

G3139, a Bcl-2 antisense oligodeoxynucleotide, induces clinical responses in VAD refractory myeloma

NWCJ van de Donk¹, O de Weerd², G Veth², M Eurelings³, E van Stralen¹, SR Frankel⁴, A Hagenbeek³, AC Bloem¹ and HM Lokhorst³

¹Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; ²St Antonius Hospital, Nieuwegein, The Netherlands; ³Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands; and ⁴Genta Incorporated, Berkeley Heights, NJ, USA

Expression of Bcl-2 in multiple myeloma is associated with resistance to chemotherapeutic drugs. Conversely, suppression of Bcl-2 enhanced the chemosensitivity of myeloma cells *in vitro*. G3139 is an antisense oligodeoxynucleotide targeted to the first six codons of the Bcl-2 mRNA open reading frame. In this study, G3139 was delivered as a continuous intravenous infusion for 7 days at a fixed dose of 7 mg/kg/day in combination with VAD (vincristine, adriamycin, and dexamethasone) chemotherapy. In total, 10 heavily pretreated patients with refractory myeloma participated in this trial, including eight patients with VAD refractory disease. The combination of G3139 and VAD was feasible and well tolerated. Seven patients (70%) responded including four patients (40%) with a partial response and three patients (30%) with a minor response. Median progression-free survival was 6 months (range, 2–7+ months) and median overall survival has not been reached. G3139 downregulated Bcl-2 protein levels in peripheral blood circulating myeloma cells, B cells, T cells, and monocytes. These results indicate that G3139 may overcome classical resistance and restore sensitivity of myeloma tumor cells to VAD chemotherapy.

Leukemia (2004) 18, 1078–1084. doi:10.1038/sj.leu.2403363
Published online 15 April 2004

Keywords: multiple myeloma; Bcl-2; antisense oligodeoxynucleotide; chemosensitization; drug resistance

Introduction

Multiple myeloma is characterized by the accumulation of monoclonal plasma cells in the bone marrow. The emergence of drug-resistant disease is the primary cause of treatment failure in myeloma. Well-established mechanisms of drug resistance for myeloma tumor cells *in vitro* are mutations or altered expression of target genes,^{1–4} increased drug metabolism,⁵ changes in drug accumulation,^{6–11} and increased repair of drug-induced damage.¹² Recent studies have demonstrated that defects in apoptotic pathways contribute significantly to resistance of cancer cells to chemotherapeutic drugs.^{13–15} Tumor cells, which are unable to undergo programmed cell death, are therefore resistant to chemotherapeutic agents.

Bcl-2 is a potent enhancer of cell viability through the inhibition of apoptotic death in various cell lines. Bcl-2 is expressed in myeloma cell lines and in myeloma patients' samples despite the absence of molecular evidence of Bcl-2 rearrangements.^{16–22} Bcl-2 inhibits apoptosis by forming inactivating heterodimers with proapoptotic Bcl-2 family proteins, and by preventing the collapse of the mitochondrial transmem-

brane potential, the release of cytochrome c and Smac/DIABLO from mitochondria into the cytosol, and the activation of caspases.^{23–26} Overexpression of Bcl-2 in transgenic mice prolongs the lifespan of plasma cells and in combination with abnormal expression of other oncogenes such as c-Myc, contributes to the development of immature pre-B-cell lymphoma, large-cell lymphoma or plasmacytoma in 40–60% of the mice.^{27,28} This suggests a role for Bcl-2 in the etiology of myeloma. Gene transfer-mediated overexpression of Bcl-2 in myeloma cells has been reported to block the process of chemotherapy-induced apoptosis.^{29,30} In addition, Bcl-2 protein levels increased following exposure of cell lines to doxorubicin, which may account for the relative resistance to a second doxorubicin exposure.²⁹ We and others have shown that specific downregulation of Bcl-2 protein by Bcl-2 antisense oligodeoxynucleotide (ODN) restored chemosensitivity in chemoresistant myeloma cell lines and in primary myeloma cells from patients.^{31–33}

G3139 is an 18-mer fully phosphorothioated antisense ODN targeted to the first six codons of the Bcl-2 mRNA. Preclinical studies showed that G3139 was effectively taken up by myeloma cells, decreased Bcl-2 mRNA levels and protein levels, and sensitized myeloma cells to chemotherapeutic agents including dexamethasone and doxorubicin.^{32,33} Furthermore, G3139 as a single agent has resulted in tumor regression in patients with relapsed non-Hodgkin's lymphoma (NHL)^{34,35} and chronic lymphocytic leukemia.³⁶ Studies in patients with metastatic melanoma,³⁷ chemorefractory small-cell lung cancer,³⁸ metastatic hormone-refractory prostate cancer,³⁹ colorectal cancer,⁴⁰ and in refractory/relapsed acute leukemia⁴¹ showed that G3139 can be administered safely in combination with chemotherapy.⁴² Based upon these data, we initiated a phase 2 study of G3139 in combination with vincristine, adriamycin, and dexamethasone (VAD) in patients with heavily pretreated multiple myeloma including patients with disease resistant to VAD, thalidomide, and other chemotherapy regimens.

Patients, materials and methods

Criteria for enrollment

Patients up to 75 years of age with multiple myeloma refractory to or relapsing after completion of at least two lines of chemotherapy that included VAD chemotherapy and with satisfactory venous access for 7-day infusion were eligible for the study. Criteria for exclusion were World Health Organization (WHO) performance status 4; severe cardiac, pulmonary, neurologic, metabolic, or psychiatric disease; absolute neutrophil count <1000/mm³ and platelets <50000/mm³; need for transfusion support; inadequate liver function; creatinine

Correspondence: Dr HM Lokhorst, Department of Hematology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands; Fax: +31 30 2511893; E-mail: h.lokhorst@azu.nl

Received 11 November 2003; accepted 18 February 2004; Published online 15 April 2004

clearance <40 ml/min; prior malignant disease except non-melanoma skin tumors or stage 0 cervical carcinoma; therapy for myeloma within 3 weeks prior to start G3139 in combination with VAD; active uncontrolled infections; known hypersensitivity to phosphorothioate-containing oligodeoxynucleotides, dexamethasone, or doxorubicin; and use of any investigational therapy within 4 weeks prior to registration. Approval was obtained from the University Medical Center Utrecht institutional review board for these studies (01/227-E). Written informed consent was obtained from all patients before inclusion. The study was performed according to the Helsinki agreement.

Study protocol

G3139 (Genasense™, oblimersen sodium; Genta Inc., Berkeley Heights, NJ, USA; 5'-TCT CCC AGC GTG CGC CAT-3') was delivered as a continuous intravenous infusion using a portable pump on days 1–7 at a dose of 7 mg/kg/day. Infusion sites were changed when early signs of inflammation were observed. VAD was started at day 4 of the G3139 infusion. The VAD regimen consisted of daily doses of 0.4 mg vincristine and 9 mg/m² doxorubicin on days 4–7. G3139 infusion was temporarily interrupted when doxorubicin and vincristine were administered by rapid intravenous infusion⁴³ through the same line. Dexamethasone (40 mg) was given orally on days 4–7 on even cycles and on days 4–7, 12–15, and 20–23 on odd cycles of VAD. The treatment cycles were repeated at 4-week intervals. Three courses of treatment were planned per patient, but additional courses could be administered in the case of response. Antibacterial and antifungal prophylaxis was given according to the local guidelines. Adverse events were graded according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTC) version 2.0 (<http://ctep.info.nih.gov>).

Evaluation of response

Response evaluation was performed according to the Blade criteria.⁴⁴ Partial response (PR) was defined as 50% or more reduction of monoclonal immunoglobulins (M-protein) in serum and/or urine or more than 50% reduction of bone marrow infiltration in nonsecretory myeloma. Minor response (MR) was defined as 25–50% reduction of M-protein in serum and/or urine or 25–50% reduction of bone marrow infiltration in nonsecretory myeloma. Complete response (CR) was defined as no M-protein measurable in serum and/or 10 times concentrated urine by immunofixation analysis and less than 5% plasma cells that had to be polyclonal by immunofluorescence staining. Patients with a reduction of less than 25% of M-protein in serum and/or urine or of bone marrow infiltration were considered to be refractory. Relapse from CR was defined as recurrence of M-protein in serum and/or urine measured by immunofixation on at least two occasions. Progression from PR or MR was defined as an increase in serum and/or urine M-protein levels or bone marrow infiltration by more than 25% on two consecutive measurements or any increase of M-protein in the presence of clinical evidence of disease progression.

Immuno-FISH analysis

To detect a (partial) deletion of chromosome 13, FISH analysis using the commercially available SpectrumOrange-conjugated

probe LSI13 (Rb1) (Vysis, Downers Grove, IL, USA) was performed according to the manufacturer's guidelines with minor modifications and combined with immunological staining of the cytoplasmatic Ig light chains to positively identify plasma cells as described previously.⁴⁵ Signals of the probe were detected by standard procedures. We intended to analyze a total of 100 plasma cells in each patient. A patient was considered as having deletion of chromosome 13 when the percentage of plasma cells with a deletion exceeded 20%.

Quantification of Bcl-2 by flow cytometry

Bcl-2 protein levels were determined in myeloma plasma cells (CD38⁺CD138⁺CD45[±]) in bone marrow prior to start of G3139 treatment and in peripheral blood circulating myeloma cells (CD38⁺, CD138⁺, CD45[±]), CD3⁺ cells, CD19⁺ cells, and CD14⁺ cells at days 0 and 4 of the treatment cycle by analysis of at least 5000 positively gated cells by flow cytometry. However, in circulating myeloma cells Bcl-2 protein levels were determined by analysis of 1500 positively gated cells, but when the percentage of circulating myeloma cells was low at least 300 cells were analyzed. Peripheral blood or bone marrow mononuclear cells, which were obtained by Ficoll-Paque (Amersham; Pharmacia Biotech AB, Uppsala, Sweden) density centrifugation, (1×10^6) were fixed and permeabilized using the FACS Lysing Solution (FLS, 10% in aqua destillata; Becton Dickinson, Erembodegem, Belgium (BDIS)) and subsequently incubated with anti-Bcl-2-FITC (IgG1, Dako, Glostrup, Denmark) antibody or the isotype- and subclass-matched control antibody. All incubations were performed at room temperature for 30 min. Cells were then incubated with mouse serum for 15 min at room temperature, and subsequently incubated with CD3-PE (BDIS), CD14-APC (BDIS), and CD19-Percp Cy5.5 (BDIS) or with CD38-APC (BDIS), CD138-PE (Immunotech, Marseille, France), and CD45-PERCP (BDIS) for 15 min at room temperature, and analyzed on a FACSCalibur (BDIS). Between different steps the cells were washed with PBS. The mean fluorescence ratio (MFR), defined as the ratio of the mean fluorescence intensities (MFI) of primary antibody and isotype control stained cells was used as a measure for Bcl-2 expression.

Flow cytometric analysis of lymphocyte subsets

Lymphocyte populations were analyzed using four-color flow cytometry at days 0, 4, and 7 of each treatment cycle. Peripheral blood, collected in sodium heparin, was stained at room temperature with a panel of antibodies, lysed using FACS Lysing Solution (BDIS), washed with PBS, and analyzed on a FACSCalibur (BDIS). The following antibody combinations were used to analyze the lymphocyte populations: CD3, CD16 + 56, CD45, CD19, and CD8, CD4, CD45, CD3. CD3, CD4, CD8, CD19, and CD45 were obtained from BDIS and CD16 + 56 was derived from Immunotech. B cells were defined as CD3⁻CD19⁺, natural killer cells as CD3⁻CD16⁺/CD56⁺, CD8 cells as CD3⁺CD8⁺, and CD4 cells as CD3⁺CD4⁺. Absolute counts of a certain lymphocyte population were calculated by multiplying the total white blood cell (WBC) count by the frequency of that population in the lymphocyte gate and by the fraction of total WBCs included in the lymphocyte gate.

Statistical analysis

Data analysis was performed using the SPSS statistical software package (SPSS, Chicago, IL, USA). Correlations were calculated

by using the Spearman test. Correlations were considered significant when $P < 0.05$.

Results

Patient characteristics

In total, 10 patients were enrolled in this study. Clinical characteristics are shown in Table 1. There were five male and five female patients. Median age was 56 years, with a range of 39–64 years. All patients were heavily pretreated and had received a median of four previous chemotherapy regimens (range, 2–6) (Tables 1 and 2). Eight patients (80%) had VAD refractory disease (Table 2). Deletion of chromosome 13 (del13) was present in seven out of nine evaluable patients (77.8%). The percentage of Ki-67-positive myeloma cells ranged from 0 to 70% (median 2%). In nine patients, Bcl-2 was determined in

bone marrow myeloma cells by flow cytometry prior to start of therapy. No material for analysis was available from patient 2. Bcl-2 protein levels, expressed as mean fluorescence ratio, varied among patients (median 12.2; range: 2.4–27.4).

Treatment

A total of 29 cycles of G3139 and VAD have been administered (Table 1). Five patients received only two cycles: three patients because they were refractory, one responsive patient because of progression just before the third course, and one patient because of pulmonary aspergillosis (patient 10). Three cycles were administered to three patients. Two patients received four and six treatment cycles, respectively.

Disease response

Seven patients (70%) responded to the combination of G3139 and VAD, including four patients (40%) with a partial response and three (30%) with a minor response (Table 2). Five of the seven responders had VAD refractory disease, including three patients who sequentially received VAD alone followed by the combination of G3139 and VAD (patients 5, 9, and 10) (Table 2). In addition, three of the seven responding patients had thalidomide/dexamethasone refractory disease and one had thalidomide refractory disease. Patient 2 had VAD and thalidomide/dexamethasone refractory disease and obtained only a minor response after allogeneic stem cell transplantation, but achieved a partial response on the current protocol (Table 2). Three patients did not respond. Del13 was present in five out of six responding and in two out of three nonresponding patients. No obvious differences were observed in the Ki-67 growth fraction and Bcl-2 protein levels of myeloma cells between responding and nonresponding patients. Among the responding patients, five had anemia at baseline. Hemoglobin levels increased in three patients, decreased in one patient, and remained unchanged in one patient. Responses were associated with increases in Karnofsky's performance-status scores in four (57%) patients and improvement of pain in seven (100%) patients. Four out of seven responding patients relapsed, whereas response persists in three patients. Median progression-free survival of these patients was 6 months (range, 2 to more than 7 months) without any further therapy before relapse

Table 1 Clinical characteristics

Characteristic	
Total number of patients enrolled	10
Gender	
Male	5
Female	5
Age (years)	
Median	56
Range	39–64
Deletion of chromosome 13 (%) ^a	77.8
Ki-67 (%)	
Median	2
Range	0–70
Number of prior chemotherapy regimens	
2	1
3	1
4	4
5	2
6	2
Number of cycles completed	
2	5
3	3
4	1
6	1

^aData available for nine patients (90%).

Table 2 Previous treatment and outcome of G3139 combined with VAD

Patient	Cycles	Previous therapy and outcome of G3139+VAD therapy	PFS	OS
1	2	VAD PR – IDM refr – VAD refr – CVP refr – Thal+dexa refr – G3139+VAD refr		6
2	3	VAD refr – Thal+dexa refr – alloSCT MR – G3139+VAD PR	6	16+
3	6	VAD refr – IDM refr – autoSCT PR – Thal+dexa refr – G3139+VAD PR	7	15+
4	2	VAD PR – IDM PR – autoSCT PR – Thal+dexa refr – G3139+VAD MR	2	3
5	3	VAD refr – IDM PR – autoSCT CR – VAD refr – G3139+VAD PR	6	11+
6	2	VAD refr – IDM PR – TAD PR – autoSCT PR – Thal+dexa refr – G3139+VAD refr		9+
7	4	VAD PR – IDM PR – autoSCT CR – VAD PR – autoSCT PR – Thal refr – G3139+VAD PR	7+	7+
8	2	VAD PR – IDM CR – VAD refr – Thal+dexa refr – G3139+VAD refr		3
9	3	VAD PR – IDM PR – alloSCT PR – TAD PR – DLI refr – VAD refr – G3139+VAD MR	3+	3+
10	2	MP refr – VAD refr – G3139+VAD MR	3+	3+

Cycles indicates the total number of cycles of G3139 in combination with VAD, which was administered; VAD, vincristine, doxorubicin, and dexamethasone; IDM, intermediate-dose melphalan; CVP, cyclophosphamide, vincristine, and prednisone; Thal, thalidomide; dexa, dexamethasone; alloSCT, allogeneic stem cell transplantation; autoSCT, autologous stem cell transplantation; TAD, thalidomide, doxorubicin, and dexamethasone; DLI, donor lymphocyte infusion; MP, melphalan and prednisone; MR, minor response; PR, partial response; CR, complete response; refr, refractory; PFS, progression-free survival in months; OS, overall survival in months.

occurred. The median overall survival for all patients to date exceeds 12 months (range, 3 to more than 16 months). Three patients have died of progressive disease (Table 2).

Toxicity

At the start of treatment, anemia was present in eight patients (six grade 1, and two grade 2), leucopenia was present in four (one grade 1, three grade 2), neutropenia in four (one grade 1, two grade 2, one grade 3), lymphopenia in two (one grade 1, one grade 2), and grade 1 thrombocytopenia in two patients. Hematological toxicity during the first cycle and during all cycles is shown in Table 3.

Nonhematological adverse events are shown in Table 4. There were no grade 4 nonhematological adverse events. The most common events were fatigue in five patients (grade 1 in three patients, and grade 2 in two patients). Nine patients had a grade 1 local inflammatory reaction at some time during treatment around the peripheral infusion site. One patient (patient 5) developed skin and subcutaneous tissue necrosis (grade 3) during administration of G3139 before start of chemotherapy. This was likely the consequence of extravasation of G3139 and the patient failed to seek medical attention despite symptoms for more than 24 h. The local skin inflammation around the infusion site could be prevented if the infusion site was changed every 2 or 3 days in all patients. Furthermore, three patients (5, 9, 10) underwent placement of a central venous catheter. One of these patients (patient 5) developed a catheter-related venous thrombosis and patient 10 had a catheter-related infection. Patients 4 and 10 developed grade 3 infections.

Table 3 Hematological toxicity in patients treated with G3139 and VAD

No. of patients with event of common toxicity criteria during first cycle (all cycles; n = 29)

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Leucopenia	0 (6)	1 (4)	1 (2)	1 (3)
Neutropenia	0 (3)	1 (1)	1 (3)	1 (3)
Lymphopenia	1 (2)	1 (1)	3 (8)	1 (2)
Thrombocytopenia	3 (7)	1 (1)	0 (4)	1 (2)
Anemia	7 (17)	2 (6)	0 (1)	0 (0)

Table 4 Nonhematological toxicity in patients treated with G3139 and VAD

No. of patients with event of common toxicity criteria during first cycle (all cycles; n = 29)

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Fatigue	3 (13)	2 (4)	0 (0)	0 (0)
Thrombosis	0 (0)	0 (0)	0 (1)	0 (0)
Catheter-related infection	0 (0)	0 (0)	0 (1)	0 (0)
Infection	0 (0)	0 (0)	0 (2)	0 (0)
Renal	2 (5)	0 (0)	0 (0)	0 (0)
Injection site reaction	9 (23)	0 (0)	1 (1)	0 (0)

Effect of treatment on Bcl-2 protein levels in peripheral blood circulating myeloma cells, B cells, T cells, and monocytes

In peripheral blood from patients 3, 4, 5, 6, 9, and 10 more than 0.01% circulating myeloma cells were detected (range: 0.01–4.0%), enabling analysis of Bcl-2 protein levels in these cells. In the other patients, the level of peripheral blood infiltration was too low, to reliably quantify Bcl-2 protein expression. Bcl-2 protein expression was determined by flow cytometry at day 0 before treatment and at day 4 of G3139 infusion prior to initiation of VAD. After 4 days of G3139 treatment, Bcl-2 protein levels in circulating myeloma cells were reduced in four out of six patients (median change: –13.3%) (Table 5). Bcl-2 protein expression was also determined by flow cytometry in peripheral blood T cells (CD3⁺), B cells (CD19⁺), and monocytes (CD14⁺). This analysis was performed during cycle 1 (patients 1, 2, 6, 7, 9, and 10) or cycle 2 (patients 3, 5, and 8). Treatment for 4 days with G3139 resulted in a reduction of Bcl-2 protein levels in B cells in nine out of nine patients when compared with baseline values (median change: –25.0%). In monocytes, Bcl-2 protein levels were reduced in eight out nine patients on day 4 (median change: –21.8%). Bcl-2 protein levels were reduced in T cells in six out of nine patients on day 4 (median change: –16.4%) (Table 5).

Effect of treatment on circulating leucocytes and thrombocytes

In eight patients, the effect of treatment on numbers of circulating lymphocytes, monocytes, granulocytes, and thrombocytes in peripheral blood was determined on days 0, 4, 7, and 28 of cycle 1 (patients 2, 4, 6, 7, 9, and 10) or cycle 2 (patients 3 and 8) (Table 6). The largest effect was observed in B cells, which showed mean reductions of 50.9% on day 4 before start of VAD and 36.5% on day 7, when compared with baseline numbers. There was a minor reduction of T cells on day 4 and a more profound reduction on day 7. This was due to a decrease of both CD4⁺ and CD8⁺ T cells. Natural killer (NK) cells either increased (n=5) or decreased (n=3) after 4 days of G3139 treatment. However, on day 7 NK cells were reduced in all patients. Both on days 4 and 7, a reduction in monocytes was observed. G3139 did not affect granulocytes on day 4, but on day 7 there was a significant increase in the number of granulocytes, which was probably related to administration of dexamethasone. Thrombocytes were reduced on days 4 and 7. T cells (CD4 and CD8) and monocytes returned to baseline or near-baseline values on day 28 prior to start of the following

Table 5 Effect of G3139 on Bcl-2 protein levels at day 4 (presented as the change from baseline (day 0) in %)

Patient	B cells	T cells	Monocytes	Myeloma cells
1	–16.3	13.8	–21.8	
2	–3.6	–5.1	–11.1	
3	–62.8	–19.3	–8.3	5.5
4				1.9
5	–27.6	–16.4	–22.2	–19.7
6	–25.0	–25.1	–25.0	–25.6
7	–5.3	2.0	–19.5	
8	–14.8	15.5	15.7	
9	–33.6	–18.0	–27.7	–26.4
10	–37.4	–19.3	–47.3	–6.9

Table 6 Effect of G3139 combined with VAD on numbers of circulating leucocytes and thrombocytes (presented as the change from baseline in %)

Cell type	Day 4		Day 7		Day 28	
	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)
B cells	-50.9	-90 to -1.1	-36.5	-95.8 to 3.4	-32.8	-88.8 to 60.0
T cells	-20.4	-78.5 to 54.1	-43.4	-78.9 to 36.8	-7.5	-55.3 to 63.2
CD4 ⁺ T cells	-11.6	-65.3 to 66.1	-45.4	-84.2 to 31.3	-1.1	-59.8 to 107.1
CD8 ⁺ T cells	-21.5	-95.9 to 49.6	-37.4	-71.6 to 47.6	5.4	-54.2 to 71.2
NK cells	3.3	-51.3 to 54.4	-40.3	-62.5 to -13.2	22.8	-65.0 to 100.0
Granulocytes	-5.3	-50.0 to 26.4	172.5	4.1 to 293.1	55.7	0.0 to 122.4
Monocytes	-27.1	-67.7 to 72.2	-28.9	-45.2 to -12.3	6.3	-20.9 to 43.1
Thrombocytes	-14.9	-26.8 to 2.6	-20.5	-38.9 to 3.6	-19.3	-75.9 to 1.6

course of G3139 and VAD. However, B cells and thrombocytes did not fully recover, and NK cells and granulocytes were increased on day 28. Although not significant, there was a trend for correlation between Bcl-2 protein reduction in B or T cells and decrease in circulating B cells (day 4: $R=0.643$; $P=0.119$, day 7: $R=0.657$; $P=0.156$) or T cells (day 4: $R=0.739$; $P=0.058$, day 7: $R=0.771$; $P=0.072$). No association was observed between Bcl-2 downregulation in monocytes and reduction of monocytes.

Discussion

We evaluated the effect of Bcl-2 antisense in combination with VAD chemotherapy in heavily pretreated multiple myeloma patients who failed previous therapy. Most patients had additional unfavorable prognostic factors like deletion of chromosome 13 or a high Ki-67 growth fraction. G3139 was administered as a continuous intravenous infusion from day 1 to day 7 at a dose of 7 mg/kg/day. VAD was started at day 4 of the G3139 infusion. The combination and sequential application of G3139 and VAD was based on our preclinical data that showed that the maximum decrease in Bcl-2 protein expression occurred after 4 days of treatment with G3139, which, although did not directly induce apoptosis, significantly enhanced sensitivity of myeloma tumor cells to chemotherapeutic agents.³² In this study, seven of the 10 patients (70%) responded including four patients (40%) with a partial response and three (30%) with a minor response. Five of the seven responders had VAD refractory disease, including three patients who sequentially received VAD followed by the combination of G3139 and VAD. Responses were associated with increased hemoglobin levels and performance status, and improvements in disease symptoms including pain. These data indicate that Bcl-2 antisense may overcome classical drug resistance and restore the sensitivity of myeloma tumor cells to VAD chemotherapy. Median progression-free survival was 6 months, which is usual for this category of patients,^{46,47} and the median overall survival has not been reached. A larger group of patients followed prospectively will be needed to further establish the possible benefit of introducing Bcl-2 antisense in myeloma treatment.

G3139 and VAD could be administered safely in heavily pretreated myeloma patients. The safety profile is similar to experiences reported in patients with refractory/relapsed acute leukemia,⁴¹ metastatic melanoma,³⁷ chemorefractory small-cell lung cancer,³⁸ or metastatic hormone-refractory prostate cancer³⁹ treated with G3139 and different cytotoxic agents. The combination of G3139 with chemotherapy was feasible and the toxicity pattern was not substantially different from that

observed with the chemotherapy drugs alone. In the current study, the only unexpected event was a local inflammatory reaction around peripheral intravenous infusion sites. Particular attention needs to be paid to monitoring the intravenous sites if patients are receiving G3139 and doxorubicin in peripheral veins. Hematological toxicity may be primarily attributable to VAD chemotherapy and was predominantly observed in patients with cytopenias at baseline. Patient 8 developed grade 2 anemia, grade 3 leucopenia, neutropenia, and thrombocytopenia, and grade 4 lymphopenia, which were probably due to advanced infiltration of bone marrow. Reduction of absolute numbers of platelets and fatigue were observed during G3139 infusions that precede VAD chemotherapy and are adverse events that were also reported in trials evaluating other phosphorothioate oligodeoxynucleotides,^{48,49} suggesting that these effects resulted from nonsequence specific effects and can be attributed to the phosphorothioate backbone of the oligodeoxynucleotide molecule.

G3139 treatment for 4 days moderately reduced Bcl-2 protein levels in peripheral blood circulating myeloma cells, B cells, T cells, and monocytes in the majority of the patients. Similar moderate reductions of Bcl-2 were found in other studies evaluating G3139.^{35,37,41} However, although the number of patients is small, there seems to be no correlation between Bcl-2 protein downregulation and response to G3139 in combination with VAD. G3139 administration alone preceding chemotherapy resulted in a transient reduction of absolute numbers of circulating lymphocytes, monocytes, and thrombocytes, but did not alter the number of circulating neutrophils or NK cells. We further show for the first time that the decline in circulating lymphocytes is due to reduction of B cells and to a lesser extent of T cells (CD4 and CD8). After completion of the treatment course, there was a full recovery of the number of circulating T cells and monocytes and a partial recovery of platelets and B cells. This is in agreement with studies indicating that Bcl-2 is not essential for survival of pluripotent hematopoietic stem cells,⁵⁰ and therefore Bcl-2 downregulation by G3139 treatment will not result in a loss of renewal potential for normal hematopoietic cells. The transient reduction of B cells and T cells by G3139 is likely related to a sequence-specific Bcl-2 downregulation in these cells. First, survival of mature B and T (CD4 and CD8) cells has been shown to be critically dependent on Bcl-2 protein expression.⁵⁰ Furthermore, there was a trend towards a correlation between downregulation of Bcl-2 protein in B and T cells and decline of circulating lymphocytes. Moreover, although lymphopenia was also reported in other studies evaluating G3139,^{35,37,39} there have been no reports of lymphopenia in clinical trials of other phosphorothioate antisense molecules developed to inhibit other targets.^{48,49} This

decrease in B and T cells together with the responses observed in refractory myeloma patients suggests that the Bcl-2 protein reduction induced by G3139 treatment was functionally relevant.

In conclusion, G3139 induced significant clinical responses when combined with VAD chemotherapy in patients with (VAD) refractory disease. The combination of Bcl-2 antisense and VAD chemotherapy was feasible and well tolerated. The results of this trial warrant exploration of Bcl-2 antisense in earlier-stage disease.

Acknowledgements

This study was financially supported by a grant from the Dutch Cancer Society (KWF). One of the authors (S.R.F.) is employed by a company (Genta) whose product was studied in the present work.

References

- Moalli PA, Pillay S, Weiner D, Leikin R, Rosen ST. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor mRNA. *Blood* 1992; **79**: 213–222.
- Ishikawa H, Kawano MM, Okada K, Tanaka H, Tanabe O, Sakai A *et al*. Expressions of DNA topoisomerase I and II gene and the genes possibly related to drug resistance in human myeloma cells. *Br J Haematol* 1993; **83**: 68–74.
- Krett NL, Pillay S, Moalli PA, Greipp PR, Rosen ST. A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. *Cancer Res* 1995; **55**: 2727–2729.
- Valkov NI, Sullivan DM. Drug resistance to DNA topoisomerase I and II inhibitors in human leukemia, lymphoma, and multiple myeloma. *Semin Hematol* 1997; **34** (4 Suppl 5): 48–62.
- Linsenmeyer ME, Jefferson S, Wolf M, Matthews JP, Board PG, Woodcock DM. Levels of expression of the *mdr1* gene and glutathione *S*-transferase genes 2 and 3 and response to chemotherapy in multiple myeloma. *Br J Cancer* 1992; **65**: 471–475.
- Dalton WS, Grogan TM, Meltzer PS, Scheper RJ, Durie BG, Taylor CW *et al*. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol* 1989; **7**: 415–424.
- Epstein J, Xiao HQ, Oba BK. P-glycoprotein expression in plasmacytoma myeloma is associated with resistance to VAD. *Blood* 1989; **74**: 913–917.
- Sonneveld P, Durie BG, Lokhorst HM, Marie JP, Solbu G, Suciu S *et al*. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON. *Lancet* 1992; **340**: 255–259.
- Grogan TM, Spier CM, Salmon SE, Matzner M, Rybski J, Weinstein RS *et al*. P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. *Blood* 1993; **81**: 490–495.
- Raaijmakers HG, Izquierdo MA, Lokhorst HM, de Leeuw C, Belien JA, Bloem AC *et al*. Lung-resistance-related protein expression is a negative predictive factor for response to conventional low but not to intensified dose alkylating chemotherapy in multiple myeloma. *Blood* 1998; **91**: 1029–1036.
- Filipits M, Drach J, Pohl G, Schuster J, Stranzl T, Ackermann J *et al*. Expression of the lung resistance protein predicts poor outcome in patients with multiple myeloma. *Clin Cancer Res* 1999; **5**: 2426–2430.
- Spanswick VJ, Craddock C, Sekhar M, Mahendra P, Shankaranarayana P, Hughes RG *et al*. Repair of DNA interstrand crosslinks as a mechanism of clinical resistance to melphalan in multiple myeloma. *Blood* 2002; **100**: 224–229.
- Solary E, Droin N, Betteieb A, Corcos L, Dimanche-Boitrel MT, Garrido C. Positive and negative regulation of apoptotic pathways by cytotoxic agents in hematological malignancies. *Leukemia* 2000; **14**: 1833–1849.
- O'Gorman DM, Cotter TG. Molecular signals in anti-apoptotic survival pathways. *Leukemia* 2001; **15**: 21–34.
- Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002; **108**: 153–164.
- Hamilton MS, Barker HF, Ball J, Drew M, Abbot SD, Franklin IM. Normal and neoplastic human plasma cells express bcl-2 antigen. *Leukemia* 1991; **5**: 768–771.
- Pettersson M, Jernberg-Wiklund H, Larsson LG, Sundstrom C, Givol I, Tsujimoto Y *et al*. Expression of the bcl-2 gene in human multiple myeloma cell lines and normal plasma cells. *Blood* 1992; **79**: 495–502.
- Sangfelt O, Osterborg A, Grander D, Anderbring E, Ost A, Mellstedt H *et al*. Response to interferon therapy in patients with multiple myeloma correlates with expression of the Bcl-2 oncoprotein. *Int J Cancer* 1995; **63**: 190–192.
- Ong F, van Nieuwkoop JA, Groot-Swings GM, Hermans J, Harvey MS, Kluin PM *et al*. Bcl-2 protein expression is not related to short survival in multiple myeloma. *Leukemia* 1995; **9**: 1282–1284.
- Harada N, Hata H, Yoshida M, Soniki T, Nagasaki A, Kuribayashi N *et al*. Expression of Bcl-2 family of proteins in fresh myeloma cells. *Leukemia* 1998; **12**: 1817–1820.
- Puthier D, Pellat-Deceunynck C, Barille S, Robillard N, Rapp MJ, Juge-Morineau N *et al*. Differential expression of Bcl-2 in human plasma cell disorders according to proliferation status and malignancy. *Leukemia* 1999; **13**: 289–294.
- Renner S, Weisz J, Krajewski S, Krajewska M, Reed JC, Lichtenstein A. Expression of BAX in plasma cell dyscrasias. *Clin Cancer Res* 2000; **6**: 2371–2380.
- Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999; **13**: 1899–1911.
- Kroemer G, Reed JC. Mitochondrial control of cell death. *Nat Med* 2000; **6**: 513–519.
- Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *EMBO J* 2001; **20**: 6627–6636.
- Debatin KM, Poncet D, Kroemer G. Chemotherapy: targeting the mitochondrial cell death pathway. *Oncogene* 2002; **21**: 8786–8803.
- McDonnell TJ, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the *t(14; 18)*. *Nature* 1991; **349**: 254–256.
- Strasser A, Harris AW, Cory S. E *mu*-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. *Oncogene* 1993; **8**: 1–9.
- Tu Y, Xu FH, Liu J, Vescio R, Berenson J, Fady C, Lichtenstein A. Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. *Blood* 1996; **88**: 1805–1812.
- Gazit Y, Fey V, Thomas C, Alvarez R. Bcl-2 overexpression is associated with resistance to dexamethasone, but not melphalan, in multiple myeloma cells. *Int J Oncol* 1998; **13**: 397–405.
- Derenne S, Monia B, Dean NM, Taylor JK, Rapp MJ, Harousseau JL *et al*. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood* 2002; **100**: 194–199.
- van de Donk NW, Kamphuis MM, Van Dijk M, Borst HP, Bloem AC, Lokhorst HM. Chemosensitization of myeloma plasma cells by an antisense-mediated downregulation of Bcl-2 protein. *Leukemia* 2003; **17**: 211–219.
- Liu Q, Gazit Y. Potentiation of dexamethasone-, paclitaxel-, and Ad-p53-induced apoptosis by Bcl-2 antisense oligodeoxynucleotides in drug-resistant multiple myeloma cells. *Blood* 2003; **101**: 4105–4114.
- Webb A, Cunningham D, Cotter F, Clarke PA, di Stefano F, Ross P *et al*. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet* 1997; **349**: 1137–1141.
- Waters JS, Webb A, Cunningham D, Clarke PA, Raynaud F, di Stefano F *et al*. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 2000; **18**: 1812–1823.
- Rai KR, O'Brien S, Cunningham C, Turkin AG, Ochoa L, Frankel SR. Genasense (Bcl-2 Antisense) monotherapy in patients with

- relapsed or refractory chronic lymphocytic leukemia: Phase 1 and 2 results. *Blood* 2002; **100**: 384a.
- 37 Jansen B, Wacheck V, Heere-Ress E, Schlagbauer-Wadl H, Hoeller C, Lucas T *et al*. Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet* 2000; **356**: 1728–1733.
 - 38 Rudin CM, Otterson GA, Mauer AM, Villalona-Calero MA, Tomek R, Prange B *et al*. A pilot trial of G3139, a bcl-2 antisense oligonucleotide, and paclitaxel in patients with chemorefractory small-cell lung cancer. *Ann Oncol* 2002; **13**: 539–545.
 - 39 Chi KN, Gleave ME, Klasa R, Murray N, Bryce C, Lopes de Menezes DE *et al*. A phase I dose-finding study of combined treatment with an antisense Bcl-2 oligonucleotide (Genasense) and mitoxantrone in patients with metastatic hormone-refractory prostate cancer. *Clin Cancer Res* 2001; **7**: 3920–3927.
 - 40 Ochoa L, Kuhn J, Salinas R, Hammond L, Hao D, Denis L. A phase I, pharmacokinetic, and biologic correlative study of G3139 and irinotecan (CPT-11) in patients with metastatic colorectal cancer. *Proc Am Soc Clin Oncol* 2001; **20**: 75a.
 - 41 Marcucci G, Byrd JC, Dai G, Klisovic MI, Kourlas PJ, Young DC *et al*. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. *Blood* 2003; **101**: 425–432.
 - 42 Klasa RJ, Gillum AM, Klem RE, Frankel SR. Oblimersen Bcl-2 antisense: facilitating apoptosis in anticancer treatment. *Antisense Nucleic Acid Drug Dev* 2002; **12**: 193–213.
 - 43 Segeren CM, Sonneveld P, van der HB, Baars JW, Biesma DH, Corneliussen JJ *et al*. Vincristine, doxorubicin and dexamethasone (VAD) administered as rapid intravenous infusion for first-line treatment in untreated multiple myeloma. *Br J Haematol* 1999; **105**: 127–130.
 - 44 Blade J, Samson D, Reece D, Apperley J, Bjorkstrand B, Gahrton G *et al*. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. *Br J Haematol* 1998; **102**: 1115–1123.
 - 45 Boersma-Vreugdenhil GR, Peeters T, Bast BJ. Translocation of the IgH locus is nearly ubiquitous in multiple myeloma as detected by immuno-FISH. *Blood* 2003; **101**: 1653.
 - 46 Blade J, Esteve J. Treatment approaches for relapsing and refractory multiple myeloma. *Acta Oncol* 2000; **39**: 843–847.
 - 47 Richardson PG, Schlossman RL, Weller E, Hideshima T, Mitsiades C, Davies F *et al*. Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. *Blood* 2002; **100**: 3063–3067.
 - 48 Bishop MR, Iversen PL, Bayever E, Sharp JG, Greiner TC, Copple BL *et al*. Phase I trial of an antisense oligonucleotide OL(1)p53 in hematologic malignancies. *J Clin Oncol* 1996; **14**: 1320–1326.
 - 49 Glover JM, Leeds JM, Mant TG, Amin D, Kisner DL, Zuckerman JE *et al*. Phase I safety and pharmacokinetic profile of an intercellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302). *J Pharmacol Exp Ther* 1997; **282**: 1173–1180.
 - 50 Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 1993; **75**: 229–240.