Cellular Uptake and Intracellular Levels of the Bcl-2 Antisense G3139 in Cultured Cells and Treated Patients with Acute Myeloid Leukemia

Guowei Dai,1 Kenneth K. Chan,1,3,4 Shujun Liu,4 Dale Hoyt,2,3 Susan Whitman,4 Marko Klisovic,4 Tian Sheng Shen,4 Michael A. Caligiuri,3,4 John Byrd,3,4 Michael Grever,3,4 and Guido Marcucci,3,4,5

Abstract Purpose: Down-regulation of Bcl-2 by the antisense G3139, currently under clinical evaluations, could restore chemosensitivity in otherwise resistant malignant cells. To date, the mechanism of intracellular accumulation of G3139 following in vivo administration remains to be elucidated. This study aimed to assess whether detectable intracellular concentrations of G3139 are achievable in vivo and how these relate to Bcl-2 down-regulation.

Experimental Design: Cellular uptake of G3139 was studied in leukemia myeloid cell lines and blasts collected from treated patients using a newly developed, novel, and highly sensitive ELISA-based assay. Real-time reverse transcription-PCR was used to quantify Bcl-2 mRNA changes in treated cells.

Results: The assay was fully validated and showed a limit of quantification of 50 pmol/L. When exposed to 0.33 to 10 μmol/L G3139, K562 cells exhibited intracellular concentrations in the range of 2.1 to 11.4 pmol/mg protein. When G3139 was delivered with cationic lipids, a 10- to 25-fold increase of the intracellular concentrations was observed. There was an accumulation of G3139 in the nuclei, and the ratio of nucleus to cytoplasm was increased 7-fold by cationic lipids. Intracellular concentrations of G3139 were correlated with Bcl-2 mRNA down-regulation. Robust intracellular concentrations of G3139 were achieved in vivo in bone marrow (range, 3.4-40.6 pmol/mg protein) and peripheral blood mononuclear cells (range, 0.47-19.4 pmol/mg protein) from acute myeloid leukemia patients treated with G3139.

Conclusions: This is the first evidence that measurable intracellular levels of G3139 are achievable in vivo in acute myeloid leukemia patients and that Bcl-2 down-regulation is likely to depend on the achievable intracellular concentrations rather than on plasma concentrations.

The antisense oligodeoxynucleotides are sequences of 16 to 29 bases of ssDNAs that hybridize to specific mRNA by Watson-Crick base pairing (1). Following hybridization, the oligodeoxynucleotide-mRNA duplex becomes a substrate for intracellular RNase H that catalyzes mRNA degradation while allowing the oligodeoxynucleotide to recycle for another base pairing event with the next target mRNA molecule. The net result of this process is a sustained decrease in translation of the target mRNA into the corresponding protein (2, 3).

The use of antisense strategies in cancer is based on the potentials of these oligodeoxynucleotide compounds to down-regulate oncogenic proteins that drive malignant transformation. The phosphorothioate antisense currently available for clinical use contains a sulfur in place of an oxygen in the phosphodiester bond between two subsequent nucleotides and seems to have favorable pharmacologic characteristics, such as nuclease resistance and the ability to recruit and activate RNase H (4–6), which makes it suitable for in vivo administration (7–10).

G3139 (Table 1), an 18-mer phosphorothioate antisense oligonucleotide designed to bind to the first six codons of the human Bcl-2 mRNA, is currently being evaluated in several phase I to III clinical trials for both solid tumors and hematologic malignancies (11, 12). As high levels of the antiapoptotic protein Bcl-2 were found to be associated with chemoresistance in malignant cells (13–15), it has been hypothesized that by down-regulating Bcl-2 the antisense decreases the apoptosis threshold, thereby restore chemotherapeutic sensitivity in otherwise resistant cells. In our initial OSU 9977 protocol study, G3139 was given as continuous i.v. infusion (CIVI) for 10 days with fludarabine/cytarabine chemotherapy starting on day 6 in patients with refractory or relapsed acute leukemia (16). A response rate of 45% and evidence of target down-regulation in ~75% of the analyzed patients were observed. Based on these results, we have now incorporated G3139 in the upfront therapy for untreated elderly acute myeloid leukaemia (AML; refs. 17, 18).

Despite these encouraging clinical results, a recurrent motif in the G3139 trials has been the lack of correlations among plasma...
drug levels, Bcl-2 down-regulation, and disease response. This problem stems in part from the low sensitivity of the previously reported analytic methods, the uncertainty of whether significant levels of G3139 uptake occur in malignant cells, and how the intracellular distribution of the drug relates to down-regulation of its target. Thus, to address these questions, we developed a novel, highly sensitive, and specific ELISA-based assay for quantification of G3139 in various biological matrices. This assay allowed us to follow plasma drug decay over a much longer period of time than previously reported methods, achieve a more accurate definition of the antisense pharmacokinetics, distinguish the parent compound from its chain-shortened metabolites, and, more importantly, quantify the intracellular drug concentrations in treated patients. Herein, for the first time, we reported that robust levels of G3139 were achievable in blood and bone marrow mononuclear cells from patients with AML treated with the Bcl-2 antisense, and these might determine levels of Bcl-2 down-regulation.

Materials and Methods

Antisense and reagents. G3139 was supplied by the National Cancer Institute (Bethesda, MD). The putative metabolites shorter of 1, 2, or 3 nucleotides (N-1, N-2, or N-3) were obtained as follows: N-1 of G3139 was a gift from Dr. William Tong (Memorial Kettering Cancer Center, New York, NY); 3’ N-2, 3’ N-3, 5’ N-2, mismatch control, reverse control (Table 1), and 5’-fluorescein-labeled G3139 (FITC-G3139) were purchased from Integrated DNA Technologies (Coralville, IA). The purity (> 95%) and identity of each oligomer was examined by elution sequence of capillary gel electrophoresis and by high-performance liquid chromatography (HPLC)-UV-mass spectrometry (model LCQ, Finnigan Corp., San Jose, CA).

Fluorogenic ELISA assay procedures. The assay principle is illustrated in Fig. 1. Briefly, the capture oligodeoxynucleotide 5’-GAA-TACCGAATGCGCGCTCGGGA/biotin-3’ (Integrated DNA Technologies) was first diluted in assay buffer [60 mmol/L phosphate buffer (pH 7.4), 1.0 mol/L NaCl, 5 mmol/L EDTA, 0.3% Tween 20] at a concentration of 200 nmol/L, heated at 95°C for 5 minutes, and mixed with plasma or cell lysates containing G3139. Triton X-100 [final concentration, 0.25% (w/v)] was added into the plasma sample and digoxigenin-modified at the 3’ end, phosphorylated at the 5’ end and digoxigenin modified at the 3’ end, Integrated DNA Technologies) was diluted with ligation buffer [66 mmol/L Tris-HCl (pH 7.6), 10 mmol/L MgCl2, 10 mmol/L DTT, 1 mmol/L/L ATP] containing 5 units/mL T4 DNA ligase (Amersham Biosciences, Piscataway, NJ). The mixture (150 μL) was dispensed into each well of a 96-well plate and incubated at 18°C for overnight. To remove the excess amount of probe oligodeoxynucleotide bound to capture oligodeoxynucleotide, 30 units of St nuclease (Invitrogen, Carlsbad, CA) in 30 mmol/L sodium acetate (pH 4.6), 1.0 mmol/L zinc acetate, 150 mmol/L NaCl, and 5% glycerol were added into each well for 60 minutes at room temperature, and the plate was then washed five times with washing buffer. Subsequently, anti-digoxigenin-alkaline phosphatase (150 μL) diluted 1:2,500 with bovine serum albumin block buffer in TBS (Roche, Indianapolis, IN) was added into each well. Following 0.5-hour incubation at 37°C, the plate was again washed with washing buffer. AttoPhos substrate (150 μL, Promega, Madison, WI) in diethanolamine buffer prepared as recommended by the manufacturer was added to each well. Fluorescence intensity was measured at excitation 430/emission 570 (filter = 550 nm) using a Gemini XS plate reader (Molecular Devices, Sunnyvale, CA) following incubation at 25°C for 30 minutes.

Validation studies. Linearity, within-day and between-day assay accuracies, and precision of the method were assessed from blank human plasma concentrations of 50 pmol/L (limit of quantification), 100 pmol/L (low-quality control), 500 pmol/L (medium-quality control), and 2,000 pmol/L (high-quality control). Between-day and within-day validation in cell lysate was also done. The specificity of the assay was evaluated using human plasma from three healthy donors (Red Cross, Columbus, OH) as well as from K562 cell extracts to assess possible interference from endogenous substances and by cross-reactivity studies with putative metabolites (see below).

Cross-reactivity analysis. To evaluate the cross-reactivity of the assay with putative metabolites, various concentrations of 3’ N-1, N-2, and N-3 oligomers from 50 pmol/L to 1,000 mmol/L were added into human blank plasma, and concentration-response curves were constructed. Additionally, different concentrations of 5’ N-2, reverse control, and mismatch control oligomers (Table 1), ranging from 50 pmol/L to 100 nmol/L in cell lysate, were also evaluated for cross-reactivity. All concentration-response curves were fitted to the sigmoid $E_{\text{max}}$ model. The maximal response produced by each compound ($E_{\text{max}}$) and the concentration that produced 50% of the maximal response ($E_{50}$) were obtained by nonlinear regression analysis using WinNonLin version 3.1 (Pharsight Corp., Mountain View, CA). The cross-reactivity was calculated as $E_{50}$ of parent compound divided by $E_{50}$ of each metabolite or analogue (19).

Cross-validation with the high-performance liquid chromatography-UV method. Samples ($n = 45$) from AML patients treated on a previous clinical phase I study (16) were analyzed by both the ELISA assay and the established HPLC-UV method.

| Oligonucleotide sequence of putative metabolites of G3139 and control oligonucleotides used in the cross-reactivity studies |
|-----------------|-----------------|-----------------|
| **Name**        | **Oligonucleotide sequence (5’-3’)** | **Cross-reactivity (%)** |
| G3139           | TCTCCCAAGCTGGCCCAT | 100  |
| 3’ N-1          | TCTCCCAAGCTGGCCA | 6.1  |
| 3’ N-2          | TCTCCCAAGCTGGCC   | 0.30 |
| 3’ N-3          | TCTCCCAAGCTGGCC   | <0.01|
| 5’ N-2          | TCCCGAGCTGGCCCAT  | 32   |
| Reverse control | TACCGGTCGACCCTCT   | 0    |
| Mismatch control (G4126) | TCTCCCAACGATGTGCACCAT | 25   |

**NOTE:** All control oligonucleotides are fully phosphorothioate oligonucleotides.
glutamine (Life Technologies, Carlsbad, CA) and 10% heat-inactivated FBS. Cells were incubated with 200 ng/ml of G3139 on the OSU 9977 K562 cell line was cultured in RPMI 1640 supplemented with L-glutamine and 10% FBS. The supernatant was used as the nuclear fraction. Cytosolic lactate dehydrogenase was used as a cytotoxic marker, and cross-contamination between cytoplasmic and nuclear fractions was determined using lactate dehydrogenase kit (Roche).

**Plasma pharmacokinetics in patients with acute myeloid leukemia.** Plasma pharmacokinetics of G3139 in patients whose cellular levels of G3139 in blood and bone marrow mononuclear cells were measured was also studied. Eight patients, five on CIVI at 4 mg/kg and two at 7 mg/kg with available material, were studied during the 10-day infusion and 4 hours following infusion using the newly developed ELISA method. Relevant pharmacokinetic variables were computed following curve fitting to an appropriate model via WinNonLin computer software version 3.1.

**Cellular uptakes of G3139 complexed with cationic liposomes.** All transfection experiments were done in Opti-MEM medium (Invitrogen). Stock solutions of Oligofectamine reagent (Invitrogen) and G3139 were prepared using Opti-MEM as the diluent. The appropriate amount of G3139 was diluted in 100 μL Opti-MEM to result in final concentrations of G3139 of 100 nmol/L, 200 nmol/L, 330 nmol/L, 1.0 μmol/L, 3.3 μmol/L, and 10 μmol/L. For 100, 200, and 330 nmol/L G3139, 1.8, 3.6, and 6 μL of Oligofectamine reagent were used. For 1.0, 3.3, and 10 μmol/L G3139, 20 μL of Oligofectamine were used. To avoid cytotoxicity due to Oligofectamine, its final concentration was kept below 20 μL/mL in all transfection experiments. These solutions were incubated at room temperature for 10 to 20 minutes to allow lipid-oligo(gene) nucleotide complex formation. Then, 200 μL of each of these complex solutions were overlaid on the cells seeded at a density of 2 × 10⁶ cells per well in 0.8 mL medium on six-well plates for 4 to 5 hours. Another cationic liposome consisting of dimethyl-dioctadecylammonium bromide (DDAB) and L-α-diethyl phosphatidylethanolamine (a generous gift by Dr. Robert Lee, The Ohio State University, Columbus, OH) was also used and was prepared as reported previously (21). The mean ± SD particle size of the cationic liposomes was determined to be 94 ± 48 nm. Because each DDAB molecule carries 1 positive charge, whereas one G3139 molecule possesses 17 negative charges, a preliminary cellular uptake study was first carried out to optimize the charge ratio of cationic lipid to G3139 to achieve the highest uptake value. The optimal ratio was found to be 1.43 on K562 cells when 0.33 μmol/L G3139 was used with various amounts of cationic liposomes. Therefore, 8, 24, and 60 μmol/L DDAB/L-α-diethyl phosphatidylethanolamine were mixed with 0.33, 1, and 3.3 μmol/L G3139, and the DDAB/G3139 complexes were prepared in the similar fashion as for Oligofectamine. Following 4- to 5-hour incubation with Oligofectamine or DDAB, 3 mL of medium containing 10% fetal bovine serum were added to each well and the cells were then gently mixed. Then, the mixture was incubated for another 20 hours before cell lysis for total RNA isolation and G3139 quantification.

**Quantification of Bcl-2 mRNA levels.** Quantification of Bcl-2 RNA was done by real-time reverse transcription-PCR as reported previously (22). Briefly, total cellular RNA and cDNAs were prepared as described previously (22). Each cDNA sample was used as a template in a PCR amplification reaction on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The measured Bcl-2 levels were normalized to the internal control of 18S.

**Flow cytometry studies.** K562 cells (0.5 × 10⁶) were exposed to 0.3 or 0.5 μmol/L FITC-G3139 in the presence or absence of delivery vehicle at 37°C for 24 hours. Then, the cells were harvested, washed thrice by cold PBS/1% fetal bovine serum, and analyzed by flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed and displayed with CellQuest software (Becton Dickinson) as histograms.

**Data analyses.** Means and SDs were computed for all variables using standard methods. All graphs are plotted as mean ± SD. Two-sided multiple comparison method was done to compare group mean difference with family-wise error at 0.01 (23). Differences were significant when a p value was less than 0.05.
considered statistically significant when $P < 0.05$. For cross-validation with the HPLC-UV method, Pearson correlation was obtained using S-Plus software (24). Equivalence test was done with the Wilcoxon signed-rank test (25).

**Results**

**Validation of the ELISA-based assay of G3139 in plasma and cell extracts.** In human plasma, we determined the limit of detection (defined as 10 times signal-to-background noise; ref. 14) to be 25 pmol/L and the limit of quantification to be 50 pmol/L, equivalent to 0.15 and 0.3 ng/mL, respectively. The assay was linear from 25 to 2,500 pmol/L after log transformation (Fig. 2A). The linear concentration range of the assay could be extended to 500 nmol/L (a 4-log magnitude of dynamic range), as serial dilution from 500 nmol/L yielded a nearly identical calibration curve (data not shown). The mean within-day coefficients of variation (CV) of the assay at 50, 100, 500, and 2,000 pmol/L were found to be 13%, 6%, 6%, and 3% (all $n = 6$), respectively (Table 2). The corresponding accuracy values were 73%, 103%, 107%, and 82% of the nominal concentrations of the standards. The between-day CVs of the assay were found to be 10%, 5%, and 10% for the 50, 500, and 2,000 pmol/L standards, respectively, with the corresponding accuracy values of 94.2%, 112%, and 94.7% of the nominal concentrations.

Similar validation studies were also conducted in cell lysate added to known concentrations of G3139. A limit of detection of 25 pmol/L and a limit of quantification of 50 pmol/L, equivalent to 2.5 and 5.0 fmol/100 µL cell lysate, respectively, were obtained. A linear calibration curve of the fluorescence signal versus concentration within the range of 25 to 4,000 pmol/L was obtained (Fig. 2B). The mean within-day precision CVs of the assay in cell lysate at 50, 100, 500, and 2,000 pmol/L were 7%, 5%, 7%, and 3%, with corresponding accuracy values of 109%, 90%, 103%, and 93% (Table 2). The

![Fig. 2.](image-url) Validation of the ELISA-based assay of G3139. A, representative standard curve of G3139 in human plasma. Each concentration was run in duplicates, and average was used for linear regression analysis. The mean fluorescence signal was plotted against G3139 concentrations (pmol/L). B, representative standard curve of G3139 in cell lysate. Each concentration was run in duplicates, and average was used for linear regression analysis. The mean fluorescence signal was plotted against G3139 concentrations (pmol/L). C, concentration-response curves of G3139 and its possible 3' metabolites. G3139 (●), 3' N-1 (○), 3' N-2 (■), and 3' N-3 (△) oligomers at the 3' end ranging from 0.05 to 1,000 nmol/L were added into human blank plasma, and dose-response curves were constructed as described in Materials and Methods. Points, average of three replicates; bars, SD. The maximal response (fluorescence intensity) produced by each compound ($E_{\text{max}}$) and the concentration that produced 50% of the maximal response ($EC_{50}$) was determined by nonlinear regression analysis.
The within-run and between-run precision and accuracy of the ELISA assay of G3139 in human plasma and K562 cell lysate are shown in Table 2.

<table>
<thead>
<tr>
<th>Nominal concentration (pmol/L)</th>
<th>Measured mean (SD), pmol/L</th>
<th>Precision (CV %), n = 6</th>
<th>Accuracy (% nominal)**</th>
<th>Measured mean (SD), pmol/L</th>
<th>Precision (CV %), n = 6</th>
<th>Accuracy (% nominal)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-run precision and accuracy in plasma (n = 6)</td>
<td>Between-run precision and accuracy in plasma (n = 5 d)</td>
<td>Within-run validation in cell lysate (n = 6)</td>
<td>Between-run validation in cell lysate (n = 5 d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>36.7 (4.8)</td>
<td>13</td>
<td>73</td>
<td>471 (4.5)</td>
<td>10</td>
<td>94.2</td>
</tr>
<tr>
<td>100</td>
<td>103.1 (6.1)</td>
<td>6</td>
<td>103</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>532.8 (31.1)</td>
<td>6</td>
<td>107</td>
<td>562 (29.5)</td>
<td>5</td>
<td>112.0</td>
</tr>
<tr>
<td>2,000</td>
<td>1,646 (44.4)</td>
<td>3</td>
<td>82</td>
<td>1,894 (196)</td>
<td>10</td>
<td>94.7</td>
</tr>
</tbody>
</table>

*Concentrations calculated from the linear least-squares regression curve (n = 6).
**Expressed as \((\text{mean observed concentration / nominal concentration}) \times 100\).

Insignificant background was found in human plasma and cell lysate. The specificity of the assay was assessed by measuring the fluorescence signal generated by four putative G3139 metabolites and two control oligodeoxynucleotides. The concentration-response curves of G3139 and the 3'-end metabolites in human plasma from 50 to 1,000 pmol/L are shown in Fig. 2C. The cross-reactivities, calculated as the ratio of EC50 of the parent compound to EC50 of each metabolite, are shown in Table 1. The cross-reactivities of 3'-N-1 and N-2 were estimated to be 6.3% and 3.4%, respectively. The 3'-N-1 metabolite at 5 nmol/L gave a fluorescence signal <10% of that of G3139 at the same concentration. The 3'-N-3 metabolite gave extremely low fluorescence (<0.04%). In contrast, the cross-reactivity of the 5'-N-2 was similarly evaluated (figure not shown), and the value was estimated to be 41% (Table 1). There was essentially no fluorescence signal when reverse control oligodeoxynucleotide was used (Table 1), but mismatch control oligodeoxynucleotide (figure not shown) gave a cross-reactivity of 26% (Table 1). Similar results were found for G3139 in cell lysate (Table 1).

Comparison of the ELISA-based assay with high-performance liquid chromatography-UV method. Cross-validation of our novel ELISA-based assay with the published HPLC-UV method was done using 45 plasma samples obtained from acute leukemia patients treated in our clinical study (OSU 9977). Because of the low sensitivity of the HPLC-UV method (88 nmol/L or 500 ng/mL), only plasma samples with sufficiently high drug levels were selected. The correlation between the results attained by the two methods was linear with a Pearson correlation coefficient of 0.968 (P < 0.001; Fig. 3). The Wilcoxon signed-rank test also showed that the two methods were equivalent at the 98% confidence level. Importantly, the advantage of the new assay was its detection sensitivity at least 3 orders of magnitude higher than the HPLC-UV method (50 versus 88,000 pmol/L).

Cellular uptake of noncomplexed and cationic lipid complexed G3139. Intracellular drug levels using our ELISA assay were quantified in K562 cell lysate following incubation with G3139 alone or in the presence of cationic lipid vehicle. Exposure to 0.33 to 10 nmol/L G3139 without delivery vehicle for 24 hours resulted in a concentration-dependent intracellular drug concentration in the range of 2.1 to 11.4 pmol/mg protein (Table 3). The uptake in K562 cells was estimated to be ~0.2% to 0.6% of the exposed drug. This is the first chemical measurement showing intracellular levels of G3139 when cells were exposed to the free drug. In contrast, exposure to G3139 complexed with

---

**Table 2. Within-run and between-run precision and accuracy of the ELISA assay of G3139 in human plasma and K562 cell lysate**

**Fig. 3.** Correlation curve of G3139 plasma concentrations measured by HPLC-UV and ELISA methods. Y axis, ELISA-based assay; X axis, HPLC-UV assay.
cationic lipids, such as Oligofectamine and DDAB/l-α-dioleyl phosphatidylethanolamine, resulted in a 25- and 50-fold increase, respectively, in antisense cellular uptake (Table 3). Notably, at a concentration as high as 10 μmol/L noncomplexed G3139, the cellular uptake of G3139 was even significantly lower than that measured when the cells were exposed to lower concentrations (i.e., 0.33, 1, and 3.3 μmol/L) of the antisense complexed with DDAB or Oligofectamine (P < 0.01). The amount of lipids used was not considered to impart significant toxicity, as IC₅₀ values for Oligofectamine were determined to be 30 μL/mL of the original lipid and 60 μmol/L for DDAB, all using 48-hour exposure.

To further validate our results and confirm that we indeed measured levels of the internalized G3139, we used a fluorescein-labeled G3139, free or complexed with Oligofectamine, to incubate with K562 cells. By flow cytometric analysis, we showed that the mean fluorescence in cells treated with G3139 and cationic lipids was ~5-fold greater than that in cells treated with G3139 alone, supporting the results obtained with our ELISA-based assay (Fig. 4A). Confocal microscopy indicated that the membrane-bound antisense was negligible compared with the internalized FITC-G3139 (data not shown). As flow cytometry and intracellular concentration determination do not provide information about intracellular distribution, we also examined differential drug distribution of different G3139 formulations in K562 cells by subcellular fractionation. Cell uptake for noncomplexed G3139 was found to be quite low (Fig. 4B), and 60% to 80% of the internalized full-length G3139 were found in the cytoplasmic fraction with a nucleus to cytoplasm drug ratio of 0.33 ± 0.053. In contrast, G3139 complexed with Oligofectamine resulted not only in higher intracellular levels but also in a 7-fold higher nucleus to cytoplasm drug ratio (i.e., 0.33 ± 0.053 versus 2.5 ± 0.017; Fig. 4B).

### Table 3. Comparison of intracellular uptake of G3139 antisense in the absence or presence of liposomal vehicle in K562 leukemic cells

<table>
<thead>
<tr>
<th>Concentration of G3139 (μmol/L)</th>
<th>Free drug, pmol/mg (μmol/L)</th>
<th>Complex with Oligofectamine, pmol/mg (μmol/L)</th>
<th>Complex with DDAB, pmol/mg (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>7.23 ± 1.35 (0.51 ± 0.094)</td>
<td>50.17 ± 5.34 (3.51 ± 0.37)</td>
<td>101.67 ± 14.52 (712 ± 101)</td>
</tr>
<tr>
<td>0.2</td>
<td>15.51 ± 3.97 (1.09 ± 0.28)</td>
<td>75.54 ± 6.94 (5.29 ± 0.48)</td>
<td>143.67 ± 24.03 (10 ± 1.68)</td>
</tr>
<tr>
<td>0.33</td>
<td>2.08 ± 0.47 (0.146 ± 0.33)</td>
<td>131.49 ± 25.34 (9.2 ± 1.77)</td>
<td>161.0 ± 29.82 (11.27 ± 2.09)</td>
</tr>
<tr>
<td>1</td>
<td>3.46 ± 0.22 (0.242 ± 0.015)</td>
<td>50.17 ± 5.34 (3.51 ± 0.37)</td>
<td>101.67 ± 14.52 (712 ± 101)</td>
</tr>
<tr>
<td>3.3</td>
<td>9.16 ± 0.59 (0.64 ± 0.041)</td>
<td>50.17 ± 5.34 (3.51 ± 0.37)</td>
<td>101.67 ± 14.52 (712 ± 101)</td>
</tr>
<tr>
<td>5</td>
<td>8.84 ± 1.28 (0.62 ± 0.09)</td>
<td>50.17 ± 5.34 (3.51 ± 0.37)</td>
<td>101.67 ± 14.52 (712 ± 101)</td>
</tr>
<tr>
<td>10</td>
<td>11.37 ± 1.97 (0.796 ± 0.138)</td>
<td>261.16 ± 30.11 (18.28 ± 2.10)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Intracellular concentration is expressed as pmol/mg protein and molar concentration (in parentheses). A measured conversion factor of 1 × 10⁶ cells equal to 1 ± 0.1 μL of cell volume and 70 ± 15 μg protein was used for calculation. Mean ± SD (n = 3 per group).

*P < 0.01, significant difference between the groups in comparison with the free drug group.
exclude the possibility of cytoplasm to nucleus contamination, the lactate dehydrogenase content in cytoplasm, nuclei wash fraction, and nuclear fraction were measured, and lactate dehydrogenase in nuclei fraction and nuclei wash fraction was found to be <10% of that in cytoplasmic fraction, suggesting minimal cytoplasm to nucleus contamination.

Correlation of G3139 intracellular levels with Bcl-2 down-regulation in acute myeloid leukemia cells in vitro. To determine whether intracellular concentrations of G3139 correlate with down-regulation of its target Bcl-2, K562 cells were again exposed to various concentrations of G3139 alone or to G3139-Oligofectamine complex for 24 hours. As shown in Fig. 5A, at G3139 concentrations between 0.1 and 10 μmol/L in the presence of cationic lipids, down-regulation of Bcl-2 mRNA as measured by real-time reverse transcription-PCR occurred efficiently. Nonlinear regression analysis of the dose-response curve showed that the G3139 concentration that produced 50% of Bcl-2 down-regulation (IC50) was ~0.29 μmol/L, and maximum Bcl-2 down-regulation (79% decrease relative to the control group) was observed at 10 μmol/L relative to the control group (Fig. 5B). The IC50 of 0.29 μmol/L corresponds to an intracellular G3139 concentration of 37 pmol/mg protein, which was not achievable even at the highest concentration of G3139 (10 μmol/L) when applied alone (Table 3). Further, exposure to 3.3 μmol/L G3139 without lipids failed to result in any significant suppression of Bcl-2 RNA (93% compared with control group). Exposure to 3.3 and 10 μmol/L mismatch control (G4126) and reverse control oligonucleotides complexed with Oligofectamine failed to show any significant Bcl-2 down-regulation, confirming specific target down-regulation (Fig. 5C).

Intracellular levels of G3139 in acute myeloid leukemia cells in vivo. To probe drug uptake in vivo, we measured levels of G3139 in bone marrow and blood mononuclear cells collected following 72 hours (day 3) and 120 hours (day 5) of G3139 CIVI before FLAG administration in AML patients treated on the protocol OSU 9977 (Tables 4 and 5). Paired bone marrow samples and peripheral mononuclear cells for determination of drug levels and Bcl-2 mRNA were only available from eight patients treated with G3139 (16). G3139 levels ranging from 3.4 to 40.6 pmol/mg protein in bone marrow mononuclear cells and intracellular levels ranging from 0.47 to 19.4 pmol/mg protein in blood mononuclear cells were found. Intracellular drug levels in bone mononuclear cell measured at 120 hours were significantly higher than those measured at 72 hours despite unchanged or even decreased plasma levels, suggesting a slower intracellular clearance of the drug over time. Of note, intracellular levels of G3139 measured following 120 hours of G3139 CIVI were found to be higher in bone marrow mononuclear cells than in blood mononuclear cells, suggesting a site-specific preferential uptake of the drug (Tables 4 and 5).

Because only few pairs of plasma and viable cell samples were available, a statistical analysis between plasma and intracellular drug concentration could not be made at this time. However, a direct correlation between drug plasma concentrations and cell uptakes did not seem to occur, as higher concentrations of G3139 were achieved in patients in whom lower plasma concentrations of the antisense were measured. Similarly, no linear correlation was found between intracellular levels of G3139 and Bcl-2 down-regulation, although it appeared that decrease of the Bcl-2 mRNA was more likely with a measured intracellular concentration of G3139 >5.0 pmol/mg protein (Table 4).

Plasma pharmacokinetics of G3139 in acute myeloid leukemia patients. Figure 6 shows the profiles of eight patients with
AML whose blood and bone marrow intracellular drug levels were monitored. Plasma G3139 levels reached steady-state concentration from 24 hours and declined biexponentially when infusion was stopped. Because the protocol was designed based on the lower assay sensitivity of the previous HPLC-UV method, plasma samples to only 4 hours post-infusion were collected, and the 4-hour levels showed a range of 22.8 to 84 nmol/L, readily measurable by the ELISA method. Based on these data and curve fitting to a two-compartment model, the relevant pharmacokinetic variables were computed and shown in Table 6. Mean steady-state concentration and area under the curve values were proportional to the infusion dose. The total clearance and half-lives are similar to those reported previously (16).

Discussion

One of the limitations in assessing the clinical activity of G3139 or other antisense therapeutics has been the inability to obtain information on the fate of the drug following in vivo administration. Specifically, it is unknown whether detectable intracellular concentration can be achieved and how these relate to the drug plasma levels and if any stoichiometric relationship between intracellular levels of the antisense and baseline Bcl-2 levels is necessary to attain a clinically significant down-regulation of the target. To date, none of the assay methods available for G3139 were either specific or sensitive enough to measure intracellular levels of the drug (12, 16, 18, 26–29). To overcome these problems and to quantify Bcl-2 antisense in different biological matrices, we developed and validated a sensitive and specific ELISA assay for G3139 and used it to measure the intracellular levels of the drug in cell extracts of blood and bone marrow samples from in vivo–treated patients. Although this method used an approach conceptually similar to those published for other antisense oligodeoxynucleotides (30–32), it also presents substantial differences. The addition of S1 nuclease specific for ssDNA following the second hybridization step seems to decrease the excess amount of probe oligodeoxynucleotide bound to capture oligodeoxynucleotide, thereby reducing the background fluorescence in biological samples and enhancing the linearity of the assay. Further, the addition of Triton X-100 at low concentration plays a role in disrupting the nonspecific interaction between antisense and cellular or plasma protein and seems quite effective at improving assay precision.

Herein, we showed that our assay is highly sensitive and specific toward the parent compound and its metabolites, as it detects only the 3'-end intact sequence of G3139 at a single nucleotide resolution, with little or no cross-reactivity with the putative 3'-end metabolites (33, 34). In contrast, the assay was not selective toward 5' metabolites; however,

<table>
<thead>
<tr>
<th>Unique patient number</th>
<th>G3139 plasma concentration (µmol/L)</th>
<th>G3139 intracellular concentration, pmol/mg protein (µmol/L)</th>
<th>Bcl-2 mRNA percentage change compared with baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.29</td>
<td>40.64 (1.00)</td>
<td>↑</td>
</tr>
<tr>
<td>17</td>
<td>1.16</td>
<td>11.89 (0.29)</td>
<td>↓</td>
</tr>
<tr>
<td>16</td>
<td>1.32</td>
<td>11.35 (0.28)</td>
<td>↓</td>
</tr>
<tr>
<td>18</td>
<td>0.70</td>
<td>9.46 (0.23)</td>
<td>←</td>
</tr>
<tr>
<td>12</td>
<td>0.20</td>
<td>5.94 (0.14)</td>
<td>↓</td>
</tr>
<tr>
<td>14</td>
<td>0.72</td>
<td>5.06 (0.12)</td>
<td>↓</td>
</tr>
<tr>
<td>2</td>
<td>0.77</td>
<td>4.18 (0.10)</td>
<td>↑</td>
</tr>
<tr>
<td>9</td>
<td>0.42</td>
<td>3.38 (0.08)</td>
<td>↑</td>
</tr>
</tbody>
</table>

NOTE: ↑, >20% decrease compared with baseline; ←, no change; ↓, >20% increase compared with baseline.

<table>
<thead>
<tr>
<th>Unique patient number</th>
<th>G3139 intracellular concentration, pmol/mg protein (µmol/L)</th>
<th>G3139 plasma concentration (µmol/L)</th>
<th>Time point (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>8.25 (0.20)</td>
<td>0.16</td>
<td>72</td>
</tr>
<tr>
<td>18</td>
<td>19.44 (0.47)</td>
<td>0.62</td>
<td>120</td>
</tr>
<tr>
<td>16</td>
<td>3.0 (0.73)</td>
<td>1.32</td>
<td>72</td>
</tr>
<tr>
<td>16</td>
<td>6.3 (0.15)</td>
<td>1.32</td>
<td>120</td>
</tr>
<tr>
<td>8</td>
<td>0.47 (0.11)</td>
<td>0.13</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>8.77 (0.21)</td>
<td>0.14</td>
<td>120</td>
</tr>
<tr>
<td>17</td>
<td>3.18 (0.078)</td>
<td>1.09</td>
<td>72</td>
</tr>
<tr>
<td>17</td>
<td>4.05 (0.10)</td>
<td>1.16</td>
<td>120</td>
</tr>
</tbody>
</table>
5’-end metabolism has not been considered a major pathway (34–36). Preliminary metabolism data in our laboratory (not shown) supported this contention. The design of the capture oligodeoxynucleotide was based on the sequence of G3139 in a way that its 3’ terminal sequence is complementary to the G3139 and the 5’ terminal overhang is complementary to the sequence of the probe oligodeoxynucleotide. The probe oligodeoxynucleotide had a predetermined random sequence with no similar match from BLAST database (http://www.ncbi.nlm.nih.gov/BLAST). The length and sequence of probe oligodeoxynucleotide were not critical in terms of assay specificity or sensitivity, and it is likely that probe oligodeoxynucleotides with different designs might work equally well as long as the sequence of the capture oligodeoxynucleotide is complementary to the probe oligodeoxynucleotide at the 5’ terminal sequence.

The higher sensitivity and selectivity of the ELISA assay permitted monitoring of drug decay for a longer time period and revealed a second slower declined phase with a mean half-life of over 3 hours; however, due to the design of the current protocol, only samples to 4 hours postinfusion were obtained. A better pharmacokinetic characterization for this drug in another protocol with longer sampling time will be obtained. More importantly, the high sensitivity of our assay allowed us to measure intracellular concentrations of G3139. This is a critical point, because assessment of intracellular drug levels could provide us with currently unavailable information on cell uptake and the fate of the antisense, once it has been internalized following in vivo administration. To date, the process through which oligodeoxynucleotide cellular uptake occurs remains to be elucidated, although adsorptive and fluid-phase endocytosis seems involved (37–39). Once internalized, oligodeoxynucleotides are sequestered in the endosomal-lysosomal compartment, and only small proportion of oligodeoxynucleotides could escape from the degradation vesicles and reached the intended targets either in the cytosol or in the nucleus. However, pharmacologic activity of antisense could be limited if insufficient concentrations are attained. Using our sensitive assay, we were able to show that indeed low uptake and no antisense activity occurred in K562 cells exposed to 3.3 μmol/L G3139 in the absence of cationic lipids. This is consistent with previously published data showing that an excess amount of antisense without cationic lipids was needed to achieve the desired target down-regulation (40, 41). In contrast, using cationic lipids (Oligofectamine and DDAB/1-α-dioleyl phosphatidylethanolamine) as delivery vehicles, marked concentration-dependent intracellular G3139 levels were observed with a more effective target down-regulation. The increase in intracellular availability was 10- to 25-fold using Oligofectamine and 20- to 50-fold by DDAB/1-α-dioleyl phosphatidylethanolamine compared with free G3139. This difference may be due to the intrinsic difference in uptake behaviors between noncomplexed G3139 and G3139 complexed with cationic lipids generally considered for antisense oligodeoxynucleotide (42, 43). Alternatively, the lower uptake of noncomplexed G3139 in cells may be due to its high binding with proteins in the cell culture medium. Of note, using FITC-labeled G3139, we showed only a 5-fold increase of cellular uptake of oligodeoxynucleotide by Oligofectamine compared with free antisense. The discrepancy may be related to quenching due to the protein binding of fluorescence-labeled oligonucleotide, a small alteration in uptake behavior of labeled G3139, or difference in methodology.

![Graph showing logarithmic plasma concentration versus time profiles during and after infusion of two different doses of G3139 (4 and 7 mg/kg). Unique patient numbers (UPN) 17 and 18 were given 7 mg/kg G3139 CIVI and the rest 4 mg/kg. Symbols represent the measured concentrations.](image)

Table 6. Relevant pharmacokinetic variables of G3139 in plasma in acute leukemia patients treated on two different doses of G3139 CIVI (4 and 7 mg/kg)

<table>
<thead>
<tr>
<th>Variables (units)/dose</th>
<th>4 mg/kg, mean ± SD or mean (range)</th>
<th>7 mg/kg, mean ± difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(0→t) (μmol/L h)</td>
<td>124.2 ± 32.5</td>
<td>204.1 ± 21.5</td>
</tr>
<tr>
<td>Css (μmol/L)</td>
<td>0.5 ± 0.11</td>
<td>0.88 ± 0.14</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>4.45 ± 0.84</td>
<td>3.97 ± 0.85</td>
</tr>
<tr>
<td>t1/2a (h)</td>
<td>0.3(0.2-0.5)</td>
<td>0.33 ± 0.1</td>
</tr>
<tr>
<td>t1/2b (h)</td>
<td>3.8(1.29-7.90)</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>Vss (L)</td>
<td>6.68 ± 3.88</td>
<td>5.42 ± 1.44</td>
</tr>
</tbody>
</table>

NOTE: All variables were estimated by fitting to a two-compartment method. Five patients at 4 mg/kg G3139 CIVI and two patients at 7 mg/kg were included in the calculation. AUC(0→t), area under the plasma concentration-time curve calculated by linear trapezoid rule; Css, plasma steady-state concentration; CL, total body clearance; Vss, steady-state volume of distribution.
Cationic lipids not only enhanced the rate and amount of G3139 uptake into K562 cells but also might alter the intracellular distribution of G3139 as reported previously. It is widely accepted that cationic liposomes deliver oligodeoxynucleotide into cells through an endocytotic pathway (44) followed by dissociation between the oligodeoxynucleotides and the cationic lipids (45). Here, we showed that the presence of cationic lipids enhanced nuclear accumulation and concentration-dependent down-regulation of Bcl-2 RNA of the antisense in K562 cells. Our results for the intracellular localization of G3139 in K562 cells are consistent with the previous finding in which >70% of the radiolabeled phosphorothioate oligonucleotides were found to be associated with the cytoplasmic fraction, and various nucleuses to cytoplasm ratios ranging from 0.146 to 0.34 were found for different sequences (46). Because the cytoplasmic fraction obtained with hypotonic lysis comprised membranes, cytosol, and endosome/lysosome, except the nuclei, the level of G3139 in cytoplasmic fraction might still be somewhat underestimated. Nevertheless, the distinct accumulation of G3139 in the nuclei by cationic liposome suggests that intranuclear content of G3139 may correlate with our observed concentration-dependent Bcl-2 down-regulation, because RNase H is enriched in the nuclei (47).

Interestingly, whereas in vitro antisense activity required the use of cationic liposomes, G3139 in aqueous saline solution showed pharmacologic activity in vivo as shown by target down-regulation. Thus, it seems that cationic liposomes are not required to achieve adequate intracellular levels of antisense in vivo. In fact, for the first time, we showed that a significant cellular uptake of G3139 occurs in mononuclear cells from patients’ bone marrow and blood mononuclear cell samples collected following 72 to 120 hours of CIVI of the antisense. Of eight patients evaluated, four of six patients who had intracellular drug concentrations >5 pmol/mg proteins showed down-regulation of Bcl-2 mRNA in bone marrow. This result may suggest a threshold concentration for the pharmacologic effect. However, given the small size of the sample population, a larger-sized study needs to be conducted to validate these preliminary results. To our surprise, there was no correlation between plasma steady-state concentration of G3139 and intracellular levels of the drug or Bcl-2 down-regulation. Further, although G3139 was infused in patients in aqueous solution without any delivery vehicle, the cellular uptakes of G3139 in bone marrow or blood samples were significantly higher than those observed in leukemia cell lines treated in vitro in the absence of cationic lipids. These results, therefore, suggest that additional unidentified factors or conditions in vivo might be responsible for an efficient internalization of the antisense into mononuclear cells. Further studies to recognize such factors or conditions are important to optimize G3139 uptake in vivo.

In conclusion, using a novel, highly sensitive hybridization-based ELISA method developed in our laboratory, we have found for the first time evidence that measurable intracellular levels of Bcl-2 antisense G3139 are achievable in vivo in AML patients when a noncomplexed form of the drug was given and that Bcl-2 down-regulation is likely to depend on the achievable intracellular concentration rather than on plasma concentrations.

References


Cellular Uptake and Intracellular Levels of the Bcl-2 Antisense G3139 in Cultured Cells and Treated Patients with Acute Myeloid Leukemia


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/11/8/2998

Cited articles
This article cites 43 articles, 18 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/11/8/2998.full#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/11/8/2998.full#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, use this link:
http://clincancerres.aacrjournals.org/content/11/8/2998.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.