SPC3042: a proapoptotic survivin inhibitor

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Abstract
The ability to regulate the cellular homeostasis of a higher organism through tight control of apoptosis and cell division is crucial for life. Dysregulation of these mechanisms is often associated with cancerous phenotypes in cells. Optimal cancer therapy is a fine balance between effective cancer cell killing and at the same time minimizing, or avoiding, damage to the surrounding healthy tissue. To obtain this, it is necessary to identify and inhibit molecular targets on which the cancer cells are strongly dependent. Survivin represents such a target, and it has been published previously that peptide vaccines, the small-molecule YM155, and the antisense molecule LY2181308/ISIS23722, via different mechanisms, have been used as survivin inhibitors. In this article, a new potent antisense inhibitor of survivin, SPC3042, is presented, and the properties of SPC3042 are compared with the previously published antisense drug, LY2181308/ISIS23722. SPC3042 is a 16-mer locked nucleic acid (LNA) oligonucleotide and designed as a fully phosphorothioated gapmer containing 7 LNA nucleotides in the flanks. The LNA nucleotides in SPC3042 provide nuclease stability and higher potency for survivin mRNA inhibition compared with earlier generations of antisense reagents. It is shown that the down-regulation of survivin with SPC3042 leads to cell cycle arrest, pronounced cellular apoptosis, and down-regulation of Bcl-2. It is also shown that SPC3042 is a sensitizer of prostate cancer cells to Taxol treatment in vitro and in vivo. [Mol Cancer Ther 2008;7(9):2736–45]

Introduction
Survivin is a 17-kDa protein and a key regulator of apoptosis and cell cycle progression (1, 2). Survivin plays a dual biological function, as it protects cells from apoptosis downstream of the intrinsic apoptotic pathway, and is involved in regulation of mitosis (1, 2), as the protein selectively accumulates and localizes to various components of the mitotic apparatus during the mitotic spindle formation. Despite this dual role, survivin has been classified to belong to the family of inhibitors of apoptosis proteins (IAP), which also include the human members XIAP, cIAP1, cIAP2, NAIP, ML-IAP, and apollon. These are characterized by the presence of baculovirus IAP repeat domains and are able to interfere with regulation of apoptosis through either direct or indirect interaction with caspases (pro-caspase-9, caspase-3, and caspase-7; refs. 3–5). Survivin is characterized by the presence of only one baculovirus IAP repeat domain and the lack of a RING finger domain present in other IAPs. The central role of survivin in cell cycle regulation, where it forms a complex with the inner centromere protein and Aurora-B kinase to promote chromosome segregation and cytokinesis in the Gap2 mitosis (G2-M) phase of the cell cycle, has been shown in several publications (6–8).

Survivin is abundantly expressed during embryonic development and plays an important role in the controlled cell divisions of normal growth and tissue differentiation. In adult healthy tissues, survivin plays a more reduced role and is only expressed in thymocytes (9), basal colonic epithelium (9), endothelial cells (9), CD45+ bone marrow-derived stem cells (9), and endometrial cells (9).

However, during carcinogenesis, survivin is often activated and can play a key role in aberrantly controlled cell division. It therefore becomes important for generating and maintaining the malignant phenotype. Furthermore, expression of survivin in tumor tissue is found to be closely linked with a poor prognosis in several cancers [e.g., colorectal cancer (10, 11), acute myeloid leukemia (12), and prostate cancer (13, 14)].

Because survivin is almost exclusively expressed in cancer cells, it is regarded as one of the most cancerspecific targets, and inhibition of survivin expression has therefore been of interest for its anticipated valuable therapeutic outcomes for many years.

Oligonucleotide-based approaches targeting survivin mRNA by either small interfering RNA (15–18) or antisense oligonucleotides (19–22) are important rational strategies to silence its expression. Small interfering RNAs are potent inhibitors in vitro but cannot be used directly in vivo due to their low nuclease resistance and the lack of cellular uptake. However, chemically modified singlestranded oligonucleotides are taken up by cells in vivo and thus offer a therapeutic platform.

Previously, the most frequently used chemical modification was phosphorothioate in which one oxygen of the internucleoside phosphate is replaced with sulfur. Today, the phosphorothioate modification is used in combination with other chemical modifications (23). Classic chemical
modifications are the “2’ modifications”. Examples of this class are 2’-O-Me and 2’-O-methoxymethyl (MOE). A representative of the latter is the antisense drug LY2181308/ISIS23722 (1, 19, 24, 25), which is currently being tested in a phase II trial for treatment of hepatocellular carcinoma.

Locked nucleic acids (LNA) represent a new class of antisense oligonucleotides. In the LNA nucleoside, the furanose is chemically modified by the insertion of a 2’-O-CH2-4’ linkage that transforms the furanose to a bicyclic structure. The bicyclic structure of LNA nucleosides locks the ribose conformation leading to an unprecedented high nucleic acid affinity of oligonucleotides comprising LNA nucleosides. The LNA modification substantially improves nuclease resistance, permits reduction in length, and maintain the affinity in combination with PS. LNA oligonucleotides are frequently used for antisense (26–30) and 14- to 16-mer LNAs typically have IC50 values, in classic cell-based assays, in the low to subnanomolar range, thus exhibiting similar potencies to small interfering RNA (31).

In this study, we report the properties of SPC3042. The compound is designed as an antisense 16-mer LNA gapmer targeting the region comprising the stop codon of the open reading frame of the survivin transcript. We compare the antisense properties of SPC3042 with ISIS23722, an oligonucleotide that targets the 3’-untranslated region of the survivin transcript and SPC2822, which has an identical sequence to ISIS23722 but where the 2’-O-MOE nucleotides are replaced with LNA nucleotides. The sequence of SPC3042 is also prepared as the “3042MOE,” in which the LNA nucleotides are replaced with 2’-O-MOE nucleotides.

Materials and Methods

Oligonucleotides and Thermal Denaturation Measurements

LNA oligonucleotides were synthesized according to methods described by Frieden et al. (32) The 2’-O-MOE amidites were prepared according to published procedures (33, 34), except for the final phosphorylation step that was done according to Pedersen et al. (35). Oligonucleotides were synthesized using the phosphoramidite approach on an Expedite 8900/Multiple Oligonucleotide Synthesis System synthesizer at a 1 μmol scale. At the end of the synthesis, the oligonucleotides were cleaved from the universal solid support using aqueous ammonia for 1 to 2 h at room temperature and further deprotected for 4 h at 65°C. The oligonucleotides were purified by reverse-phase high-performance liquid chromatography and were then characterized by liquid chromatography-mass spectrometry (reverse phase and electrospray ionization-mass spectrometry).

The melting temperature of oligonucleotide/RNA duplexes was determined by using UV spectrometry. Duplexes of oligonucleotide and cRNA oligonucleotide (1.5 μmol/L concentration) were prepared in thermal denaturation (Tm) buffer [100 nmol/L NaCl, 0.1 nmol/L EDTA, 10 nmol/L NaP (pH 7.0)] for analysis by heating to 95°C for 3 min and cooled at room temperature for 30 min.

Melting temperature (Tm) values were measured in a Lambda 40 UV-visible spectrophotometer (Perkin-Elmer) and data were collected and analyzed using TempLab software (Perkin-Elmer). The instrument was programmed to heat the oligonucleotide duplex sample from 20°C to 95°C and afterwards to cool the sample to 25°C. During this process, the absorbance at 260 nm was recorded. The melting curves were used to calculate Tm values.

Nuclease Stability

Mouse or human plasma was defrosted and part of the sample was preincubated for 24 h at 37°C before assaying; to 45 μL plasma from stock solution (not preincubated) was added 5 μL oligonucleotide to a final concentration of 20 μmol/L. Samples were incubated at 37°C for 0 to 72 h. At different time points, samples were collected and “snap frozen” in liquid N2 before storage at −80°C. When all samples are collected, they are analyzed by electrophoresis on PAGE gels (16%; 8 mol/L urea).

Cell Culturing

The prostate cancer cell line 15PC3 was used in all in vitro studies (kindly provided by Dr. F. Baas, Neurozintuigen Laboratory). Cells were kept as frozen stocks, thawed, and grown in DMEM (Sigma) plus 10% fetal bovine serum (Biochrom), 1% GlutaMAX I 100× (Invitrogen), and 0.05% gentamicin (Sigma) at 37°C in 5% CO2.

Transfection

Cells were seeded 2 days before transfection in 175 flasks or 12-well plates (Nunc) at a density of 0.8 × 10^6 or 0.9 × 10^6 per flask or well, respectively. At 60% to 70% confluence, cells were transfected with oligonucleotides at final concentrations varying from 0.04 to 25 nmol/L. In mock transfections, sterile water was used instead of oligonucleotide. At the time of transfection, the cells were washed in Dulbecco’s PBS (Sigma) followed by addition of 5 μg/mL LipofectAMINE 2000 (Invitrogen) in Opti-MEM I + GlutaMAX I (Life Technologies). The cells were incubated for 7 min at room temperature. Oligonucleotides at varying concentrations dissolved in Opti-MEM I + GlutaMAX I were added and the cells were incubated for 4 h. The cells were washed in PBS, fresh medium was added, and cells were incubated until harvested.

Cells were harvested after 24, 48, or 72 h by washing in PBS and trypsinizing, which was quenched with culture medium. The cells were collected in 15 or 50 mL tubes by centrifugation. Cell pellets were washed in PBS and separated for further analysis.

Propidium Iodide Staining

Transfected cells were harvested as described above and washed twice in PBS. Approximately 10^6 cells were fixed in ice-cold ethanol (70%) for 30 min at 4°C. The fixed cells were washed twice in PBS to remove all ethanol. A nuclear staining solution [33 μg/mL propidium iodide, 1 mg/mL RNase A (Sigma) in PBS] was then added. The cells were incubated in the dark at room temperature for 30 min and...
analyzed by flow cytometry using a FACSCalibur (Becton Dickinson). Fluorescence intensity was measured and plotted against cell count.

**Total RNA Purification**

Total RNA was isolated from transfected cells using the RNaseasy Mini Kit (Qiagen) according to the manufacturer’s recommendations. The total RNA concentration was measured by UV spectroscopy at 260 nm. The ratio of 260/280 nm was used as a measure of RNA purity. Isolated RNA was stored at –80°C.

**cDNA Synthesis**

First-strand cDNA was synthesized from 0.5 μg total RNA using OmniScript Reverse Transcriptase kit (Qiagen) according to the protocol provided by the manufacturer.

**Quantitative PCR**

The relative amounts of total survivin mRNA in transfected cells were measured by quantitative PCR using primers amplifying a 121-bp fragment of survivin exon 1. Template cDNA was diluted 5-fold before mixing with a Platinum Quantitative PCR SuperMix uracil DNA glycosylase (Invitrogen) and a primer-probe mix containing 0.6 μmol/L forward primer (5′-AAGGACACCGC-CATCTCTACA-3′), 0.9 μmol/L reverse primer (5′-CCAAAGTCTGCTGTTCTCAGT-3′), and 0.1 μmol/L TaqMan probe (5′-FAM-GAGGCTGGCTTCATCCACTGCC-TAMRA-3′) according to manufacturer’s protocol.

A commercially available human glyceraldehyde 3-phosphate dehydrogenase (20×) primer-probe mix (Applied Biosystems) was used for glyceraldehyde 3-phosphate dehydrogenase measurements. For human Bcl-2 mRNA, a 20× primer-probe mix containing 0.3 μmol/L forward primer (5′-CATGTGTGTTGAGAGCGTTCA-3′), 0.6 μmol/L reverse primer (5′-GCCGGTTACGG-TACTCAGTCA-3′), and 0.1 μmol/L TaqMan probe (5′-FAM-CCTGCGACACATACGCCCCGTG-TAMRA-3′) was used.

Quantitative PCR was done on an iCycler instrument (Bio-Rad) using 50°C for 2 min, 95°C for 5 min followed by 40 cycles of 95°C for 30 s and 60°C for 1 min.

Data obtained from quantitative PCR analysis were analyzed using the iCycler iQ Real-time Detection System software (Bio-Rad). Data were correlated to a cDNA standard curve made from nontransfected 15PC3 cells. The survivin mRNA level was normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA levels and plotted relative to an average value of the mock samples from the same transfection, which was set to 100%.

**Survivin Protein Detection-ELISA**

Survivin protein levels were detected by ELISA using a DuoSet IC Human Total Survivin kit (R&D Systems). Harvested cells were lysed in a solution consisting of PBS added 1 mmol/L EDTA, 0.5% Triton X-100 (Sigma), 6 mol/L urea (Sigma), and protease inhibitors. Total protein was measured by BCA protein assay kit (Fierce) using an albumin standard according to manufacturer’s protocol. The ELISA was carried out as described in manufacturer’s protocol using 100 μL of a 45× dilution of each lysed sample. Survivin protein concentrations were detected by spectrophotometer at 450/540 nm correlated to a 2-fold serial standard dilution. Data were normalized to total amount of protein and plotted relative to an average value of mock samples from same transfection, which was set to 100%.

**Caspase-3/7 Activity**

The activity of caspase-3 and -7 was measured using a luminogenic Caspase-Glo-3/7 substrate assay (Promega). 15PC3 cells were seeded to a density of 12,000 per well in white 96-well plates (Nunc) in growth medium 1 day before transfection (see above). Cells were transfected as described above. Camptothecin (1 μmol/L; Sigma) was used as positive control. A serial dilution of cell density was added 200 nmol/L staurosporine (Sigma) as a control of assay viability.

At 24, 48, or 72 h after transfection, Caspase-Glo-3/7 reagent was added to the medium and the cells were incubated for additional 1 h at room temperature. The activity of the caspases was measured as relative light units per second in a Luminoskan Ascent instrument (Thermo Labsystems). Data were correlated, plotted, and normalized to an average value of the mock samples. Data from the staurosporine-treated serial dilution were plotted and evaluated for linearity.

**PC3 Xenograft Model and Statistics**

PC3 prostate cancer cells were cultured as described above for 15PC3 cells. PC3 cells (3 × 10⁶ per tumor) were mixed with 300 μL Matrigel (Becton Dickinson) and injected s.c. in the flank of female 8- to 10-week-old BALB/c nu/nu mice (Taconics). The group size was 5, 8, or 10 mice per group depending on the experiment. Saline or drug dissolved in saline (0.9%) was injected i.p. in the mice. The animals were observed daily for any reactions related to the treatment or any deviations from normal health. The day of cell implantation was designated day 0. Tumor volume was calculated for each animal based on tumor length and width measurement, assuming spherical growth. At sacrifice, the tumors, livers, and spleens were excised and weighed. The data from each mouse in each experiment were normalized relative to the mean of the saline control group.

Statistical analysis was done using the nonparametric Kruskal-Wallis test with Dunn’s Multiple Comparison post-test using GraphPad Prism 4 software.

**Results**

**Thermal Denaturation**

The Tₘ of the oligonucleotides (Fig. 1) was determined against their cRNA sequences. SPC3042 gave rise to the highest Tₘ of all the tested duplexes, whereas the Tₘ of the corresponding 2′-O-MOE gapmer (3′042MOE) was 59°C. The Tₘ of the unmodified 18-mer phosphorothioate (SPC2823), isosequential with ISIS23722, was only 44°C, whereas the Tₘ of ISIS23722 was 51°C. Thus, the eight 2′-O-MOE nucleotides increase the Tₘ by 7°C, but substituting the 2′-O-MOE nucleotides with LNA residues resulted in a increase of 19°C. The scrambled control did not show any Tₘ (Fig. 1).
Survivin mRNA Inhibition

All oligonucleotides were transfected into the prostate cancer cell line 15PC3 at concentrations of 1, 5, 12.5, 25, 100, and 250 nmol/L. After 4 h, the cells were transferred to growth medium and cultured for 24, 48, or 72 h, respectively. Survivin mRNA expression was measured by quantitative PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA levels. The approximate IC₅₀ values of each oligonucleotide at the different time points are shown in Fig. 1. The IC₅₀ of SPC3042 at 72 h was not possible to determine due to pronounced cellular apoptosis, but significant survivin down-regulation could be detected after 72 h for both SPC2822 and ISIS23722, with IC₅₀ values of 1 and 10 nmol/L, respectively. Increased potency followed increased affinity \((T_m)\) when compared with the identical sequence. SPC3042 is more potent than MOE3042, and SPC2822 is more potent than ISIS23722, which is more potent than SPC2823. However, among the sequences, a higher \(T_m\) is not necessarily associated with higher potency (e.g., SPC3042 versus SPC2822). The maximum potency for all oligonucleotides was found after 24 h (Fig. 2). At low concentrations, SPC2822 was found to be the most potent, but SPC3042 was found to be the most effective to inhibit survivin at higher concentrations (100 and 250 nmol/L; Fig. 2). In fact, SPC3042 was the only oligonucleotide capable of >95% inhibition of survivin. The scrambled control SPC3046 showed no significant survivin mRNA down-regulation at any time point (Figs. 1 and 2). The results were confirmed by measuring the protein level using a survivin-specific ELISA (Table 1).

Nuclease Stability

We assessed the in vitro plasma nuclease stability of the oligonucleotides to evaluate if the antisense activity in the cellular assay was related to differences in nuclease resistance. The oligonucleotides were incubated in mouse plasma for 1, 2, 4, 24, 48, and 72 h. As shown in Fig. 1, both 2’-O-MOE and LNA nucleotides almost entirely protect SPC3042, ISIS23722, SPC2822, and 3042MOE against nuclease degradation. However, the SPC2823 (18-mer phosphorothioate) is degraded more rapidly, with a half-life of approximately 24 h. The oligonucleotides were also tested in human plasma and the same patterns were found (data not shown).

Apoptosis

The significance of survivin in the intrinsic apoptotic pathway has been described (36), and a clear correlation between survivin inhibition and induction of apoptosis in prostate cancer cells has been documented (37). We wanted to determine if this also was the case in 15PC3 cells. Accordingly, we followed induction of apoptosis as a function of caspase-3 and -7 activation. Caspase activation was measured at 24, 48, and 72 h after the six oligonucleotides were transfected into 15PC3 cells at concentrations of 0.2, 1, 5, and 25 nmol/L (Fig. 3). Cells treated with transfection medium without oligonucleotide were used as a reference. SPC3042 was the only oligonucleotide that effectively activated caspases. Treatment with 5 and 25 nmol/L SPC3042 led to programmed cell death that peaked after 48 h (12-fold activation at 25 nmol/L), and pronounced cell death occurred after 72 h leading to reduction in the detection of caspase activation (Fig. 3). The proapoptotic activity of SPC3042 could be observed at a concentration of 5 nmol/L. Despite being the most potent, survivin inhibitor SPC2822 showed only slight apoptosis induction at 25 nmol/L. Minimal activation is also seen for the control oligonucleotide SPC3046 (Fig. 3). Oligonucleotides with 2’-O-MOE substitutions (3042MOE and ISIS23722) and the all phosphorothioate modified (SPC2823) showed no induction of apoptosis at 25 nmol/L and even at concentrations as high as 500 nmol/L, no significant induction was observed (data not shown). The induction of apoptosis was confirmed by the Annexin V staining of cells treated with SPC3042 and SPC3046 (data not shown).

The lack of caspase activation by the LNA version of ISIS23722 (SPC2822) indicates that the strong caspase activation observed for SPC3042 might be a combined effect of the high survivin target potency and a non-sequence-specific effect based on the particular sequence.
A general “LNA effect” can be ruled out, because neither SPC2822 nor SPC3046 is reactive.

**Cell Cycle Arrest**
We then studied the cell cycle following survivin down-regulation (1, 15, 18, 38, 39) for the oligonucleotides described here. To this point, only ISIS23722 has been noted to confer cell cycle arrest (in G2-M; ref. 24). Propidium iodide staining of the 15PC3 cells was used to assess cell cycle arrest and was measured 24, 48, and 72 h post-transfection with 5 nmol/L oligonucleotide. Cellular staining was analyzed by flow cytometry (fluorescence-activated cell sorting). SPC2822 showed significant cell cycle arrest during the entire period, whereas LY2181308/ISIS23722 arrested the cells only slightly at 24 h with a minor shift toward the G2-M phase (Fig. 4A and B).

The 3042MOE and the control SPC3046 did not alter the proportion of cells in the various phases of the cell cycle and gave rise to identical histograms as the mock-treated cells (Fig. 4A). However, cells transfected with SPC3042 revealed, after 24 h, an increase in cells in the G2-M phase (Fig. 4A). The fraction of cells in the sub-G1 peak seen at the 48 h time point in cells treated with the control SPC3046 was not consequently observed in repeated experiments.

LY2181308/ISIS23722 and 3042MOE have much lower potency than SPC3042 and SPC2822, and to compensate this, the concentration was increased to 100 nmol/L. At this concentration, LY2181308/ISIS23722 arrested cells in the

### Table 1. Effect on survivin protein of the oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Survivin protein inhibition (% inhibition ± SD relative to mock)</th>
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<tbody>
<tr>
<td></td>
<td>1 nmol/L</td>
</tr>
<tr>
<td>SPC3042</td>
<td>0 ± 9</td>
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<tr>
<td>3042MOE</td>
<td>0 ± 5</td>
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<tr>
<td>SPC2822</td>
<td>80 ± 1</td>
</tr>
<tr>
<td>LY2181308/ISIS 23722</td>
<td>0 ± 10</td>
</tr>
<tr>
<td>SPC3046</td>
<td>ND</td>
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**NOTE:** 15PC3 cells were treated with the oligonucleotides for 24 h before harvesting and survivin protein was analyzed by ELISA. ND, not determined.

Figure 2. Survivin mRNA. Survivin mRNA inhibition (normalized to mock control) in 15PC3 cells at 24 h post-transfection. Mean of three independent *in vitro* transfections with 1, 5, 12.5, 25, 100, and 250 nmol/L concentration of antisense oligonucleotides. Mock represents saline-treated cells. Bars, SD.
G2-M phase to a similar extent as did SPC2822 at a 20-fold lower concentration (5 nmol/L). Both SPC2822 (5 nmol/L) and LY2181308/ISIS23722 (100 nmol/L) halted mitosis and led to accumulation of chromosomes over a 72 h period (Fig. 4A). This was confirmed by 4',6-diamidino-2-phenylindole staining, which showed multinucleated cells.

**SPC3042 Inhibits Bcl-2 Selectively**

The data presented above show that down-regulation of Survivin by the selected oligonucleotides produced different cellular responses including the induction of apoptosis (SPC3042). To understand the reason for this phenotype, we examined the effects of the oligonucleotides against other targets. We showed that SPC3042 also down-regulated the Bcl-2 mRNA in a dose-dependent manner (Fig. 5). Other Bcl-2 family members, like Bcl-x, were not affected (data not shown). The effect on Bcl-2 mRNA was not linked to survivin down-regulation, because neither SPC2822, LY2181308/ISIS23722, nor the published anti-survivin proapoptotic small interfering RNA (siRNA_S1; ref. 15) had any effect on Bcl-2 expression even at concentrations of 250 nmol/L (data not shown). The control oligonucleotide SPC3046 did not produce an effect on Bcl-2 mRNA expression at the doses where SPC3042 was active (data not shown).

This effect on Bcl-2 was thus related to the specific sequence of SPC3042, and the observation that the less potent 3042MOE at higher concentrations (>25 nmol/L) also down-regulated Bcl-2 mRNA expression supported this hypothesis (Fig. 5). Furthermore, although this effect is solely linked to the sequence, it is not linked to the chemical modifications employed (LNA versus MOE). Bcl-2 inhibition was confirmed at protein level by Western blotting.

The SPC3042 sequence does not hybridize to the Bcl-2 mRNA by Watson-Crick hybridization. The closest matching complementarities of SPC3042 to the Bcl-2 mRNA sequence has a T_m below 25°C.

**Sensitizing for Taxol**

Studies have shown that down-regulation of Survivin sensitizes cancer cells to chemotherapeutic agents (40, 41). Having identified SPC3042 as a potent survivin inhibitor that is associated with the induction of apoptosis, it was of interest to determine if this agent also sensitized cancer cells toward Taxol. This mitotic spindle inhibitor has been reported to synergize with survivin down-regulation (42, 43).

We used the induction of apoptosis as a measure of a synergistic effect in 15PC3 cells (Fig. 6A). Taxol (10 nmol/L) produced no significant induction of apoptosis, but SPC3042 (10 nmol/L) produced a 6-fold increase in caspase-3/7 activation (after 48 h). However, in combination, a 10-fold increase was observed (Fig. 6A). It could also be detected that the number of cells blocked in the G2-M phase of the cell cycle was significantly increased when SPC3042 was combined with Taxol (data not shown).

**In vivo Tumor Response**

Based on the cellular responses with SPC3042, the in vivo properties of SPC3042 were examined in combination with Taxol in a PC3 prostate cancer xenograft mouse model (Fig. 6B). Animals were treated with either saline, Taxol, SPC3042, or Taxol and SPC3042. Animals treated...
Figure 4. A and B, fluorescence-activated cell sorting analysis of the cell cycle distribution. Propidium iodide-stained cells. The cell cycle is measured by and fluorescence-activated cell sorting. Data are collected from oligonucleotides transfected 15PC3 cells at 24, 48, and 72 h. Each peak represents accumulation of cells within different phases of the cell cycle (see B). The results show cell counts (5,000 cells per sample) as a function of chromosomal mass. B, fluorescence-activated cell sorting analysis illustration. Schematic presentation of cells analyzed by fluorescence-activated cell sorting after propidium iodide staining. The different phases of the cell cycle are depicted (G1, S, G2-M, and >G2-M). The >G2-M represents cells that have more than two sets of chromosomes. Normal distribution of 15PC3 cells at 24 h.
with Taxol were dosed (10 mg/kg i.p.) on days 8, 14, and 22 with or without SPC3042. Animals treated with SPC3042 were dosed (20 mg/kg i.p.) on days 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, and 24. Compared with saline, animals treated with Taxol and SPC3042 alone had a marginal reduction in the tumor weights. However, a significant reduction in tumor weight was observed in the animals with combined Taxol and SPC3042 treatment ($P < 0.001$; Fig. 6B). This shows the synergistic effect of the Taxol/SPC3042 combination. The compounds, either alone or in combination, were well tolerated by the mice. No adverse effects, as measured by body weight loss and macroscopic autopsy, were detected.

Discussion

It is well established that LNA can improve the potency and efficacy of antisense oligonucleotides (44). Thus, SPC3042 was identified with the purpose of finding more potent survivin inhibitor. In this study, we compare isosequential LNAs with 2'-O-MOE oligonucleotides and show that they have significantly better potency (Figs. 1 and 2). All oligonucleotides tested, except SPC2823, are nuclease resistant, so the increased potency is directly driven by the better affinity provided by LNA. However, affinity is not the only driver, because SPC2822, which has a lower $T_m$ compared with SPC3042, has higher potency. This indicates that other factors, such as target accessibility on the mRNA, and perhaps the rate of RNase H recruitment, are of importance as well. For a specific sequence, the affinity is decisive, and this is why an isomodified sequence with either LNA or 2'-O-MOE will favor the LNA with respect to potency. Although the target site for SPC3042 is less accessible than the target site for SPC2822, the high affinity of SPC3042 compensates to a certain extent. This is illustrated by the fact that SPC3042 has a 5-fold higher IC$_{50}$ value (at 48 h) for that target site compared with SPC2822, but for the corresponding 2'-O-MOE oligonucleotides the IC$_{50}$ increase is larger than 10-fold (48 h). This suggests that LNA oligonucleotides will be able to address many more target sites on the mRNA with high potency compared with, for example, 2'-O-MOE or 2'-O-Me modified oligonucleotides.

Survivin plays an important role in the control of apoptosis (1, 4, 5, 41, 43, 45, 46), and we show here that the survivin inhibitor SPC3042 has strong proapoptotic activity in cancer cells. This was shown by potent induction of caspase-3 and -7 activation that peaked at about 48 h after treatment. However, SPC2822 and its 2'-MOE analogue, which also inhibit the expression of survivin, did not induce apoptosis. The apoptotic phenotype was not related to the LNA modification per se because neither SPC2822 nor the 16-mer scrambled control SPC3046 (Fig. 3) induced apoptosis. Likewise, it was found that none of the 2'-O-MOE-modified oligonucleotides elicited apoptosis in concentrations comparable with SPC3042.

The nonapoptotic survivin inhibitors arrested the cells predominantly in the G2-M phase of the cell cycle. However, the irreversible apoptotic pathway, which is induced by SPC3042, could scatter the observations of cell cycle arrest simply because the arrested cells are dying.

The difference in proapoptotic activity was not found to be due to differences in the survivin splice variant expression profile (data not shown). Surprisingly however, we found that Bcl-2 mRNA and protein was downregulated after treatment with SPC3042. This effect cannot be caused by an antisense effect via Watson-Crick hybridization because there is no sequence homology.
Furthermore, basic Local Alignment Search Tool studies of the SPC3042 sequence did not provide additional RNA or DNA targets for SPC3042. However, the effect was related to the specific sequence of SPC3042, because the less potent 2'-O-MOE variant at higher concentrations (>100 nmol/L) also downregulated Bcl-2 expression.

A link between transcription control of survivin and Bcl-2 is not evident, so we anticipate that the observed effect on the Bcl-2 transcript is not a direct result of survivin inhibition. The mechanism behind this is currently uncertain, but evidently it is related to the specific sequence of SPC3042. Irrespective of the molecular mechanism, the marked proapoptotic phenotype of SPC3042 is likely to be a combination of multiple effects.

LY2181308/ISIS23722 has been tested in antitumor models. It has been administrated intratumorally into xenografted human tumor cells or pretransfected into cells before transplantation (19), and antitumor responses have been observed. However, the effects on tumors, following systemically administration of this drug, have not yet been published.

Because survivin inhibitors are known to synergize with Taxol in antitumor models, we evaluated if the potent inhibition of survivin by SPC3042 could lead to the same effect following systemically administration.

We evaluated SPC3042 in combination with Taxol in the 15PC3 prostate cancer cell line and found that adding Taxol to SPC3042-treated cells increased the induction of caspase-3/7 by 2-fold. The administered 20 mg/kg SPC3042 (i.p.) combined with 10 mg/kg Taxol to xenografted mice showed reductions of PC3 tumor weights by 40% compared with the saline control (%0.0001; Fig. 6B). Neither 10 mg/kg Taxol nor SPC3042 alone reduced tumor weights, to any significant effect. These data show that SPC3042 can significantly reduce tumor growth of prostate tumors in combination with Taxol and improve the therapeutic effect of both. SPC3042 has been assessed in investigational new drug enabling toxicology studies in rodents and primates. The maximum tolerated dose was determined in rats and it was found to be >1,000 mg/kg. A comprehensive presentation of the toxicology is beyond the scope of this article but will be reported in due course.

We find the antitumor activity and safety encouraging for further oncologic exploration of SPC3042 in a clinical setting.

Disclosure of Potential Conflicts of Interest
All authors were employees of Santaris Pharma at the time of the study.

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