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TITLE: Predictive Biomarkers of Response to Bcl-2 Biomodulation
         by G3139 and Docetaxel in Hormone-Refractory Prostate
         Cancer

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13. ABSTRACT (Maximum 200 Words)
Background: The specific aims of this grant are to demonstrate (1) that *bcl-2* overexpression in prostate cancer specimens is a predictive biomarker for enhanced responsiveness to G3139 (oblimersen), and antisense oligonucleotide targeting Bcl-2, and docetaxel; (2) that the degree of *bcl-2* downregulation in normal tissue surrogate (peripheral blood mononuclear cells [MNC]) will predict prostate cancer responsiveness to oblimersen and docetaxel; and (3) whether the pharmacokinetic parameters of oblimersen and docetaxel are predictive of *bcl-2* biomodulation and antitumor activity, respectively. **Results:** Oblimersen steady-state concentrations are a predictive determinant of PSA response to the combination of oblimersen and docetaxel in patients with hormone-refractory prostate cancer. Although the majority of patients had marked decrements in Bcl-2 protein expression in MNCs following treatment with oblimersen, there was no relationship between the decrement in Bcl-2 expression in MNC and response to therapy or Oblimersen Css. Bcl-2, Bax and Bcl-X$_L$ expression in the patient's original tumor block specimens was not predictive of response to therapy with oblimersen and docetaxel. **Conclusions:** oblimersen Css is a significant predictor of PSA response to therapy with this combination. Oblimersen at the current recommended dose of 7 mg/kg/day in solid tumor studies may provide inadequate Css for a significant proportion of patients treated that may lead to suboptimal effectiveness in some clinical studies.

14. SUBJECT TERMS
Biomarkers, Bcl-2, Bax, Apoptosis, Pharmacokinetics, Clinical Research, and Hormone Refractory Prostate Cancer

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Introduction:

The central hypothesis of this grant is that *bcl-2* protein overexpression confers intrinsic resistance to chemotherapy in patients with hormone-refractory prostate cancer (HRPC), therefore downregulation of *bcl-2* protein by G3139 will enhance the antitumor activity of docetaxel in patients HRPC. We further hypothesized and the focus of the current grant application is that patients whose tumors utilize *bcl-2* overexpression as a mechanism to escape the apoptotic events of chemotherapy will benefit from G3139 biomodulation, whereas tumors that exhibit other mechanisms for impaired apoptosis, such as diminished Bax expression, will fail to respond or fail to have a durable response. The specific aims of this project are to demonstrate (1) that *bcl-2* overexpression in prostate cancer specimens is a predictive biomarker for enhanced responsiveness to G3139 and docetaxel therapy; (2) that the degree of *bcl-2* downregulation in normal tissue surrogate (peripheral blood mononuclear cells) will predict prostate cancer responsiveness to G3139 and docetaxel; and (3) whether the pharmacokinetic parameters of G3139 and docetaxel are predictive of *bcl-2* biomodulation and antitumor activity, respectively.

Objectives:

Specific Aim 1: Demonstrate that *bcl*-2 overexpression in prostate cancer specimens is a predictive biomarker of enhanced responsiveness to G3139 and docetaxel therapy.

Specific Aim 2: Demonstrate that the degree of *bcl*-2 downregulation in normal tissue surrogate (peripheral blood mononuclear cells) will predict for the responsiveness of prostate cancer to G3139 and docetaxel.

Specific Aim 3: Determine whether the pharmacokinetic parameters of G3139 and docetaxel are predictive of *bcl*-2 biomodulation and antitumor activity, respectively.

Body:

Significant Accomplishments Year 3:

1. Immunohistochemical detection of protein expression for selected members of the Bcl-2 family (Bcl-2, Bax, Bcl-X$_L$) completed on the 28 patients.
2. Examined the expression of these biomarkers and clinical outcome.

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3. Analyze and determine the relationship of these protein biomarkers as well as pharmacokinetic markers and response to Bcl-2 biomodulation by oblimersen and docetaxel.

Reportable Outcomes - Final Report (1-36 months):

The long-term goal of this grant proposal is to identify the biomarkers and pharmacologic markers that will predict which HRPC patients will respond and attain the most benefit from this mechanism-based therapeutic strategy. This proposal addresses an unmet need for the development of new therapeutic strategies for HRPC and seeks to identify which subset of patients will most benefit from this investigational therapy. Furthermore, the identification of patient subsets that would not benefit from that this therapeutic strategy would permit alternative therapeutic approaches to be developed and patients would be spared the toxicity of ineffective therapy.

A total of 31 patients were entered into the clinical study entitled, *A Phase II pharmacokinetic and biologic correlative study of G3139 (antisense oligonucleotide directed to bcl-2) and docetaxel in patients with hormone-refractory prostate cancer.* Accrual of new patients is complete with updated response and survival data available up to November 2004. Twenty-eight patients are evaluable for the work performed in the grant. The initial 3 patients received a different schedule of oblimersen (5 day infusion) with docetaxel prior to the study protocol being amended and therefore these patients were excluded in the analysis to ensure that the treatment the population received was homogenous. The correlative biologic and pharmacokinetic studies are the companion to the clinical study, funded by the current Department of Defense Grant (PC010504). The accomplishments of all funded years are described below in order of the Objectives and Tasks described in the original grant application.

Specific Aim 1: Demonstrate that *bcl*-2 overexpression in prostate cancer specimens is a predictive biomarker of enhanced responsiveness to G3139 and docetaxel therapy.

To meet this objective the following tasks were undertaken.

Task 1: Immunohistochemical Detection of bcl-2, bcl-X$_L$, and Bax from patients entered onto Phase II study of G3139 and docetaxel for HRPC

    a. Obtain primary tissue blocks (paraffin embedded) from each patient entered on Phase II study (30 patients total) for banking, section paraffin blocks for representative tumor, and perform immunohistochemical staining for bcl-2, bcl-X$_L$, and Bax staining. 24 months.

    b. Pathologic scoring of all immunohistochemical stained specimens will be complete by end of year 2. 24 months.

All 28 specimens were collected, sectioned and underwent immunohistochemistry. Twenty-three specimens had tumor adequate for staining. The results of Bcl-2, Bcl-X$_L$, and Bax staining for the 23 patients are demonstrated in Table 1.

Table 1: Expression[*] of Predictive Biomarkers in Prostate Cancer Patients

Patient #	Bcl-2 N	Bcl-2 I	Bcl-2 (NxI)	Bax N	Bax I	Bax (NxI)	Bcl-X$_L$ N	Bcl-X$_L$ I	Bcl-X$_L$ (NxI)
1	0	0	0	0	0	0	0	0	0
2	1	1	1	0	0	0	0	0	0
3	0	0	0	1	1	1	3	2	6
4	0	0	0	0	0	0	3	2	6
5	0	0	0	0	0	0	1	1	1
6	0	0	0	0	0	0	3	1	3
7	0	0	0	0	0	0	1	1	1
8	0	0	0	0	0	0	1	1	1
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	2	1	2
11	3	3	9	0	0	0	3	1	3
12	2	3	6	0	0	0	3	2	6
13	1	1	1	0	0	0	0	0	0
14	2	2	4	0	0	0	0	0	0
15	0	0	0	0	0	0	3	1	3
17	2	3	6	0	0	0	3	2	6
18	2	3	6	0	0	0	3	2	6
19	3	3	9	2	3	6	3	3	9
20	0	0	0	0	0	0	2	1	2
22	1	3	3	0	0	0	0	0	0
26	2	2	4	0	0	0	2	1	2
27	2	3	6	0	0	0	2	1	2
28	1	2	2	3	1	3	3	2	6

*NxI: Number of cells multiplied by intensity. N represents the frequency of expressing cells within the tumor specimen was scored 1-3 with the following grades 0= no cells staining positive, 1 = 10-20%, 2 = 20-50%, 3 = >50%. The letter "I" reflect intensity scored 1-3. The numerical value "0" represents absent staining.

The frequency of any level of expression (NxI) of the Bcl-2, BAX, and Bcl-X$_L$ protein in the primary tumor specimens was 12/23 (52%), 3/23 (23%), and 17/23 (74%), respectively. This level of expression is concordant with other published reports although the level of Bcl-2 expression in this study population is modestly higher than reported by other investigators examining unselected prostate cancer populations. Since patients that entered into this study had developed hormone-refractory prostate cancer this sample may represent a selected population that exhibits this known negative prognostic indicator.

Bcl-2, Bax, and Bcl-X$_L$ Protein Expression as Predictive Biomarkers of Response

To determine if Bcl-2, Bax, Bcl-X$_L$ protein expression were predictive of response to Bcl-2 biomodulation by oblimersen when combined with docetaxel for the treatment of hormone-refractory prostate cancer the relationship of Bcl-2, Bax and Ncl-X$_L$ protein expression, as detected by immunohistochemistry, and the response to therapy with docetaxel and oblimersen were analyzed using contingency tables using three criteria: a) negative versus positive expression and PSA response; b) low ($\leq$3+) versus high ($\geq$4+) expression and PSA response; and c) low versus high expression on overall survival.

Analysis included both frequency (N), intensity of staining (I), as well as the product (NxI). Representitive contingency tables and statistical analysis for Bcl-2 expression are depicted below in Tables 2 A & B.

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Table 2: Contingency table analysis of Bcl-2 expression versus PSA response. Bcl-2 expression positive versus negative (A), high versus low (B), and the analysis by Fisher's exact test

A

Table of BCL-2 (NI) by RESPONSE			
BCL-2 (NI)	RESPONSE		
Expression	No Response	Response	Total
Negative	6 54.55	5 45.45	11
Positive	3 25.00	9 75.00	12
Total	9	14	23

Fisher's Exact Test	
Cell (1,1) Frequency (F)	6
Left-sided Pr <= F	0.9709
Right-sided Pr >= F	0.1535
Table Probability (P)	0.1244
Two-sided Pr <= P	0.2138

B

Table of BCL- 2 (NI) by RESPONSE			
BCL-2 (NI)	RESPONSE		
Expression	No Response	Response	Total
0-3	6 40.00	9 60.00	15
4+	3 37.50	5 62.50	8
Total	9	14	23

Fisher's Exact Test	
Cell (1,1) Frequency (F)	6
Left-sided Pr <= F	0.7104
Right-sided Pr >= F	0.6326
Table Probability (P)	0.3430
Two-sided Pr <= P	1.0000

The same analysis was performed for Bax and Bcl-X_L. The expression of Bcl-2, Bax, and Bcl-X_L protein as detected by immunohistochemistry was not predictive of PSA response to therapy with oblimersen and docetaxel in this study, with no statistically significant relationship determined for either frequency, intensity of expression, or the product (NxI).

The relationship of Bcl-2 expression and survival was next determined. In this analysis, Bcl-2 expression was grouped as either positive versus negative and survival curves plotted (Figure 1). Although the curves are separate, there is no significant difference between the two groups for survival at present. The limited number of events as of November 2004 may limit the ability to detect a difference currently, and analysis with longer follow-up will occur. Bcl-2 expression has been previously described as a negative prognostic variable for survival in prostate cancer.

Figure 1: Representitive Kaplan-Meier survival curve of Bcl-2 positive versus negative patients entered into study, $p > 0.05$, Log Rank test.
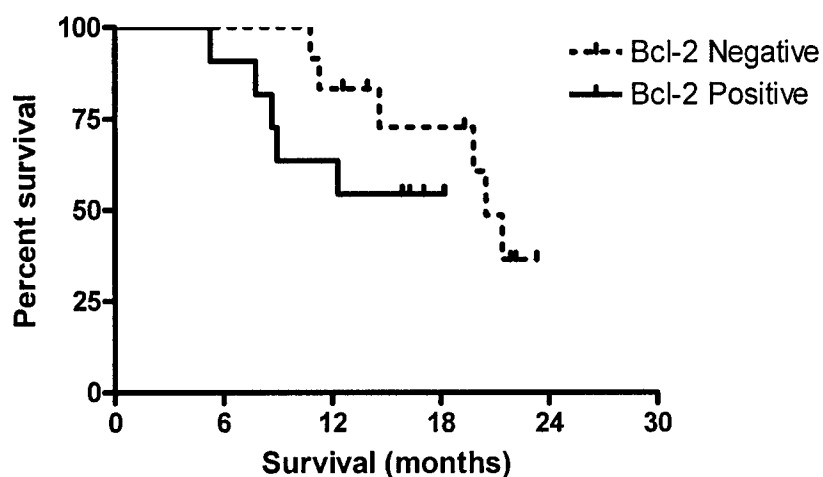
A similar analysis of Bax and Bcl-X_L expression did not demonstrate a relationship between these two proteins and survival following treatment with oblimersen and docetaxel.

Specific Aim 2: Demonstrate that the degree of *bcl*-2 downregulation in normal tissue surrogate (peripheral blood mononuclear cells) will predict for the responsiveness of prostate cancer to G3139 and docetaxel.

Task 2: Quantification of G3139 mediated bcl-2 downregulation in peripheral blood
   mononuclear cells.
   a. Obtain isolate blood mononuclear cells (MNCs) from all patients (30
      patients) at the two time points (prior to G3139 therapy and on day 5),
      isolate protein 18-24 months.
   b. Perform western assay for bcl-2 protein.

Task 4: Examine the predictive pharmacokinetic and biomarkers for response to bcl-2
   biomodulation by G3139 and docetaxel.
   a. Examine relationships between MNCs and G3139 steady-state
      concentrations, and patient clinical outcome (e.g. response rate, time to
   *progression, survival).*

A total of 28 patients had paired mononuclear specimens were obtained from the 28 evaluable patients entered into this study. Peripheral blood mononuclear cells (MNCs) were obtained day 1 prior to the initiation of the antisense molecule oblimersen and again following 5 days (12 hours) of continuous intravenous administered oblimersen (protocol day 6), immediately prior to the initiation of docetaxel. Following separation of the MNCs, protein was isolated from the samples and Bcl-2 protein quantified by Western Blot analysis as outline in the methodology section. The measured Bcl-2 values were normalized to actin expression.

There was considerable interpatient variability in the absolute value of Bcl-2 protein obtained and this was reflected in the median normalized ratio (0.286) and the wide range of ratios (0.017-15.159).

The decrement of normalized Bcl-2 from baseline to day 6 was calculated and 19 patients had a net decrease in Bcl-2 expression whereas 9 patients had a net increase in Bcl-2 expression. The median percent decrement was 49.9% with a range of 95% decrease to

444% increase. The individual patient's percent decrements in normal Bcl-2 values are depicted in Figure 2.

Figure 2: The percent decrement of Bcl-2 protein expression following treatment with G3139 in peripheral blood mononuclear cells at Day 6 compared to pretreatment. The Y axis was truncated at +100 with 3 patients having increases of 120, 131, 444%.
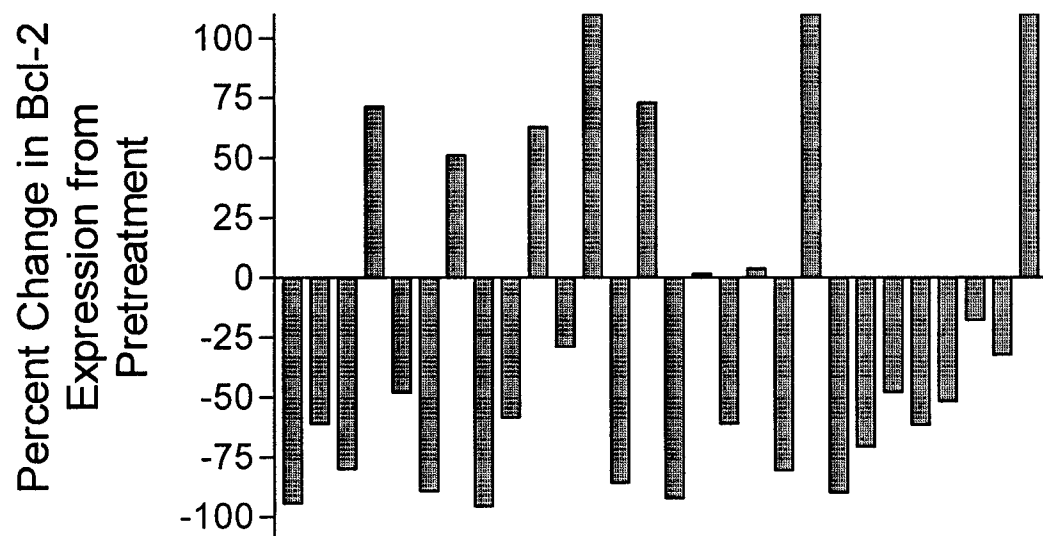

To determine if the decrement of MNC Bcl-2 protein may be a function of oblimersen steady state concentration (C_{ss}), the relationships of these two parameters were evaluated. The mean C_{ss} ±SD of G3139 for the patients who had a decrease versus an increase in Bcl-2 expression was 5.3 ± 1.4 versus 6.1 ± 2.0 µg/mL, respectively. The difference of these means were not statistically significant (p = 0.24). Furthermore, no linear or non-linear relationship could be derived for the values of C_{ss} and decrements in Bcl-2 expression (Figure 3).

Figure 3: Scatterplot of decrement of Bcl-2 expression day 6 versus pretreatment in MNC as a function of G3139 steady state concentrations. The Y-axis was limited at 200% increase therefore 1 patient at 444% not shown
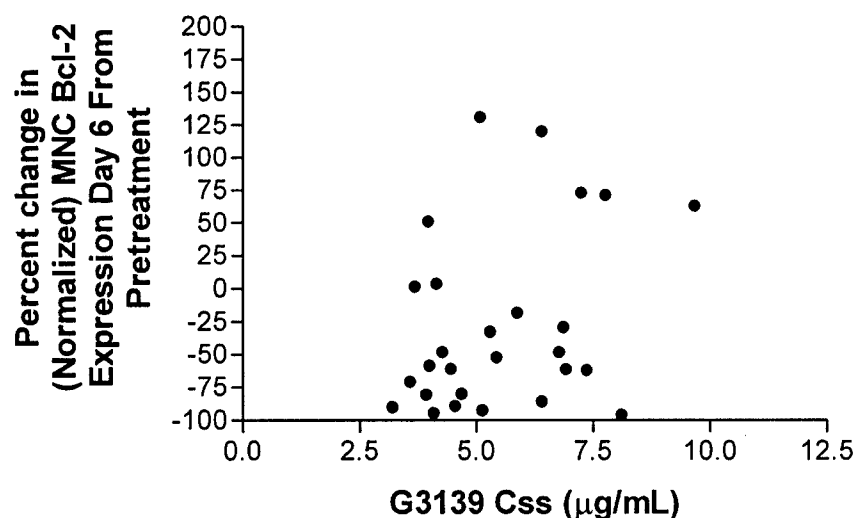
To evaluate whether the decrement in Bcl-2 levels in MNCs would be a predictive biomarker for clinical outcome the percent decrement in Bcl-2 was evaluated in the context of the response to therapy (PSA response), time to progression, and survival. There was no evidence that the decrement in Bcl-2 expression predicted response to therapy as demonstrated in Figure 4 (means comparison $p > 0.05$).

Figure 4: Distribution of percent Bcl-2 expression decrements in MNC Expression in patients that responded versus no response
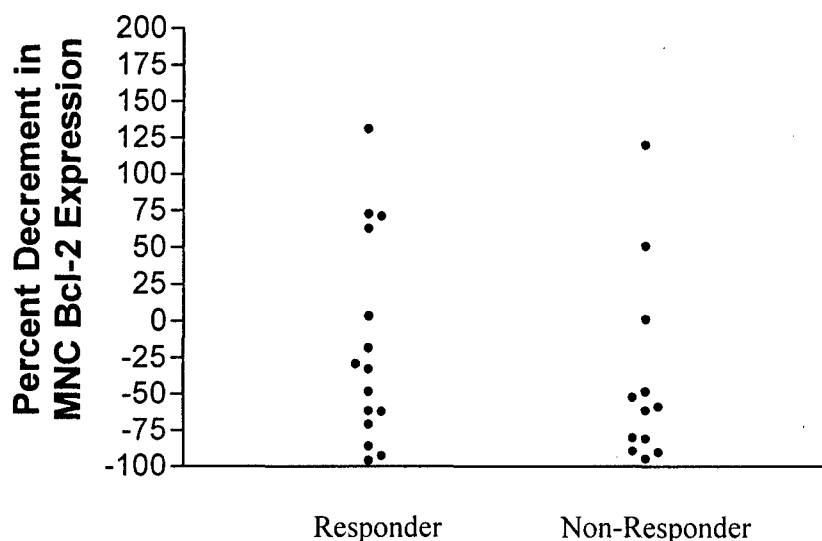
There was no evidence that MNC Bcl-2 expression predicted either improvement in time to progression or overall survival (data not shown).

The results of this study indicate that, although the majority of patients had decrements in MNC Bcl-2 expression following oblimersen treatment, the decrement in MNC Bcl-2 expression as measured in this study is not a function of oblimersen steady-state concentrations, nor a predictive biomarker for the relevant clinical outcomes of response and survival following treatment with oblimersen and docetaxel.

Alternative hypothesis and unanticipated results: The use of peripheral blood MNC remains an un-validated marker of oblimersen drug effect. Two potential confounding aspects may influence these results: 1) the marked lymphopenia observed in patients that receive oblimersen may lead to an enrichment of remaining MNCs that express higher levels of Bcl-2, thereby alter the expression of Bcl-2 measured in this study, or 2) induction of apoptosis may result in degradation of actin resulting in imprecise quantification of Bcl-2 when normalized for actin. This latter hypothesis, recently

suggested by other investigators examining oblimersen, is based on the detection of protein bands indicative of actin fragments on western blot assays. This may be a consideration for future study but at the current time MNCs appear not to be a valid biomarker for clinical studies of oblimersen.

Specific Aim 3: Determine whether the pharmacokinetic parameters of oblimersen and docetaxel are predictive of *bcl*-2 biomodulation and antitumor activity, respectively.

Plasma specimens were obtained in all 28 patients for the determination of oblimersen and docetaxel pharmacokinetic parameters. Plasma concentrations for both oblimersen and docetaxel were determined using high-performance liquid chromatography methods outlined in the grant submission.

Mean ($\pm$SD) G3139 steady state concentrations for the entire group treated at 7 mg/kg/day was 5.6 ($\pm$1.6) μg/mL.

The premise of the clinical study is that Bcl-2 downregulation will enhance the antitumor efficacy of docetaxel. To determine if the C_{ss} of oblimersen is predictive of clinical outcome, the relationship between oblimersen C_{ss} and antitumor response (PSA Response), time to progression, and survival were explored.

There was a significant difference between the mean C_{ss} for patients who responded compared to those patients who did not respond. The mean ($\pm$SEM) C_{ss} for patients who responded was 6.2 ± 0.4 compared to 4.8 ± 0.3 μg/mL for patients who failed to respond to treatment (p = 0.015). The distribution of Css and response is depicted in Figure 5.

Figure 5: Distribution of oblimersen Css for responding versus non-responding patients. Means depicted above each column.
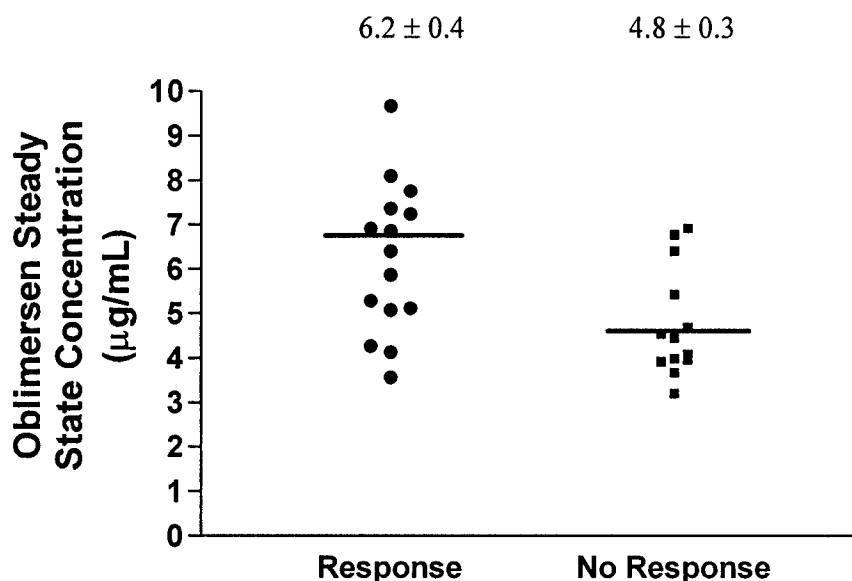
Furthermore, if the subsets of patients are divided into those patients that achieve oblimersen plasma Css $\geq$ 5 μg/mL, the response rate for this cohort of patients was 12/16 (75%) patients whereas in the patients whose Css was less than 5 μg/mL the response rate was 3/12 (25%). Analysis of the impact of Css on survival indicates that the group with Css $\geq$ 5 μg/mL had a median survival of 689 days versus 595 days for the patients with G3139 Css less than 5 μg/mL, although the difference did not reach statistical significance in this low powered Phase II study (Logrank test p > 0.05).

Since Oblimersen steady-state concentrations and PSA decrements are continuous variables, the relationship between these two variables for course 1 were analyzed.

Figure 6: Scatterplot of individual patients percent change in PSA from baseline after 1 course of treatment as a function of oblimersen C_{ss}. The curve represents the best fit for a sigmoidal dose-response equation ($R^2 = 0.375$).
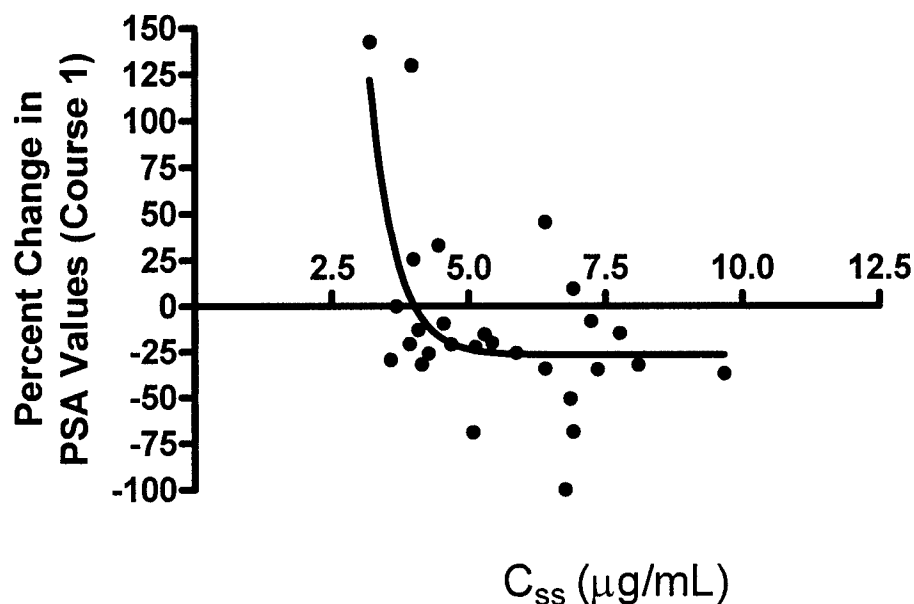
These results indicate that the likelihood of response during the first course is, at least in part, a function of oblimersen steady-state concentration. Further analysis of Bcl-2 expression, oblimersen C_{ss}, and response did not demonstrate a relationship between these multiple variables. Each subset, however, was of small numbers and therefore insufficiently powered to draw conclusions.

Oblimersen Css is a strong determinant of response in the patient population treated with the oblimersen and docetaxel combination. These results further indicate that a significant proportion of patients treated at the recommended oblimersen dose of 7mg/kg/day do not reach optimal steady state concentrations, which based on the aforementioned data appear to be at or greater than 5 µg/mL.

Summary and Discussion:

In the current grant that examined the predictive biomarkers of response associated with treatment with oblimersen and docetaxel, pharmacokinetic considerations (oblimersen steady state concentrations) were the strongest and only significant predictor of response to this therapy. Bcl-2, Bax and Bcl-X_L protein expression as detected by immunohistochemistry from the original tumor blocks and the pharmacodynamic endpoint of Bcl-2 inhibition in a normal tissue surrogate, PBMNCs, were not significant predictors of response to this therapy in hormone-refractory prostate cancer.

There are several important implications of this finding. As stated before and an original finding from this project, oblimersen C_{ss} is a statistically significant predictive of PSA response, and those patients with greater oblimersen C_{ss} exhibited a trend to increased survival. Furthermore, patients who achieved oblimersen $C_{ss} \geq 5\mu g/mL$ had a 75% response rate to therapy. However, despite the administration of oblimersen at the recommended dose for solid tumor studies of 7 mg/kg/day a significant minority of patients (44% of patients) did not achieve a C_{ss} of 5 $\mu g/mL$ or greater and may have failed to reach optimal Bcl-2 inhibitory concentrations. This may suggest that the current recommended dose used for oblimersen is suboptimal. Recent results of randomized studies in a solid tumor (melanoma) failed to reach the primary endpoint (improved survival) using the recommended dose of 7 mg/kg/day. Moreover, the premise and selection of 7 mg/kg/day in solid tumor studies may be flawed since this recommended dose was derived from a single agent Phase I study that did not determine a maximum tolerated dose, was arbitrary, and used reductions in Bcl-2 protein in PBMNCs in a limited sample of patients to justify, at least in part, the recommended dose. This unfortunately begs the question whether further clinical exploration and dose finding studies should be performed to redefine the optimal dose of oblimersen, or failing that, establishing a maximum tolerated dose prior to new clinical studies.

Additional implications of a suboptimal dose finding may confound ones ability to determine biologic predictive biomarkers such as Bcl-2 expression in the patient's original tumor tissue. If the therapy designed to inhibit Bcl-2 was not optimal, the ability

to determine a predictive biomarker will no doubt be compromised. The rational next step to address this provocative finding, our group has proposed further clinical studies of oblimersen in combination with docetaxel to determine the maximum tolerated dose of oblimersen with a fixed dose of docetaxel and then determine the activity of this combination.

Key Research Accomplishments:

- Oblimersen (antisense oligonucleotide to Bcl-2) steady-state concentrations are a predictive determinant of PSA response for patients with hormone-refractory prostate cancer treated with the combination of oblimersen and docetaxel

- A relationship exists between oblimersen steady-state concentrations (Css) and PSA decrement in the first course following treatment with this combination

- Identified suboptimal oblimersen Css, at the heretofore recommended dose level of 7 mg/kg/day, as a possible etiology for recent negative results in randomized studies of oblimersen combined with chemotherapy in solid tumor studies.

- Bcl-2 protein in peripheral blood mononuclear cells (PBMNC) decreases markedly in the majority of patients receiving oblimersen, however no relationship between this decrement and oblimersen Css or PSA response could be determined and PBMNC Bcl-2 expression is not a predictive biomarker of response to this combination therapy

- Expression of Bcl-2 family members Bcl-2, Bax, and Bcl-X_L protein in the original tumor blocks is not predictive of response to therapy with oblimersen and docetaxel in hormone-refractory prostate cancer patients

Reportable Outcomes:

KN Chi, RN Murray, ME Gleave, J Kuhn, E Izbicka, K Berg, EK Rowinsky, AW Tolcher. *A Phase II study of oblimersen sodium (G3139) and docetaxel in patients with prostate cancer* (HRPC) Proceedings of the American Society of Clinical Oncology 22: 2003 (Abstract 1580)

Conclusions:

For patients with hormone-refractory prostate cancer treated with 7mg/kg/day days 1-8 of G3139 (antisense oligonulcleotide to Bcl-2) and docetaxel 75 mg/kg day 6, G3139 steady-state concentration levels that equaled or exceeding 5 ug/mL was a significant predictor of PSA responsiveness. This finding has important implications: 1) at the recommended dose (7mg/kg/day) of G3139 for this and other Phase II studies and the ongoing or recently completed pivotal Phase III studies may not be optimal for Bcl-2 biomodulation and antitumor activity due to the marked interpatient variablility of G3139 steady state concentrations; 2) are there other factors that hasten the clearance of G3139 and may permit optimal dosing of individual patients (e.g. serum albumin, total protein levels, or renal function); 3) the basis for the recommended dose from the original Phase I study may be incorrect since an MTD was not established, and therefore reinforces the need for rigorously performed Phase I studies with targeted therapies(2).

Peripheral blood mononuclear cells are poor surrogates for anti-Bcl-2 activity since the decrement in Bcl-2 protein cannot be related to either G3139 concentrations, or antitumor activity.

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