The Novel Sequence-Specific DNA Cross-Linking Agent SJG-136 (NSC 694501) Has Potent and Selective In vitro Cytotoxicity in Human B-Cell Chronic Lymphocytic Leukemia Cells with Evidence of a p53-Independent Mechanism of Cell Kill

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ABSTRACT

SJG-136 (NSC 694501) is a novel DNA cross-linking agent that binds in a sequence-selective manner in the minor groove of the DNA helix. It is structurally novel compared with other clinically used DNA cross-linking agents and has exhibited a unique multilog differential pattern of activity in the NCI 60-cell line screen (i.e., is COMPARE negative to other cross-linking agents). Given this profile, we undertook a preclinical evaluation of SJG-136 in primary tumor cells derived from 34 B-cell chronic lymphocytic leukemia (B-CLL) patients. SJG-136 induced apoptosis in all of the B-CLL samples tested with a mean LD50 value (the concentration of drug required to kill 50% of the cells) of 9.06 nmol/L. Its cytotoxicity was undiminished in B-CLL cells derived from patients treated previously, those with unmutated VH genes, and those with p53 mutations (P = 0.17; P = 0.63; P = 0.42, respectively). SJG-136-induced apoptosis was associated with the activation of caspase-3 that could be partially abrogated by the caspase-9 inhibitor Z-LEHD-FMK. Furthermore, SJG-136 did not trigger the phosphorylation of p53 or the up-regulation of GADD45 expression in B-CLL cells whereas the cross-linking agent chlorambucil elicited both of these effects. This suggests that SJG-136 cross-linking adducts are not subject to p53-mediated DNA excision repair mechanisms in B-CLL cells. Taken together, these data demonstrate a novel mechanism of action for SJG-136 that appears to circumvent the effects of poor prognostic markers. This unique cytotoxicity profile warrants further investigation and supports the evaluation of this agent in Phase I clinical trials for patients with B-CLL.

INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in western countries and is responsible for 5,000 deaths in the United States alone each year (1). The clinical course of CLL is variable, with some patients having normal age-adjusted survival whereas the median survival for those patients with advanced-stage disease is only 36 months (2). Although the introduction of therapeutic agents such as fludarabine has resulted in higher rates of remission, the development of drug resistance is a common complication and to-date there has been no improvement in overall survival for patients with this condition. Consequently, there is much interest in elucidating the biological mechanisms of drug resistance and in identifying novel therapies designed to overcome this pivotal clinical problem.

SJG-136 (NSC 694501; Fig. 1A) is a pyrrolobenzodiazepine dimer that represents a new family of sequence-selective DNA-interstrand cross-linking agents. It comprises two pyrrolobenzodiazepine monomeric units (3, 4) joined through their C8-positions via a propyldioxy linker, with each pyrrolobenzodiazepine C-ring containing a C2-extomethylene functionality (5, 6). The molecule has been shown to interact in the minor groove of DNA, spanning a total of 6 bp and alkylating the N2-positions of guanine bases situated on opposite strands of the DNA but separated by 2 bp. NMR, molecular modeling and gel electrophoresis-based studies on SJG-136 and related analogous suggest that it prefers to bind to Pu-GATC-Py sequences (Pu = purine; Py = pyrimidine), a feature that can be explained by hydrogen bonding interactions between the drug and certain molecular features of the DNA bases (Fig. 1B; ref. 7–9). The SJG-136 adduct provides a high degree of stabilization toward melting of duplex DNA as evidenced by energy calculations and an observed 33.6°C increase in the thermal denaturation of calf thymus DNA after incubation for 18 h at 37°C with SJG-136 (10, 11). In vitro transcription studies have demonstrated that SJG-136 can block transcription at preferred Pu-GATC-Py binding sites. A National Cancer Institute (NCI) COMPARE analysis has shown that, although SJG-136 compares in general terms with DNA-binding agents, it does not fit within any of the clusters of known agents, including anthracycin and bizelesin (12, 13).

Results from the NCI 60-cell-line screen indicate that SJG-136 is very potent in vitro with a GI50 range of 0.14 to 324 nmol/L (mean = 7.41 nmol/L), and also has a multilog differential pattern of activity. The molecule retains full potency in a number of resistant cell lines, which is thought to be because of the resistance of SJG-136 adducts to repair (12). It scored a total of 54 in the NCI Hollow Fiber assay with subcutaneous and intraperitoneal scores of 14 and 40, respectively, and has now been evaluated in over 40 human tumor xenograft models in which it has demonstrated broad-spectrum antitumor activity with little schedule dependency. Most importantly, in initial toxicity studies, SJG-136 has displayed neither the cardiotoxic effects of the structurally related anthracycins (i.e., pyrrolobenzodiazepine monomers; ref. 3) nor the discrepancy between human and murine bone marrow sensitivity observed with the AT-selective minor groove DNA interstrand cross-linking agent of similar length and molecular weight, bizelesin (12, 13). SJG-136 is currently undergoing Phase I evaluation in both the United States (through the NCI) and United Kingdom (through Cancer Research United Kingdom).

MATERIALS AND METHODS

Cells and Clinical Details of Patients. Peripheral blood samples from 34 patients with B-CLL (20 untreated and 14 treated) and 10 age-matched normal controls were obtained with the informed consent of patients. B-CLL was defined by clinical criteria, cellular morphology, and the coexpression of CD19 and CD5 in lymphocytes simultaneously displaying restriction of light-chain rearrangement. Staging was based on the Binet classification system. None of the patients treated previously had received therapy for at least 3 months before this study, VH gene mutational status was determined for all 34 patients using the method described previously (15). The resulting PCR products were sequenced and considered unmutated if they showed homology of ≥98% with the closest germ line sequence. The clinical characteristics of the patient cohort are summarized in Table 1.

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Primary B-CLL Cell Culture Conditions. Freshly isolated peripheral blood lymphocytes (1 x 10^6/ml) were cultured in Eagle’s medium (Invitrogen, Paisley, United Kingdom) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Lymphocytes were incubated at 37 °C in a humidified 5% carbon dioxide atmosphere in the presence of SJG-136 (10^-7 mol/L). Parallel experiments using chlorambucil (10^{-6.5} – 10^{-5} mol/L) and fludarabine (10^{-7} – 10^{-6} mol/L) were also performed to assess the comparative intra- and inter-sample in vitro cytotoxicity. In addition, control cultures were carried out to which no drug was added to normal and leukemic lymphocytes. Cells were subsequently harvested by centrifugation and analyzed by flow cytometry using the methods outlined below. Experiments were performed either in duplicate or triplicate.

Cell Lines. We investigated the ability of SJG-136 to induce apoptotic cell death in the p53 non-expressing/mutant leukemic cell lines, K562 (chronic myelogenous leukemia) and MOLT-4 (T-cell acute lymphoblastic leukemia) containing a G>A mutation at codon 248 of the p53 gene. Cells were maintained in RPMI 1640 (Invitrogen) with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO₂. Cells were cultured for 48 h in the presence or absence of SJG-136, chlorambucil or fludarabine at the concentrations given previously. Apoptosis was measured by Annexin V labeling (Dako, Ely, United Kingdom) and was quantified using flow cytometry.

Measurement of In vitro Apoptosis. In this study, changes in forward light scatter (FSC) and side light scatter (SSC) characteristics were used to quantify apoptotic and viable cell populations as described previously (16–18). Typically, lymphocytes show a reduction in FSC (a function of cytoplasmic shrinkage) and an increase in SSC (attributable to increased granularity) when they undergo apoptosis (19). The quantitation of apoptosis using an FSC/SSC gating strategy in conjunction with back gating of R-phycoerythrin cyanine 5-labeled CD19⁺ (Dako) or R-phycoerythrin-labeled CD3⁺ (Dako) lymphocytes allowed simultaneous acquisition of data in viable and apoptotic B-lymphocyte and T-lymphocyte subpopulations, respectively. All LD_{50} and LD_{90} values (the concentration of SJG-136 required to kill 50 and 90% of cells, respectively) were derived from the dose-response curves. Duplicate samples were assessed using fluorescein (FITC)-labeled Annexin V to confirm the presence of apoptotic cells in the cell cultures and to validate the FSC/SSC quantitation method (20).

SJG-136 Induced Caspase-3 Activation. B-CLL cells were incubated at 37 °C in a humidified 5% carbon dioxide atmosphere in the presence of SJG-136 (10^{-10}–10^{-7} mol/L) or fludarabine (10^{-7}–10^{-5} mol/L) for 12, 24,
and 48 h. Cells were then harvested by centrifugation and labeled with CD19 R-phycocerythrin cyanine 5-conjugated antibody. Subsequently the cells were incubated for 1 h at 37 °C in the presence of the PhiPhiLux G1,D2 substrate (Calbiochem, Nottingham, United Kingdom). The substrate contains two fluorophores separated by a quenching linker sequence that is cleaved by active caspase-3. Once cleaved, the resulting products fluoresce green and can be quantified using flow cytometry. In addition experiments, the caspase-8 inhibitor, Z-IETD-FMK, or the caspase-9 inhibitor, Z-LEHD-FMK (Cambridge Bioscience, Cambridge, United Kingdom) were added to SJG-136-treated cell cultures (final concentration 2 μM) to determine whether either of these inhibitors was able to abrogate the apoptotic effects of SJG-136 in B-CLL cells.

**Activation of p53 and the Induction of GADD45 Expression in B-CLL Cells.** GADD45 protein expression is up-regulated in response to DNA damage and is responsible for orchestrating nucleotide excision repair. In this study, we compared the cellular response of B-CLL cells to chlorambucil and SJG-136 to determine whether these two cross-linking agents both induced phosphorylation of p53 and activated downstream nucleotide excision repair. B-CLL cells were cultured for 4 and 48 hours in the presence or absence of one of the drugs under investigation. Cells were harvested by centrifugation and incubated with 10 μL of anti-CD19-R-phycocerythrin cyanine 5-conjugated antibody. Subsequently the cells were washed and then prepared for intracellular staining of phosphorylated p53 and GADD45 (Santa Cruz Biotechnology, Santa Cruz, CA) using a commercially available kit (DAKO, Ely, United Kingdom). A FITC-labeled secondary antibody was added to the cells (DAKO), and after a final washing step, the cells were resuspended in 0.5 ml of 1% paraformaldehyde before flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

**Statistical Analysis.** The data obtained in these experiments were evaluated using the equal variance and paired Student’s t test, and correlation coefficients were calculated from least squares linear regression plots. LD_{50} values were calculated from line of best-fit analysis of the dose-response curves. All statistical analyses were performed using Graphpad Prism 3.0 software (Graphpad Software Inc., San Diego, CA).

**RESULTS**

**Induction of Apoptosis in B-CLL Cells.** A flow cytometry-based **in vitro** apoptosis detection assay was used to determine whether SJG-136 could induce apoptotic cell death in B-CLL cells. The characteristic changes in the forward- and side-light scatter resulting from cellular shrinkage described previously were used to define apoptosis (19). In addition, Annexin V labeling was also performed to verify the light-scatter data. Apoptosis was induced in all 34 of patient samples after exposure to SJG-136 with a mean LD_{50} value (±SD) of 9.06 nmol/L (±3.2 nmol/L) and a mean LD_{90} value (±SD) of 43.09 nmol/L (±26.1 nmol/L; Fig. 2A and B, respectively). There was no significant difference in the LD_{50} values between the treated and untreated patient groups (Fig. 3A). Similarly, there was no significant difference in LD_{90} values when the patient cohort was analyzed according to mutational status (Fig. 3B). Furthermore, two of the patients in the treated patient group had a known p53 mutation and showed a high degree of in vitro resistance to fludarabine but demonstrated similar sensitivity to SJG-136 when compared with the remaining patient samples.

**Induction of Apoptosis in Normal Age-Matched Control B and T Cells and T Cells from B-CLL Patients.** B and T lymphocytes from 10 healthy normal controls were assessed for their sensitivity to SJG-136-induced apoptosis. In addition, the T lymphocytes from 12 B-CLL patients from the untreated patient group whose T-lymphocyte population was >5% of the total lymphocyte population were also analyzed to determine whether SJG-136 had differential cytotoxic effects on the various lymphocyte subpopulations. None of the treated patient samples met this criterion and were therefore not analyzed. The T cells from the B-CLL samples showed consistently higher LD_{50} values than their corresponding malignant B-cell clones (P = 0.0006; paired t test). In addition, the healthy normal control B- and T-lymphocytes demonstrated higher LD_{50} values than the B-CLL cells (P < 0.0001 and P < 0.0001, respectively). The relative sensitivities of the various lymphocyte populations to SJG-136 are illustrated in Fig. 4.

**Comparison of the Cytotoxic Effects of SJG-136 and Fludarabine in Treated and Untreated Patients.** The single largest obstacle to the successful clinical management of B-CLL is the development of drug resistance. All of the currently used treatment options suffer from diminished cytotoxicity in subsequent treatment rounds. Furthermore,
pleiotropic drug resistance is a common phenomenon where previous treatment with one agent causes resistance to others. This is graphically illustrated in Fig. 5; the patient treated previously subset demonstrated a significantly higher mean LD50 value for fludarabine when compared with the untreated subset ($P < 0.0001$). In contrast, previous treatment appeared to have no effect on the cytotoxicity of SJG-136 in cells taken from the same patient cohort ($P = 0.17$).

p53 Non-expressing/Mutant Cell Lines. Both the p53 non-expressing cell line, K562, and the p53 mutant cell line, MOLT-4, showed marked in vitro sensitivity to SJG-136 yielding LD50 values of 16.4 and 24.8 nmol/L, respectively. In contrast, both cell lines were relatively resistant to chlorambucil and fludarabine-induced cell killing with LD50 values at least 3 logs higher than SJG-136 (data not shown).

SJG-136 Induced Apoptosis Is Dependent on Caspase-3 Activation. To investigate whether SJG-136 mediates its cell killing effects through the induction of caspases, time course experiments were conducted in the presence or absence of SJG-136. The B-CLL cells were harvested by centrifugation and labeled with CD19 R-phycocerythrin cyanine 5-conjugated antibody. The cells were then resuspended in flow cytometry buffer together with the PhiPhiLux G1 D2 substrate (Calbiochem) and incubated for 1 h at 37°C. In the presence of active caspase-3 the substrate is cleaved resulting in the emission of a green fluorescence signal that can be detected by flow cytometry. SJG-136 induced a time- and concentration-dependent increase in the activation of caspase-3. Figure 6 shows the concentration-dependent induction of activated caspase-3 in SJG-136-treated B-CLL cells after 48 h in culture. The activation of caspase-3 was partially abrogated by the addition of the caspase-9 inhibitor, Z-LEHD-FMK, indicating that SJG-136-induced apoptosis is predominantly mediated through the intrinsic apoptotic pathway (data not shown).

p53 Activation and the Induction of DNA Excision Repair in B-CLL Cells. Two of the patients in our B-CLL cohort had known p53 mutations that were associated with both clinical and in vitro resistance to fludarabine. In contrast, these patients showed similar SJG-136 in vitro sensitivity to the rest of the patient cohort indicating that p53 activation was probably not required for effective SJG-136 cell killing. Because SJG-136 was designed as a DNA minor groove interstrand cross-linking agent, we investigated whether SJG-136 induced the phosphorylation of p53 and stimulated downstream nucleotide excision repair in B-CLL cells as evidenced by the induction of GADD45. None of the B-CLL patients ($n = 9$) phosphorylated p53 or up-regulated GADD45 expression after exposure to SJG-136. In contrast, when the same patient samples were treated with the cross-linking agent chlorambucil, they showed an increase in phosphorylated p53 (Fig. 7A) and a marked increase in GADD45 expression over the same culture period (Fig. 7B).

**DISCUSSION**

This report represents the first preclinical evaluation of the novel DNA minor-groove interstrand cross-linking agent SJG-136 (NSC 694501) in B-CLL cells. Our data demonstrate that SJG-136 is extremely cytotoxic to B-CLL cells in vitro, exhibiting LD50 values more than two logs lower than fludarabine, the current treatment of choice for this condition. Furthermore, SJG-136 exhibited differential cytotoxicity because it was less effective at inducing apoptosis in normal B and T lymphocytes and T lymphocytes derived from B-CLL patients. Whether this finding can be extrapolated beyond the in vitro cell culture system used in this study into a preferential antileukemic effect in vivo will need to be tested in suitable animal models and/or in a prospective clinical trial.
The mode of SIG-136-induced cell killing was shown to be dependent on caspase-3 activation, and the up-stream events that led to the processing of this effector caspase were predominantly mediated through caspase-9 signaling via the intrinsic apoptotic pathway. Because SIG-136 is a DNA cross-linking agent it might be assumed that p53-mediated detection of these adducts would result in the up-regulation of nucleotide excision repair mechanisms. However, the binding of SIG-136 to genomic DNA did not appear to elicit these responses in B-CLL cells, and the specific mechanism of apoptosis induction remains obscure. Corroborative evidence for a novel, p53-independent mechanism of cell killing was derived from the fact that two of the patients in our B-CLL cohort possessed known, function impairing, p53 mutations that presumably contributed toward their clinical and in vitro resistance to chlorambucil and fludarabine. Leukemic cells derived from these patients showed similar sensitivity to SIG-136 when compared with the rest of the patient cohort. Furthermore, the p53 non-expression cell line, K562, and the p53 mutant cell line, MOLT-4, were relatively sensitive to SIG-136 induced-apoptosis. In contrast, both of these cell lines showed marked resistance to both chlorambucil and fludarabine. In addition, we found no significant difference in sensitivity to SIG-136 when we analyzed our patient cohort in terms of V_H gene mutational status (mutated versus unmutated) and previous exposure to chemotherapeutic drugs (treated versus untreated).

Taken together our data indicate that SIG-136 might be a useful therapeutic option for the treatment of B-CLL, particularly in cases of refractory disease or where poor prognostic markers are in evidence. Given that B-CLL remains an incurable disease with a median survival for advanced-stage patients of only 3 years (2), the progression of SIG-136 toward early Phase I clinical evaluation would appear justified.

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