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


1 Cancer Research UK Drug-DNA Interactions Research Group and 2 Cancer Research UK Targeting and Imaging Research Group, Department of Oncology, Royal Free and University College Medical School, London, United Kingdom; 3 Cancer Research UK Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, Surrey, United Kingdom; 4 Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland; and 5 Cancer Research UK Gene Targeted Drug Design Research Group, School of Pharmacy, University of London, London, United Kingdom

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 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 anthracyclins, DC-81 (Fig. 1), tomatamycin, and sibimycin (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...
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 $10^3$ through $10^5$ 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 trypan blue and counted with an automated cell counter. The percentage of cell kill was calculated from the number of colonies in the drug-treated groups divided by the number of colonies in the untreated, irradiated control. The percent decrease in tail moment ($\text{TMDi - Tmcu}$) was calculated from the comet (comet) assay to measure DNA interstrand cross-linking. The comet (comet) assay was performed on single-cell suspensions of drug-treated and drug-free samples. Single-cell suspensions were prepared for each image by holding the sample at $4^\circ$C for 15 min and incubated at 37°C for 2 hours.

**CELLULAR PHARMACOLOGY OF SJG-136**

**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 ImmunoGen (South San Francisco, CA); recombinant 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 µL) or 2.5 x 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 1X 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 x (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 narrow specimens flanking the IC_{50}/IC_{90} intercepts. Regression analyses relating drug concentration and colony inhibition were derived from CFU-GM data from four narrow 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 sodium 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 lysis buffer, changed every 15 minutes. Slides were then incubated in alkali buffer [50 mMol/L NaOH, 1 mMol/L sodium 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 mMol/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 tail end and tail head. The tail moment was compared with irradiated controls calculated by the formula:

$\text{percent decrease in tail moment} = [1 - \frac{\text{TMDi} - \text{Tmcu}}{\text{Tmcu} - \text{Tcu}}] \times 100$

where TMDi = tail moment of drug-treated irradiated sample; Tmcu = tail moment of untreated, unirradiated control; and Tmcu = 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 x 10^6 cells/mL). Cells were centrifuged and resuspended in 6 mL of serum-free medium. After the distribution of the sample size-time 1 mL aliquots of cells were centrifuged, resuspended in 1 mL of cold PBS, fixed with 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 RNase, 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.
GI50 was 7.4 nmol/L with a range of 0.14 to 320 nmol/L. Although agents. As shown in Fig. 2, the average concentration required to inhibit pine dimer, SJG-136 (Fig. 1), resulted from an ongoing program to which most cell lines would show very similar GI50, 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 pattern of cell line sensitivity (18), suggest that this agent confers a multifactor, differential effect upon cell lines rather than exerting a nonspecific cytotoxicity in which most cell lines would show very similar GI50, total growth inhibition, and/or LC50 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 pattern of cell line sensitivity (18), suggest that this agent confers a multifactor, differential effect upon cell lines rather than exerting a nonspecific cytotoxicity in which most cell lines would show very similar GI50, total growth inhibition, and/or LC50 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 pattern of cell line sensitivity (18), suggest that this agent confers a multifactor, differential effect upon cell lines rather than exerting a nonspecific cytotoxicity in which most cell lines would show very similar GI50, total growth inhibition, and/or LC50 indices.
in the G2-M phase is observed by 24 hours. At sub-GI50 doses, this accumulation is reversed at later times, and at 0.001 mol/L, the cells have returned to a normal cell cycle distribution by 96 hours. At a dose of 0.05 mol/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. A 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 GI50, total growth inhibition (TGI), and LC50 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 (GI50, TGI, and LC50). 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 (16).

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

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Cell type</th>
<th>Pharmacologic indices, pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro cell line cancer screen</td>
<td>Human leukemia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• HL-60 TB</td>
<td>GI50: 0.240 ± 0.122 TGI: 2.39 ± 0.88 LC50: 28.2 ± 17.4</td>
</tr>
<tr>
<td></td>
<td>• Molt-4</td>
<td>1.04 ± 0.003</td>
</tr>
<tr>
<td>In vitro soft agar colony formation assay</td>
<td>Human leukemia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• HL-60 TB</td>
<td>IC50: 1.19 ± 0.070 TGI: 1.76 ± 0.11 LC50: 2.95 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>• Molt-4</td>
<td>1.50 ± 0.065</td>
</tr>
<tr>
<td>Ex vivo bone marrow colony formation assay</td>
<td>Granulocytes and macrophages</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>IC50: 111 ± 13 TGI: 218 ± 26 LC50: 536 ± 61</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>162 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>21.3 ± 12.9</td>
</tr>
</tbody>
</table>

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 GI50, TGI, LC50 and the IC50, IC75, IC90.
0.3 mg/kg given i.v., SJG-136 causes a significant growth delay to this tumor (Fig. 6A). 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. 6B). 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. 6C). 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 cytotoxic 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 monooalkylation, 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-
istration 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. 3B and 6A) 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-G150 doses, cells can eventually overcome a significant G2-M block. The DNA damage induced by SJG-136 clearly activated a G2-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).

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.

REFERENCES


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


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/64/18/6693

Cited articles  This article cites 37 articles, 10 of which you can access for free at: http://cancerres.aacrjournals.org/content/64/18/6693.full.html#ref-list-1

Citing articles  This article has been cited by 20 HighWire-hosted articles. Access the articles at: /content/64/18/6693.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.