Novel single-stranded oligonucleotides that inhibit signal transducer and activator of transcription 3 induce apoptosis in vitro and in vivo in prostate cancer cell lines

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Abstract

Signal transducers and activators of transcription (STAT) were originally discovered as components of cytokine signal transduction pathways. Persistent activation of one of these transcription factors, STAT3, is a feature of many malignancies, including hormone-resistant prostate cancer. In this regard, malignant cells expressing persistently activated STAT3 become dependent on it for survival, thus rendering STAT3 a potential molecular target for therapy of hormone-resistant prostate cancer. Previously, we reported that antisense oligonucleotides specific for STAT3 were better at inducing apoptosis than inhibitors of JAK1 or JAK2, the upstream activating kinases of STAT3. Here, we report that novel single-stranded oligonucleotides, which putatively block STAT3-DNA binding, were better at inducing hormone-resistant prostate cancer apoptosis than antisense STAT3 oligonucleotides. We observed that the novel STAT3-inhibiting oligonucleotides induced apoptosis by a mitochondrial-dependent pathway involving the activation of caspase-3. Prostate cell lines not expressing persistently activated STAT3 did not become apoptotic after treatment with these same oligonucleotides. Scrambled-sequence control oligonucleotides had none of the effects of the active sequence oligonucleotides on any variable measured. Furthermore, the novel STAT3-inhibiting oligonucleotides, but not scrambled-sequence control oligonucleotide, significantly reduced the volume of s.c. DU145 tumors in vivo. Histologic examination of the tumors revealed no infiltrate of mononuclear or granulocytic cells, which would be indicative of evocation of a nonspecific immune response by the oligonucleotides. We conclude that single-stranded oligonucleotides based on the binding sequences of STAT3 are an additional strategy to design inhibitors for this molecular target and that these inhibitors should be useful as experimental therapeutics for hormone-resistant prostate cancer. [Mol Cancer Ther 2004;3(10):1183–91]

Introduction

Prostate cancer is the most frequently diagnosed noncutaneous malignancy of males in the United States, affecting 35% of all American men (1, 2). Androgens play a critical role in the tumorigenic process, with activity mediated by the androgen receptor. Initially, prostate cancer is androgen sensitive, and most patients respond to androgen-ablation therapy. However, there are side effects to this therapy that make it unpleasant for the patient (3). Even with androgen-ablation therapy, the disease often recurs in androgen-refractory form (4). Androgen-ablation therapy may benefit the patient in the short-term, but it apparently has no effect on relapse-free long-term survival (3). When prostate cancer recurs, treatment consists of cytotoxic chemotherapeutic agents, which due to their high nonspecific cytotoxicity have narrow therapeutic indices and many undesirable side effects. Moreover, androgen-resistant prostate cancer does not respond well to cytotoxic agents and has a high mortality rate. Therefore, finding a therapy that (a) does not have the side effect of androgen-ablation therapy and (b) is effective on the recurring, androgen-resistant tumor would have major impact on the prostate cancer patient population.

Transcription factors are proteins that bind to the genome and either induce or repress gene expression. After activation, transcription factors bind to specific sequences on the genome upstream or near the promoter region of the gene regulated by the transcription factor. Signal transducers and activators of transcription (STAT) are part of the signal transduction pathway of many growth factors and cytokines and are activated by phosphorylation of tyrosine and serine residues by upstream kinases (5). For example, signaling by IL-6 generally induces phosphorylation of STAT3 (5). In benign cells, the signaling by STAT3 is under tight regulation, so that the signal is transient. However, aberrant signaling by STAT3 is found in many types of malignancies: multiple myeloma, head and neck cancer, breast cancer, prostate cancer, etc. (6–10).
Malignant cells expressing persistently activated STAT3 become dependent on it for survival; disruption of activation or expression of STAT3 resulted in apoptosis (7–9, 11–13). STAT3 binds to two known sequences, HSIE and GAS (14–18), through which its antiapoptotic and oncogenic effects are directed (19, 20).

Recently, we showed that human and rat prostate cancer lines were sensitive to treatment with antisense STAT3 oligonucleotides; 48 hours after treatment, most of the cells were apoptotic, but few cells treated with sense oligonucleotide were affected (13). However, antisense as a therapeutic category has shown a propensity for nonspecific activation of the immune system, mainly through unmethylated CpG motifs that are recognized erroneously as bacterial or viral in origin (21). Therefore, we decided to create a new strategy for inhibiting STAT3 activity, not activation. Because STAT3 binds to discrete, known sequences on the genome, we thought to develop a novel anticancer therapeutic based on the STAT3 binding sequence. We postulated that introducing an excess amount of either strand of a consensus binding sequence, in the form of a single-stranded oligonucleotide, could induce apoptosis. In this article, we report that both complementary strands of the binding sequence of STAT3 induced apoptosis in prostate cancer cells in vitro and furthermore reduced mean tumor volumes in vivo. This article describes an exciting, novel approach to prostate cancer therapy.

Materials and Methods

Oligonucleotides

Oligonucleotides were synthesized using phosphorothioate chemistry by the Molecular Resources Facility at University of Medicine and Dentistry-New Jersey Medical School (Newark, NJ). The sugar moieties of 5 bp at both 5' and 3' ends were modified with 2'-O-methoxymethyl groups to increase stability of the oligonucleotides and to provide higher hybridization affinity (22). For determinations of transfection efficiencies, a fluorescent oligonucleotide labeled with FITC was synthesized; it was included in every experiment to calculate transfection efficiencies. The sequences of the active oligonucleotides were based on the consensus sequence published previously by Mora et al. (23).

Cells

NRP-154 cells were the gift of Dr. David Danielpour (Case Western Reserve School of Medicine, Cleveland, OH; ref. 24). DU145 cells were the gift of Dr. Linda Mora (University of South Florida, Tampa, FL; ref. 23). These cells were maintained in either DMEM/Ham’s F12 or RPMI 1640 (Invitrogen, Carlsbad, CA) plus 10% newborn bovine serum (Hyclone, Logan, UT). LNCaP cells were also the gift of Dr. Mora and were maintained in RPMI 1640 (Invitrogen) plus 10% fetal bovine serum (Hyclone). LNCAp cells were derived by transfecting the 53c gene (on pIRESEGFP, Clontech, Palo Alto, CA; ref. 25) for constitutive STAT3 expression into LNCaP cells, which do not express constitutively activated STAT3 (10), using ClonFectin (Clontech). After selection, survivors were checked for enhanced green fluorescent protein (EGFP) expression by flow cytometry. Cell viability was determined using fluorescein diacetate (Sigma Chemical Co., St. Louis, MO; ref. 26) and a Universal RIII fluorescence microscope (Zeiss, Jena, Germany).

Transfection of Oligonucleotides into Prostate Cancer Lines

LipofectAMINE 2000 transfection reagent (Invitrogen) was used to transfect oligonucleotides into the prostate cancer lines as described previously (13). We routinely observed >85% transfection efficiency using this protocol. Briefly, cells plated in six-well plates were grown to 50% confluence. Oligonucleotides were diluted in Opti-MEM I (Invitrogen) appropriately. Next, LipofectAMINE 2000 was diluted in Opti-MEM I (2 μL LipofectAMINE 2000 to 250 μL Opti-MEM I per well). The diluted LipofectAMINE 2000 was allowed to incubate for 5 minutes at room temperature; then, 250 μL of it per well were mixed with 250 μL of diluted oligonucleotide. The liposome-oligonucleotide mixture remained at room temperature for 20 minutes before the 500 μL of liposome-oligonucleotide mixture were added to each well of cells. Cells were incubated with the liposome-oligonucleotide mixture for 6 hours at 37°C; then 1.5 mL of medium per well containing 30% serum was added to each well. Additional medium containing 10% serum was added each of the following days until the experiment was ended.

Apoptosis Determinations

FITC- or phycoerythrin (PE)-Annexin V and propidium iodide or 7-aminoactinomycin D staining (Caltag, Burlingame, CA) were used to measure the induction of apoptosis by the test compounds after incubation for the appropriate amount of time. Harvested cells were washed twice in buffer; then, 5 × 10⁶ cells in 1 mL of buffer, containing at least 40 mmol/L Ca²⁺, were put into each tube (Falcon Plastics, Franklin Lakes, NJ). Five microliters of FITC-Annexin V and propidium iodide or 7-aminoactinomycin D were put into each tube as well. Fluorescence was quantified on a FACSscan (Becton Dickinson, San Jose, CA) for at least 10,000 events.

Determination of Mitochondrial Transmembrane Potential

The JC-1 dye assay was used for determination of reduction in mitochondrial transmembrane potential during apoptosis (27, 28). Briefly, 10⁶ cells in 1 mL were stained with 1 μL of JC-1 (Molecular Probes, Eugene, OR) at 1 mg/mL in DMSO, according to manufacturer’s instructions at various times following treatment with plus or minus HSIE oligonucleotides or control oligonucleotide. After incubating with JC-1 for 15 minutes at 37°C, cells were analyzed for the decrease in red-orange fluorescence on a FACScan flow cytometer; at least 10,000 events were collected for analysis.

Measurement of Caspase-3 Activation

To measure caspase activation, the caspase-3 kit from Becton Dickinson PharMingen (La Jolla, CA) was used
according to the manufacturer’s directions. The PE-labeled antibody in this kit recognizes the active (cleaved) form of caspase-3, and a control immunoglobulin was used to determine the amount of nonspecific staining in each experiment. Treated cells were harvested at various times after transfection, fixed, and permeabilized with the reagents in the kit. Cells were incubated with PE-labeled anti-activated caspase-3 antibody or with control immunoglobulin for 30 minutes at room temperature and then washed twice with the wash buffer from the kit. Fluorescence was analyzed on a Becton Dickinson FACSCan flow cytometer.

**STAT3-DNA Binding Assay**

A reporter gene plasmid was used to investigate STAT3-DNA binding in the presence of 13410 in living cells. BPH-1 cells were grown to 50% confluency. They were cotransfected with 132410 or 13778 oligonucleotide and 2 μg/well purified pEGFP-survivin (kind gift of Dr. Y. Aoki, NIH, Bethesda, MD; ref. 29) using LipofectAMINE 2000 as described above. EGFP was quantified by flow cytometry on a Becton Dickinson FACSCan 24 hours after transfection.

**Determination of In vivo Activity**

To determine the effect of the novel oligonucleotides on tumor growth in vivo, DU145 cells (5 × 10⁶ per mouse) were resuspended in Matrigel (Becton Dickinson, Mansfield, MA) in ice in 300 μL aliquots and injected s.c. into the right flanks of male 6-week-old severe combined immunodeficient (SCID) mice (Charles River Laboratories, Raleigh, NC). Tumor growth was monitored externally by palpation weekly and tumor sizes were measured in two perpendicular diameters using engineer’s calipers (Mitsutoyo America Corporation, Aurora, IL). Tumor volumes were calculated using the following formula: tumor volume = (0.52)a²b, where a is the smaller diameter. Oligonucleotides were given at 5 μmol/L final concentration in saline by a single intratumor injection using a 25 gauge needle; tumor diameters were then measured either 1 week later or 24, 48, and 72 hours later. At 1 week or 72 hours following the single intratumor injection of oligonucleotides or vehicle, the mice were euthanized. The tumors were removed, fixed in formalin, embedded in paraffin, and stained with H&E. Microscopic examination was done on an Eclipse light microscope (Nikon, Tokyo, Japan) equipped with a digital camera using FastBus imaging software.

**Statistical Analysis**

The graphing software program Kaleidograph (Synergy Software, Reading, PA) and the statistical program InStat3 (GraphPad Software, San Diego, CA) were used for data analyses.

**Results**

**Novel Anti-STAT3 Oligonucleotides Induce Apoptosis in Prostate Cancer Cells**

We hypothesized that novel single-stranded STAT3 oligonucleotides based on the STAT3 binding sequence would induce apoptosis in prostate cancer cell lines. To test this hypothesis, each of the two strands of a modified HSIE STAT3 binding sequence (13410 and 13411; Table 1; ref. 23) and the scrambled sequences for each (13778 and 13779, respectively; Table 1) were synthesized and then transfected into prostate cancer cell lines. Apoptosis was quantified by staining with FITC-Annexin V and 7-aminoactinomycin D or propidium iodide and then quantifying the fluorescence on a flow cytometer. We found that both 13410 and 13411 induced apoptosis in the hormone-resistant prostate cancer cell lines tested, whereas the scrambled-sequence oligonucleotides did not induce apoptosis in the same cells. Figure 1 shows the results of one representative experiment in DU145 cells. Transfection of 13410 resulted in ~67% apoptosis of DU145 cells (Fig. 1A) 48 hours after transfection, whereas transfection of 13411 resulted in 61% apoptosis of DU145 cells (Fig. 1B). Transfection of control scrambled-sequence oligonucleotide for 13410 (oligonucleotide 13778) at the same time resulted in 86% viability of DU145 cells (Fig. 1C). Similar results were obtained with the scrambled sequence of 13411 (Fig. 1D): 92% of the cells remained viable, indicating that the effects observed were specific due to the sequences of 13410 and 13411.

Table 2 summarizes the results obtained with these oligonucleotides in the various prostate cancer cell lines tested thus far. Note that the non-STAT3-dependent line LNCaP (10) was not killed by either 13410 or 13411, but that the subline of LNCaP derived by transfecting a gene for constitutive STAT3 expression (25) into it (thus rendering this LNCaP cell subline STAT3 dependent) was killed by 13410 and 13411, thereby providing evidence that 13410 and 13411 induce apoptosis in hormone-refractory lines. In this and other sets of experiments, we noted more activity for 13410 than for 13411.

**Table 1. Oligonucleotides and sequences used in experiments**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>13410</td>
<td>5'-AGCTTCATTTCGCCGTAATCCCTA-3'</td>
<td>Consensus STAT3 binding site</td>
</tr>
<tr>
<td>13411</td>
<td>5'-TAGGGATTTACGGGAAATGAAGCT-3'</td>
<td>Complementary strand</td>
</tr>
<tr>
<td>13778</td>
<td>5'-TATGATCTCCTCCCTGAACCTCTCA-3'</td>
<td>Scrambled 13410</td>
</tr>
<tr>
<td>13779</td>
<td>5'-TAAGTACGAGCAGCTTTGGGATATA-3'</td>
<td>Scrambled 13411</td>
</tr>
<tr>
<td>Fluorescent oligonucleotide</td>
<td>5'-GTGCTCATGTGGTGACGTTGCT-5'</td>
<td>Transfection efficiency</td>
</tr>
</tbody>
</table>

NOTE: All oligonucleotides were made using phosphorothioration chemistry. In addition, the 5 bp at each end were 2'-O methoxylated.
Table 2. Summary of results on prostate cancer lines tested

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Oligonucleotide</th>
<th>% Average Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>100 nmol/L 13410</td>
<td>83 ± 1</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13411</td>
<td>78.2 ± 7.7</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13378</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13779</td>
<td>&lt;20</td>
</tr>
<tr>
<td>NRP-154</td>
<td>100 nmol/L 13410</td>
<td>52.3 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13411</td>
<td>47.6 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13778</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13779</td>
<td>&lt;10</td>
</tr>
<tr>
<td>LNCaP</td>
<td>100 nmol/L 13410</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13411</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13411</td>
<td>&lt;10</td>
</tr>
<tr>
<td>LNCaP-cS3</td>
<td>100 nmol/L 13410</td>
<td>49.05 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>100 nmol/L 13411</td>
<td>40 ± 10</td>
</tr>
</tbody>
</table>

NOTE: 13410, 13411, 13778, and 13779 were transfected into cells using LipofectAMINE 2000. Forty-eight hours after transfection, cells were harvested and stained using PE-Annexin V and 7-aminoactinomycin D to enumerate the apoptotic cells.

Novel Anti-STAT3 Oligonucleotide Inhibitors Induce Apoptosis through a Mitochondrial-Dependent Pathway

To show that prostate cancer cell lines underwent apoptosis associated with decreased mitochondrial membrane potential when transfected with novel anti-STAT3 oligonucleotides, the fluorescent dye JC-1, which measures mitochondrial transmembrane potential, was used (28).

The results are presented in Table 3. Transfection with 200 nmol/L of each novel oligonucleotide but not scrambled oligonucleotide for 48 hours decreased JC-1 fluorescence by ~45% in NRP-154 and by ~33% in DU145 cells. At 72 hours following transfection with 200 nmol/L novel anti-STAT3 oligonucleotides, the decreases in JC-1 fluorescence were even more marked: JC-1 fluorescence was decreased by >63% in NRP-154 cells and by >50% in DU145 cells (data not shown). These data reveal that both novel oligonucleotides were capable of decreasing mitochondrial membrane potential in prostate cancer cells, which is something that occurs in apoptotic cells in many systems.

To determine if activation of caspases was involved in the apoptotic pathway induced by treatment with novel anti-STAT3 oligonucleotides, the caspase-3 kit from Becton Dickinson PharMingen was used. The antibody included in the kit recognizes the active form of human caspase-3 but not the precursor form. DU145 cells transfected with 200 nmol/L 13410 or 13778 were harvested, fixed, and permeabilized and then stained with antibody to activated caspase-3 48 hours after transfection. The results of a representative experiment are shown in Fig. 2. In both panels, the thick line shows the mean fluorescence intensity of DU145 cells stained with PE-labeled anti-activated caspase-3, whereas the thin line shows the mean fluorescence intensity of cells stained with the control immunoglobulin. When transfected with 200 nmol/L 13410 anti-STAT3 oligonucleotide, DU145 cells exhibited activated caspase-3 in >80% of the cells (Fig. 2A); in contrast, when transfected with 200 nmol/L scrambled-sequence control oligonucleotide 13778, only 25% of the DU145 cells exhibited activated caspase-3 (Fig. 2B). A summary of results obtained in three independent experiments are presented in Table 4. These results show that treatment with 13410 induced a caspase-dependent apoptosis pathway in DU145 prostate cancer cells, whereas treatment with the scrambled sequence of it (13778) did not.

Table 3. Novel anti-STAT3 inhibitors decreased mitochondrial transmembrane potential

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Rx</th>
<th>% JC-1 ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>0 nmol/L</td>
<td>94.4 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>33.1 ± 11.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>48.5 ± 22.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>93.2 ± 6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>93.1 ± 6.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NRP-154</td>
<td>0 nmol/L</td>
<td>87.9 ± 5.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>39.7 ± 21.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>42.7 ± 0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>85.9 ± 4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>93.4 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Forty-eight hours after transfection, cells were washed and incubated with JC-1 (20 µg/mL). Fluorescence after 15-minute incubation at 37°C was quantitated on a FACSscan flow cytometer. The control oligonucleotides used were 13778 and 13779. The average percentage of cells fluorescing due to JC-1 were calculated from three experiments; one-way ANOVA was used to calculate the P's.
Novel Anti-STAT3 Oligonucleotides Inhibit STAT3-Regulated Gene Expression

To elucidate the mechanism of action of the novel STAT3-inhibiting oligonucleotides, we did a series of experiments to see if they inhibited STAT3-regulated gene-expression. Survivin, an antiapoptotic gene product, is reported to be the direct regulator for the Bcl family of proteins, notably Bcl-2 and Bcl-XL, in several malignancies (29, 30). Recently, investigators showed that survivin expression was regulated directly by STAT3 (29). We decided to use survivin expression in a reporter gene plasmid in which the endogenous cytomegalovirus immediate early gene promoter has been excised and replaced with bp /C0 1,512 to +70 of the human survivin gene on the plasmid pEGFP-N3. When STAT3 binds to the survivin promoter, the transfected cells fluoresce with EGFP. The amount of STAT3 bound to the plasmid therefore correlates with EGFP fluorescence, which is monitored by flow cytometry.

Therefore, we cotransfected the reporter gene plasmid pEGFP-survivin (29) along with 13410 or control oligonucleotide into BPH-1 cells. Table 5 shows the results of these experiments. Transfection of the control oligonucleotide 13778 into the cells had no significant effect on EGFP expression. In contrast, cotransfection of 200 nmol/L 13410 greatly reduced EGFP expression in BPH-1 cells by an average of >70% (P < 0.0001, repeated ANOVA). We concluded from these results that 13410 but not 13411 interfered with STAT3-DNA binding activity.

Novel Anti-STAT3 Oligonucleotides Induce Apoptosis in Prostate Cancer Cells In vivo

Efficient uptake of oligonucleotides was observed in the absence of transfection reagent. Because the novel inhibitors are modified ribonucleotides, which are only partially 2'-O methoxylated, studies were undertaken to ascertain the extent to which they would manifest in vivo activity locally before committing ourselves to generate the large quantities required for systemic in vivo studies. Prior to performing these in vivo experiments, we needed to establish that there would be high enough efficiency of oligonucleotide uptake by DU145 cells in the absence of a transfection reagent such as LipofectAMINE 2000, because we planned to administer the oligonucleotides without one. Therefore, we added fluorescent control oligonucleotide to DU145 cells in medium alone and then quantified the fluorescence intensity that resulted in the cells 2 days later. Figure 3A shows that by increasing the concentration of oligonucleotide to 2 μmol/L (from 500 nmol/L, the highest we had formerly used in vitro), we achieved an

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Oligonucleotide</th>
<th>% Positive for Caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200 nmol/L 13410</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L 13778</td>
<td>13.4</td>
</tr>
<tr>
<td>2</td>
<td>200 nmol/L 13410</td>
<td>81.8</td>
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<tr>
<td></td>
<td>200 nmol/L 13778</td>
<td>25.4</td>
</tr>
<tr>
<td>3</td>
<td>200 nmol/L 13410</td>
<td>42.7</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L 13778</td>
<td>17.8</td>
</tr>
</tbody>
</table>

NOTE: Forty-eight hours after transfection, DU145 cells were harvested, fixed, and permeabilized using the reagents supplied in the caspase activation kit. Cells were stained either with PE anti-caspase-3 antibody or with PE isotype control immunoglobulin for 30 minutes, washed, and analyzed for fluorescence on a FACScan flow cytometer using CellQuest Pro software for acquisition and analysis.

Table 5. 13410 inhibited STAT3-regulated survivin expression

<table>
<thead>
<tr>
<th>Rx</th>
<th>% EGFP Positive</th>
<th>% Inhibition ± SD</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>24.5</td>
<td>0</td>
</tr>
<tr>
<td>200 nmol/L 13410</td>
<td>6.9</td>
<td>71.6 ± 1.5*</td>
</tr>
<tr>
<td>200 nmol/L 13778</td>
<td>24.7</td>
<td>1.7 ± 3</td>
</tr>
</tbody>
</table>

NOTE: BPH-1 cells were cotransfected with pEGFP-survivin and 13410 or 13778. Twenty-four hours later, they were harvested and analyzed for EGFP fluorescence by flow cytometry.

*p < 0.0001, ANOVA.

Table 4. Novel anti-STAT3 oligonucleotides induced caspase activation in DU145 cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Oligonucleotide</th>
<th>% Positive for Caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200 nmol/L 13410</td>
<td>48.7</td>
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</tbody>
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NOTE: Forty-eight hours after transfection, DU145 cells were harvested, fixed, and permeabilized using the reagents supplied in the caspase activation kit. Cells were stained either with PE anti-caspase-3 antibody or with PE isotype control immunoglobulin for 30 minutes, washed, and analyzed for fluorescence on a FACScan flow cytometer using CellQuest Pro software for acquisition and analysis.

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uptake efficiency of 92% at 48 hours after addition of oligonucleotide. We concluded that in the absence of a transfection reagent we would have sufficient uptake of oligonucleotides by the tumor cells to see an effect in vivo.

**Novel Anti-STAT3 Oligonucleotides Induce Apoptosis in Prostate Cancer Cells In vivo**

Novel oligonucleotides slowed growth of established DU145 tumors in vivo. To test for antitumor activity exhibited by the novel STAT3 inhibitors in vivo, we devised a model in which we made xenografts of DU145 cells suspended in Matrigel collagen. The cells in Matrigel were then injected s.c. into the right flanks of SCID mice. Tumor growth was monitored weekly using engineer’s calipers until the average tumor diameter size was 3 to 4 mm. In the first experiment (Fig. 3B), tumor volumes were determined 1 week after a single intratumor treatment with oligonucleotides. We found that intratumor injection of either 13410 or 13411 slowed the increase in tumor volume compared with injection of vehicle. Figure 3B shows the significant decrease in tumor volume of treated mice compared with vehicle-treated mice: the five mice treated with 13410 had a mean tumor volume of 100 μL (P < 0.01, one-way ANOVA) and the eight mice treated with 13411 had a mean tumor volume of 270 μL (P < 0.05, one-way ANOVA) compared with the five vehicle-treated mice, which had a mean tumor volume of 590 μL. In the second experiment (Fig. 3C), tumor volumes were determined 24, 48, and 72 hours following intratumor injection with oligonucleotides. The results shown in Fig. 3C confirmed those of Fig. 3B; moreover, injection of the scrambled oligonucleotide for 13410 had the same effect as the vehicle (Dulbeco’s PBS), whereas 13410 significantly reduced the tumor volume by 72 hours (P < 0.001, one-way ANOVA).

The tumors removed from the sacrificed mice were prepared for histology and H&E-stained slides were examined for apoptotic or necrotic cells under ×100 magnification (Fig. 4). Two representative microscopic fields are shown in Fig. 4 from mice treated with either 13410 or vehicle. As seen in Fig. 4A, the morphology of the majority of DU145 tumor cells treated with 13410 in vivo was atypical, with many pyknotic nuclei observed. In contrast, tumor cells given only vehicle in vivo displayed normal morphology; moreover, dividing cells were seen (Fig. 4B). We found that, whereas untreated tumor tissue from mice of experiment 1 was essentially devoid of apoptotic cells (less than one third of slide; Fig. 4B), all the slides from tumors treated with both 13410 and 13411 STAT3 inhibitors displayed moderate apoptotic cells (up to two thirds of slide; Fig. 4A). We would like to point out that late apoptosis morphologically resembles necrosis on a cellular level (31) and that the tumors were removed 72 hours or 1 week after treatment so that apoptotic cells would by that time be difficult to distinguish from necrotic cells. No monocytic or polymorphonuclear cell infiltrates were observed in the slides from treated tumors, indicating that the novel oligonucleotides did not induce nonspecific immune or inflammatory responses, something that can occur with deoxynucleotides bearing unmethylated CpG.
sequences (21). These photomicrographs confirm the data presented in Fig. 3, namely, that treatment in vivo with both novel oligonucleotide STAT3 inhibitors promote tumor regression. The histologic data corroborated our gross morphometric findings and support the conclusion that these novel STAT3 inhibitors contribute positively to a reduction of tumor growth in vivo.

**Discussion**

We have designed novel oligonucleotides that inhibit STAT3 activity. These oligonucleotides were based on the modified HSIE sequence reported previously (ref. 23; Table 1). Initially, we had made and tested double-stranded STAT3 binding decoys for apoptotic activity in various prostate cancer lines but observed to our surprise that the unannealed single-stranded oligonucleotides had more activity than the annealed oligonucleotide (annealed oligonucleotides induced apoptosis in 69% of DU145 cells at 500 nmol/L versus an average of 83% for single-stranded 13410 at 100 nmol/L in DU145 cells) and more activity than antisense STAT3 oligonucleotides on which we have recently reported (13). Because of the lower activity of the annealed oligonucleotide, we focused our attention on the single-stranded oligonucleotides, because they are a novel type of STAT3 inhibitor.

We observed that both single-stranded oligonucleotides, 13410 and 13411, induced apoptosis in hormone-refractory prostate cancer lines but not in the androgen-sensitive line LNCaP (Fig. 1; Table 2). Moreover, we found that one of the oligonucleotides (13410) was somewhat more potent than the second one (13411). Scrambled-sequence oligonucleotide controls had no more activity than cell culture medium (Table 2). As for the apoptotic pathway induced by 13410 and 13411, we have evidence that the pathway is at least partially mitochondrial dependent, based on the data obtained using JC-1, which reveals changes in the mitochondrial transmembrane potential (Table 3). These data were confirmed by those shown in Fig. 2 and Table 4: the novel oligonucleotide STAT3 inhibitors induced caspase-3 activation, which was not induced by the scrambled-sequence control oligonucleotides. As for the mechanism of action of 13410 and 13411, we postulated that they inhibited STAT3-DNA binding; a model of this putative mechanism appears in Fig. 5. Evidence for our hypothesis is based in part on the data shown in Table 5 in which expression of STAT3-regulated survivin was reduced by >70% in BPH-1 prostate cells by 13410, whereas 13778 had no effect. In the plasmid used here, pEGFP-survivin, the endogenous cytomegalovirus immediate early gene promoter, was replaced by bp

![Figure 4](image_url)  
**Figure 4.** Treatment with 13410 induced apoptosis in vivo. Tumors were removed from mice described in A (1 week after injection of 13410) and subjected to histopathologic examination by light microscopy. A, representative field from a section of a tumor treated with 5 μmol/L 13410. Note the atypical morphology of the nuclei of the majority of cells. Arrows, pyknotic nuclei. B, representative field from a section of vehicle-treated tumor. Note the absence of pyknotic nuclei and the presence of dividing cells (arrows). Magnification, ×400. Male SCID mice, 6 weeks old, were injected s.c. with DU145 cells and treated with oligonucleotide as described. When the mice were sacrificed 1 week after receiving either 13410 or vehicle, the tumors were removed, fixed in formalin, embedded in paraffin, sectioned on a microtome, and stained with H&E. Slides were analyzed on a light microscope using FastBus imaging software to capture images to the digital camera.

![Figure 5](image_url)  
**Figure 5.** A model of the proposed mechanism of action of STAT3-inhibiting oligonucleotides 13410 and 13411. In the absence of 13410 or 13411, activated and dimerized STAT3 translocates from the cytoplasm to the nucleus, thereby regulating target gene expression. Novel oligonucleotides 13410 and 13411 bind to STAT3 binding sites; therefore, STAT3-regulated gene expression is reduced.
STAT3 is occupied. Recently, a double-stranded STAT3-dimerized STAT3, so that the genome docking site on have been described (39), which bind to phosphorylated, decoys that inhibit STAT3 from binding to its binding site apoptosis (Table 5). Double-stranded cis-quently, target gene regulation is reduced. In prostate constitutive STAT3 expression in prostate cancer cell lines prostate cancer (33, 35, 36). Finally, there is a correlation chemosensitive; this would certainly expand the treatment of Bcl-XL expression, which is a consequence of STAT3 (34), and to antisense STAT3 oligonucleotides (13). Inhibi-
AG490, which inhibits JAK2-mediated activation of STAT3 cancer, human prostate cancer cell lines are sensitive to binding activity in DU145 cells (33). Moreover, for prostate injection of the scrambled-sequence oligonucleotide had therapeutic efficacy by use of antisense oligonucleotide had therapeutic efficacy with pEGFP-survivin reduced EGFP expression by >70% (P < 0.0001), whereas cotransfection of 13778 had no significant effect on EGFP expression. Thus, the most plausible explanation for the reduction of EGFP we observed following 13410 treatment is that 13410 interfered with STAT3-DNA binding. Because these data are evidence but not proof of the mechanism of action of these novel STAT3-inhibiting oligonucleotides, we are undertaking more experiments to elucidate how they work.

Using a xenograft model of s.c. DU145 tumors in SCID mice, we observed significant decreases in tumor volume following intratumor injection with 13410 and 13411 (Fig. 3); injection of the scrambled-sequence oligonucleotide of 13410 did not affect the mean tumor volume (Fig. 3C). Histologic examination of the tumors excised from the mice revealed that there was no mononuclear cell or granulo-
cytic infiltrate; therefore, there was no nonspecific immune response evoked by treatment with the oligonucleotides. Rather, there were higher numbers of apoptotic or necrotic cells (at 7 days, it is difficult to distinguish between the two by H&E staining alone; ref. 31) and pyknotic cells seen following treatment with either 13410 or 13411, which were not seen in the tumors treated with either Dulbecco's PBS or with scrambled-sequence 13410 oligonucleotide (Fig. 4).

There is abundant evidence showing that inhibition of STAT3 leads to cessation of tumor cell growth and to apoptosis. Head and neck squamous cell carcinoma lines showed sensitivity to dominant-negative STAT3: when transfected with a plasmid bearing the gene, the squamous cell carcinoma lines failed to divide, whereas transfection with dominant-negative STAT1 did not have the antiproliferative effect (6). In other sets of studies, blocking STAT3 by use of antisense oligonucleotide had therapeutic efficacy in a melanoma model (32) and inhibited STAT3-DNA binding activity in DU145 cells (33). Moreover, for prostate cancer, human prostate cancer cell lines are sensitive to AG490, which inhibits JAK2-mediated activation of STAT3 (34), and to antisense STAT3 oligonucleotides (13). Inhibition of Bcl-XL expression, which is a consequence of STAT3 inhibition, renders some prostate cancer cell cancer lines chemosensitive; this would certainly expand the treatment options of relatively chemoresistant hormone-refractory prostate cancer (33, 35, 36). Finally, there is a correlation between the loss of androgen sensitivity and the gain of constitutive STAT3 expression in prostate cancer cell lines (10, 37, 38).

Lately, strategies have emerged to block the function of STAT3 by inhibiting its binding to the genome; conse-
sequently, target gene regulation is reduced. In prostate cancer, for example, such a strategy would be expected to limit the expression of survivin, thereby resulting in apoptosis (Table 5). Double-stranded cis-promoter element decoys that inhibit STAT3 from binding to its binding site have been described (39), which bind to phosphorylated, dimerized STAT3, so that the genome docking site on STAT3 is occupied. Recently, a double-stranded STAT3-
DNA decoy for the treatment of head and neck squamous cell carcinoma was described (40) with an efficacy is comparable with the in vitro efficacy of G-quartet oligonucleotides, which have an IC50 of 7 μmol/L (41). G-quartets are believed to act somewhat differently: destabilization of STAT3 dimers bound to DNA through binding of the G-quartet oligonucleotides to the SH2 domains of STAT3 is their purported mechanism (41). G-quartet oligonucleoti-
des need a reagent to facilitate cellular uptake, which may limit their therapeutic usefulness in vivo (41). Clearly, more types of anti-STAT3 therapeutic agents need to be identified and evaluated for efficacy in prostate cancer.

Because we were aware of the limitations associated with the current roster of STAT3 inhibitors, we decided to try a different approach for inhibiting STAT3. We thought that modified single-stranded ribonucleotides, with sequences based on a consensus binding sequence for STAT3, might prevent STAT3-DNA binding by annealing to DNA per-
haps by strand invasion, a strategy currently under in-
vestigation for control of gene expression (42) and one shown to work in MCF-7 breast cancer cells (43). Therefore, one of the published STAT3 binding sites [HSIE sequence (23, 44)] was the basis for the design of the two oligo-
ribonucleotides, which were the two strands of this consen-
sus binding site. Ribonucleotides were synthesized using phosphorothioration modifications at each phospho-
diester linkage, the 2′-O positions had methoxyl groups attached to the 5′p at each end of the oligonucleotide. Both modifications have been reported to increase the stability and in vitro half-life of oligoribonucleotides (45). Moreover, by only modifying the terminal 5′p at either end with the 2′-O-methoxyl group, we allowed for the possibility of the activation of RNase H as an additional mode of action (45, 46). We have not yet optimized the sequences we used for the most potent possible; we expect to improve on our observed efficacy both in vitro and in vivo. Therefore, we created novel STAT3 oligonucleotide inhibitors, which retained activity in vitro and showed activity at concentra-
tions lower than those reported for double-stranded decoys and G-quartet oligonucleotides in vitro. Our novel oligonucleotides exhibited in vivo activity when given in buffer, which will facilitate development of them for therapy. In our future studies, we plan to investigate in detail the mechanism of action of these STAT3 oligonucleo-
tide inhibitors and examine the effect of sequence modifications on their potencies.

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Correction

Article on anti-STAT3 and prostate cancer

In the article on anti-STAT3-inducing apoptosis of prostate cancer cells in the October 2004 issue (1), Markus Meyenhofer’s name was spelled incorrectly as Marcus Meyenhofen. In addition, the second sentence in Materials and Methods should read “The sugar moieties of 5 bp at both 5’ and 3’ ends were modified with 2’-O-methyl groups to increase stability of the oligonucleotides and to provide higher hybridization affinity (22).”

Reference

Molecular Cancer Therapeutics

Novel single-stranded oligonucleotides that inhibit signal transducer and activator of transcription 3 induce apoptosis in vitro and in vivo in prostate cancer cell lines

Beverly E. Barton, Thomas F. Murphy, Ping Shu, et al.


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