A Novel Antisense Oligonucleotide Targeting Survivin Expression Induces Apoptosis and Sensitizes Lung Cancer Cells to Chemotherapy

Robert A. Olie, A. Paula Simões-Wüst, Bettina Baumann, Siân H. Leech, Doriano Fabbro, Rolf A. Stahel, and Uwe Zangemeister-Wittke

Division of Oncology, Department of Internal Medicine, University Hospital Zurich, CH-8044 Zurich [B. A. O., A. P. S.-W., S. H. L., R. A. S., U. Z.W.]; Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8092 Zurich [B. B.]; and Department of Oncology Research, Novartis Pharma AG, CH-4002 Basel [D. F.], Switzerland

Abstract
Survivin, an inhibitor of apoptosis protein, deserves attention as a selective target for cancer therapy because it lacks expression in differentiated adult tissues but is expressed in a variety of human tumors. We designed 20-mer phosphorothioate antisense oligonucleotides targeting different regions of survivin mRNA and investigated their ability to down-regulate survivin mRNA and induce apoptosis in the lung adenocarcinoma cell line A549. Oligonucleotide 4003, which targets nucleotides 232–251 of survivin mRNA, was identified as the most potent compound. As measured by real-time PCR, 4003 down-regulated survivin mRNA in a dose-dependent manner with an IC50 of 200 nM. Its maximum effect was achieved at a concentration of 400 nM, at which mRNA was down-regulated by 76%. As revealed by increased caspase-3-like protease activity, nuclear condensation and fragmentation, and trypan blue uptake, treatment with 4003 induced apoptosis and sensitized tumor cells to the chemotherapeutic agent etoposide. Oligonucleotide 4003 did not reduce the viability of normal blood leukocytes with marginal levels of survivin mRNA.

Introduction
Diminished apoptosis plays a critical role in tumor initiation, progression, and drug resistance. Several proteins that inhibit apoptosis have been identified, including bcl-2 family members bcl-2 and bcl-XL and the IAPs.3 Certain members of the latter family directly inhibit terminal effector caspases engaged in the execution of cell death (1). The gene encoding the IAP survivin was cloned recently, and the protein was characterized (2). Survivin is expressed during embryonal development but lacks expression in terminally differentiated adult tissues (2, 3). Interestingly, it becomes reexpressed in transformed cell lines and in a variety of human tumors (1, 2). Survivin is expressed in the G2-M phase in a cell cycle-regulated manner, and its interaction with the mitotic spindle apparatus is essential for antiapoptotic function (4). This could imply that the IAP survivin counteracts a default induction of apoptosis in the G2-M phase of the cell cycle. Overexpression of survivin has oncogenic potential because it may overcome the G2-M-phase checkpoint to enforce progression of cells through mitosis. Because survivin inhibits processing of downstream effector caspase-3 and -7 in cells receiving an apoptotic stimulus (1), its overexpression in tumors is implicated in the resistance to a variety of apoptotic stimuli, including chemotherapy.
Lung and breast cancer are leading causes of cancer death, and their incidence continues to rise. The main reasons for the unfavorable prognosis of these tumors is their propensity to metastasize early and develop resistance to a wide range of functionally unrelated anticancer agents. Interestingly, lung and breast cancer cells express the highest levels of survivin found in human tumors (1, 2), and in agreement with its biological function, survivin expression is correlated with shorter survival in patients with non-small cell lung cancer (5). Although survivin has been widely recognized as an attractive target for cancer therapy (2–10), the use of antisense cDNA and oligonucleotides to inhibit its expression has only recently been described (11–13). Whereas these studies were designed to unravel the biological function of survivin, the promise of survivin antisense to facilitate apoptosis of tumor cells and overcome chemoresistance in cancer therapy remains to be determined.

In the present study, we developed a series of 20-mer phosphorothioate oligonucleotides targeting various regions of survivin mRNA. Using real-time PCR and the survivin-overexpressing lung adenocarcinoma cell line A549, one antisense oligonucleotide was identified that most efficiently down-regulated survivin mRNA levels and directly induced apoptosis. Moreover, in a combination experiment with the chemotherapeutic agent etoposide, evidence is provided that antisense-mediated down-regulation of survivin has the potential to sensitize tumor cells to chemotherapy.

Materials and Methods

Antisense Oligonucleotides. The secondary structure of the 1619-bp survivin mRNA (GenBank accession number NM001168) was predicted by the RNADraw program (14) to identify target sites putatively presenting in single-stranded conformation and thus likely to be accessible to antisense oligonucleotides (15, 16). Based on the identified sites, a series of six 20-mer antisense sequences targeting different regions of survivin mRNA were designed. Antisense oligonucleotide sequences are shown in Table 1. A 3-base mismatch control to the most potent antisense oligonucleotide was also used. Oligonucleotides were provided by Genset SA (Paris, France) in the form of phosphorothioate oligonucleotides. A BLASTN search of a database containing all sequences of GenBank, European Molecular Biology Laboratory, DDBJ: DNA Data Base of Japan, and Protein Data Base was performed to exclude homology of the antisense and control oligonucleotides to other human genes.

Tumor Cell Line and PBMCs. The lung adenocarcinoma cell line A549, which is reported to express high levels of survivin (1), was obtained from American Type Culture Collection and maintained in RPMI 1640 supplemented with 2 mM l-glutamine and 10% FCS at 37°C in a humidified atmosphere containing 5% CO2. PBMCs, which are reported to lack survivin expression (2), were isolated from the heparin-treated blood of three healthy donors and used in transfection experiments directly upon isolation using a Ficoll-Hypaque solution (Biochrom KG, Berlin, Germany). Treatment of Cells with Antisense and Etoposide. One day before transfection, A549 cells were plated in 6-, 24-, or 96-well tissue culture plates. Oligonucleotides were delivered in the form of complexes with Lipofectin.
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Table 1  Sequences of antisense oligonucleotides and their target sites on the survivin mRNA

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Target site on survivin mRNA (nucleotide n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4018</td>
<td>GGACCGATGGGAGGGGAGG</td>
<td>39–58</td>
</tr>
<tr>
<td>4002</td>
<td>TGAGATTAGAGATGCGG</td>
<td>97–116</td>
</tr>
<tr>
<td>4003</td>
<td>CCGAGGTCCTGACGTGGTTG</td>
<td>232–251</td>
</tr>
<tr>
<td>4007</td>
<td>TACCAGGGTTATTCTCC</td>
<td>329–348</td>
</tr>
<tr>
<td>4008</td>
<td>CTGTGAAGGCCCTCAAAACCA</td>
<td>821–840</td>
</tr>
<tr>
<td>4007</td>
<td>GAGGGCAATCAAACTCCATC</td>
<td>1461–1480</td>
</tr>
</tbody>
</table>

The 1619-bp mRNA (GenBank accession number NM001188) was used.

Measurement of Cell Growth and Viability. Growth inhibition of A549 cells was determined by use of the colorimetric MTT cell viabili/proliferation assay as described previously (20, 21). Cells were transfected for 20 h at a final concentration of 1.5 μg/ml. PMBCs were plated immediately before transfaction in 24-well tissue culture plates. In contrast to the treatment of A549 cells, the transfection medium for PMBCs was not replaced before harvest of the cells 64 h after the start of transfection.

Real-Time PCR. Total RNA was isolated from cells by using the RNeasy Mini Kit (QIAGEN Inc., Basel, Switzerland). For cDNA synthesis, 0.5 μg extracted RNA/sample and Taqman Reverse Transcription Reagents (including random hexamers) were used, as described in the user’s manual of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). This system was used for real-time monitoring of PCR amplification of the cDNA following the Taqman Universal PCR Master Mix protocol (Perkin-Elmer; Refs. 17–19). The amplification of survivin cDNA was performed using two sets of primers and Taqman probes referred to as S2 and S3. S2 consisted of 5′-AGTGAAGGGGAAAAGGAAGC-3′ as a forward primer, 5′-ATCCATGGGAGGGGACGTGC-3′ as a reverse primer, and 5′-CGGGCAAAAGACTGTCCAGCTTCAAGA-3′ as the Taqman probe. S3 consisted of 5′-ATGCGGCGCCCCGCAGCT-3′ as a forward primer, 5′-AATG- CCGGAGGCAGGTCGCTTCTT-3′ as a reverse primer, and 5′-CCCCCTGCTG- GCCAGCCCTTCTC-3′ as the Taqman probe. The region in which S2 interacts with the survivin cDNA is distinct from the sites where the antisense oligonucleotides hybridize on the mRNA; therefore, this set of primers and probe was used to screen for the most potent antisense oligonucleotide. Because the sequence of the survivin gene amplified by S2 is identical to part of the EPR-1 gene, in principle S2 may also amplify the EPR-1 cDNA. Therefore, S3, which amplifies a region of the survivin gene not shared with the EPR-1 gene, was used in addition to confirm the data obtained with S2 for the most potent antisense oligonucleotide. The survivin probes were labeled at the 5′ end with the reporter molecule 6-carboxy-fluorescein and labeled at the 3′ end with the quencher molecule 6-carboxy-tetramethylrhodamine. Relative quantification of gene expression was performed as described in the above-mentioned user’s manual, using rRNA as internal standard. Briefly, ribosomal cDNA was amplified separately as indicated in the Taqman rRNA Control Reagents protocol (Perkin-Elmer). The comparative Ct (cycle threshold) method was used for relative quantification of survivin mRNA using S2, after confirming that survivin cDNA and ribosomal cDNA were amplified with the same efficiency. Using S3, survivin cDNA was not amplified with the same efficiency as ribosomal cDNA; therefore, the calibration curve method was used for relative quantification of survivin mRNA.

Measurement of Caspase Activation. Caspase-3-like protease activity in cells was analyzed by use of a colorimetric test system. Detached and attached cells were harvested as described above and pooled and lysed in buffer by freezing/thawing essentially as described previously (22). Lysates were centrifuged at 17,500 × g at 4°C for 15 min. Cytosolic protein (40 μg) was mixed with 80 μM of the caspase-3-specific substrate DEVD-p-nitroanilide (Bachem, Dübedorf, Switzerland) and incubated at 37°C. Subsequently, substrate cleavage was monitored at 405 nm using a SPECTRAMax 340 microplate reader and analyzed using SOFTmax PRO software (Molecular Devices). To confirm that substrate cleavage was due to caspase activity, extracts were incubated in the presence of 10 μM of the caspase-3-specific inhibitor DEVD-CHO (Bachem) for 30 min at 37°C, before the addition of substrate. The value (in arbitrary absorbance units) of the absorbance signal of the inhibited sample was subtracted from that of the noninhibited sample.

Nuclear Staining. Detached cells harvested from the culture medium by centrifugation at 1000 rpm (170 × g) for 5 min were pooled with attached cells collected in a similar way after trypsinization. Cells were pelleted, resus-

Fig. 1. Survivin mRNA level in A549 cells on treatment with antisense oligonucleotides. A, cells were treated with Lipofectin or 600 nM antisense oligonucleotide 4018, 4002, 4003, 4004, 4006, or 4007 and harvested after a 20-h incubation. B, cells were treated with an increasing concentration of antisense oligonucleotide 4003 or 600 nM mismatch control oligonucleotide 4003mis and harvested after a 20-h incubation. Real-time PCR quantification of the relative amount of survivin transcript was performed using the S2 primers and probe set as described in “Materials and Methods,” using rRNA as internal standard. Use of the survivin-specific primers and probe set S3 confirmed the data obtained with S2 for 600 nM oligonucleotide (insert). Real-time PCR quantification of the relative levels of survivin transcript was performed with S2 or S3 as described in “Materials and Methods,” using rRNA as internal standard. All data are presented as the mean ± SD of three independent experiments; the level of survivin mRNA relative to rRNA in untreated cells maintained under identical experimental conditions was taken as 100%.
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Results

Down-Regulating Effects of Antisense Oligonucleotides on Survivin mRNA. With the aim of down-regulating expression of the antiapoptotic protein survivin in tumor cells, the RNAdraw program was used to design a series of 20-mer phosphorothioate oligonucleotides targeting various sites of survivin mRNA (Table 1). By using real-time PCR with the S2 primers and probe set, antisense oligonucleotides were examined for their effects on survivin mRNA in A549 cells, which overexpress the survivin protein (1). Antisense oligonucleotide 4003, which targets nucleotides 232–251, revealed the strongest effect, and at a concentration of 600 nM, it reduced the survivin mRNA level by 70% at 20 h after the start of transfection (Fig. 1A). Based on this finding, only oligonucleotide 4003 was used in the following experiments.

To further characterize the potency of oligonucleotide 4003, the dose dependency of its effect on survivin mRNA in A549 cells was examined using the S2 primers and probe set. As shown in Fig. 1B, oligonucleotide 4003 down-regulated the survivin mRNA level in a dose-dependent manner with an IC_{50} of about 200 nM. At a concentration of 400 nM, a maximum down-regulation to 30% of the initial mRNA level was achieved. A further increase in oligonucleotide concentration did not result in increased antisense efficacy. The mismatch control oligonucleotide was comparatively low. Oligonucleotide 4003 induced death in A549 cells, as revealed by detachment from the culture surface (Fig. 2, B–D).

The coding strand of the survivin gene shows extensive complementarity to the EPR-1 gene (11), and S2 amplifies a region that is identical in both genes. Therefore S2 is also likely to amplify EPR-1 cDNA, which may lead to a false positive survivin signal in cases where only EPR-1 is expressed or to an underestimation of survivin down-regulation on antisense treatment if both genes are coexpressed. The expression of both genes, however, is reported to be mutually exclusive (2, 3), and the use of antisense oligonucleotide 4003 strongly reduced the survivin mRNA signal measured by real-time PCR. This suggests that the S2-amplified transcript corresponds to the survivin mRNA and not the EPR-1 mRNA. To confirm the data obtained with S2, the effect of the most potent antisense oligonucleotide, 4003, at a dose of 600 nM was also analyzed using the S3 primers and probe set, which amplifies a region unique to survivin and not shared with EPR-1. As shown in the inset in Fig. 1B, the data obtained with S3 confirm the ability of 4003 to specifically down-regulate survivin mRNA expression in A549 cells.

Inhibition of Cell Growth by Oligonucleotide 4003. To analyze the biological effect associated with the down-regulation of survivin expression, the growth of A549 cells on treatment with oligonucleotide 4003 was investigated by use of the MTT assay. As shown in Fig. 2A, 64 h after the start of transfection, oligonucleotide 4003 had reduced A549 cell growth dose dependently, with an IC_{50} of 300 nM. The unspecific growth-inhibitory effect of the mismatch control oligonucleotide was comparatively low. Oligonucleotide 4003 induced death in A549 cells, as revealed by detachment from the culture surface (Fig. 2, B–D).

Induction of Apoptosis by Oligonucleotide 4003. Having demonstrated that down-regulation of survivin expression reduced the viability of A549 cells, we analyzed whether cell death was due to the...
induction of apoptosis. As shown in Fig. 3, lysates from oligonucleotide 4003-treated cells revealed increased caspase-3-like protease activity compared with untreated cells. Moreover, on treatment with oligonucleotide 4003, cells contained nuclei with condensed and fragmented chromatin, providing further evidence for the induction of apoptosis as a consequence of survivin antisense treatment (inset in Fig. 3). In cultures treated with the mismatch control oligonucleotide, very few cells showed signs of apoptosis.

To verify that the induction of apoptosis in A549 cells was due to the down-regulation of survivin mRNA, the effect of oligonucleotide 4003 was also examined on PBMCs, which represent a normal cell population reported to lack survivin expression, as analyzed by Northern blotting (2). In agreement with this finding, we found PBMCs to express marginal levels of survivin mRNA as analyzed by real-time PCR using the survivin-specific S3 primers and probe set (inset in Fig. 4A). Trypan blue exclusion assays revealed that PBMCs, in contrast to A549 cells, were not susceptible to death induction by oligonucleotide 4003 (Fig. 4A).

Oligonucleotide 4003 Sensitizes A549 Cells to Etoposide. To investigate whether down-regulation of survivin expression has the potential to sensitize A549 cells to chemotherapy, a combination treatment with antisense oligonucleotide 4003 and etoposide was performed. Fig. 4B shows that in cultures treated with a combination of 600 nm antisense oligonucleotide and 1.5 μg/ml etoposide, approximately 85% of the cells showed signs of cell death 64 h after the start of transfection. Compared to treatments with either oligonucleotide or etoposide alone, this represents about twice or even six times the number of dead cells, respectively. Similarly, nuclear fragmentation and condensation were also increased in combination-treated cells compared with single-agent treated cells (data not shown).

Discussion

The IAP survivin deserves attention as a target for cancer therapy due to its differential expression in tumors versus normal tissues. It is expressed during embryonal development, lacks expression in terminally differentiated adult tissues, and becomes reexpressed in transformed cell lines and a variety of human tumors, with highest levels being found in breast and lung cancer (1). The expression of survivin in tumors is correlated with drug resistance and/or shorter survival of patients with non-small cell lung cancer (5), colorectal cancer (7), and neuroblastoma (9). Despite recognition that survivin represents an attractive target for cancer therapy (2–10), mainly survivin antisense cDNA fragments (11, 12) and very recently also antisense oligonucleotides (13) have been used to elucidate the role of survivin during cell division and apoptosis. In the present study, we describe an antisense oligonucleotide approach to down-regulate survivin expression in the lung adenocarcinoma cell line A549 and demonstrate its ability to induce apoptosis and sensitize tumor cells to chemotherapy.

Most antisense approaches rely on empirical targeting of oligonucleotides against the translation initiation site of mRNAs, where the ATG start codon lies. The rationale for choosing this site is that it likely presents in single-stranded conformation and thus should be accessible to antisense oligonucleotides. In a previous bcl-2 antisense study, we have shown that oligonucleotides hybridizing to the coding region of the mRNA may offer additional targeting options that are equally or even more effective (20). To predict putative binding sites likely to present in single-stranded confirmation, the RNAdraw program (14) was used as described previously (15). One of the phosphorothioate antisense oligonucleotides developed in the present study targets the ATG start codon site (4018), whereas the others were designed to hybridize to sequences located in the coding region. From these oligonucleotides, 4003 most efficiently down-regulated the survivin mRNA level in A549 cells, achieving its maximum effect at a
mitochondrial pathway. Whether it also increases cell death induced of survivin expression sensitizes cells to death induction via the cytochrome c apoptosis pathways, the survivin antisense approach has the potential to directly blocks the processing and activation of effector caspase-3 and cytotoxic proteins bcl-2 and bcl-xL block the apoptotic event of mitochondrial cytochrome c release into the cytosol and have been shown to inhibit mainly the first of these two pathways. We have previously shown that in lung cancer cells, antisense oligonucleotides down-regulating bcl-2 or bcl-xL expression induce apoptosis and synergistically interact with chemotherapy (20, 21, 23). Because survivin directly blocks the processing and activation of effector caspase-3 and -7, which act as a common downstream part of the two major apoptosis pathways, the survivin antisense approach has the potential to facilitate apoptosis through both pathways. Using oligonucleotide 4003 in combination with etoposide, which triggers cell death via cytochrome c release (24), we provide evidence that down-regulation of survivin expression sensitizes cells to death induction via the mitochondrial pathway. Whether it also increases cell death induced by the death receptor pathway and whether it sensitizes other survivin-overexpressing tumor cells to chemotherapy are currently under investigation.

We demonstrate that down-regulation of survivin expression by use of the novel antisense oligonucleotide 4003 facilitates apoptosis and sensitizes lung cancer cells to chemotherapy. Oligonucleotide 4003 does not induce death in a normal cell population lacking survivin expression. Our data suggest that the use of oligonucleotide 4003 deserves further investigation as a novel approach to selective cancer therapy.

Acknowledgments

We thank Dr. T. Bächli (Laboratory for Electron Microscopy, University of Zürich, Zürich, Switzerland) for assistance with the use of a confocal laserscan microscope.
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