Background: Increased expression of the bcl-2 gene has been observed in prostate cancer cells after androgen withdrawal and has been associated with the development of androgen independence and chemoresistance. The objective of this study was to determine whether antisense Bcl-2 oligodeoxynucleotides could enhance paclitaxel cytotoxicity and delay androgen-independent progression. Methods: Northern and western blot analyses were used to measure changes in Bcl-2 expression in mouse Shionogi tumor cells after treatment with antisense Bcl-2 oligodeoxynucleotides and/or paclitaxel. Growth inhibition and induction of apoptotic cell death were assessed with the use of standard methods. All P values are two-sided. Results: Treatment of Shionogi tumor cells with 500 nM antisense Bcl-2 oligodeoxynucleotides decreased expression of Bcl-2 messenger RNA (mRNA) by approximately 85%. Paclitaxel treatment induced Bcl-2 protein phosphorylation but did not alter Bcl-2 mRNA expression. Antisense Bcl-2 oligodeoxynucleotide treatment substantially enhanced paclitaxel chemosensitivity in a dose-dependent manner. Characteristic apoptotic DNA laddering and cleavage of poly(adenosine diphosphate-ribose) polymerase were demonstrated only after combined treatment. Adjuvant in vivo administration of antisense Bcl-2 oligodeoxynucleotides and micellar paclitaxel following castration resulted in a statistically significant delay of castration-independent, recurrent tumors compared with administration of either agent alone (P<.001, Mantel–Cox log-rank test). Combination therapy also statistically significantly inhibited the growth of established hormone-refractory tumors compared with treatment with either agent alone (P<.001, Student’s t test). Conclusions. Combined treatment with antisense Bcl-2 oligodeoxynucleotides and paclitaxel could be a novel and attractive strategy to inhibit progression to androgen-independent disease as well as growth of hormone-refractory prostate cancer through deprivation of Bcl-2 function. [J Natl Cancer Inst 2000;92:34–41]
senger RNA (mRNA) regions of a target gene that can inhibit
gene expression by forming RNA–DNA duplexes, thereby re-
ducing the activity of the target gene products (14). Phosphor-
othioate oligodeoxynucleotides are stabilized to resist nuclease
digestion by substituting one of the nonbridging phosphoryl
oxygen of DNA with a sulfur. Antisense oligodeoxynucleotides
targeting several oncogenes have been reported to specifically
inhibit expression of these genes and to delay tumor progression
(15–18). However, since numerous genes mediate tumor pro-
gression, inhibition of a single target gene is likely insufficient to
completely suppress tumor progression. Although there have
been no reports demonstrating complete responses of established
tumors in vivo with the use of antisense oligodeoxynucleotides
alone, combined use of antisense oligodeoxynucleotides with
chemotherapeutic agents has been demonstrated to improve re-
sponse rates in some tumor model systems (17,18).

Conventional chemotherapy in advanced prostate cancer is
ineffective for various reasons, including inherent chemoresis-
tance, pharmaceutical mechanism of chemotherapeutic action,
and the inability of elderly patients to tolerate its toxicity. Al-
though paclitaxel has significant cytotoxicity in prostate cancer
cells in vitro, results from clinical studies (2) in which paclitaxel
is used as a single agent in hormone-refractory disease have been
disappointing. However, paclitaxel is known to phosphorylate
and to inactivate Bcl-2 (7); therefore, we undertook this study to
test whether the cytotoxic effects of paclitaxel are enhanced by
antisense Bcl-2 oligodeoxynucleotide treatment and to deter-
mine whether adjuvant use of antisense Bcl-2 oligodeoxynucleo-
tide and paclitaxel after castration delays progression to andro-
gen dependence.

Materials and Methods

Shionogi tumor growth. The Toronto subline of the transplantable Shionogi
SC-115 androgen-dependent mouse mammary carcinoma was used in all ex-
periments (19). Shionogi tumor cells were maintained in Dulbecco’s modified
Eagle medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD)
supplemented with 5% heat-inactivated fetal calf serum. For
in vivo study, approximately 5 × 10^6 cells of the Shionogi carcinoma were injected subcuta-
naneously into adult male DD/S strain mice. When Shionogi tumors reached 1–2
cm in diameter, usually 2–3 weeks after injection, castration was performed
through an abdominal incision in the animals while under methoxyflurane
anesthesia. Details of the maintenance of mice, tumor stock, and surgical pro-
cedures have been described previously (20). Mice were maintained in accordance
with institutional accredited guidelines of the University of British Columbia.

Antisense Bcl-2 oligodeoxynucleotide. Phosphorothioate oligodeoxynu-
cleotides used in this study were supplied Dr. Brett P. Monia (Isis
Pharmaceuticals, Carlsbad, CA). The sequence of antisense Bcl-2 oligodeoxynu-
cleotides corresponding to the mouse bcl-2 translation initiation site was 5’-
TCTCCCGGCTTGCGCCAT-3’ (antisense) for Bcl-2 and 5’-
ATGGTGAAGGTCGGTGTGAACGGAT-3’ (sense) and 5’-
TTATCCTGGATCCAGTGTGAGAT-3’ (antisense) for G3PDH and
5’-AAAG1-16TGTCATGGAGCTAACC-3’ (antisense) for G3PDH. The density of the bands
for Bcl-2 mRNA was normalized against that of G3PDH by densitometric analy-
sis.

Western blot analysis. The expression of Bcl-2 and poly(adenosine di-
phosphate-ribose) polymerase (PARP) protein in cultured Shionogi cells and/or
Shionogi tumor tissues was determined by equal loading analysis as described
previously (22). Briefly, samples containing equal amounts of protein (15 μg)
were subjected to electrophoresis on a sodium dodecyl sulfate (SDS–)
polyacrylamide gel and transferred to a nitrocellulose filter. The filters were
blotted in PBS containing 5% nonfat milk powder at 4 °C overnight and
then incubated for 1 hour with a 1: 200-diluted anti-human Bcl-2 mouse monoclonal
antibody that reacts with mouse Bcl-2 (Santa Cruz Biotechnology Inc., Santa
Cruz, CA) or anti-human PARP mouse monoclonal antibody that reacts with mouse PARP (Pharmingen, Mississauga, Canada). The filters were then
incubated for 30 minutes with horseradish peroxidase-conjugated anti-mouse IgG
antibody (Amersham Life Science Inc.), and specific proteins were detected with
the use of an enhanced chemiluminescence system (Amersham Life Science Inc.).

In vitro cell growth assay. The in vitro growth-inhibitory effects of antisense
Bcl-2 oligodeoxynucleotide and/or paclitaxel on Shionogi tumor cells were as-
sessed in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
(MTT) assay as described previously (22). Briefly, 1 × 10^5 cells were seeded in
each well of 96-well microtiter plates and allowed to attach overnight. The cells
were then treated once daily with various concentrations of oligodeoxynucleo-
tide for 2 days. After oligodeoxynucleotide treatment, the cells were treated with
various concentrations of paclitaxel. After 48 hours of incubation, 20 μL of 5
mg/mL MTT (Sigma Chemical Co.) in PBS was added to each well, followed by
incubation for 4 hours at 37 °C. The formazan crystals were dissolved in DMSO.
The optical density was determined with a microculture plate reader (Becton
Dickinson Labware, Lincoln Park, NJ) at 540 nm. Absorbance values were
normalized to the values obtained for the vehicle-treated cells to determine
the percent survival. Each assay was performed in triplicate.

DNA fragmentation analysis. The nucleosomal DNA degradation was ana-
yzed as described previously with a minor modification (22). Briefly, 1 × 10^5
Shionogi tumor cells were seeded in 5-cm culture dishes and allowed to adhere
overnight. After the treatment with oligodeoxynucleotide and/or paclitaxel under
the same schedule as described above, cells were harvested and then lysed in
a solution containing 100 mM NaCl, 10 mM Tris (pH 7.4), 25 mM EDTA, and
0.5% SDS. After centrifugation at 10 000g for 10 minutes at 4 °C, the superna-
tants were incubated with 300 μg/mL proteinase K for 5 hours at 65 °C and
extracted with phenol-chloroform. The aqueous layer was treated with 0.1
volume of 3 M sodium acetate, and the DNA was precipitated with 2.5 volumes of
95% ethanol. After treatment with 100 μg/mL ribonuclease A for 1 hour at
37 °C, the sample was subjected to electrophoresis on a 2% agarose gel and
stained with ethidium bromide.

Assessment of in vivo tumor growth. For the determination of whether the
combined treatment with antisense Bcl-2 oligodeoxynucleotide and paclitaxel
delays the time to androgen-independent recurrence after castration compared
with treatment with either agent alone, male DD/S mice bearing the Shionogi
tumor were castrated and randomly selected for treatment with antisense Bcl-2
oligodeoxynucleotide alone (group 1), antisense Bcl-2 oligodeoxynucleotide plus
paclitaxel (group 2), or mismatch control oligodeoxynucleotide plus pacli-
taxel (group 3). Each experimental group consisted of six mice. Beginning 1 day
after castration, 12.5 mg/kg antisense Bcl-2 or mismatch control oligodeoxynu-
ucleotide was injected intraperitoneally once daily into each mouse for 14 days.
From 10 to 14 days after castration, 0.5 mg of polymeric micellar paclitaxel was administered once daily by intravenous injection in groups 2 and 3. A second set of experiments was designed to evaluate the effects of combined treatment on established androgen-independent, recurrent tumors. Castrated male DDS mice bearing androgen-independent Shionogi tumors that were approximately 1 cm in diameter were randomly selected to receive one of the three treatment regimens as described above. The tumor volume was measured twice weekly and calculated by the formula length × width × depth × 0.5236 (21). Data points were reported as average tumor volumes ± standard deviations.

Statistical analysis. The in vitro cytotoxic effects of antisense or mismatch oligodeoxynucleotide and paclitaxel were analyzed with the use of a repeated-measure analysis of variance (ANOVA) model. Androgen-independent, recurrence-free survival curves were calculated by the method of Kaplan–Meier and evaluated with the Mantel–Cox log-rank test. Synergy between antisense Bcl-2 oligodeoxynucleotide and paclitaxel was analyzed by calculation of the fractional product parameter according to the fractional product method as previously described (23). The other data were analyzed by Student’s t test. The levels of statistical significance were set at P<.05 (two-sided), and all statistical calculations were done by use of the Statview 4.5 software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Effects of Antisense Bcl-2 Oligodeoxynucleotide and Paclitaxel Treatment on Bcl-2 mRNA and Protein Expression

Northern blot analysis was used to determine the effect of treatment with antisense Bcl-2 oligodeoxynucleotide and paclitaxel on Bcl-2 mRNA expression in Shionogi tumor cells. As shown in Fig. 1, A, treatment of Shionogi tumor cells with 500 nM antisense Bcl-2 oligodeoxynucleotide decreased Bcl-2 mRNA by approximately 85% compared with those cells treated with 500 nM mismatch control oligodeoxynucleotide; however, Bcl-2 mRNA expression was not affected by paclitaxel treatment. Western blotting was then used to analyze changes in Bcl-2 protein expression in Shionogi tumor cells after treatment with antisense Bcl-2 oligodeoxynucleotide, paclitaxel, or both agents. Fig. 1, B, shows that treatment of Shionogi tumor cells with antisense Bcl-2 oligodeoxynucleotide resulted in a substantial decrease in Bcl-2 protein and that paclitaxel treatment induced the expression of the slow-migrating (i.e., phosphorylated) form of the Bcl-2 protein. In addition, incubation of the cell lysates after paclitaxel treatment with λ protein phosphatase, which has specificity for cleavage of phosphate groups appended to the amino acids serine, threonine, or tyrosine (24), resulted in the absence of the slowly migrating form of the Bcl-2 protein. These findings confirm that paclitaxel treatment results in Bcl-2 phosphorylation, which has been reported to interfere with Bcl-2 dimerization to Bax in several cell types and consequently to increase Bax homodimerization, resulting in enhanced induction of apoptotic cell death (7).

Synergistic Cytotoxicity of Antisense Bcl-2 Oligodeoxynucleotide and Paclitaxel Treatment in Shionogi Tumor Cell Growth In Vitro

To determine whether treatment with antisense Bcl-2 oligodeoxynucleotide enhances the cytotoxic effect of paclitaxel, we treated Shionogi tumor cells with various concentrations of antisense Bcl-2 or mismatch control oligodeoxynucleotide once daily for 2 days and then incubated them with various concentrations of paclitaxel for 2 days. The MTT assay was then performed to determine cell viability. As shown in Fig. 2, A, treatment with antisense Bcl-2 oligodeoxynucleotide statistically significantly enhanced paclitaxel chemosensitivity in a dose-dependent manner (two-sided P = .018, ANOVA), reducing the IC_{50} (i.e., the concentration that reduces cell viability by 50%) of paclitaxel by 1 log (100 nM to 10 nM), whereas mismatch control oligodeoxynucleotide had no effect. The combined effects between antisense Bcl-2 oligodeoxynucleotide and paclitaxel were synergistic, as determined by an analysis that utilized the fractional product method (23). We also observed synergistic cytotoxic effects between antisense Bcl-2 oligodeoxynucleotide and paclitaxel by increasing the concentration of antisense Bcl-2 oligodeoxynucleotide while keeping the concentration of paclitaxel constant at 10 nM (two-sided P<.044, ANOVA) (Fig. 2, B).

A DNA fragmentation assay was performed to compare the effects of combined treatment with antisense Bcl-2 oligodeoxynucleotide (500 nM) and paclitaxel (10 nM) on induction of apoptotic cell death. With the use of the same treatment schedule described above, the characteristic apoptotic DNA ladder was observed only after combined treatment with antisense Bcl-2 oligodeoxynucleotide plus paclitaxel (Fig. 3, A). Furthermore,
Fig. 2. Effect of combined treatment with antisense Bcl-2 oligodeoxynucleotide (ODN) and paclitaxel (Taxol) on Shionogi tumor cell growth. A) Shionogi tumor cells were treated daily with 500 nM antisense Bcl-2 or mismatch control ODN for 2 days. After ODN treatment, the medium was replaced with medium containing various concentrations of paclitaxel. After 48 hours of incubation, cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Each data point represents the mean of three independent experiments with standard deviations. The cytotoxic effects of paclitaxel on Shionogi cells were statistically significantly enhanced by antisense Bcl-2 ODN treatment (two-sided \( P = .018 \), analysis of variance). B) Shionogi tumor cells were treated with various concentrations of antisense Bcl-2 ODN or mismatch control ODN and then incubated for 48 hours with medium alone or medium containing 10 nM paclitaxel, and the cell viability was determined by MTT assay. Each data point represents the mean of three independent experiments with standard deviations. Treatment of Shionogi tumor cells with antisense Bcl-2 ODN statistically significantly enhanced the sensitivity to paclitaxel (two-sided \( P < .044 \), analysis of variance). * indicates a synergistic effect between antisense Bcl-2 ODN and paclitaxel, as determined by the fractional product method.

Fig. 3. Effect of combined treatment with antisense Bcl-2 oligodeoxynucleotide (ODN) and paclitaxel (Taxol) on apoptotic death of Shionogi tumor cells. A) Shionogi tumor cells were treated daily with 500 nM antisense Bcl-2 ODN or mismatch control ODN for 2 days. After ODN treatment, the medium was replaced with medium containing various concentrations of paclitaxel. After 48 hours of incubation, DNA was extracted from culture cells, subjected to electrophoresis in a 2% agarose gel, and visualized by ethidium bromide staining. M = molecular weight markers (1-kilobase ladder; Life Technologies, Inc.). B) Proteins were extracted from Shionogi tumor cells after the same treatment as described in panel A and analyzed by western blotting with an anti-poly(adenosine diphosphate-ribose) polymerase (PARP) protein antibody. Uncleaved intact PARP, molecular weight \( (M_f) = 116 \) kilodalton (kD); cleaved PARP, \( M_c = 85 \) kD.
cleavage of the PARP protein, a substrate of the caspase activated during the process of apoptotic execution (25), was examined by western blotting. The 116-kilodalton (kD) intact form of PARP was observed in all of the samples examined, whereas the 85-kD PARP cleavage fragment was detected only after combined treatment with antisense Bcl-2 oligodeoxy nucleotide plus paclitaxel (Fig. 3, B).

**Delayed Progression to Androgen Independence of Shionogi Tumors In Vivo by Combined Treatment With Antisense Bcl-2 Oligodeoxy Nucleotide Plus Paclitaxel**

Male mice with Shionogi tumors that were between 1 and 2 cm in diameter were randomly selected for treatment with either antisense Bcl-2 oligodeoxy nucleotide alone, antisense Bcl-2 oligodeoxy nucleotide plus paclitaxel, or mismatch control oligodeoxy nucleotide plus paclitaxel. The mean tumor volume was similar at the beginning of treatment in the three treatment groups. Beginning 1 day after castration, 12.5 mg/kg antisense Bcl-2 or mismatch control oligodeoxy nucleotide, diluted with PBS, was injected intraperitoneally once daily for 14 days. Beginning 10 days after castration, 0.5 mg of polymeric micellar paclitaxel was administered intravenously once daily for 5 days. Fig. 4, A, illustrates the changes in the mean tumor volume after castration and adjuvant therapy. By 40 days after castration, the mean tumor volume in the group treated with antisense Bcl-2 oligodeoxy nucleotide plus paclitaxel was 91% and 86% lower than that of the group treated with antisense Bcl-2 oligodeoxy nucleotide or that of the group treated with mismatch control oligodeoxy nucleotide and micellar paclitaxel, respectively (two-sided P <.001, Student’s t test). Fig. 4, B, illustrates the differences in recurrence-free survival after castration and adjuvant therapy. Androgen-independent tumors recurred in three of six mice after a median of 37 days in the group treated with antisense Bcl-2 oligodeoxy nucleotide plus micellar paclitaxel, while androgen-independent tumors recurred in all mice after a median of 23 or 28 days in the group treated with antisense Bcl-2 oligodeoxy nucleotide or in the group treated with

**Fig. 4.** Effects of adjuvant administration of antisense Bcl-2 oligodeoxy nucleotide (ODN) and polymeric micellar paclitaxel (Taxol) on Shionogi tumor growth in mice after castration. A) Mice were treated with either antisense Bcl-2 ODN alone, antisense Bcl-2 ODN plus micellar paclitaxel, and mismatch control ODN plus micellar paclitaxel. Beginning 1 day after castration, antisense Bcl-2 ODN or mismatch control ODN (12.5 mg/kg body weight) was injected intraperitoneally once daily for 14 days. Beginning on day 10 after castration, 0.5 mg of micellar paclitaxel was injected intravenously once daily for 5 days. The tumor volume was measured twice weekly and calculated by the formula length x width x depth x 0.5236. Each point represents the mean tumor volume in each experimental group containing six mice with standard deviations. The mean tumor volume in mice treated with antisense Bcl-2 ODN plus micellar paclitaxel 40 days after castration was statistically significantly smaller than that in mice treated with antisense Bcl-2 ODN alone or mismatch control ODN plus micellar paclitaxel (two-sided P <.001, Student’s t test). B) Androgen-independent, recurrence-free survival curves in mice treated as described in panel A. The time of progression to androgen independence in mice treated with antisense Bcl-2 ODN plus micellar paclitaxel was statistically significantly delayed compared with that in mice treated with antisense Bcl-2 ODN alone or mismatch control ODN plus micellar paclitaxel (two-sided P <.001, Mantel–Cox log-rank test). The androgen-independent, recurrence-free survival probabilities for mice at days 20, 30, and 40 are 67%, 0%, and 0%, respectively, for antisense Bcl-2 ODN treatment alone; 100%, 50%, and 0%, respectively, for mismatch control ODN plus micellar paclitaxel treatment; and 100%, 100%, and 67%, respectively, for combined antisense Bcl-2 ODN plus micellar paclitaxel treatment.
mismatch control oligodeoxynucleotide and micellar paclitaxel, respectively (two-sided $P < 0.01$, Mantel–Cox log-rank test). These data demonstrate that antisense Bcl-2 oligodeoxynucleotide and paclitaxel prolong time to progression to androgen independence when combined in an adjuvant manner with androgen ablation.

Efficacy of Combined Antisense Bcl-2 Oligodeoxynucleotide plus Paclitaxel in Treatment of Established Androgen-Independent, Recurrent Shionogi Tumors

Approximately 3–4 weeks after castration, androgen-independent Shionogi tumors recur and grow rapidly, with a doubling time of 72 hours (19). When androgen-independent tumors reached 1 cm in diameter, the mice were randomly selected for treatment with either antisense Bcl-2 oligodeoxynucleotide alone, antisense Bcl-2 oligodeoxynucleotide plus micellar paclitaxel, or mismatch control oligodeoxynucleotide plus micellar paclitaxel, and the treatment was administered under the same schedule as described above. The mean tumor volume was similar at the beginning of treatment in the three treatment groups. Untreated mice with androgen-independent Shionogi tumors require sacrifice within 2–3 weeks after recurrence because their tumor mass became larger than 10% of their body weight or because of weight loss, tumor ulceration, or gait disturbance (data not shown). Hence, time to sacrifice was delayed in all three treatment groups; however, combined treatment with antisense Bcl-2 oligodeoxynucleotide plus paclitaxel resulted in the most statistically significant delay in tumor progression of the three treatment groups, producing a mean tumor volume that was 50%–70% lower at day 38 than that in the other two treatment groups (two-sided $P < 0.001$, Student’s $t$ test) (Fig. 5, A). During a 38-day observation period, the mice treated with antisense Bcl-2 oligodeoxynucleotide plus micellar paclitaxel averaged a 1.6-fold increase in tumor volume compared with a 2.9-fold or 2.6-fold increase in the mice treated with antisense Bcl-2 oligodeoxynucleotide or control oligodeoxynucleotide plus micellar paclitaxel, respectively.

![Graph](https://i.imgur.com/1234567.png)

**Fig. 5.** Effects of combined treatment with antisense Bcl-2 oligodeoxynucleotide (ODN) and polymeric micellar paclitaxel (Taxol) on androgen-independent Shionogi tumors. (A) Mice with androgen-independent, recurrent Shionogi tumors were randomly selected for treatment with antisense Bcl-2 ODN alone, antisense Bcl-2 ODN plus micellar paclitaxel, and mismatch control ODN plus micellar paclitaxel. Treatments and measurement of tumor volume were performed with the use of the methods described in the legend to Fig. 4. Each data point represents the mean tumor volume plus standard deviations in each experimental group containing six mice. The mean tumor volume at day 38 after the initial treatment in mice treated with antisense Bcl-2 ODN plus micellar paclitaxel was statistically significantly lower than that in mice treated with antisense Bcl-2 ODN alone or mismatch control ODN plus micellar paclitaxel (two-sided $P < 0.001$, Student’s $t$ test). (B) After completion of treatment as described in panel A, polyadenylated RNA was extracted from androgen-independent Shionogi tumors, and Bcl-2 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) messenger RNA (mRNA) levels were analyzed by northern blotting. Lane 1, androgen-independent tumors without treatment; lane 2, androgen-independent tumors treated with antisense Bcl-2 ODN alone; lane 3, androgen-independent tumors treated with mismatch control ODN and micellar paclitaxel; and lane 4, androgen-independent tumors treated with antisense Bcl-2 ODN and micellar paclitaxel. Bcl-2 mRNA levels were decreased by antisense Bcl-2 ODN treatment (lanes 2 and 4) but not by micellar paclitaxel treatment (lane 3). (C) After completion of treatment as described in panel A, the proteins were extracted from androgen-independent Shionogi tumors and analyzed by western blotting with an anti-Bcl-2 antibody. Lane 1, androgen-independent tumors without treatment; lane 2, androgen-independent tumors treated with antisense Bcl-2 ODN alone; lane 3, androgen-independent tumors treated with mismatch control ODN and micellar paclitaxel; and lane 4, androgen-independent tumors treated with antisense Bcl-2 ODN and micellar paclitaxel. Bcl-2 protein levels were decreased by antisense Bcl-2 ODN treatment (lanes 2 and 4). Although the Bcl-2 protein levels were not affected by micellar paclitaxel, micellar paclitaxel treatment resulted in Bcl-2 protein phosphorylation (lanes 3 and 4). (D) The same protein samples as described in panel C were analyzed by western blotting with an anti-poly(A)-diphosphate-ribose) polymerase (PARP) protein antibody. Lane 1, androgen-independent tumors without treatment; lane 2, androgen-independent tumors treated with antisense Bcl-2 ODN alone; lane 3, androgen-independent tumors treated with mismatch control ODN and micellar paclitaxel; and lane 4, androgen-independent tumors treated with antisense Bcl-2 ODN and micellar paclitaxel. Un cleaved intact PARP, molecular weight ($M_r$) = 116 kilodalton (kD); cleaved PARP, $M_r$ = 85 kD. Cleaved PARP fragments were detected in androgen-independent Shionogi tumors only after combined treatment with antisense Bcl-2 ODN plus micellar paclitaxel (lane 4).
The effects of combined in vivo treatment with antisense Bcl-2 oligodeoxynucleotide and paclitaxel on Bcl-2 mRNA expression, Bcl-2 phosphorylation, and cleavage of PARP protein in Shionogi tumors were examined with the use of northern or western blot analysis. Androgen-independent tumors were harvested after completion of the same treatment schedule described above. Treatment with antisense Bcl-2 oligodeoxynucleotide resulted in a substantial reduction in Bcl-2 mRNA and protein levels, while micellar paclitaxel induced Bcl-2 phosphorylation in androgen-independent Shionogi tumors (Fig. 5, B and C). Furthermore, the 85-kd PARP cleavage fragment was detectable in androgen-independent Shionogi tumors only after combined treatment with antisense Bcl-2 oligodeoxynucleotide and micellar paclitaxel (Fig. 5, D).

**DISCUSSION**

Although Bcl-2 expression in normal prostatic epithelial cells is low or absent, Bcl-2 is highly elevated in prostate cancer cells after androgen withdrawal and during progression to androgen independence (8,9). Accumulating evidence strongly suggests that Bcl-2 overexpression protects prostate cancer cells from apoptotic cell death induced by several therapies, including androgen withdrawal and cytotoxic chemotherapy, thereby accelerating progression to androgen independence and conferring chemoresistance. For example, introduction of Bcl-2 cDNA into LNCaP human prostate cancer cells increases in vivo tumorigenic potential and renders the cells highly resistant to androgen ablation (11). Repression of Bcl-2 expression in LNCaP cells blocks the protective effect of androgens on etoposide cytotoxicity (10). Taken together, these findings suggest that inhibition of increased Bcl-2 expression precipitated by androgen withdrawal may enhance castration-induced apoptotic cell death and delay androgen-independent progression of prostate cancer and may also render cells more sensitive to cytotoxic chemotherapy. This hypothesis has not, however, been confirmed in in vivo models, which is critical when evaluating the mechanism of castration-induced apoptotic cell death and androgen resistance, which are complicated processes that are lost in cells in in vitro culture.

Antisense oligodeoxynucleotide therapy offers one strategy to specifically target bcl-2 gene expression. Phosphorothioate oligodeoxynucleotides are water-soluble, stable agents manufactured to resist nuclease digestion. After parenteral administration, phosphorothioate oligodeoxynucleotides become associated with high-capacity, low-affinity, serum-binding proteins (26). Various reports have shown that antisense Bcl-2 oligodeoxynucleotides induce apoptotic cell death in various types of malignant cell lines in vitro, including small-cell lung cancer (16), myeloma (27), leukemia (28), lymphoma (29), and cholangiocarcinoma (30). Furthermore, combined use of antisense Bcl-2 oligodeoxynucleotide with chemotherapeutic agents resulted in a more than additive inhibition of small-cell lung cancer cells in vitro (18) and melanoma cells in vitro and in vivo (17).

Recently, a novel polymeric micellar paclitaxel, which is characterized by high drug payload and long circulation time in the blood compared with conventional Cremophor paclitaxel, has been developed (31,32) and has been demonstrated to induce complete responses in androgen-independent LNCaP tumors (Gleave ME: unpublished data). We have previously shown that the antisense Bcl-2 oligodeoxynucleotides used in these experiments decrease Bcl-2 expression levels in Shionogi tumor cells in a dose-dependent manner, enhance castration-induced apoptotic cell death, and delay time to androgen-independent progression (33). The objective of this study was to determine whether combined treatment with antisense Bcl-2 oligodeoxynucleotide plus paclitaxel after castration delays androgen-independent progression beyond that achieved with either agent alone. Because of its androgen-dependent behavior, the Shionogi tumor model is particularly useful to study the androgen action, the molecular mechanism regulating castration-induced apoptotic cell death, and the progression to androgen independence, as well as therapeutic approaches to delay or avert tumor progression (3).

In this study, phosphorothioate antisense Bcl-2 oligodeoxynucleotides, corresponding to the mRNA bcl-2 translation initiation site, inhibited expression of Bcl-2 mRNA and protein in Shionogi tumor cells, whereas two-base mismatch Bcl-2 oligodeoxynucleotides had no effects on Bcl-2 expression levels. Although paclitaxel did not affect Bcl-2 expression levels, it did induce Bcl-2 phosphorylation in Shionogi tumor cells in a dose-dependent manner. Bcl-2 phosphorylation has been demonstrated to result in the decreased ability to form heterodimers with Bax protein (7). These findings suggest that combined treatment with antisense Bcl-2 oligodeoxynucleotides and paclitaxel cooperatively inhibits Bcl-2 function. Indeed, antisense Bcl-2 oligodeoxynucleotide enhanced paclitaxel-induced apoptotic cell death and decreased the IC50 of paclitaxel by one order of magnitude. In vivo administration of antisense Bcl-2 oligodeoxynucleotides plus micellar paclitaxel delayed the time to progression to androgen independence compared with either agent alone and cooperatively inhibited established androgen-independent Shionogi tumor growth. We also documented an in vivo decrease in Bcl-2 mRNA expression and phosphorylation of Bcl-2 protein by antisense Bcl-2 oligodeoxynucleotides and micellar paclitaxel, respectively. These findings illustrate that systemic administration of antisense Bcl-2 oligodeoxynucleotides and micellar paclitaxel cooperatively inhibits Bcl-2 function in tumor cells. Enhanced cleavage of the PARP protein in androgen-independent Shionogi tumors by combined treatment suggests that inhibition of Bcl-2 function results in increased apoptotic cell death in tumor tissues.

Several hundred nonhormonal therapies for prostate cancer have been traditionally evaluated in patients with advanced hormone-refractory disease; when used in this end-stage setting, none has demonstrated improved survival (2). A more rational strategy to improve survival would be to combine antisense agents earlier with androgen ablation to target adaptive changes in gene expression precipitated by androgen withdrawal in order to enhance castration-induced apoptotic cell death and delay emergence of hormone-refractory disease. A second strategy would be to try to enhance the sensitivity to conventional chemotherapy agents by use of antisense agents that target cell survival genes mediating chemoresistance. Our study confirms that the inhibition of Bcl-2 function with the use of antisense Bcl-2 oligodeoxynucleotides plus paclitaxel causes a delay in progression to androgen independence as well as inhibition of established androgen-independent tumor growth in the Shionogi tumor model. These preclinical data provide support for clinical studies with antisense Bcl-2 oligodeoxynucleotides plus paclitaxel for patients with prostate cancer.


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NOTES

A. Tolcher holds stock in and is currently conducting research sponsored by Genta Inc., Boston, MA.

Supported in part by grant 009002 from the National Cancer Institute of Canada.

We thank Mary Bowden and Virginia Yago for their excellent technical assistance.

Manuscript received March 10, 1999; revised October 12, 1999; accepted October 29, 1999.