°. Chart 1B shows that spirogermanium was more effective in reducing the colony-forming ability of cells in logarithmic growth than those in stationary phase, with ID50 values differing by a factor of 6. However, at the higher doses of spirogermanium, significant cell kill (approximately 50%) of these quiescence cells could still be achieved.

Spirogermanium was also effective in reducing the survival of a range of established human cell lines (Chart 2). In all cases, after a 24-hr drug exposure, there was a logarithmic reduction in colony-forming ability with increasing drug concentration. It should also be noted (a) that there was a shoulder to each of these graphs, which was most marked for HN-1 cells, suggesting that perhaps a critical drug concentration was required before cell kill occurred and (b) that concentrations in excess of 3 μg/ml for 24 hr resulted in extensive detachment of cell monolayers from the culture dishes and complete cell lysis for all cell types consistent with zero survival.

Table 1 summarizes the ID50 and ID90 values for spirogermanium using a 24-hr drug exposure for the various mammalian cells examined. It is noticeable that the range of values is small, with the ID90 values differing only by a factor of approximately 2 and, with the exception of the squamous cell carcinoma line which proved least sensitive to spirogermanium, the consistency of ID90 values among the various lines is most marked.

**Synchronized Cells.** To determine whether spirogermanium showed any cell cycle-specific cytotoxicity, NIL 8 cells, synchronized by mitotic selection, were drug treated for 1 hr with 15 μg/ml at all stages of the cell cycle. Chart 3 shows that spirogermanium appeared to be equally effective at reducing cell survival at all phases of the cycle.

**Effects of Spirogermanium on Cell Cycle Distribution**

FMF analyses of NIL 8 cells and COLO 205 cells treated with spirogermanium confirmed the lack of cycle-dependent effects of this drug. Using drug concentrations and exposure times which allowed greater than 90% cell survival, there were no changes from the control pattern in the DNA distribution histo-
Chart 1. Lethal effects of spirogermanium on NIL 8 cells as determined by colony formation. A, 1-hr exposure of logarithmically growing cells at 4, 25, 37, or 40°. B, 24-hr exposure of logarithmically growing or stationary-phase cells at 37°. Points, mean of 4 assays; bars, S.E.

Chart 2. Lethal effects of a 24-hr exposure of a series of logarithmically growing established human cell lines to varying concentrations of spirogermanium. Survival was determined by colony-forming assays. Points, means of 4 assays; bars, S.E.

Table 1
Summary of the lethal effects of a 24-hr drug exposure to spirogermanium on a range of established mammalian cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Source</th>
<th>ID₅₀ (µg/ml)</th>
<th>ID₉₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIL 8</td>
<td>Syrian hamster ovary</td>
<td>0.55</td>
<td>1.8</td>
</tr>
<tr>
<td>CHP100</td>
<td>Human neuroblastoma</td>
<td>0.55</td>
<td>1.55</td>
</tr>
<tr>
<td>COL0 205</td>
<td>Human colon carcinoma</td>
<td>0.74</td>
<td>1.18</td>
</tr>
<tr>
<td>LOVO</td>
<td>Human colon carcinoma</td>
<td>0.52</td>
<td>1.49</td>
</tr>
<tr>
<td>HN-1</td>
<td>Human squamous cell carcinoma</td>
<td>2.20</td>
<td>2.65</td>
</tr>
</tbody>
</table>

Effects of Spirogermanium on the Incorporation of ³H-labeled Precursors into DNA, RNA, and Protein

The results of exposure of COLO 205 cells to spirogermanium (0.75 µg/ml) over a 24-hr period were monitored in terms of effects on cell volume; DNA, RNA, and protein content; the incorporation of ³H-labeled precursors into these macromolecules; and cell survival. Data presented in Table 2 show that, under these conditions, in the surviving cells neither cell volume, nor DNA, nor RNA per cell were significantly altered, and protein levels were only marginally reduced. In the studies using ³H-labeled precursors (see Table 3), the most marked effect of spirogermanium was on the incorporation of [³H]leucine, and this was both time and dose dependent. While the lowest concentration tested (0.5 µg/ml) was without effect on survival or [³H]thymidine or [³H]uridine incorporation, [³H]leucine incorporation was inhibited after the 24-hr drug exposure. At the higher concentrations of spirogermanium (0.75 and 1.5 µg/ml), a reduction in [³H]leucine incorporation was evident within 2 hr of drug addition, although cell survival remained high. Increasing the duration of exposure further reduced [³H]
DISCUSSION

These results provide evidence that spirogermanium is cytotoxic to a range of mammalian cell lines in vitro and confirm earlier reports using other cell types (10, 11). Exposure of all cell lines to spirogermanium for 24 hr produced exponentially shaped survival curves, suggesting that spirogermanium is a Class III agent according to the Kinetic Classification of Antitumor Agents (1). These lethal effects appear to show no phase specificity. There is no evidence of any effects of the drug on the progression of cells through the cycle. At drug concentrations resulting in a >2-log cell kill, rapid and complete cell lysis occurred.

The similarity of the ID50 doses in the human tumor cell lines and the presence of shoulders on the dose-response curves suggested that spirogermanium may have quite a nonspecific

Effects of Spirogermanium on Vital Dye Exclusion

Chart 4 shows that the ability of COLO 205 cells to exclude trypan blue is affected by treatment with spirogermanium. This effect on the cell membranes is both time and dose dependent but noted only under conditions of drug treatment which result in extensive cell death.

Effects of Spirogermanium on Survival of Rat Hypothalamic Neurons

The toxic response of cultured rat neurons to a 24-hr exposure to a range of spirogermanium concentrations is shown in Chart 5. Neurotoxicity is marked at levels in excess of 1 µg/ml, which is approximately equivalent to the concentration range which resulted in cytotoxicity in the other permanent cell lines tested (see above).
mode of action. Indeed, the membrane damage elicited by the drug as measured rather crudely by trypan blue exclusion would also be consistent with this proposal. Further studies on the effects of spirogermanium on model membranes are now under way. The clinical observation that, after i.m. dosing, patients complained of severe pain at the injection site with sterile abscesses developing in 2 patients (15) may also be associated with these apparent cytolytic effects.

However, there must also be other factors involved in the mechanism of action of spirogermanium since cell kill was both temperature and time dependent and, while the drug exerted some cytotoxicity on stationary-phase culture, it preferentially killed logarithmically growing cells. Our data show that, of the main cell macromolecules, synthesis of protein was most readily interfered with by spirogermanium. This agrees with the results of Rice et al. (11), who suggested that the induction of cytolyis in vitro by spirogermanium was via protein synthesis inhibition. Effects on DNA and RNA synthesis, implicated by Schein et al. (12) in the mode of action of the drug, appeared only after drug exposure resulting in marked cell death.

Phase I and II clinical studies with spirogermanium are now being considered. Preliminary clinical studies showed an absence of hematologial, renal, and hepatic toxicities (12, 15). This lack of hematological toxicity confirms preclinical toxicological evaluations (10) and is further supported by a recent study showing spirogermanium to have no effect on marrow colony-forming cells in mice, dogs, or humans (8). The principal clinical toxicity encountered with spirogermanium was neurological (9). In this respect, the toxic effects reported here on cultured rat neurons are of interest. In addition to inhibiting neurite formation, spirogermanium was also noted to exert lethal effects on the glial cells in this experimental system. These results therefore suggest that the range of spirogermanium concentrations which are cytotoxic to tumor cell lines and those cytotoxic against normal central nervous system neurons are similar. This may be related to the proposed nonspecificity of the action of the drug, but clearly this might limit the clinical usefulness of the drug. This experimental model system is now being evaluated further to establish its use in vitro in screening for neurotoxic side effects of drugs.

In summary, spirogermanium exerts cytotoxicity against a range of established mammalian tumor cell lines; the effect appears to be markedly dependent on dose, time, temperature, and the proliferative state of the cell. The marked ability of the drug to cause extensive cell lysis at high drug concentrations is a novel one which may be related to direct effects on the cell membrane or mediated via an inhibition of protein synthesis.

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REFERENCES

Cytotoxic Effects and Biological Activity of 2-Aza-8-germanspiro[4,5]decane-2-propanamine-8,8-diethyl- N,N-dimethyl Dichloride (NSC 192965; Spirogermanium) \textit{in Vitro}

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