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SJG-136 (NSC 694501), a Novel Rationally Designed DNA Minor Groove Interstrand Cross-Linking Agent with Potent and Broad Spectrum Antitumor Activity. Part 1: Cellular Pharmacology, *In vitro* and Initial *In vivo* Antitumor Activity

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ABSTRACT

SJG-136 (NSC 694501) is a rationally designed pyrrolobenzodiazepine dimer that binds in the minor groove of DNA. It spans 6 bp with a preference for binding to purine-GATC-pyrimidine sequences. The agent has potent activity in the National Cancer Institute (NCI) anticancer drug screen with 50% net growth inhibition conferred by 0.14 to 320 nmol/L (7.4 nmol/L mean). Sensitive cell lines exhibit total growth inhibition and 50% lethality after treatment with as little as 0.83 and 7.1 nmol/L SJG-136, respectively. COMPARE and molecular target analysis of SJG-136 data *versus* that of >60,000 compounds tested in the NCI 60 cell line screen shows that, although the agent has similarity to other DNA binding agents, the pattern of activity for SJG-136 does not fit within the clusters of any known agents, suggesting that SJG-136 possesses a distinct mechanism of action. Testing in the NCI standard hollow fiber assay produced prominent growth inhibition in 20 of 24 i.p. and 7 of 24 s.c. test combinations with 5 of 12 cell lines exhibiting cell kill. In addition, SJG-136 produced antitumor activity in mice bearing CH1 and CH1cisR xenografts, a cisplatin-resistant human ovarian tumor model, and also in mice bearing LS174T xenografts, a human colon tumor model. SJG-136 produces DNA interstrand cross-links between two *N*-2 guanine positions on opposite strands and separated by 2 bp. In human tumor cell lines, the cross-links form rapidly and persist compared with those produced by conventional cross-linking agents such as nitrogen mustards. In mice bearing the LS174T human colon xenograft, DNA interstrand cross-links can be detected in tumor cells using a modification of the single cell gel electrophoresis (comet) assay after administration of a therapeutic dose. Cross-links in the tumor increase with dose and are clearly detectable at 1 hour after i.v. administration. The level of cross-linking persists over a 24-hour period in this tumor in contrast to cross-links produced by conventional cross-linking agents observed over the same time period.

INTRODUCTION

The pyrrolo[2,1-*c*] [1,4]benzodiazepines (PBDs) are a family of naturally occurring antitumor antibiotics, which includes anthramycin, DC-81 (Fig. 1), tomaymycin, and sibiromycin (1). They exert their biological activity through covalent binding to the exocyclic N2 group of guanine in the minor groove of DNA and block transcription in a sequence-specific manner (2). These PBD monomers span three DNA bp and have a preference for binding to purine-G-purine triplets (3).

The PBDs have been used as a scaffold to attach EDTA and epoxide moieties, leading to novel sequence-selective DNA cleaving and cross-linking agents, respectively (4, 5). In addition, as part of a rational approach to producing more efficient and selective DNA interstrand cross-linking agents, two PBD monomers have been linked together (6). For example, the C8-diether-linked PBD dimer DSB-120 (Fig. 1) demonstrated potent *in vitro* cytotoxicity and enhanced DNA binding affinity and sequence specificity compared with the monomer DC-81 (7–9). DSB-120 is a highly efficient DNA interstrand cross-linker in both naked DNA and intact cells (8, 9). Nuclear magnetic resonance spectroscopy and molecular modeling studies indicate that DSB-120 spans 6 bp in the minor groove, actively recognizing and cross-linking a 5′-GATC sequence (10, 11).

DSB-120 was not developed further because of its high reactivity with thiol-containing molecules *in vivo* (12). Second generation molecules were designed and synthesized, which were unsaturated at the C2/C2′ positions (13) in an attempt to produce lower electrophilicity at the N10-C11 positions within the molecule to decrease deactivation by cellular nucleophiles (14). A resulting C2-*exo*-methylene PBD dimer, SJG-136 (Fig. 1), was found to be significantly more cytotoxic than DSB-120 in a number of human cancer cell lines and was a highly efficient DNA interstrand cross-linker when studied using a plasmid-based gel electrophoresis assay (15). This article reports the cellular pharmacology of this novel agent and the initial *in vitro* and *in vivo* antitumor activity data, which resulted in its selection for additional preclinical and clinical development. The accompanying paper describes some of the extensive *in vivo* data on this molecule produced by the United States National Cancer Institute (NCI).

MATERIALS AND METHODS

Synthesis of SJG-136. The synthesis and chemical characterization of SJG-136 have been documented elsewhere (13, 15). Stock solutions, unless otherwise indicated, were prepared in analytical grade methanol and stored dry at −20°C.

***In vitro* 60 Cell Line Cancer Screen.** The methods used for the 60 cell line panel have been described elsewhere (16, 17). Briefly, compounds are solubilized in DMSO at 200×. The compounds are diluted into RPMI 1640

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Note: L. Kelland is presently at the Antisoma Laboratory, Department of Basic Medical Sciences, St. George's Hospital Medical School, Cranmer Terrace, London, United Kingdom; In May 2003, Beaufour-Ipsen (now Ipsen) acquired the rights to develop SJG-136 as a clinical antitumor agent through Spirogen, Ltd., in which J. Hartley, P. Howard, and D. Thurston have equity interests; The data contained in this paper have been presented in part at the annual meeting of the American Association for Cancer Research (40, 41).

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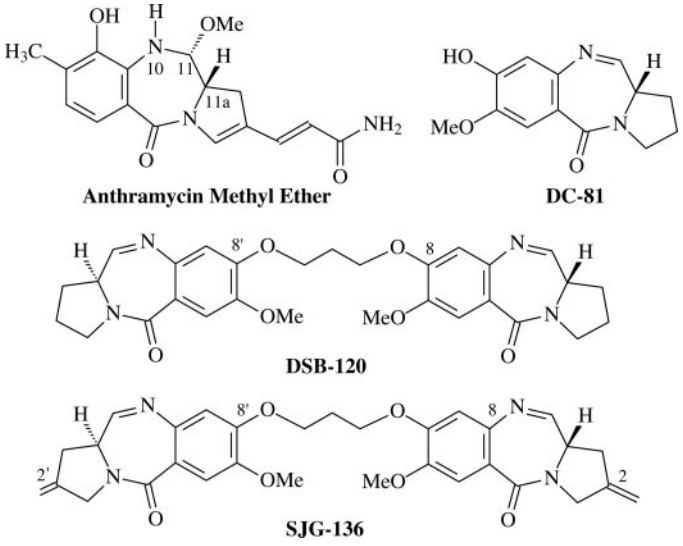

Fig. 1. Structures of the PBD monomers, anthramycin and DC-81, and the PBD dimers, DSB-120 and SJG-136.

containing 5% fetal bovine serum and serial 10-fold dilutions are prepared for a total of five concentrations. Generally, the working range for initial testing of a compound is 10^{-4} through 10^{-8} molar. The compounds are added to 24-hour old cultures of each of the 60 cell lines used in the panel. After an 48-hour incubation, the media are removed, the cells are fixed and stained with sulforhodamine B, and the total stain quantitated by absorbance determinations. Through the use of a time 0 cell control, the cell growth can be determined for each cell line thus allowing calculations of the 50% growth inhibitory concentration GI_{50}, the total growth inhibition, and the 50% lethal concentration (LC_{50}). These data are then plotted as mean bar graphs and as dose-response curves. COMPARE and molecular target analyses were performed as a means to identify similarities with other known or experimental chemotherapeutic agents (18) and to determine whether there is evidence for alignment with patterns of cell line gene expression (19).

Colony Formation Assay of Leukemia Cell Lines. Bilayer soft agar colony formation assays of leukemia cell lines were performed using RPMI 1640 containing 10% fetal bovine serum and 10,000 cells/2 mL culture on day 0, as described previously (20). For drug sensitivity assays, 0.1 mL of culture medium containing drug ($n = 3$/each of 6 drug concentrations) and/or drug vehicle ($n = 6$) were applied to cultures on day 1. Cultures were then incubated until day 7, stained with 1 mg/mL methylthiazoletetrazolium for 4 hours, stabilized and clarified with 2.5% protamine sulfate buffer for 16 hours, and then analyzed by computerized image analysis (20). Percentage of vehicle control (%C) values were calculated for each drug concentration as well as IC_{50}, IC_{75}, and IC_{90} indices from regression analysis of colony-forming unit (CFU) data associated with drug concentrations spanning the IC_{50}, IC_{75}, and IC_{90} intercepts.

***Ex vivo* Bone Marrow (CFU-GM) Colony Formation Assays.** Methods for the isolation and culture of hematopoietic cells from fresh human, canine (beagle dog), and murine (CD2F1) bone marrow in the presence of chemotherapeutic agents have been described previously (21–23). Recombinant cytokines were obtained as follows: human granulocyte-macrophage colony-stimulating factor from Immunex (Seattle, WA); human interleukin 3, murine GM-CSF, and murine interleukin 2 from R&D Systems (Minneapolis, MN); and Epogen from Amgen (Thousand Oaks, CA). In brief, 400 μL of murine, canine, or human marrow mononuclear cells [2.0 (murine) or 2.5×10^5 (canine and human) cells/mL] in Iscove's modified Dulbecco's medium containing 20% FBS, 10 units/mL recombinant murine GM-CSF (murine), 25 ng/mL recombinant human GM-CSF (canine and human), 5 ng/mL recombinant human interleukin 3 (canine only), 10% of a 10× drug solution or control solution, and 0.3% agarose were pipetted into microwells containing a 0.4-mL underlayer of Iscove's modified Dulbecco's medium and 0.3% agarose. The cultures were allowed to gel at 4°C for 15 min and incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 7 days (murine) or 14 days (canine and human). CFU-GM colonies [aggregates of ≥40 cells (murine) or 50-μm diameter or larger for canine and human] were counted with an inverted microscope using phase contrast. Percent survivals were calculated as 100 × (the number of colonies in the drug-treated groups divided by the number of colonies in the vehicle control-treated group). IC_{50}, IC_{75}, and IC_{90} values were determined from regression analysis of CFU data from multiple marrow specimens flanking the IC_{50}/IC_{90} intercepts. Regression analyses relating drug concentration and colony inhibition were derived from CFU-GM data from four marrow specimens for each species.

NCI Standard Hollow Fiber Assay. This initial assessment of *in vivo* activity using cells transferred to polyvinylidene fluoride fibers and grown in the i.p. and s.c. compartments of mice was conducted as described previously by Hollingshead *et al.* (24, 25). SJG-136 was evaluated in the standard hollow fiber assay after i.p. administration daily for 4 days with treatment starting on the third or fourth day after fiber implantation. All assays included a vehicle control group consisting of six mice. The test groups consisted of three mice treated with one of two dose levels (0.5 or 0.4 mg/kg). Replicate fibers containing human tumor cells were implanted in the i.p., as well as the s.c. compartments of each mouse. Group body weights were recorded daily as an index of compound toxicity.

Determination of DNA Interstrand Cross-Linking. The details of the Single Cell Gel Electrophoresis (comet) assay to measure DNA interstrand cross-links are described in detail elsewhere (26, 27). All procedures performed on the sample single cell suspension were carried out on ice and in subdued lighting. All chemicals used were obtained from Sigma Chemical Co.(Poole, United Kingdom) unless otherwise stated. Immediately before analysis, cells were irradiated (10 Gy) to deliver a fixed number of random DNA strand breaks. After embedding cells in 1% low melting temperature agarose on a precoated microscope slide, the cells were lysed for 1 hour in lysis buffer [100 mmol/L disodium EDTA, 2.5 mol/L NaCl, 10 mmol/L Tris-HCl (pH 10.5)] containing 1% Triton X-100 added immediately before analysis and then washed for 1 hour in distilled water, changed every 15 minutes. Slides were then incubated in alkali buffer [50 mmol/L NaOH, 1 mmol/L disodium EDTA (pH 12.5)] for 45 minutes followed by electrophoresis in the same buffer for 25 minutes at 18 V (0.6 V/cm), 250 mA. The slides were finally rinsed in neutralizing buffer [0.5 mol/L Tris-HCl (pH 7.5)] then saline.

After drying, the slides were stained with propidium iodide (2.5 μg/mL) for 30 minutes, then rinsed in distilled water. Images were visualized using a NIKON inverted microscope with a high-pressure mercury light source, a 510 to 560-nm excitation filter, and 590-nm barrier filter at ×20 magnification. Images were captured using an on-line charge-coupled device camera and analyzed using Komet Analysis software (Kinetic Imaging, Liverpool, United Kingdom). For each duplicate slide, 25 cells were analyzed. The tail moment for each image was calculated using the Komet Analysis software as the product of the percentage DNA in the comet tail and the distance between the means of the head and tail distributions based on the definition of Olive *et al.* (28). Cross-linking was expressed as the percentage decrease in tail moment compared with irradiated controls calculated by the formula:

$$\text{percent decrease in tail moment} = \left[1 - \left(\frac{\text{TMdi} - \text{Tmcu}}{\text{Tmci} - \text{Tmcu}}\right)\right] \times 100$$

where TMdi = tail moment of drug-treated irradiated sample; TMcu = tail moment of untreated, unirradiated control; and TMci = tail moment of untreated, irradiated control.

Cell Cycle Analysis. K562 cells were incubated for 1 hour with the appropriate concentration of SJG-136 in 2 mL of serum-free RPMI 1640 (1×10^6 cells/ml). Cells were centrifuged and resuspended in 6 mL of drug-free medium. After the appropriate postincubation time, 1-mL aliquots of cells were centrifuged, resuspended in 1 mL of cold PBS, fixed by the addition of cold 70% ethanol, and stored at 4°C until all of the samples had been collected. For cell cycle analysis, fixed cells were centrifuged, washed with cold PBS, and resuspended in 1 mL of cold PBS containing 250 μg/mL RNAase, and 50 μg/mL propidium iodide. DNA content was quantified by detecting red fluorescence using a Becton Dickinson FACScan.

***In vivo* Xenograft Studies.** All procedures were within local institute and national ethical guidelines and were in compliance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia.

CH1 Human Ovarian Cancer. The CH1 human ovarian cancer parental cell line and the acquired cisplatin-resistant line CH1cisR (29) were established as s.c. xenografts by injection of 5×10^6 cells into the flanks of adult female athymic nude (nu/nu) mice. When palpable tumors arose, 2-mm^2 pieces were transplanted by surgical incision under anesthesia to other mice.

Drug treatment did not start until tumors had reached an average largest diameter of 6 to 8 mm, whereupon mice were randomized into groups (= day 0). There were at least five animals in each control or treatment group. Mice were treated i.v. with either 4 mg/kg cisplatin in 0.9% NaCl or 0.2 mg/kg SJG-136 in 1% DMSO/0.9% NaCl on days 0, 4, and 8. Route, schedule, and doses were selected on the basis of maximum-tolerated dose determination experiments for each drug in non-tumor-bearing mice before drug testing in tumor-bearing mice. Tumor size was then determined once or twice weekly using caliper measurements, and tumor volumes were calculated.

LS174T Human Colon Cancer. This animal model consisted of MF1 nude mice and the s.c. growing LS174T human colon xenograft tumor (30). Tumors were maintained by s.c. passage into the flank of the animal. Tumor starting sizes were 0.2 cm^3 for antitumor efficacy experiments and 0.5 cm^3 for DNA cross-linking experiments.

For efficacy experiments, animals were randomized into test and control groups with six animals in each. Animals received i.v. injections of 0.3 mg/kg SJG-136 on days 0, 4, and 8. SJG-136 was prepared in PBS containing 0.05% Tween 80 in a final volume of 0.1 mL per 10 g body weight. The control groups were injected with the vehicle only at the appropriate time points. Tumor volume was calculated every 4 days as [(length $\times$ width $\times$ height)/2], and results were expressed as tumor volume in cm^3.

For DNA cross-linking studies, animals were injected i.v. with a single dose of 0.30 or 0.45 mg/kg SJG-136 made up in PBS containing 0.05% Tween 80 in a final volume of 0.1 mL per 10 g body weight. Two mice were used at each dose level. Tumors were collected before dosing and 1, 3, and 24 hours posttreatment.

Once collected, the entire tumor was placed in a small volume of ice-cold RPMI 1640. Using two scalpel blades, the tumor was finely chopped using a crosscutting action until a suspension of cells was formed. The cells were suspended in 5 mL and centrifuged at 200 $\times$ *g* for 5 minutes at 4°C. The supernatant was discarded, and the cells resuspended in 1.5 mL of RPMI 1640 containing 20% FCS and 10% DMSO and frozen at −80°C until analyzed in the comet assay.

RESULTS

***In vitro* Antitumor Activity of SJG-136.** The pyrrolobenzodiazepine dimer, SJG-136 (Fig. 1), resulted from an ongoing program to develop more efficient and selective DNA interstrand cross-linking agents. As shown in Fig. 2, the average concentration required to inhibit GI_{50} was 7.4 nmol/L with a range of 0.14 to 320 nmol/L. Although >1000 nmol/L (10^{-6} mol/L) was required to achieve LC_{50} in the majority of human tumor cell lines, cell lines sensitive to SJG-136 exhibited an LC_{50} with as little as 7.1 nmol/L and total growth inhibition (TGI) with as little as 0.83 nmol/L. The $>10^3$ range in the GI_{50}, the $>10^3$ range in the total growth inhibition, and the $>10^2$ range in the LC_{50} drug concentrations among cell lines together, with the mean-graph pattern of cell line sensitivity (18), suggest that this agent confers a multilog, differential effect upon cell lines rather than exerting a nonspecific cytotoxicity in which most cell lines would show very similar GI_{50}, total growth inhibition, and/or LC_{50} indices.

A comparison of SJG-136 mean bar graph profiles by pattern recognition analysis (COMPARE) with that of 60,000 compounds tested in the NCI 60 cell line screen indicated that the agent has an activity pattern similar to some DNA binding agents. However, the activity pattern of SJG-136, a pyrrolobenzodiazepine dimer, differed from three chemically related pyrrolobenzodiazepine monomer compounds (COMPARE- negative patterns of activity). In addition, the mean graph activity pattern of SJG-136 did not align with the gene expression cluster patterns associated with any known chemotherapeutic agents, suggesting that SJG-136 possesses a unique mechanism of action.

Comparison of SJG-136 in Colony Formation Assays of Selected Leukemia *versus* Normal Cell Types. SJG-136 appears to confer a preferential cytostatic and/or cytocidal effect upon leukemia cells *versus* normal bone marrow cells based on results of colony formation assays summarized in Table 1. Although the assays were performed according to different methodologies, it is clear that HL-60 TB and Molt-4 cells (continuous drug exposure/7-day assay) are at least one order of magnitude more sensitive to SJG-136 than are bone marrow cells derived from the mouse, dog, and man (continuous drug exposure/7 to 14-day assays). Furthermore, the drug concentrations observed to confer GI_{50}, and total growth inhibition activity in the NCI cell line screen (48-hour drug exposure/3-day assay) are substantially lower than the concentrations required to confer IC_{50}, IC_{75}, and IC_{90} activity in the *ex vivo* bone marrow assay.

***In vivo* Antitumor Activity of SJG-136.** SJG-136 was tested in the standard NCI hollow fiber assay against NCI-H522, NCI-H23, LOX IMVI, UACC-62, OVCAR-3, OVCAR-5, MDA-MB-435, MDA-MB-231, CoLo-205, SW-620, U-251, and SF-295. Treatment with SJG-136 at doses of 0.5 and 0.4 mg/kg given once daily for 4 days produced >50% growth inhibition in 83% of the cell lines growing in the i.p. fibers and in 29% of the cell lines growing in the s.c. fibers (total score = 54 of 96). Cell kill (a reduction in cell mass below the input mass) was observed in 5 of the 12 cell lines: NCI-H522 lung adenocarcinoma (13% kill); LOX IMVI melanoma (10% kill); UACC-62 melanoma (10% kill); MDA-MB-435 breast carcinoma (29% kill); and OVCAR-3 ovarian adenocarcinoma (47%). The average body weight losses in treated mice were ≤9% with no drug-related deaths, suggesting that SJG-136 treatments were well tolerated. Furthermore, SJG-136 is one of the most active compounds (top 5%) tested in this assay to date.

Subsequent testing in ovarian cancer xenograft models demonstrated prominent *in vivo* antitumor activity. In the cisplatin-sensitive CHI tumor model, cisplatin at 4 mg/kg and SJG-136 at 0.2 mg/kg gave comparable levels of tumor growth delay on the schedule tested (i.v., days 0, 4, and 8; Fig. 3*A*). In contrast, in the cisplatin-resistant CH1cisR tumor, cisplatin was ineffective at 4 mg/kg, whereas SJG-136 produced a significant growth delay at 0.2 mg/kg (Fig. 3*B*).

Cellular Pharmacology of SJG-136. SJG-136 was designed as a DNA minor groove-interstrand cross-linking agent. Previous studies demonstrated that it is highly efficient at producing interstrand cross-links in naked DNA using an agarose gel-based method (15). Cross-linking was measured in cells using a modification of the single cell gel electrophoresis (comet) assay (27). After a 1-hour treatment of human leukemic K562 cells with SJG-136, cross-links (measured as the percent decrease in comet tail moment) were clearly detected at 0.01 μmol/L and increased with dose up to 0.3 μmol/L (Fig. 4*A*). After exposure to 0.05 μmol/L SJG-136 for 1 hour, K562 cells were incubated in fresh medium and interstrand cross-link formation was followed with time (Fig. 4*B*). Extensive cross-linking was observed immediately after the drug treatment and increased slightly with time. No evidence of cross-link loss was seen over the 48-hour postincubation period. In contrast, the peak of cross-linking observed after a 1-hour exposure of the cells to the conventional major groove cross-linking agent melphalan is ~16 hours, and clear evidence of loss of cross-links is seen at 48 hours (Fig. 4*B*). The relative potencies of these two cross-linking agents can be seen from the doses used. Even at 100 μmol/L melphalan fails to produce the level of cross-linking occurring with SJG-136 at 0.05 μmol/L. The GI_{50} values after a 1-hour exposure in the K562 cell line, as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, are 0.02 and 30 μmol/L for SJG-136 and melphalan, respectively.

The effect of SJG-136 on the cell cycle was examined in K562 cells after a 1-hour exposure (Fig. 5). A dose-dependent accumulation of cells

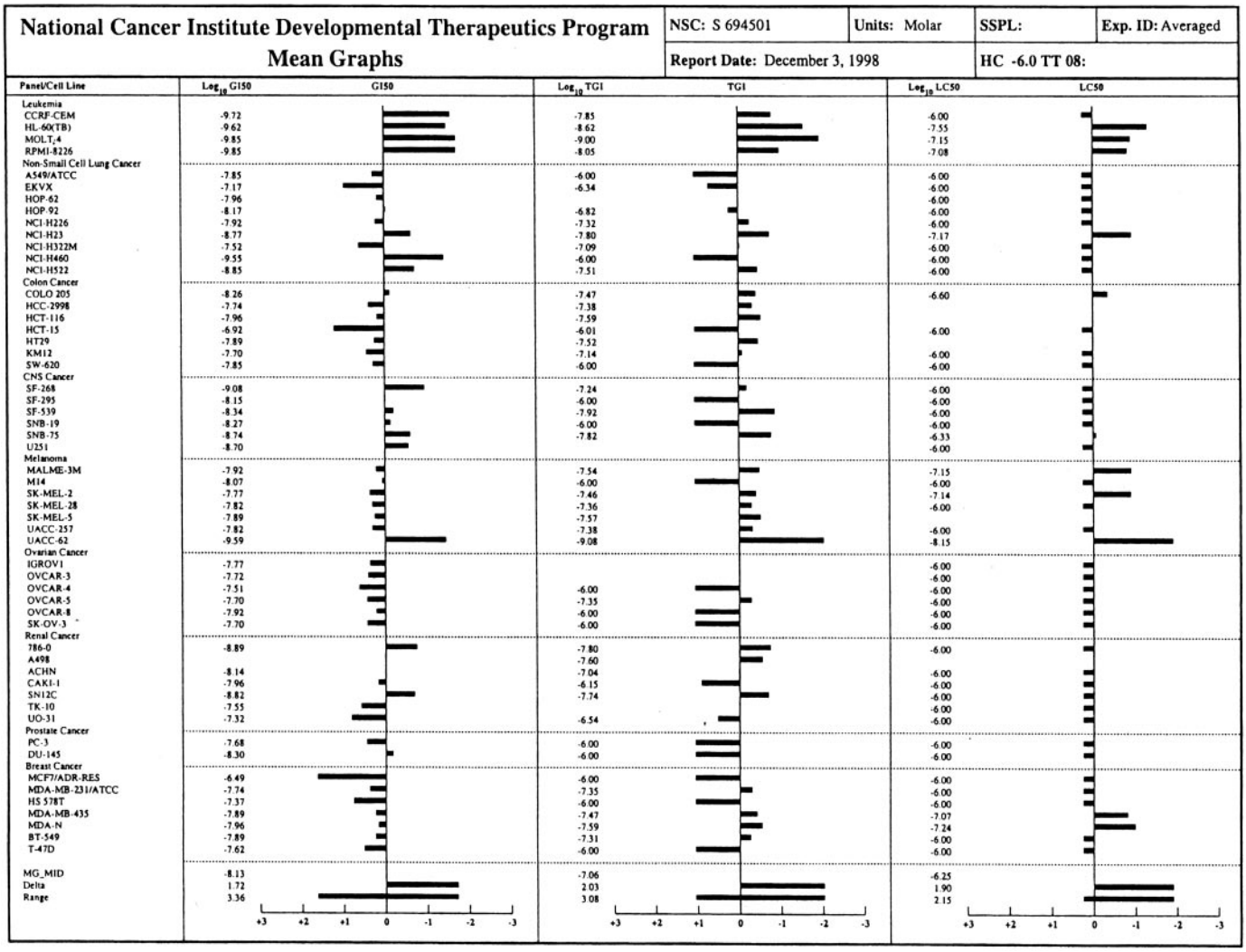

Fig. 2. Averaged mean graphs for the testing of SJG-136 (NSC 694501) in the NCI Developmental Therapeutics Program's *in vitro* 60 cell line screen on three separate occasions. The figure provides a graphic and tabular listing of the molar drug concentrations (log units) conferring GI_{50}, total growth inhibition (TGI), and LC_{50} for each cell line. The response of each cell line relative to the mean of all cell line responses is depicted by a horizontal bar extending either to the right (more sensitive) or to the left (less sensitive) of the mean (*vertical line*) for each index of activity (GI_{50}, TGI, and LC_{50}). The length of each bar is proportional to the cell line sensitivity relative to the mean in log units. Mean bar graph plots permit comparisons of individual cell line responses, as well as a "fingerprint" of all cell line responses for a particular agent. The terminology, derivation of data, and interpretation of mean graph fingerprints by pattern recognition analysis (COMPARE) have been described in detail elsewhere (18).

in the G_2-M phase is observed by 24 hours. At sub-GI_{50} doses, this accumulation is reversed at later times, and at 0.001 μmol/L, the cells have returned to a normal cell cycle distribution by 96 hours. At a dose of 0.05 μmol/L, the block is extensive and is not overcome by 96 hours.

DNA Interstrand Cross-Linking by SJG-136 *In vivo*. The comet assay was used to determine cross-linking by SJG-136 in the LS174T human colon cancer xenograft *in vivo* at a therapeutically relevant dose, *i.e.*, a dose that gives a significant antitumor effect *in vivo*. At

Table 1 *Comparison of the growth inhibitory properties of SJG-136 (NSC 694501) in cultures of selected leukemia cells and normal bone marrow cells*

Methodology	Cell type	Pharmacologic indices, pmol/L		
In vitro cell line cancer screen	Human leukemia	GI_{50}	TGI	LC_{50}
	• HL-60 TB	0.240 ± 0.122	2.39 ± 0.88	28.2 ± 17.4
	• Molt-4	0.141 ± 0.003	1.05 ± 0.104	70.8 ± 22.2
In vitro soft agar colony formation assay	Human leukemia	IC_{50}	IC_{75}	IC_{90}
	• HL-60 TB	1.19 ± 0.070	1.76 ± 0.11	2.95 ± 0.20
	• Molt-4	0.503 ± 0.065	1.13 ± 0.06	1.70 ± 0.15
Ex vivo bone marrow colony formation assay	Granulocytes and macrophages	IC_{50}	IC_{75}	IC_{90}
	• Mouse	111 ± 13	218 ± 26	536 ± 61
	• Dog	16.2 ± 4.4	41.3 ± 11.7	118 ± 29
	• Human	21.3 ± 12.9	101 ± 16	167 ± 56

NOTE. SJG-136 was evaluated in each of three standardized assays employed in preclinical drug evaluations: 60 cell line screening assay; soft agar colony formation assay; and in *ex vivo* bone marrow assays. Pharmacologic indices listed in the table (mean ± SD) were derived from testing the agent over concentration ranges verified to cover the full effective concentration range required for each assay and by regression analysis of data associated with drug concentrations, which span the GI_{50}, TGI, LC_{50} and the IC_{50}, IC_{75}, IC_{90}.

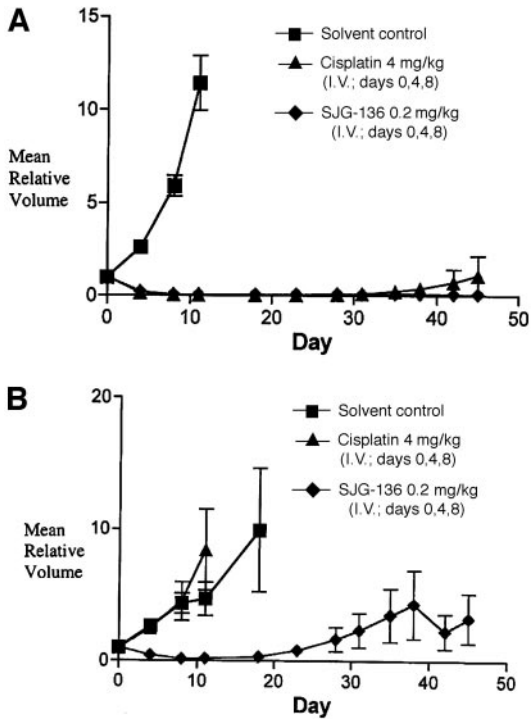

Fig. 3. *In vivo* activity of SJG-136 and cisplatin against the CH1 human ovarian tumor (*A*) and the cisplatin acquired resistant line CH1cisR (*B*). Doses were selected on the basis of prior maximum-tolerated dose determination experiments for each drug by the chosen route and schedule.

0.3 mg/kg given i.v., SJG-136 causes a significant growth delay to this tumor (Fig. 6*A*). After administration of drug, tumor samples were taken at 1, 3, and 24 hours and analyzed for cross-linking. A small but significant level of cross-linking could be detected at 1 hour, and the level of cross-linking remained relatively constant over 24 hours (Fig. 6*B*). At a higher dose of 0.45 mg/kg, more cross-links were detected at 1 hour after administration, and again, the level of cross-linking remained constant over 24 hours (Fig. 6*C*). In contrast, at a dose level of 0.2 mg/kg, which did not give a significant growth delay in this tumor, no cross-linking could be detected in tumor at any of the time points (data not shown).

DISCUSSION

SJG-136 (NSC 694501) was rationally designed to produce DNA interstrand cross-links in the minor groove of DNA. Previous studies confirmed that the compound is a highly efficient cross-linking agent of naked DNA (15), and the current study demonstrates that cross-links are formed rapidly in cultured cells and in human tumor xenografts after administration of a therapeutic dose. *In vitro*, SJG-136 conferred a preferential cytostatic and/or cytocidal effect upon leukemia cells *versus* normal bone marrow cells. *In vivo* SJG-136 treatments were well tolerated and SJG-136 was highly efficacious in each of three xenograft models, including a cisplatin-resistant tumor.

Additional findings in this study are that SJG-136 has a multilog differential pattern of activity in the NCI 60 cell line screen and that COMPARE/molecular target analyses of SJG-136 data show that, although the agent exhibits a pattern of activity similar to that of other DNA binding agents, it does not fit within any of the cluster patterns associated with other known chemotherapeutic drugs. These results suggest that SJG-136 may possess a biologically unique mechanism(s) of action. Of particular note is the significant difference in the mean graph pattern of activity for SJG-136, a PBD dimer, compared with that of the PBD monomer compounds (*e.g.*, anthramycin), which are capable of minor groove binding and monoalkylation, and from which the SJG-136 dimer structure was derived.

In addition, SJG-136 activity does not correlate with that of the minor groove cross-linking agent bizelesin. Bizelesin is a symmetrical dimer based on fragments of CC-1065 in which the linker consists of two indole subunits separated by a ureido group (31). It is unique among the cyclopropylpyrroloindole-related compounds in its bifunctional alkylating capability. In contrast to SJG-136, minor groove binding is selectively to AT-rich regions of DNA and the covalent cross-linking occurs between two adenine-N3 positions. Bizelesin has recently undergone phase I studies in patients with advanced solid tumors (32, 33). The fact that SJG-136 does not compare with bizelesin implies distinct aspects of their respective mechanisms of action or detoxification. This raises the possibility that different DNA interstrand cross-links may be created in the minor groove [*e.g.*, sequence selective adenine-adenine (in the case of bizelesin) *versus* guanine-guanine (in the case of SJG-136)] or utilization of distinct repair components that consequently evoke different biological responses.

In human tumor cells treated with SJG-136 *in vitro*, interstrand cross-links form rapidly and persist compared with those produced by more conventional DNA cross-linking agents such as the nitrogen mustards, exemplified here by melphalan. Although the repair of DNA interstrand cross-links is poorly understood in mammalian cells, it appears to require components of both nucleotide excision repair (in particular XPF and ERCC1) and homologous recombination (34, 35). In general, cross-links produced by agents such as melphalan and cisplatin cause a high degree of helical distortion. In contrast, molecular modeling of SJG-136 interstrand cross-links reveals they are relatively nondistorting for the helix (15). In a panel of normal and DNA repair defective Chinese hamster ovary cell lines, SJG-136 is highly cytotoxic compared with melphalan. The SJG-136 cellular sensitivity is much less dependent on XPF-ERCC1, and the homologous recombination factors XRCC2 and XRCC3, than is melphalan (36). Repair of DNA interstrand cross-links is an important determinant of sensitivity to DNA cross-linking drugs (35) and has recently been shown to be an important mechanism of clinically acquired drug resistance to nitrogen mustard drugs such as melphalan (37). The SJG-136-induced cross-links in the minor groove of DNA are more difficult to repair in human tumor cells than those formed by melphalan. This suggests that SJG-136 may have activity in tumors resistant to conventional DNA cross-linking drugs, as is demonstrated in the present study in an acquired cisplatin-resistant tumor.

In the present study, DNA interstrand cross-links were detected in the human colon tumor LS174T grown as a xenograft after adminis-

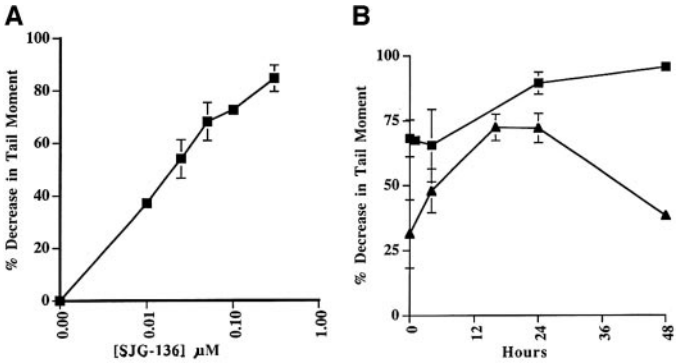

Fig. 4. *A*, DNA interstrand cross-linking in human leukemic K562 cells after a 1-hour incubation with SJG-136. Cross-linking was determined as the percent decrease in tail moment using the single cell gel electrophoresis (comet) assay. *B*, time course of cross-linking for SJG-136 (0.05 μmol/L, ■) or melphalan (100 μmol/L, ▲) after a 1-hour exposure of K562 cells.

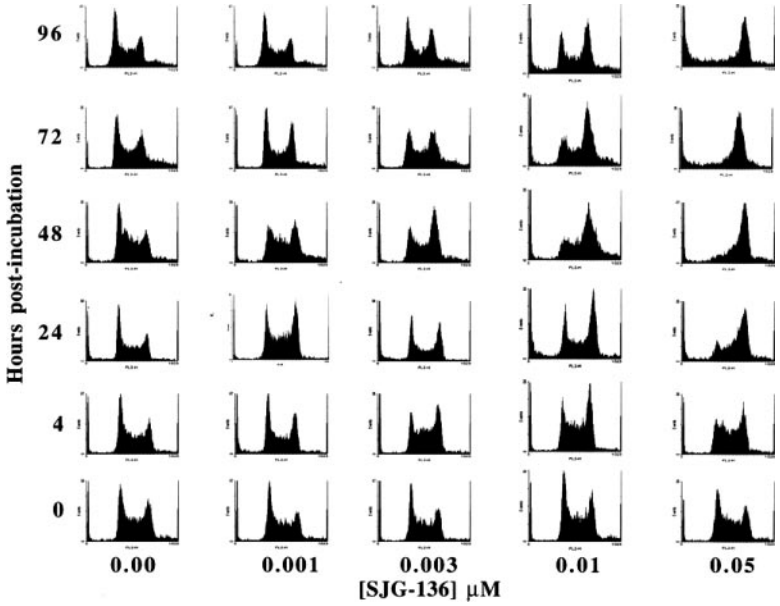

Fig. 5. Cell cycle analysis by cytofluorimetry of K562 cells after a 1-hour exposure to SJG-136 at the times and doses indicated. DNA histograms were generated by staining fixed cells with propidium iodide. The *X* axis in each plot represents the DNA content, and the *Y* axis represents the cell number. In total, 10,000 events were recorded, and the histograms were ungated.

tration of a therapeutic dose of SJG-136. After a single administration, the cross-links were detected at 1 hour using the modified comet assay (27), and the level of cross-linking remained constant over a 24-hour period. In this tumor model, significant repair of nitrogen mustard-induced cross-links was demonstrated over a 24-hour period *in vivo* (30). Similarly, it is clearly demonstrated that SJG-136-induced cross-links persist in cells *in vitro* compared with nitrogen mustard-induced cross-links. The *in vivo* experiments (Figs. 3*B* and 6*A*) suggest that, in the absence of complete eradication, tumors can regrow after drug removal after a significant growth delay. This might indicate that the cross-links can be ultimately removed or tolerated. This is also suggested from the cell cycle experiments which indicate that, at sub-GI_{50} doses, cells can eventually overcome a significant G_2-M block. The DNA damage induced by SJG-136 clearly activated a G_2-M block in the K562 (p53 mutant) cells, which has previously been observed with other PBD-based cross-linking agents (9) and other cross-linking agents (38).

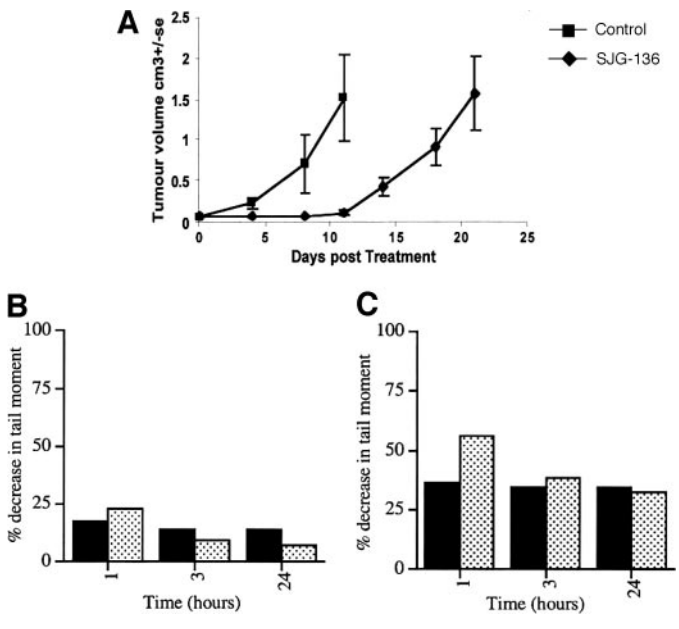

Fig. 6. DNA interstrand cross-linking by SJG-136 *in vivo*. *A*, Effect of SJG-136 given i.v. at 0.3 mg/kg on days 0, 4, and 8 on growth of the human LS174T human colon xenograft tumor. *B*, DNA cross-linking (measured as percent decrease in tail moment compared with untreated controls by the comet assay) in tumor cells at the times indicated after a single i.v. dose of SJG-136 at 0.3 mg/kg. Tumors from two separate animals were measured at each time point as indicated by the *filled* and *dotted bars*. *C*, As in *B*, except that SJG-136 was given at a dose of 0.45 mg/kg.

The comet assay allows the sensitive detection of DNA interstrand cross-links at the single cell level at pharmacologically relevant doses and has recently been applied to monitor cross-links in the clinical setting (26, 30). This method has recently been validated for the detection of SJG-136–induced cross-links in lymphocytes and tumor biopsy material as a pharmacodynamic end point in the early clinical trials of this agent. Thus, its mechanism of action as a highly efficient DNA interstrand cross-linking agent in cells and tumors is confirmed, and the cross-links are shown to persist compared with those produced by a more conventional DNA cross-linking agent.

In conclusion, the rationally designed pyrrolobenzodiazepine dimer, SJG-136, is the lead clinical candidate in a novel class of compounds that produce unique sequence selective guanine-guanine cross-links. The data in this article indicate that the compound exhibits potent and differential *in vitro* activity against cancer cells compared with normal hematopoietic cells and provides initial evidence that the compound is well tolerated and is highly active *in vivo*. On the basis of these pharmacologically desirable properties and the broad spectrum *in vivo* efficacy reported in the accompanying article (39), SJG-136 is currently undergoing more detailed preclinical pharmacology and toxicology studies through Cancer Research UK and the United States NCI in support of the phase I clinical trials underway in the United Kingdom and planned to commence shortly in the United States.

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