

Antisense 2'-Deoxy, 2'-Fluoroarabino Nucleic Acid (2'-F-ANA) Oligonucleotides: *In Vitro* Gymnotic Silencers of Gene Expression Whose Potency Is Enhanced by Fatty Acids

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Gymnosis is the process of the delivery of antisense oligodeoxynucleotides to cells, in the absence of any carriers or conjugation, that produces sequence-specific gene silencing. While gymnosis was originally demonstrated using locked nucleic acid (LNA) gapmers, 2'-deoxy-2'-fluoroarabinonucleic acid (2'-F-ANA) phosphorothioate gapmer oligonucleotides (oligos) when targeted to the Bcl-2 and androgen receptor (AR) mRNAs in multiple cell lines in tissue culture, are approximately as effective at silencing of Bcl-2 expression as the iso-sequential LNA congeners. In LNCaP prostate cancer cells, gymnotic silencing of the AR by a 2'-F-ANA phosphorothioate gapmer oligo led to downstream silencing of cellular prostate-specific antigen (PSA) expression even in the presence of the androgenic steroid R1881 (metribolone), which stabilizes cytoplasmic levels of the AR. Furthermore, gymnotic silencing occurs in the absence of serum, and silencing by both LNA and 2'-F-ANA oligos is augmented in serum-free (SF) media in some cell lines when they are treated with oleic acid and a variety of ω -6 polyunsaturated fatty acids (ω -6 PUFAs), but not by an aliphatic (palmitic) fatty acid. These results significantly expand our understanding of and ability to successfully manipulate the cellular delivery of single-stranded oligos *in vitro*.

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Introduction

Perhaps the most important and unresolved impediment to the clinical success of oligonucleotide (oligo) therapeutics is the issue of intracellular delivery. Over the decades, numerous delivery strategies, most involving lipid¹ or polyamine encapsulation² of the oligos have been evaluated, with varying *in vitro* and *in vivo* success.^{3,4} More recently^{5,6} it has been appreciated as a general principle that the intracellular delivery of antisense oligos that silence gene expression in tissue culture can be accomplished in the absence of either a delivery vehicle or molecular conjugation. This process has been termed gymnosis,⁷ and has typically employed 3'-5' locked nucleic acid (LNA) gap-mer phosphorothioate oligos (two LNAs at each molecular terminus). By virtue of these terminal LNA modifications, such oligos are highly nuclease resistant due to dramatic reduction of 3'-5' exonuclease digestion. In addition, the LNA moieties also increase the T_m of the mRNA-DNA duplex by as much as ~4–6 °C/base modification.^{8,9} Under gymnotic delivery conditions, these oligos, after only a single addition to the tissue culture medium, have been demonstrated to robustly silence gene expression in the low micromolar concentration range in numerous cell types. Examples of genes gymnotically silenced by LNA phosphorothioate gapmer antisense oligos include Bcl-2, HIF-1 α , ApoB,^{5,7} Her3, PIK3CA, β -catenin, and heat shock

protein 27.⁶ In the vast majority of cell lines examined, >90% silencing of gene expression at the protein and mRNA level, with remarkable specificity, could be routinely achieved with minimal cellular toxicity.

Non-LNA substituted all-phosphorothioate oligos, in our experience,^{5,7} do not silence gene expression when delivered without carriers (though a specific exception, G3139 a.k.a. oblimersen, an 18mer targeted to the Bcl-2 mRNA initiation codon region, has been shown to exist⁵). Nevertheless, while the gymnotic activity of the LNA gapmers is high and extremely robust in tissue culture, it is uncertain whether these molecules are uniquely competent. Damha and co-workers^{10–15} have synthesized antisense oligos that substitute 2'-deoxy, 2'-fluoro- β -D-arabinonucleic acid (2'-F-ANA: arabinose is an epimer of ribose at C2') for deoxyribose in the oligo chain. Because the 2'-fluorine atom does not significantly perturb duplex helix structure, phosphorothioate 2'-F-ANA/RNA duplexes activate RNase H.^{12,13} Furthermore, this ability may even be greater for 2'-F-ANA/DNA phosphorothioate chimeras than for the corresponding deoxyribose phosphorothioate oligos.¹² In addition, the 2'-F-ANA modification dramatically increases oligo nuclease resistance,¹⁴ and increases the thermal stability of the duplex formed with its target mRNA by ~1 °C per 2'-F-ANA substitution.¹⁵ 2'-F-ANA substitution at the molecular termini also increases intracellular oligo retention, probably due to increasing nuclease resistance.¹²

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In this work, we demonstrate that 2'F-ANA gapmer phosphorothioate oligos, when targeted to the Bcl-2 and androgen receptor (AR) mRNAs in multiple cell lines in tissue culture, are approximately as effective at gymnotic silencing of gene expression as the LNA gapmers. In LNCaP prostate cancer cells, we demonstrate that silencing of the AR by a 2'F-ANA phosphorothioate oligo leads to downstream silencing of prostate-specific antigen (PSA), even in the presence of the androgenic steroid R1881 (metribolone), which stabilizes cytoplasmic levels of the AR. We further demonstrate that gymnotic silencing occurs in the absence of serum, and that silencing by both LNA and 2'F-ANA oligos is augmented in serum-free (SF) media in some cell lines when they are treated with oleic acid and a variety of ω -6 polyunsaturated fatty acids (ω -6 PUFAs), but not by an aliphatic fatty acid. These results significantly expand our understanding of and ability to successfully manipulate the cellular delivery of single-stranded DNA molecules *in vitro*.

Results

Initial experiments to determine whether 2'F-ANA phosphorothioate gapmer oligos targeted to Bcl-2 are active in gymnotic gene silencing were performed with the FAS2 oligo (six 2'F-ANA moieties at each molecular terminus) in 518A2 melanoma cells. The Bcl-2 mRNA target (codons 1–6) and 518A2 melanoma cells were chosen to demonstrate proof-of-principle because LNA phosphorothioate gapmer oligos (two LNAs at the 3' and 5' termini) have previously been shown to be highly potent at silencing in this system.⁵ An example of the silencing by the FAS2 oligo (single treatment on day 1) is shown in **Figure 1a**. SPC2996, a 3', 5' LNA gapmer of identical sequence to FAS2 also targeting the Bcl-2 mRNA is the control sequence. Gymnotic silencing with additional anti-Bcl-2 2'F-ANA oligos (S1 thru S6) was also performed in PC3 cells (**Figure 1b**). In these cells, the incubation time necessary for maximal silencing was 8 days. The S1 and S2 2'F-ANA oligos, similar to the FAS2 oligo, contained six 2'F-ANA moieties at the 3' and 5' molecular termini. However, since 2'F-ANA is nearly transparent to RNaseH (*i.e.*, unlike LNA, 2'-MOE, and morpholino oligos, 2'F-ANAs do not block RNase H cleavage activity¹⁰), an additional 2'F-ANA moiety could be inserted within the gap with minimal effects on gene silencing (compare S1 and S2 to LNA control SPC2996 and Fas2). In addition, the 2'F-ANA moieties did not need to be present at both the 3' and 5' termini for effective silencing (see S5 and S6), although shorter gapmers with fewer 2' F-ANAs [S3 (15 nucleotide) and S4 (12 nucleotide)] were relatively ineffective at silencing, in contrast to LNA gapmers of identical length, which are active. S5 and S6 are of the "altimer" design, where short contiguous regions of unmodified and modified nucleotides are alternated throughout the strand. When the modification is 2'F-ANA, these altimers are compatible with a mechanism of gene silencing involving RNase H-mediated mRNA cleavage.^{12,13} An additional control for specificity is shown in **Figure 1c**, in which PC3 cells were treated with either S1, S5, SPC2996, or SPC3046, an LNA gapmer oligo which is a scrambled version of SPC2996. As can be seen, SPC3046 did not silence.

After proving the principle that 2'F-ANA oligos could silence Bcl-2 gene expression gymnotically in 518A2 melanoma and PC3 prostate cancer cells, we then expanded the utility of

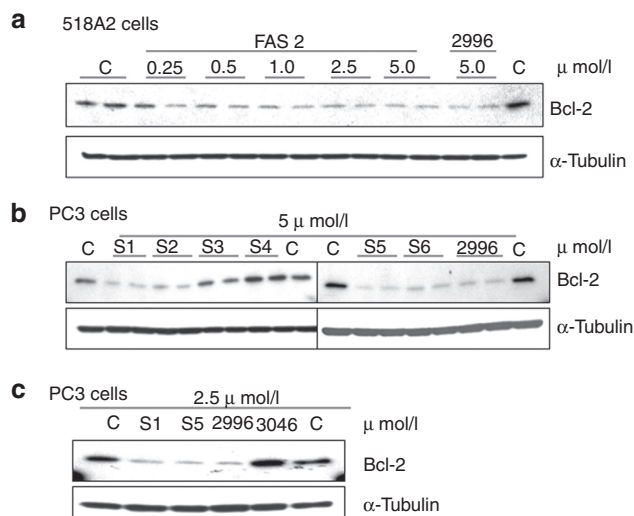


Figure 1 2'-Deoxy, 2'-fluoro- β -D-arabinonucleic acid (2'F-ANA) gap-mer phosphorothioate oligos targeted to the Bcl-2 protein are as effective at gymnotic silencing as the locked nucleic acid (LNA) gapmers. (a) Downregulation of Bcl-2 protein expression by the FAS2 2'F-ANA oligo in 518A2 melanoma cells. Cells were treated with or without increasing concentrations (0.25–5.0 μ mol/l) of FAS2 or the control SPC2996 LNA oligo and harvested on day 8, followed by western blotting. (b) Western blot analysis for Bcl-2 protein expression in PC3 cells after treatment with (S1 through S6) or without (c) 2'F-ANA oligos or the LNA gap-mer oligo SPC2996 (positive control). (c) Western blot analysis examining Bcl-2 silencing after treating PC3 cells with a 2.5 μ mol/l concentration of the most active 2'F-ANA oligos, S1 and S5. SPC2996 and SPC3046 (a scrambled version of SPC2996) were employed as control LNA oligos. In all experiments, the incubation time was 8 days and levels of α -tubulin were determined as a loading control. Decrease in Bcl-2 expression after normalization to control in: (c): S1, 84%; S5 90%; SPC3046, –17%.

these constructs by examining AR silencing in LNCaP prostate cancer cells. Translocation of the AR from the cytoplasm to the nucleus after testosterone or dihydrotestosterone binding in prostate cancer cells has been demonstrated to up-regulate the expression of pro-metastatic, pro-angiogenic, and anti-apoptosis genes.¹⁶ Inhibition of AR translocation/silencing of AR expression is currently an important goal in the experimental therapeutics of late stage clinical prostate cancer.¹⁷

Experiments were first performed in complete media, which contains small amounts of androgen. The positive control oligo for these experiments was 2629, a 20mer LNA phosphorothioate gapmer containing four LNAs at the 3' and 5' molecular termini.¹⁸ **Figure 2a** shows the concentration dependence of silencing in LNCaP cells (6-day incubation) by 2629; silencing is maximum at a concentration of 10 μ mol/l, but rapidly declines at lower oligo concentrations. The exquisite sequence dependence of silencing with 2629 is shown in **Figure 2b**. Here, a single base T-A substitution in 2629, forming 3054, is sufficient to virtually block gymnotic silencing under these experimental conditions. Scrambled versions of 2629, *e.g.*, 2882 and 2883, do not silence the AR in LNCaP cells under these conditions (5 μ mol/l, 6-day incubation), but the level of intracellular PSA protein, which is regulated by the AR at the transcriptional level, were markedly downregulated by 2629 treatment (**Figure 2c**). 2629 also potently silenced the AR mRNA at 5 μ mol/l, which is shown in **Figure 2d**.

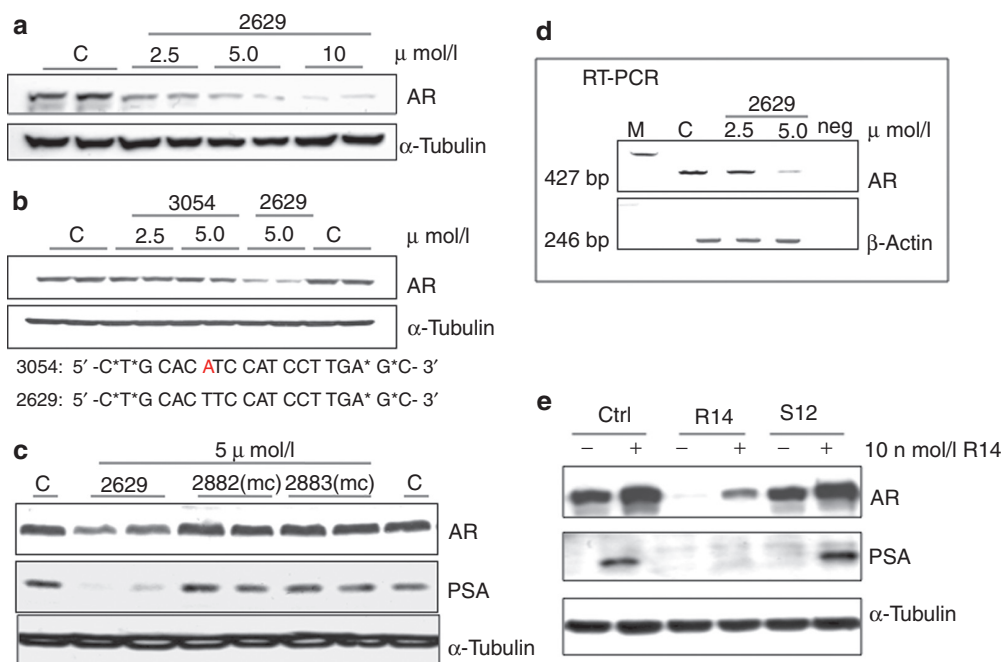


Figure 2 Gymnotic silencing of the androgen receptor (AR) in LNCaP prostate cancer cells. (a) Cells were treated with 2629 [a 20mer locked nucleic acid (LNA) phosphothioate gapmer oligo], at increasing concentrations (2.5–10 μmol/l). Western blot analyses were performed for AR protein after 6 days of incubation. α-Tubulin was the loading control. Average decrease in AR expression after normalization to control: 2.5 μmol/l, 50%; 5 μmol/l, 75%; 10 μmol/l, 90%. (b) A single base substitution in 2629 dramatically decreased the silencing of the AR. LNCaP cells were treated with or without 2629 (5 μmol/l) or with 3054 (2.5, 5 μmol/l), a single base (T-A) substitution in 2629. After a 6-day incubation, western blotting was performed for AR protein. α-Tubulin was the loading control. *LNA. (c) Western blots for AR protein and prostate-specific antigen (PSA). LNCaP cells were gymnotically treated with 2629 and with the oligos 2882, 2883 (each a scrambled version of 2629; mc = mutant control) at a concentration of 5 μmol/l and incubated for 6 days. (d) Reverse transcriptase-PCR (RT-PCR) evaluation of AR mRNA levels in LNCaP cells after gymnotic treatment with 2629 (5 μmol/l) and a 6 day incubation. (e) LNCaP cells were cultured in media containing charcoal-stripped serum media. After 16 hours of incubation the cells were treated with or without the (2′F-ANA) oligos R14 or S12 (scrambled version of R14) at a concentration of 5 μmol/l. R1881 (10 nmol/l) was then added and 6 days later western blots for AR and intracellular PSA were obtained. Average decrease in protein expression after normalization to control: AR: R14–, 99%; R14+, 65%; S12–, 10%; S12+, –5%. PSA: R14–, –15%; R14+, 70%; S12–, 70%.

In **Figure 2e**, LNCaP cells were treated with either R14 (six 2′F-ANA moieties on the 5′ terminus, and six on the 3′ terminus) or S12, a scrambled version of R14 that has no known target. Both of these oligos are 20mers. These experiments were performed in charcoal-stripped media to remove the majority of serum androgen; androgen was then added back in the form of the synthetic androgen R1881 (a.k.a metribolone; 10 nmol/l). R1881 has several functions,¹⁷ including: (i) stabilization of the AR against proteasomal degradation, thus prolonging its cytoplasmic half-life, and (ii) AR activation, involving induction of translocation of the AR from the cytoplasm to the nucleus, where it acts as a nuclear transcriptional regulatory factor for many genes and promotes PSA expression, as noted above. In the absence of R1881 (**Figure 2e**), R14 dramatically downregulated the expression of AR protein (5 μmol/l, 6 days) versus control, untreated cells. In contrast, and as expected, the scrambled oligo S12 did not produce AR silencing. Intracellular PSA expression is not observed in LNCaP cells in the absence of R1881, due to lack of AR activation (see (–) control lanes, and the (–) R1881 lanes of R14 and S12). However, when 10 nmol/l R1881 was added to these cells, PSA protein expression was easily observed (see (+) control lanes, and the (+) R1881 lane of

S12). However, PSA expression was essentially completely blocked when the LNCaP cells were treated with R14, due to the silencing of AR expression, which binds to androgen response elements in the PSA promoter. As also shown, the extent of AR protein silencing by R14 in the presence of R1881 was markedly less than in its absence. This was almost certainly due to the prolongation of the cytoplasmic half-life of the AR by this androgenic steroid.

Similar to what was done for the anti-Bcl-2 2′F-ANA oligos, anti-AR oligos were synthesized based on the sequence of R14 that contained 2′F-ANAs in the gap. As shown in **Figure 3a**, S10, which contained six 2′F-ANAs at the 3′ and 5′ termini, and one 2′F-ANA in the gap, reduced AR protein levels to 20% of control (untreated) protein expression in LNCaP cells (5 μmol/l, 8 day incubation). S12, the scrambled control, was inactive. However, S9, which also contained one 2′F-ANA in the gap, was somewhat less active under the identical conditions (reduction to 40% of control expression). Neither S11 nor S13, which contained alternating 2′F-ANA and deoxyribose phosphorothioates, were active, unlike what was observed with anti-Bcl-2 oligos of the same chemical composition. No treatment with any oligo employed in **Figure 3a** led to cleavage of PARP-1, a very sensitive measure of apoptosis induction in LNCaP cells, and an event that invariably occurs

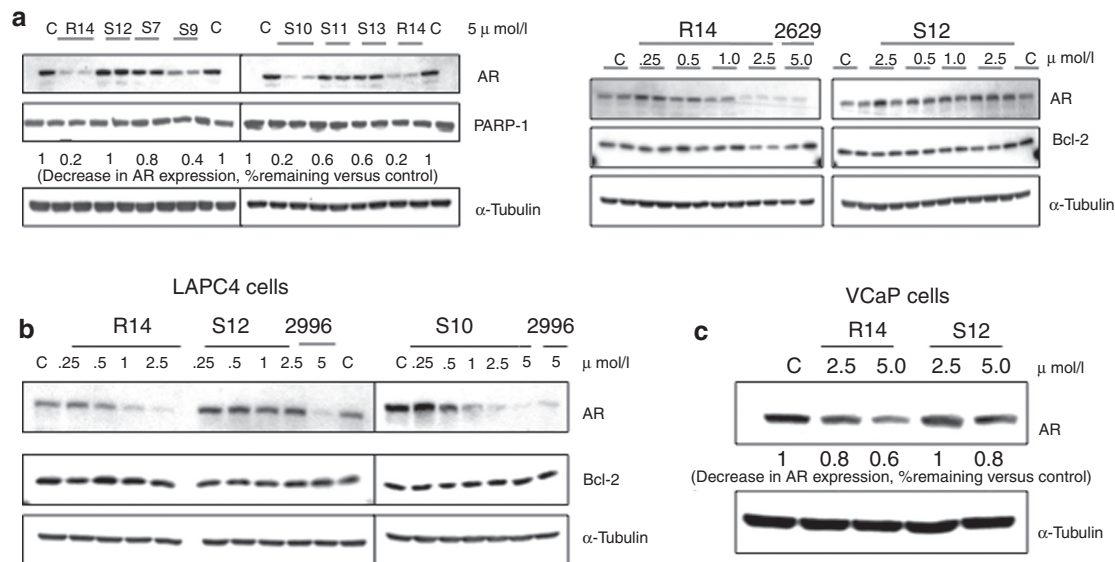


Figure 3 Effect of anti-androgen receptor (AR) 2'F-ANA oligos on AR protein expression in LNCaP, LAPC4, and VCaP cells. (a) Left panel: LNCaP cells were gymnotically treated with the additional indicated 2'F-ANA oligos at a concentration of 5 $\mu\text{mol/l}$ and incubated for 8 days. Western blots were then obtained for AR protein and PARP-1 expression. The percentage of AR inhibition was calculated by comparison with non-treated control. Right panel: Concentration dependence of the levels of AR and Bcl-2 protein expression after gymnotic delivery of R14 and S12 (scrambled R14) 2'F-ANA oligos in LNCaP cells. (b) Western blot in LAPC4 cells demonstrating the concentration dependence of the silencing of the AR protein after 8 days gymnotic delivery of the R14 and S10 2'F-ANA oligos. Average decrease in AR protein expression after normalization to control: R14: 0.25 $\mu\text{mol/l}$, 0%; 0.5 $\mu\text{mol/l}$, 10%; 1 $\mu\text{mol/l}$, 50%; 2.5 $\mu\text{mol/l}$, 80%. S12: 0.25 $\mu\text{mol/l}$, -10%; 0.5 $\mu\text{mol/l}$, -30%; 1 $\mu\text{mol/l}$, -20%; 2.5 $\mu\text{mol/l}$, -20%; SPC2996, 50%. S10: 0.25 $\mu\text{mol/l}$, -10%; 0.5 $\mu\text{mol/l}$, 30%; 1 $\mu\text{mol/l}$, 65%; 2.5 $\mu\text{mol/l}$, 80%; 5 $\mu\text{mol/l}$, 90%. (c) Same as 3b, but in VCaP prostate cancer cells (8 days gymnosis); in all experiments, α -tubulin was determined as a loading control.

in LNCaP cells when phosphorothioate antisense oligos are transfected by lipid particulates. In LAPC4 cells (Figure 3b), which express the wild-type AR (as opposed to LNCaP cells, which express a T877A mutated AR), both S10 and R14 are highly active. However, in VCaP cells, which also express the wild-type AR, and which proliferate slowly compared to other prostate cancer cell lines, R14 was only minimally active at 5 $\mu\text{mol/l}$ (Figure 3c), and demonstrated modest activity only at 7.5 $\mu\text{mol/l}$ (8 days incubation; not shown). However, this was associated with diminished α -tubulin expression (data not shown).

Gymnosis occurs under SF conditions and is stimulated by oleic acid and PUFAs

Treatment of A431 squamous carcinoma cells with 5 $\mu\text{mol/l}$ SPC2993 (the exact complement of the Bcl-2 mRNA codons 1–6) in complete media under standard gymnosis conditions very rapidly led to a sharp diminution in Bcl-2 protein expression (IC₅₀ = 1 day; IC₉₀ = 2 days; Figure 4a). Repetition of this experiment at the identical cell density in SF media with the addition of insulin/transferrin/selenium also led to an IC₉₀ of 2 days (Figure 4b). In PC3 cells in SF media to which was added either 0.25 g/dl bovine serum albumin (BSA) or 0.25 g/dl fatty acid-free albumin (FAFA), gymnotic silencing of Bcl-2 proceeded with SPC2993 to the same extent and with the same kinetics as that observed in complete media, *i.e.*, the IC₉₀ of silencing was achieved after ~6 days (Figure 4c and d). Gymnotic silencing in PC3 cells under SF conditions was also observed after treatment with the S1 anti-Bcl-2 2'F-ANA gapmer (3 and 6 days; Figure 4e).

The S5 2'F-ANA oligo was also gymnotically active under these conditions, but a scrambled version of S1 was completely inactive. As also can be seen in Figure 4e, under SF conditions, as had previously been seen in complete media, silencing is substantially vitiated below an oligo concentration of 2.5 $\mu\text{mol/l}$ (Figure 4e).

Unlike when the oligos are lipid-encapsulated, where concentrations in the nanomolar range can produce dramatic gene-silencing, low micromolar concentrations are virtually always required for gymnotic silencing. Accordingly, we then designed a series of experiments, employing both LNA and 2'F-ANA oligos that were designed to reduce the IC₅₀ of gymnotic gene silencing. Our previous work had demonstrated¹⁹ that the addition of free oleic acid and other ω -6 PUFAs to K562 and other cells in SF media-containing FAFA could increase the intracellular concentrations of 5'-fluorescein unmodified phosphorothioate oligos. Accordingly, we treated PC3 (Figure 4f) and HT1080 cells (Figure 4g) in SF media + FAFA (2.5 g/dl) \pm 150 $\mu\text{mol/l}$ oleic acid for 6 days with the anti-Bcl-2 SPC2993 before determining normalized Bcl-2 protein expression by Western blotting. In both cell lines, the IC₅₀ of gymnotic gene silencing was decreased by ~50%. In neither cell line, as determined by MTT assay, was oleic acid shown to be toxic. Experiments employing the S1 anti-Bcl-2 2'F-ANA oligo in SF media \pm 150 $\mu\text{mol/l}$ oleic acid produced similar results (Figure 4h). However, oleic acid, even at high (up to 1.5 mmol/l) concentrations, was not active in all cell lines tested (*e.g.*, A431 cells). These experiments were then repeated in PC3 cells with the arachidonic and linoleic acids, both ω -6 PUFAs, and with the aliphatic C16 fatty acid palmitic

acid. As shown in **Figure 4i**, both the ω -6 PUFAs, but not palmitic acid, increased the potency of gymnotic silencing by SPC2993 relative to control (SF media plus FAFA alone).

Discussion

This work demonstrates that 2′F-ANA gapmer oligos, in addition to LNA gapmer oligos, are also gymnotically active, and to a similar extent (based on our previous work⁵) in the cell lines we examined. Standard first generation phosphorothioate oligos that are not further chemically modified by either LNA or 2′F-ANA are in our experience¹ generally not gymnotically active. The exception to this statement is the 18mer unmodified all-phosphorothioate G3139, which forms an unusually high melting dimer with its complementary RNA strand. Gymnotically active oligos also seem to require a relatively strictly defined set of attributes: (i) The phosphorothioate modification must be present in at least 75% of all phosphate linkages, probably at least in part because high phosphorothioate content promotes adsorptive endocytosis (the intracellular delivery “mechanism”^{20,21}). This may be due to high-affinity ($K_m < 50$ nmol/l) PS oligo binding to heparin-binding proteins on the cell surface²² and subsequent formation of intracellular vesicles containing oligo (although the manner in which oligos exit the endosomes, while critical to our understanding of oligonucleotide therapeutics in general, is currently not well characterized). High phosphorothioate content also retards exo- and endonucleolytic nuclease degradation, in addition to reducing renal clearance through low-affinity plasma albumin binding.²³ (ii) However, additional chemical modification of the phosphorothioate oligo appears to be required to further diminish nuclease sensitivity. (iii) The additional chemical modification (LNA or 2′F-ANA) seems to be required to at least partially compensate for the lowering of the duplex T_m by the phosphorothioate modification. All three criteria are all met at least in part by both these two modifications, but not, for example, by 3′, 5′ substituted 2′-O-methyl or methylphosphonate gapmers, which in our hands were gymnotically inactive.

In our original gymnotic experiments,⁷ which were performed in complete media, we observed that if cells were allowed to become confluent during the course of the experiment, gene silencing was severely diminished. However, we have presented data herein that demonstrates that silencing can occur in various cell types in the absence of serum, when cell proliferation essentially ceases. This is probably because the endocytosis rate in these nonproliferating cells still remains high, permitting the delivery of the oligo to the interior of cells after robust adsorptive endocytosis. Indeed, these observations may relate to the most puzzling and difficult problem in the area of oligonucleotide therapeutics: Why, despite the proven ability of oligos to silence gene expression in the absence of carriers in tissue culture experiments, have clinical successes (with nonencapsulated or even encapsulated oligos), particularly in cancer, been so meager after decades of such great effort? As has been long noted, malignant cells in solid tumors are located in a vastly different physiologic environment than malignant cells in tissue culture. Solid tumor masses frequently have heterogeneous blood flow. The resulting tissue hypoxia causes, for example, upregulation

of the nuclear transcriptional regulatory factor Hif-1 α and its downstream effector molecule vascular endothelial growth factor.²⁴ In one model, proangiogenic vascular endothelial growth factor produces tumor neovascularization, but the new vessels are defective: tortuous, fenestrated, leaky.²⁵ These vascular properties lead to hemoconcentration, sludging of red cells in the neo-vessels, and subsequent further tissue hypoxia and hence additional vascular endothelial growth factor-mediated defective neovascularization. The leaked plasma proteins from defective neo-vessels contribute to a negative oncotic pressure surrounding the tumor mass that further retards ingress of red cells, oxygen, and blood plasma which carries nutrients and therapeutic agents (e.g., nucleic acids).²⁶ Severe hypoxia also leads eventually to decreased intratumoral pH, the removal of cells from cycling, and to cellular dormancy and eventually necrosis. All of these factors may contribute to lack of cellular uptake of oligos via changes in the density of the cell surface heparin-binding proteins required for phosphorothioate oligo binding and subsequent adsorptive endocytosis, on the intrinsic rate of endocytosis itself, on the ability of the oligo to exit the endosome and be transported to the cell nucleus, or on a combination of all these factors, in addition to those not currently recognized. The role of phosphorothioate oligo charge in the *in vivo*, as opposed to the *in vitro* internalization process is also not understood. At this point, 25 years in, there seems little doubt that an improved understanding of oligo delivery mechanisms could only help clinically rejuvenate this promising therapeutic approach for cancer.

Apropos, because the *in vitro* oligo concentrations required for optimal gene silencing are relatively high and required incubation times long with respect to what is probably physiologically relevant, we searched for ways to decrease oligo concentrations and incubation times. Felber *et al.*²⁷ have recently observed that gene silencing by 2′F-ANA oligos conjugated to lipophilic moieties (e.g., cholesterol, docosahexaenoic acid, docosanoic acid), in the absence of transfection vehicles but in the presence of BSA, can be augmented by the addition of decanoic acid. It was postulated that the decanoic acid competed for albumin-binding sites with the lipid-oligo conjugate, thus raising the local free oligo concentration and promoting cell surface-binding and adsorptive endocytosis. However, by increasing free oligo concentrations physiologically, renal clearance may also be augmented, perhaps vitiating the effect of the decanoic acid. Therefore, because of our older observations¹⁹ that ω -6 PUFAs and the ω -9 monounsaturated oleic acid increased phosphorothioate oligo internalization, we returned to this method to try to potentiate gymnotic silencing, and met with at least modest successes. However, the mechanism underlying these modest successes is undoubtedly quite complex. For example, oleic acid appears to be a direct activator of phospholipase D2 (PLD²⁸), an enzyme involved in membrane lipid remodeling during vesicular trafficking.²⁹ PLD, produces phosphatidic acid from diacyl phosphatidylglycerols; phosphatidic acid may alter membrane curvature, and is the substrate for the production of other signaling lipids in the contexts of vesicle trafficking and cytoskeletal dynamics.³⁰ PLD activity dramatically increases in the presence of a number of unsaturated fatty acids (e.g., oleate,^{28,31} arachidonate, linoleate²⁸) but not in the

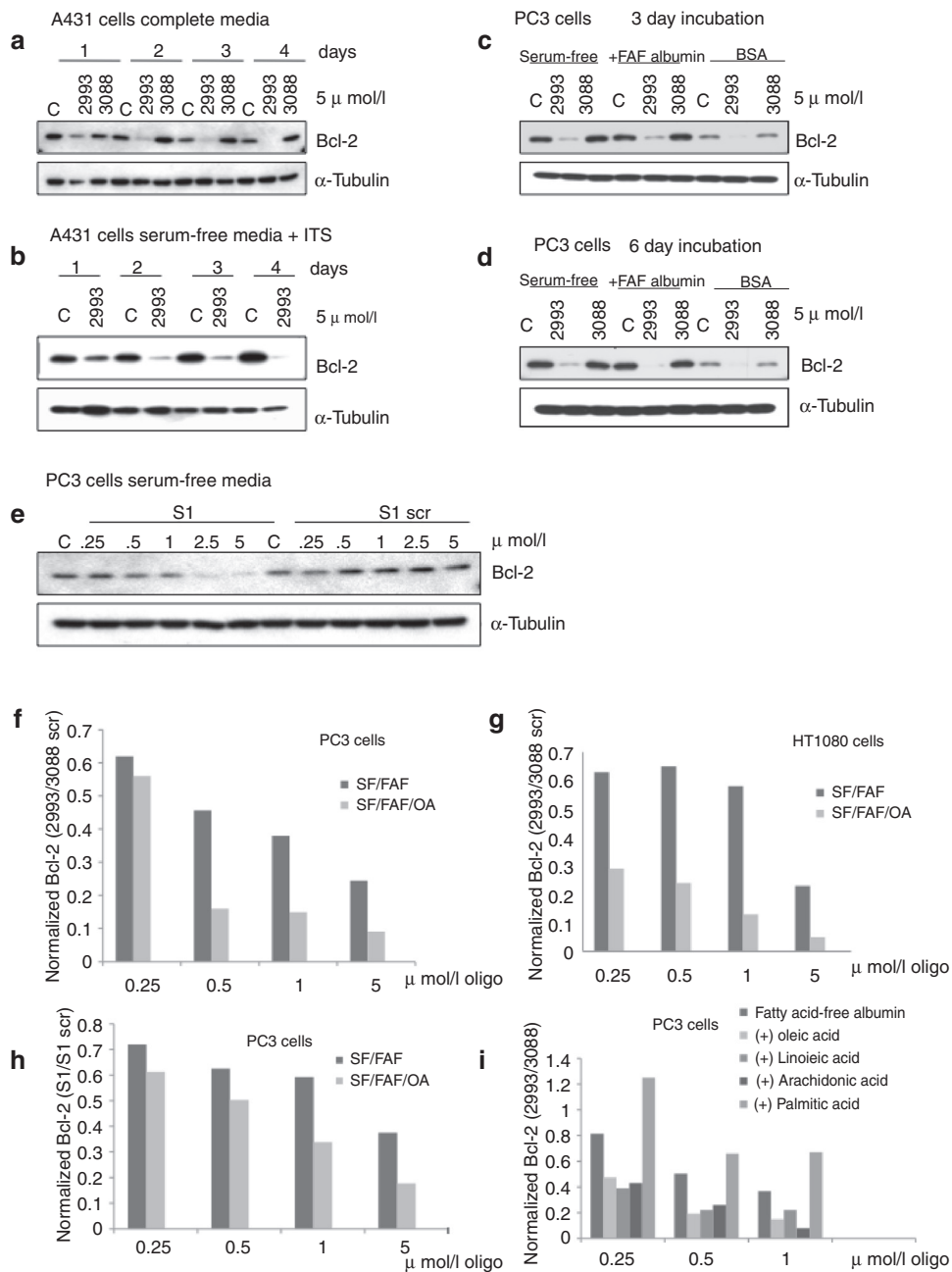
presence of saturated fatty acids (e.g., palmitate, stearate²⁸), which correlates with our observations that unsaturated, but not saturated fatty acids modulate gymnotic gene silencing. We have also shown that phosphorothioate oligo internalization can be PKC- ζ -dependent,¹⁹ and PKC- ζ activity has been in at least some cell lines shown to stimulate the activity of PLD.³² Curiously, PLD activity is high in kidney, liver, and small intestine,³³ which are the sites of probably the highest rates of physiologic oligo internalization, though correlation here does not prove causation.

In summary, gymnotic gene silencing *in vitro* is readily accomplished by 2'F-ANA phosphorothioate gapmer oligos, similar to what has been shown for LNA gapmer oligos.⁷ Gymnosis, for both the LNA and 2'F-ANA gapmers, occurs

under SF conditions, and is stimulated both by ω -6 PUFAs and the ω -9 monounsaturated fatty acid oleic acid, perhaps by a mechanism that involves PLD. A further understanding of the cellular factors that contribute to the gymnotic process will be invaluable in augmenting its gene silencing potential.

Materials and methods

Oligonucleotides. LNA oligos SPC2996, 2993, 3088, and 3046 were obtained from Santaris (Horsholm, DK). 2629, 3054, 2882, and 2883 were obtained from Jesper Wengel (U. Southern Denmark, Odense, DK).



Synthesis of 2′F-ANA antisense oligos. Standard phosphoramidite solid-phase synthesis conditions were used for the synthesis of all modified and unmodified oligonucleotides.¹ Syntheses were performed on an Applied Biosystems (ABI, Foster City, CA) 3400 DNA Synthesizer at a 1- μ mol scale using Unylink CPG as the solid support (ChemGenes, Wilmington, MA). 2′F-ANA phosphoramidites were prepared as 0.15 mol/l solutions in dry acetonitrile (ACN), and DNA phosphoramidites were prepared as 0.1 mol/l in dry ACN. 5-Ethylthiotetrazole (0.25 mol/l in ACN; ChemGenes) was used to activate the phosphoramidites for coupling. Detritylations were accomplished with 3% trichloroacetic acid in CH_2Cl_2 (DCM) for 110 seconds. Capping of failure sequences was achieved with acetic anhydride in tetrahydrofuran and 16% *N*-methylimidazole in tetrahydrofuran. Oxidation was done using 0.1 mol/l I_2 in 1:2:10 pyridine:water:tetrahydrofuran. Sulfurizations of phosphorothioate backbone oligos were accomplished using a 0.1 mol/l solution of xanthane hydride (TCI) in 1:1 vol/vol pyridine/ACN (anhydrous). The sulfurization step was allowed to proceed for 2.5 minutes, with new sulfurization reagent added to the column after 1.25 minutes. Phosphoramidite coupling times were 600 seconds for 2′F-ANA, with the exception of the guanosine phosphoramidite, which was allowed to couple for 900 seconds. DNA coupling times were 110 seconds, and 270 seconds for guanosine.

Cleavage and purification. Deprotection and cleavage from the solid support was accomplished with 1 ml of 3:1 aqueous $\text{NH}_4\text{OH}:\text{EtOH}$ for 48 hours at room temperature. The oligo solution was decanted from the solid support, vented for 2 hours, cooled on dry ice, and then cleavage solution was removed under vacuum. Purification of crude oligos was done either by preparative denaturing polyacrylamide gel electrophoresis using 24% acrylamide gels, or by reverse phase HPLC on a Waters 1525 HPLC using a Varian Pursuit 5 semipreparative reverse phase C18 column with a stationary phase of 100 mmol triethylammonium acetate in water with 5% ACN (pH 7), and a mobile phase of HPLC-grade acetonitrile (Sigma-Aldrich, St Louis, MO). In the case

of gel-purified oligonucleotides, gel bands were extracted overnight in DEPC-treated autoclaved Millipore water on a shaker, and lyophilized to dryness. Gel-purified oligos were desalted with Nap-25 Sephadex columns from GE Healthcare (Wakesha, WI) according to standard protocol before use, and all oligos were quantitated by UV (2′F-ANA oligonucleotide extinction coefficients were calculated using DNA values according to the online IDT OligoAnalyzer tool (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). Antisense oligonucleotides were characterized by LC-MS on a Waters Q-TOF2 using an ESI NanoSpray source. A CapLC (Waters, Milford, MA) with a C18 trap column was used for LC prior to injections. The sequences of all oligos used in this work are given in **Table 1**.

Cell lines. The 518A2 mycoplasma-free human melanoma cell line, a kind gift of Dr Volker Wachek (University of Vienna, Austria) and VCap cells (CRL-2876; ATCC, Rockville, MD) were grown in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen), 2 mmol/l L-glutamine, and 100 U/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate (Sigma-Aldrich). PC3, LNCaP, and A431 cells (all from ATCC) were maintained in RPMI-1640 media (Invitrogen) supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate, 1% sodium pyruvate (Sigma-Aldrich), 1% nonessential amino acids (Sigma-Aldrich) at 37 °C in a humidified 5% CO_2 incubator. LAPC-4 cells, a generous gift of Dr R. Reiter (UCLA, Los Angeles, CA), were maintained similarly, but in IMDM supplemented with 5% heat inactivated FBS. HT-1080 fibrosarcoma cells (ATCC) were grown in DMEM/F12 media (ATCC) supplemented with 10% FBS, U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, 2 mmol/l L-glutamine.

Materials. The anti-Bcl-2 monoclonal antibody and the mouse antihuman PSA antigen (PSA; clone ER-PR8) were purchased from Dako (Carpinteria, CA); the anti- α -tubulin monoclonal antibody was purchased from Sigma-Aldrich;

Figure 4 Gymnosis occurs under serum-free conditions and is stimulated by oleic acid (OA) and ω -6 polyunsaturated fatty acids (ω -6 PUFAs). (a) Time course of Bcl-2 protein silencing in A431 cells after gymnotic delivery of SPC2993 and SPC3088 locked nucleic acid (LNA) gapmer oligos at 5 μ mol/l in complete media. Downregulation (50%) of Bcl-2 protein expression was observed after 24 hours of incubation. Decrease in Bcl-2 protein expression after normalization to control: SPC2996: day 1, 35%; day 2, 55%; day 3, 65%; day 4, 97%. SPC3088: day 1, 25%; day 2, -75%; day 3, -55%; day 4, -25%. (b) Gymnotic delivery of SPC2993 (5 μ mol/l) to A431 cells cultured in serum-free media with addition of 1% ITS also demonstrated robust (IC90 = 2 days) downregulation of Bcl-2 protein expression. Decrease in Bcl-2 protein expression after normalization to control: day 1, 60%; day 2, 85%; day 3, 80%; day 4, 99%. (c) Gymnotic delivery of SPC2993 and SPC3088 at 5 μ mol/l to PC3 cells cultured either in serum-free (SF) media or in SF media plus fatty acid-free (FAF) albumin (2.5 g/dl) or in SF media plus bovine serum albumin (BSA) (2.5 g/dl). Western blots demonstrate approximately equal levels of downregulation of Bcl-2 protein expression after 3 and (d) 6 days of incubation. (e) Western blots in PC3 cells demonstrating concentration dependence of the levels of Bcl-2 protein expression after 6 days of gymnotic delivery of the S1 and S1 (scrambled) 2′F-ANA oligos incubated in serum-free media. (f) Oleic acid stimulates gymnosis in PC3 cells. Cells were cultured in complete media and then for 24 hours in serum-free media containing fatty acid-free albumin (2.5 g/dl) in the presence or absence of oleic acid (150 μ mol/l). After 2 hours, the cells were treated with the LNA gapmer oligos SPC2993 and SPC3088 at increasing concentrations for 6 days, after which time western blots for Bcl-2 protein expression were obtained. Data were analyzed by the Students dependent t-test for paired samples, $P = 0.037$. (g) Similar experiments as in (f) were performed in HT1080 cells. Data were analyzed by the Students dependent t-test for paired samples, $P = 0.009$. (h) Gymnotic delivery (6 days) of the S1- and S1-scrambled (S1-scr) 2′F-ANA oligos at increasing concentrations in PC3 cells cultured serum-free media plus fatty acid-free albumin (2.5 g/dl) in the presence or absence of oleic acid (150 μ mol/l). Levels of Bcl-2 protein expression were determined by western blotting. Data were analyzed by the Students dependent t-test for paired samples, $P = 0.02$ (i) Gymnotic silencing of Bcl-2 protein expression is stimulated in the presence of ω -6 polyunsaturated fatty acids (ω -6 PUFAs). PC3 cells were cultured in complete media for 24 hours, and then in serum-free media containing fatty acid-free albumin (2.5 g/dl) in the presence or absence of oleic acid (150 μ mol/l) or ω -6 PUFAs [linoleic acid (100 μ mol/l) or arachidonic acid (100 μ mol/l)] or the C16 aliphatic fatty acid palmitic acid (100 μ mol/l). Cells then were gymnotically treated with SPC2993 or SPC3088 at increasing concentrations, as indicated. Western blot analyses for levels of Bcl-2 protein expression were obtained after 6 days of incubation. Data (oleic versus palmitic acid) were analyzed by the Students dependent t-test for paired samples, $P = 0.03$.

Table 1 Sequences of oligonucleotides used in this work

Bcl-2 targeting		Androgen receptor targeting	
<i>LNA oligonucleotide sequences</i>			
5'- CTC CCA ACG TGC GCC A -3'	SPC2996	5'-CTG CAC TTC CAT CCT TGA GC-3'	2629
5'- CTC CCA GCG TGC GCC A -3'	SPC2993	5' -CTG CAC ATC CAT CCT TGA GC-3'	3054
5'- CGT CAG TAT GCG AAT C -3	SPC3088	5'- AGG CAC TTC CAT CCT TGA CG -3	2883
5'- CGC AGA TTA GAA ACC T -3'	SPC3046	5' -TTG CTC ACT AGG TGA CGA TA-3'	2882
<i>2' F-ANA oligonucleotide sequences</i>			
5'- TCT CCC AGC GTG CGC CAT -3'	Fas2	5' - CTG CAC TTC CAT CCT TGA GC -3'	R14
5'- TCT CCC AGC GTG CGC CAT -3'	S1	5'- TTA ATT TCA GAAATC AAG TT -3	S12
5'- TCT CCC AGC GTG CGC CAT -3'	S2	5' - CTG CAC ATC CAT CCT TGA GC -3'	S7
5'- TCT CCC AGC GTG CGC -3'	S3	5' - CTG CAC TTC CAT CCT TGA GC -3'	S9
5'- TCT CCC AGC GTG -3'	S4	5' - CTG CAC TTC CAT CCT TGA GC -3'	S10
5'- TCT CCC AGC GTG CGC CAT -3'	S5	5' - CTG CAC TTC CAT CCT TGA GC -3'	S11
5'- TCT CCC AGC GTG CGC CAT -3'	S6	5' - CTG CAC TTC CAT CCT TGA GC -3'	S13
5'- CGC AGA TTA GAA ACC TTT	S1-scr	5' - CTG CAC TTC CAT CCT TGA GC -3'	Fas5

Abbreviations: 2'F-ANA, 2'-fluoro-β-D-arabinose; LNA, locked nucleic acid. DNA, LNA, 2'F-ANA. All oligonucleotides shown have phosphorothioate backbones.

the mouse monoclonal anti-AR was obtained from BioGenex (San Ramon, CA); the mouse anti-PARP antibody (clone F-2) was from Santa Cruz Biotechnology (Santa Cruz, CA). FAFA, oleic, linoleic, arachidonic, and palmitic acids were obtained from Sigma-Aldrich. ITS (insulin/transferin/selenium) was purchased from BD Biosciences (Bedford, MA). R1881 was obtained from Perkin-Elmer (Boston, MA).

Gymnotic delivery of antisense oligos to cells. Cells were seeded in complete media the day before the treatment with either the 2'F-ANA or LNA gapmer phosphorothioate oligos in 6-well plates at low density, so as to be confluent on the last day of the experiment. Depending on the cell line and the goal of the experiment, the total incubation time before harvest was from one up to 8 days.

Gymnosis under SF condition. A431 squamous carcinoma cells were seeded in a 6-well plates at a density 8×10^4 cells/well in 2-ml complete media containing 10% FBS and incubated at 37 °C. After 24 hours the media was aspirated, and the attached cells washed with phosphate-buffered saline and then 2-ml SF RPMI-1640 media supplemented with 1% ITS were added to each well. After an overnight incubation at 37 °C, the cells were treated with either SPC2993 or SPC3088 (5 μmol/l) and allowed to incubate for the stated times before harvest. Similar conditions with the exception of the initial cell density (3×10^4 per well) and incubation time (6 days) were used also for PC3 cells. Gymnosis was also performed in SF-media containing either 2.5 g/dl BSA or 2.5 g/dl FAFA. PC3, LNCaP, LAPC4, A431, or HT1080 cell lines were seeded in 6-well plates in media containing 5–10% FBS, and after incubation for 24 hours at 37 °C, were washed with phosphate-buffered saline and 2 ml of SF media-containing either BSA or FAFA as above. In some experiments, oleic acid (150 μmol/l) were added to each well following by treatment with SPC2993,

SPC 3088, or S1 oligos at the indicated concentrations. The incubation time for all cell lines was 6 days, except for A431 (3 days). In PC3 cells, the same experiment was repeated with linoleic, arachidonic, and palmitic acids (150 μmol/l) followed by treatment with SPC2993 and SPC3088 at the indicated concentrations.

Western blot analysis. Cells, whether or not treated with oligos, were scraped off the plates, washed once with phosphate-buffered saline, and suspended in lysis buffer for 1 hour at 4 °C. Cell debris was removed by centrifugation at 14,000 rpm for 20 minutes at 4 °C. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Total amounts of protein were determined by the method of Bradford. 30–40 μg of total protein were mixed with 5× sample buffer (12.5 mmol/l Tris (pH 6.8), 10% sodium dodecyl sulfate, 0.5% bromophenol blue, 5 mmol/l dithiothreitol, 50% glycerol), denatured at 95 °C for 5 minutes, and separated on a 10% polyacrylamide gel. Proteins were then transferred onto Hybond ECL filter paper (Amersham, Arlington Heights, IL), and blots were incubated sequentially with 1% nonfat dry milk or 1% BSA at room temperature for at least 1 hour, followed by incubation at 4 °C with the antibodies of the following dilutions: a mouse monoclonal anti-AR antibody (1:250), a mouse monoclonal antihuman Bcl-2 antibody (1:500), a mouse monoclonal anti-α-tubulin antibody (1:4,000), a mouse antihuman PSA antibody (1:1), a mouse anti-PARP antibody (1:200) overnight. Blots were then washed and incubated with a secondary horse-radish peroxidase-conjugated anti-mouse immunoglobulin G (1:3,000) (Santa Cruz Biotechnology) for an 1 hour at room temperature, and evaluated using the enhanced chemiluminescence system from Amersham. Blots were scanned and quantitated by laser scanning densitometry. The typical margin error for a western blotting is at least 20–25%.

Reverse transcriptase-PCR. Total RNA was isolated from cells using QIAGEN's RNeasy kit (QIAGEN, Valencia, CA) and quantitated by UV absorbtion. One microgram of the total RNA was reverse-transcribed using SuperScript One-Step reverse transcriptase PCR with Platinum Taq (Invitrogen). cDNA fragments of AR or β -actin were amplified using the following primers: forward AR 2047/20 (5'-ATGGTGAGCAGAGTGCCTA-3'), reverse AR 2474/20 (5'-GTGGTGCTGGAAGCCTCTCC-3'); forward β -actin (5'-GAGCTGCGTGTGGCTCCCGAGG-3') and reverse β -actin (5'-CGCAGGATGGCATGGGGAGGGCATACCCC-3'). The AR and β -actin amplicons were 427 and 246 bp, respectively. Amplification was performed on a thermocycler [GeneAmp PCR System 9700 (Applied Biosystems)] for 35 cycles according to the following program: 30 seconds at 94 °C, 30 seconds at 60 °C, 1 minute at 72 °C. PCR products were separated electrophoretically in a 1.5% agarose gel. Bands were visualized by ethidium bromide staining.

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