Progression to Androgen Independence Is Delayed by Adjuvant Treatment with Antisense Bcl-2 Oligodeoxynucleotides after Castration in the LNCaP Prostate Tumor Model

Martin Gleave, Anthony Tolcher, Hideaki Miyake, Colleen Nelson, Bob Brown, Eliana Beraldi, and James Goldie


ABSTRACT

Bcl-2 has emerged as a critical regulator of apoptosis in a variety of cell systems and is up-regulated during progression to androgen independence in prostate cancer cells. The objectives of this study were to characterize changes in Bcl-2 after androgen withdrawal and during progression to androgen independence in the human prostate LNCaP tumor model and determine whether adjuvant use of antisense Bcl-2 oligodeoxynucleotides (ODNs) with androgen ablation delays progression to androgen independence. Bcl-2 expression in LNCaP cells is down-regulated to undetectable levels by androgen in vitro and up-regulated after castration in vivo. Antisense Bcl-2 ODN treatment reduced LNCaP cell Bcl-2 messenger RNA and protein levels by >90% in a sequence-specific and dose-dependent manner at concentrations >50 nM. Bcl-2 mRNA levels returned to pretreatment levels by 48 h after discontinuing treatment. Athymic male nude mice bearing SQ LNCaP tumors were castrated and injected i.p. with 12.5 mg/kg/day with two-base mismatch ODN control, reverse polarity ODN control, or antisense Bcl-2 ODN. Tumor volume in control mice gradually increased 5-fold (range, 3–6) by 12 weeks after castration compared to a 10–50% decrease in precastrate tumor volume in mice treated with antisense Bcl-2 ODN. Changes in serum PSA paralleled changes in tumor volume, increasing 4-fold faster above nadir in controls than in mice treated with antisense Bcl-2 ODN. After decreasing 70% by 1 week after castration, PSA increased 1.6-fold above precastrate levels by 11 weeks in controls while staying 30% below precastrate levels in antisense-treated mice. In a second group of experiments, LNCaP tumor growth and serum PSA levels were 90% lower (P < 0.01) in mice treated with antisense Bcl-2 ODN compared with mismatch or reverse polarity ODN controls. These results support the hypothesis that Bcl-2 helps mediate progression to androgen independence and is an appropriate target for antisense therapy.

INTRODUCTION

Prostate cancer is the most common cancer in men with over 200,000 new cases being diagnosed in North America in 1996 and <40,000 deaths/year in the United States from disseminated hormone-refractory prostate cancer (1). Androgen withdrawal therapy remains the only efficacious treatment for advanced prostate cancer, leading to tumor cell apoptosis and >90% decrease in serum PSA levels. Responses, although marked, are temporary, lasting, on average, 18–24 months. The development of androgen-independent prostate cancer is heralded by a rise in serum PSA and a return of clinical symptoms, most prominently painful bone metastases. Therapeutic options at this phase of the disease are limited and confined to palliative radiotherapy. Despite several hundred clinical studies of both experimental and approved single agents, chemotherapy has limited antitumor activity in hormone-refractory prostate cancer, with objective response rates generally below 10% and no demonstrated survival benefit (2).

Progression to androgen independence is a multifactorial process by which cells acquire the ability to both survive in the absence of androgens and proliferate using nonandrogenic stimuli for mitogenesis (3). At the molecular level, some genes initially dependent on androgens for expression, such as PSA, become constitutively expressed (4, 5), whereas many other genes become aberrantly expressed and actively participate, or passively accompany, the progression to androgen independence (3, 6). Recent evidence suggests that a critical step in progression to androgen-independent prostate carcinoma may involve overexpression of Bcl-2, which blocks apoptosis induced by androgen withdrawal. The Bcl-2 proto-oncogene belongs to a family of related genes, the proteins of which regulate a final common pathway regulating programmed cell death in both normal and abnormal cell populations (7, 8). Bcl-2-transfected cell lines exhibit prolonged survival in growth factor-deprived medium and greater resistance to heat shock stress, various chemotherapeutic agents, and irradiation (9–13). Intrinsic expression of Bcl-2 by prostate carcinoma tissue may result...
in resistance to the effects of hormone manipulation because higher proportion of nonresponders or early relapers to hormo-

nal therapy occurred in patients strongly expressing Bcl-2 (14–
16). Virtually all hormone-refractory prostate carcinomas ex-
press Bcl-2, which supports the hypothesis that Bcl-2 expression
confers resistance to androgen withdrawal by cells blocking the
usual apoptotic signal from androgen manipulation (17). Indeed,
stable Bcl-2 transfection of LNCaP cells increased in vitro
resistance to serum starvation, increased in vivo tumorigenicity,
and accelerated tumor growth in male mice after castration (18).

Accumulating evidence suggests that Bcl-2 overexpression
protects prostate cancer cells from apoptosis after androgen
withdrawal and, therefore, represents a suitable molecular target
with antisense technology. Antisense ODNs are modified
stretches of single-stranded DNA that are complementary to
mRNA regions of a target gene and thereby block translation
and protein synthesis. Phosphorothioate ODNs are stabilized to
resist nuclease digestion by substituting one of the nonbridging
phosphoryl oxygens of DNA with a sulfur (19, 20). The objec-
tives of this study were to define in vitro and in vivo effects of
androgens and androgen withdrawal on Bcl-2 gene expression in
the LNCaP tumor model, to determine the effects of antisense-
Bcl-2 ODN on Bcl-2 mRNA levels in LNCaP cells in vitro, and
to evaluate the ability of antisense-BCL-2 ODN to delay time to
androgen-independent progression of LNCaP tumors after cas-
tration.

**MATERIALS AND METHODS**

**Animals and Cell Lines.** LNCaP cells were maintained
in RPMI 1640 (Terry Fox Laboratory, Vancouver, British Co-
lumbia, Canada) with 5% FBS (Life Technologies, Inc., Burl-
ington, Ontario, Canada) as described previously (4, 21). Male
athymic nude mice (BALB/c strain), 6–8 weeks of age, were
purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN).

**Bcl-2 ODNs.** The following Bcl-2 ODNs were kindly
provided by Genta Inc. (San Diego, CA): antisense Bcl-2 ODN
(sequence, 5′-TCTCCCAAGCTTGCGACGCTTGGGGACG-3′), a two-base
mismatch control ODN (5′-TCTCCCAAGCTTGCGACGCTTGGGGAC-3′), and a
reverse control ODN (sequence, 5′-TACCGGTGCGACGCTTGGGGACG-3′).
All ODNs are 18-mer phosphorothioates targeting the
translation initiation site. ODNs were stored at −20°C at
concentration in 10 mM Tris and 1 mM EDTA.

**Evaluation of Androgen and ODN Effects on Bcl-2
mRNA Levels in Vitro.** LNCaP cells (5 × 10⁶) were plated
in 30-mm dishes (Falcon) in RPMI + 5% FBS. When LNCaP
cells reached 70%–80% confluence, the cell medium was
changed to RPMI + 1% charcoal-stripped FBS and treated with
various concentrations of antisense or control ODN +/+– 10 M
of lipofectin (Life Technologies, Inc., Gaithersburg, MD) for
6 h, or androgen (10 ng/ml R1881) for 24 h. Controls were treated with
equivalent concentrations of lipofectin (Life Technologies, Inc.)
or medium alone. One day later, total RNA was isolated
from LNCaP cells using the 4 M guanidinium thiocyanate
extraction method, as described in previous studies (22), and 4 μg
were reverse transcribed by Moloney murine leukemia virus
reverse transcriptase. For the PCR reaction, 5 μl of the synthe-
sized cDNA were added to 45 μl of a reaction solution contain-
ing 1× PCR buffer (20 mM Tris-HCl and 50 mM KCl), 2 mM
MgCl₂, 160 μM dNTPs, 1 μM each of the primers bcl2, 2.5 units
of Taq polymerase, and 70 ng Taq start antibody (23). The
upstream sequence primer beginning at nucleotide 1814 in the
human Bcl-2 complementary DNA sequence is 5′-TGACCTT-
GACCGTTTAC-3′, and the downstream primer at nucleotide
2377 is 5′-TACGCGATCGGGCGTTTAC-3′. The
gene bank accession number for Bcl-2 is M 13994. After 40 cycles
of amplification, the PCR products were analyzed by ethidium
bromide-stained agarose gel electrophoresis. GAPDH primers
were used in parallel reactions to verify the integrity of the
RNA. Experiments were repeated three times, and representa-
tive examples are shown.

**Evaluation of ODN Effects on LNCaP Cell Apoptosis in
Vitro.** When LNCaP cell cultures reached 70–80% conflu-
ence, cell medium was changed to serum-free RPMI with 100
ng antisense or control mismatch ODN with 10 μl of lipofectin
(Life Technologies, Inc.). After 6 h, cell medium was changed
to RPMI + 1% charcoal-stripped serum for 18 h. Additional 6-h
incubations were performed every 24 h for 5 days. Controls
were treated with equivalent concentrations of lipofectin (Life
Technologies, Inc.) or medium alone. After 5 days, cells were
harvested and assayed for DNA fragmentation and flow cytom-
etry. DNA fragmentation was used to detect apoptosis induced
by ODN treatment and was analyzed by agarose gel electropho-
resis (24). Flow cytometric evaluation for apoptosis was
performed as described previously (25).

**In Vitro Mitogenic Assays.** Effects of antisense Bcl-2
ODN on LNCaP cell growth in vitro was assessed using a
96-well assay based on the uptake and elution of crystal violet
dye by the cells in each well (21). Three thousand LNCaP cells
were plated/well in 96-well plates (Falcon) in RPMI with 5%
FBS, and the cell media was changed to various concentrations
of antisense or mismatch ODN (500, 1, 5, or 10 μM
plus lipofectin) 24 h later. One week later, the cells were fixed
in 1% glutaraldehyde (Sigma), stained with 0.5% crystal
violet (Sigma), and eluted with 100 μl of Sorensen’s solution (9
mg of trisodium citrate in 305 ml of distilled H₂O, 195 ml of 0.1
N HCl, and 500 ml of 90% ethanol). Absorbance of each well
was measured by a Titertek Multiskan TCC/340 (Flow Labora-
tories, McLean, VA) at 560 nm.

**Bcl-2 Western Blot Analysis.** LNCaP cells were cul-
tured in 5% FBS until 70–80% confluence, and media was
changed to RPMI + 1% charcoal-stripped FBS with 50, 100, or
500 ng antisense or control mismatch ODN plus 10 μl of
lipofectin (Life Technologies, Inc.) for 6 h, or androgen (10 ng
R1881) for 24 h. Additional 6-h incubations were performed
every 24 h for 4 days. Controls were treated with equivalent
concentrations of lipofectin or medium alone. After 5 days, cells
were harvested directly into lysis buffer containing NP40 and 40
μg of protein from each transfection were separated on 12%
acrylamide gel. After blotting onto an Immobilon membrane
(Millipore, Bedford, MD), Bcl-2 protein or β-tubulin were de-
tected using monoclonal mouse anti-human Bcl-2 antibodies
(DAKO, Mississauga, Ontario, Canada) or β-tubulin antibody
(Chemicon International Inc., Tumecula, CA).

**Northern Blot Analysis.** Northern analysis was used to
define changes in Bcl-2 expression in LNCaP tumors in vivo. Total
cellular RNA for PSA and polyA for Bcl-2 were prepared from
cultured LNCaP cells or LNCaP tumors using the 4 M guanidinium
thiocyanate extraction method. Electrophoresis, blotting, hybridization, and densitometric analyses were carried out as reported previously (21, 22). The cDNA probe for PSA was a 1.4-kb EcoRI fragment of PSA cDNA (21). The cDNA probe for Bcl-2 was generated using primers with the following sequence: sense 5′-AGATAGTGATGAAGTACATCCATTA-3′ and antisense 5′-TCCGTTATCCTGGATCCAGGTGTGCA-3′. Density of bands for PSA and Bcl-2 were normalized against that of GAPDH (sense, TGCTTTTAACTCTGGTAAAGT; antisense, ATATTTGCGCATGTTTCTTGAT).

**Assessment of in Vivo Tumor Growth.** LNCaP cells (1 × 10⁶) were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) in the flank region of male athymic nude mice, 6–8 weeks of age, via a 27-gauge needle under methoxyfluorane anesthesia. Tumors were thereafter measured twice weekly and their volumes were calculated by the formula L × W × H × 0.5236 (22). Mice bearing tumors between 100–200 mm³ in volume were castrated via a scrotal approach and randomly assigned to a treatment arm. Mice were treated beginning 1 or 7 days after castration with 12.5 mg/kg ODN i.p. twice daily in the first experiment and with 12.5 mg/kg ODN i.p. once daily for the second experiment. Tumor volume and serum PSA measurements were performed weekly. Data points for both sets of experiments were expressed as average tumor volumes ± SEs of the mean based on at least five determinations.

**Determination of Serum PSA Levels.** Blood samples were obtained with tail vein incisions of mice before treatment and then once weekly after starting ODN treatment. Serum PSA levels were determined by an enzymatic immunoassay kit with a lower limit of sensitivity of 0.2 μg/liter (Abbott IMX, Montreal, Quebec, Canada), according to the manufacturer’s protocol. PSA velocity is defined as the rate of change of serum PSA over time, whereas PSA doubling time is defined as the number of doublings of serum PSA over the treatment period. Time to androgen-independent progression was defined as the duration of time required after castration for serum PSA levels to return to or increase above precastrate levels. Data points were expressed as average tumor volumes ± SEs of the mean based on at least five determinations.

**RESULTS**

**Effects of Androgens on Basal Levels of Bcl-2 mRNA in LNCaP Cells in Vitro and in Vivo.** RT-PCR was used to determine changes in Bcl-2 mRNA levels in LNCaP cells after treatment with androgen (1, 10 nM R1881). Physiological concentrations of androgen down-regulated Bcl-2 gene expression to undetectable levels after 24 h (Fig. 1A) After 1 week of 1 nM R1881, Bcl-2 protein levels in LNCaP cells were undetectable (data not shown). Androgen-induced down-regulation of Bcl-2 mRNA was reversible; within 48 h after androgen withdrawal and culture in charcoal-stripped serum in vitro, Bcl-2 mRNA levels returned back to baseline (data not shown).

Changes in Bcl-2 expression in LNCaP tumors grown in vivo paralleled the in vitro observations. Bcl-2 expression was low in LNCaP tumors grown in intact male mice (i.e., androgen-independent tumors), but increases 3–4-fold beginning 1 week after castration and remains elevated in androgen-independent tumors (Fig. 2). LNCaP tumors stained variably positive for Bcl-2 protein, but staining was too weak and heterogenous to permit quantitative comparison between intact and castrate LNCaP tumors (data not shown). PSA gene expression decreases by 80% by 1 week after castration, but increases again in androgen-independent tumors (4, 22).

**Rapid and Reversible Suppression of Bcl-2 mRNA Levels in LNCaP Cells in Vitro by Antisense Bcl-2 ODN.** RT-PCR was used to measure changes in Bcl-2 mRNA levels in LNCaP cells after treatment with antisense, mismatch, or re-
verse polarity ODN. ODN treatment in vitro was evaluated with or without lipofectin (10 µl). Potency of antisense Bcl-2 ODN is increased 100-fold with lipofectin treatment, which permits increased uptake of the ODN into the cell (26). Without lipofectin treatment, Bcl-2 mRNA suppression was induced by antisense ODN at doses of 5 µg and higher (Lanes 3–6); with lipofectin treatment, doses of 100 nm and higher suppressed Bcl-2 mRNA to undetectable levels (Lanes 7–10).

**Effects of Bcl-2 ODN on LNCaP Tumor Growth Rates in Vitro.** In the first set of in vivo experiments, a two-base mismatch Bcl-2 ODN was compared with two groups receiving antisense Bcl-2 ODN, one beginning 1 day and the other 7 days after castration. Changes in LNCaP tumor growth and serum PSA are compared in Fig. 6. LNCaP tumor growth rates were 5-fold higher in mismatch ODN controls compared with antisense Bcl-2 ODN-treated mice. Tumor volume in mismatch controls increased 2.5-fold (range, 1.5–3.2) by 6 weeks after castration and 5-fold (range, 3–6.2) by 12 weeks after castration. In contrast, tumor volume in both antisense Bcl-2 ODN groups gradually decreased during the first 2–3 weeks after castration of treatment, with stabilization thereafter. By 6 weeks after castration, mean tumor volume in group 1 was 53% of baseline (range, 42–59%) and 61% in group 2 (range, 48–75%). By 12 weeks after castration, tumor volume remained below precastrate baseline tumor volumes in both groups (75% of baseline in group 1 and 95% in group 2). Changes in serum PSA levels paralleled changes in tumor volume. Mean pretreatment PSA levels were 156 µg/liter, 143 µg/liter, and 153 µg/liter for the mismatch ODN and the two groups receiving antisense ODN, respectively. After castration, serum PSA decreased by 60–70% in all three groups to nadir levels by 1 week after castration. In the mismatch ODN group, serum PSA increased beginning 2 weeks after castration to a mean of 400 µg/liter by 12 weeks after castration. In contrast, serum PSA levels stabilized or regressed compared with controls over the treatment period in mice treated with adjuvant antisense Bcl-2 ODN. By 12 weeks after castration, serum PSA levels were 75% lower (group 1, 92 µg/liter; group 2, 115 µg/liter) than control (400 µg/liter) levels. By 12 weeks after castration serum PSA increased 2.5-fold above precastrate levels in controls compared with remaining 30% lower than precastrate levels in the two antisense ODN groups. No significant toxicity (i.e., changes in weight, activity, death) was observed in any treatment group.

To confirm the results observed in the first set of in vivo experiments, a second set of in vivo experiments compared two control groups (mismatch and reverse polarity Bcl-2 ODN; n = 6 in each group) to antisense Bcl-2 ODN (n = 12). ODN treatment was initiated 1 day after castration. Changes in LNCaP tumor growth and serum PSA are compared in Fig. 7, A and B, respectively. As in the first set of experiments, LNCaP tumor growth rates were five times faster in mice treated with control ODN groups compared with antisense Bcl-2 ODN-treated mice (P < 0.01). LNCaP tumor volume in the mismatch and reverse polarity ODN groups increased 2.2- and 3-fold by 9 weeks after castration and by >7-fold by 14 weeks after castration. In contrast, tumor volume in the antisense Bcl-2 ODN group decreased by 40% by 9 weeks after castration and stabilized thereafter. Mean pretreatment PSA levels were 88 µg/liter, 82 µg/liter, and 80 µg/liter for the mismatch, reverse polarity, and antisense ODN groups, respectively. After castration, serum PSA decreased 70–80% in all three groups to nadir levels by 1–2 weeks after castration. In the reverse polarity and mismatch ODN groups, serum PSA began increasing again by 4–5 weeks after castration to a mean of 180 and 136 µg/liter by 14 weeks after castration. At 14 weeks after castration, mean serum PSA level (16 µg/liter) in the antisense-treated group was signifi-
cantly lower than in both control groups. No significant toxicity was observed in any treatment group.

We then examined the effects of in vivo ODN treatment on Bcl-2 mRNA expression in LNCaP tumors by Northern blotting. In this experiment, beginning 1 day after castration, five tumor-bearing mice were administered either 12.5 mg/kg antisense Bcl-2 or mismatch control ODN i.p. once daily, and tumor tissues were harvested for RNA extraction 4 days after castration. Antisense Bcl-2 ODN resulted in a 62% reduction in Bcl-2 mRNA levels in LNCaP tumors compared with mismatch control ODN-treated tumors (Fig. 8).

**DISCUSSION**

Although prostate cancer is resistant to conventional chemotherapeutic agents, androgen ablation results in tumor regression and symptomatic relief through apoptotic cell death (1, 2). The benefits of androgen withdrawal therapy, however, are usually temporary because surviving tumor cells generally progress to an androgen-independent state (1–3). Average survival from initiation of androgen ablation in patients with metastatic disease is <30 months (1). Progression to hormone-resistant disease, therefore, represents a major obstacle to effective control and cure of disseminated disease. Before we can have a significant impact on survival with the development of new therapeutic strategies, an improved understanding of the molecular pathogenesis of AI progression is needed.

Controlled investigation of the molecular mechanisms mediating androgen-independent progression has proved difficult because of a paucity of animal models that mimic the course of the clinical disease. Of the available human prostate cancer cell lines, only the LNCaP cell line is androgen responsive, PSA secreting, and immortalized in vitro (27). Like in human prostate cancer, serum PSA levels in the LNCaP tumor model are initially regulated by androgen, are directly proportional to tumor volume, and increase after prolonged periods of growth after castration to signal progression to androgen independence (4, 22). Apoptotic tumor regression does not consistently occur after castration, but tumor growth is inhibited and serum and tumor-cell PSA levels decrease by 80% for several weeks after castration, after which LNCaP tumor growth rates increase and PSA expression rises above precastrate levels. Nonandrogen-regulated PSA gene expression in LNCaP tumors after castration is one example of how other transacting factors can replace androgens and alter gene regulation during progression to androgen independence (28). Characterization of androgen-repressed genes that are up-regulated after androgen withdrawal and play a causative role in progression to androgen independence is a critical step in developing targeted therapies that may prevent hormone-refractory prostate cancer.

Bcl-2 is one gene that has emerged as a putative mediator of resistance to chemotherapy and hormonal therapies and serves as a logical therapeutic target to delay progression after castration in prostate cancer. The Bcl-2 gene was originally identified at the chromosomal breakpoint of the translocation of a portion of chromosome 18 to 14 in B-cell lymphomas (7). Bcl-2 is a critical regulator of apoptosis in a variety of cell systems and is now known to belong to a growing family of apoptosis regulatory gene products, which may either be death

---

**Fig. 4** Sequence-specific decreases in Bcl-2 mRNA levels by antisense, but not mismatch, Bcl-2 ODN treatment. RT-PCR was used to measure changes in Bcl-2 mRNA levels in LNCaP cells treated with various concentrations of antisense or mismatch Bcl-2 ODN. Cells were treated with ODN plus 10 μl of lipofectin for 6 h, and RNA was isolated 24 h later. A: Antisense, but not a two-base mismatch, Bcl-2 ODN decreased Bcl-2 expression in LNCaP cells to undetectable levels within 24 h at concentrations of 50 nm or higher. B, suppression of Bcl-2 mRNA levels was reversible, with Bcl-2 mRNA levels returning to baseline 48 h after a single 24-h treatment.
antagonists (Bcl-2, Bcl-XI, Bcl-W, Bfl-1, Brag-1, and Mcl-1) or death agonists (Bax, Bak, Bcl-XX, Bad, Bid, Bik, and Hrk; Refs. (7–10). The ratio of death antagonists to death agonists determines how a cell will respond to an apoptotic signal. In the prostate gland, Bcl-2 is expressed in the less differentiated basal cell layer of prostatic acini, but not in benign differentiated luminal cells or androgen-dependent prostate cancer cells (13–16). In prostate cancer cells, Bcl-2 is up-regulated during progression to androgen independence and may play an active role in resistance to apoptosis (17). In view of the high prevalence of Bcl-2 in androgen-independent prostate cancers and its anti-apoptosis function, we hypothesize that Bcl-2 plays a protective role in preventing castration-induced apoptosis initiated by androgen withdrawal, thereby accelerating progression to androgen independence. Hence, preventing Bcl-2 up-regulation after androgen withdrawal may enhance castration-induced apoptosis and delay progression to androgen independence.

Antisense ODNs offer one strategy to specifically target Bcl-2 gene expression. Phosphorothioate ODNs are water-soluble, stable agents manufactured to resist nuclease digestion. After parenteral administration, phosphorothioate ODNs become associated with high-capacity, low-affinity serum-binding proteins (19). Recent studies have shown that antisense Bcl-2 ODNs induce apoptosis in Bcl-2-positive small-cell lung cancer cell lines in vitro (29) and increase chemosensitivity of melanoma cells in vitro and in vivo (30). Hammerhead anti-Bcl-2 ribozyme treatment of LNCaP cells reduces Bcl-2 levels and induces apoptosis in low-Bcl-2-expressing LNCaP variants in vitro, but in vivo activity has not yet been reported (31). A Phase I dose-escalation trial using antisense Bcl-2 ODNs in nine patients with lymphoma reported objective and subjective responses with no significant toxicity (32). Taken together, the preclinical and early clinical data support the hypothesis that targeting Bcl-2 gene expression using antisense ODNs may be a

---

**Fig. 6** LNCaP tumor-bearing mice were castrated and treated with twice daily intraperitoneal injections of either a two-base mismatch or antisense ODN. Antisense Bcl-2 ODN therapy was initiated 24 h after castration in group 1 and 1 week after castration in group 2 (n = 6 in each group). A, mean tumor volume increased 5-fold (range, 3–6.2) in mismatch-treated controls by 12 weeks after castration compared with stabilization or up to 25% decrease in antisense-treated groups. B, by 12 weeks after castration, mean serum PSA was 75% lower in antisense-treated groups than mismatch-treated group.

**Fig. 7** LNCaP tumor-bearing mice were castrated and treated with once daily intraperitoneal injections of a two-base mismatch control ODN (n = 6), a reverse polarity control ODN (n = 6), or antisense Bcl-2 ODN (n = 12). All mice were treated once daily beginning 1 day after castration for the duration of the experiment. By 14 weeks after castration, mean tumor volume (A) and serum PSA levels (B) were 90% greater in controls compared with the antisense Bcl-2 ODN group (P < 0.01).
GAPDH mRNA levels were analyzed by Northern blotting. Lanes 1 and 2: ODN. Mean Bcl-2 mRNA levels were 62% lower after antisense ODN, LNCaP tumors in mice administered mismatch Bcl-2 control ODN; Lanes 3, 4, and 5, LNCaP tumors in mice administered antisense Bcl-2 ODN. Mean Bcl-2 mRNA levels were 62% lower after antisense ODN treatment.

valid therapeutic strategy. Indeed, in the androgen-dependent mouse Shionogi tumor model, which undergoes complete apoptotic regression with increased Bcl-2 gene expression after castration, followed by recurrence of androgen-refractory tumors, we have recently reported that adjuvant treatment with mouse antisense Bcl-2 ODN enhances castration-induced apoptosis and delays time to recurrence (33).

Previous studies have focused on treatment strategies combining antisense Bcl-2 ODNs with chemotherapeutic agents (30). The objectives of our studies were to evaluate the effects of androgen on Bcl-2 expression in androgen-sensitive LNCaP cells and whether the combination of antisense Bcl-2 ODN and androgen withdrawal therapy could delay progression to androgen independence. Increased levels of bcl-2 expression during progression from early untreated to hormone-refractory tumors may result from either clonal selection of Bcl-2-expressing clones or adaptive up-regulation of Bcl-2 gene expression in response to androgen depletion. Our data in LNCaP cells suggest that Bcl-2 is an androgen-repressed gene that is up-regulated after withdrawal of androgens both in vitro and in vivo. Physiological levels of androgens almost completely down-regulate Bcl-2 gene expression within 24 h. The reversible, androgen-repressed Bcl-2 expression demonstrated in this study, in addition to the sequence-specific in vitro and in vivo effects of antisense Bcl-2 ODNs in LNCaP cells, further supports the hypothesis that Bcl-2 up-regulation after androgen ablation helps mediate progression to androgen independence, presumably by inhibiting castration-induced apoptosis.

The sequence specificity of Bcl-2 mRNA suppression observed in our in vitro and in vivo studies supports an antisense mechanism of action for the antisense ODN, although additional therapeutically beneficial, sequence-independent, nonantisense interactions can not be ruled out (34, 35). For example, nonspecific phosphorothioate immunostimulation can occur via natural killer cell activation (36). Phosphorothioates have also been shown to competitively inhibit a variety of nucleases and polymerases (37, 38) and interact with heparin-binding growth factors (39). However, nonspecific in vivo activity was not observed in our studies using two similar phosphorothioate control ODNs. Despite distinct sequence-specific Bcl-2 suppression and significant in vivo activity, a cytotoxic effect of antisense Bcl-2 ODNs could not be demonstrated in vitro. Other investigators have reported induction of apoptosis after treatment with antisense bcl-2 ODN (29, 30) or ribozymes (31). This disparity may result from varying sensitivity to specific apoptotic stimuli depending on growth conditions. Tumor cell sensitivity to antisense ODNs vary from tissue type and cell subline (28–30). The relative balance between death antagonists and death agonists after androgen withdrawal may differ under in vitro and in vivo conditions. Second, androgen-regulated gene expression and growth sensitivity is significantly altered in androgen-dependent tissues when transferred to in vitro monolayer culture (40, 41). Finally, DNA fragmentation may not be a reliable marker of apoptosis in epithelial cells (42), although lack of measurable apoptosis was also observed using flow cytometry.

In summary, the data reported herein provides additional evidence that Bcl-2 helps mediate progression to androgen independence. Antisense Bcl-2 ODNs decrease Bcl-2 mRNA and protein levels in LNCaP cells in vivo and inhibit tumor growth and serum PSA increases in vivo after castration in a sequence-specific manner. These data provide the first direct evidence that targeting a putative molecular mediator of androgen-independent progression using antisense ODN can delay progression.

ACKNOWLEDGMENTS
We thank Virginia Yagoda and Dr. Marianne Sadar for excellent technical assistance and Drs. Marcel Bally and Richard Klasa for constructive advice and collaboration.

REFERENCES
Antisense Bcl-2 Delays Androgen-independent Growth

Progression to Androgen Independence Is Delayed by Adjuvant Treatment with Antisense Bcl-2 Oligodeoxynucleotides after Castration in the LNCaP Prostate Tumor Model

Martin Gleave, Anthony Tolcher, Hideaki Miyake, et al.


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/10/2891

Cited articles  This article cites 39 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/10/2891.full.html#ref-list-1

Citing articles  This article has been cited by 33 HighWire-hosted articles. Access the articles at:
/content/5/10/2891.full.html#related-urls

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

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

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