Antisense Oligonucleotides Targeting XIAP Induce Apoptosis and Enhance Chemotherapeutic Activity against Human Lung Cancer Cells in Vitro and in Vivo


ABSTRACT

Activation of programmed cell death in cancer cells offers novel and potentially useful approaches to improving patient responses to conventional chemotherapy. X-linked inhibitor of apoptosis (XIAP), is the most potent member of the IAP gene family in terms of its ability to inhibit caspases and suppress apoptosis. In this study, we investigated the effect of XIAP down-regulation by antisense oligonucleotides (AS ODNs) on human non-small cell lung cancer (NIH-H460) growth in vitro and in vivo. In cultured H460 cells, G4 AS ODN was identified as the most potent compound. It down-regulated XIAP mRNA by 55% and protein levels up to 60% as determined by real-time quantitative reverse transcription-PCR and Western blotting, respectively, and induced 60% cell death. In contrast, the scrambled control ODN caused minimal XIAP loss and less than 10% cell death. Treatment with G4 AS ODN induced apoptosis as revealed by degradation of procaspase-3 and poly(ADP-ribose) polymerase proteins with significant nuclear DNA condensation and fragmentation. In addition, G4 AS ODNs sensitized H460 cells to the cytotoxic effects of doxorubicin, Taxol, vinorelbine, and etoposide. In animal models, administration of G4 AS ODN had significant sequence-specific inhibitory effects on H460 solid tumor establishment in a xenograft model. This antitumor activity was associated with an 85% down-regulation of XIAP protein in the tumors. In addition, the combination of 15 mg/kg G4 AS ODN with 5 mg/kg vinorelbine significantly delayed tumor establishment, more than either agent alone. These studies support the contention that XIAP is a viable target for cancer therapy in human non-small cell lung cancer.

INTRODUCTION

Lung cancer is one of the leading causes of cancer death in both men and women, despite recent advances made in early diagnosis and treatment. More than 75% of patients with NSCLC prove to be potential candidates for chemotherapy at some point during the course of their disease (1). However, chemotherapy provides rather poor response rates in NSCLC patients, with rare complete remissions. Clearly, new treatments must be developed to improve overall disease-free survival in NSCLC patients. Activation of programmed cell death in cancer cells can be induced by using AS ODNs that target antiapoptotic regulatory proteins and, consequently, has been identified as a potential targeted therapy to provide such improvements in cancer patients (2–4).

Apoptosis is directly involved in tumor cell death after treatment with chemotherapeutic agents and radiation and is a process that requires the activation of proteolytic enzymes termed caspases. Moreover, resistance to apoptosis is a hallmark of cancer, and failure to properly execute apoptosis through disabling mutations provides the cancer cells with an unwanted survival advantage (5, 6). Whether a cell undergoes apoptosis or continues through the cell cycle in response to potential apoptotic stimuli is under the control of a complex set of gene and protein interactions. Several potent endogenous proteins that inhibit apoptosis have been identified, including Bcl-2, and IAP family members in mammalian cells. Certain members of the latter family directly inhibit terminal effector caspases, i.e., caspase 3 and caspase 7, engaged in the execution of cell death, as well as

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1 The abbreviations used are: NSCLC, non-small cell lung cancer; IAP, inhibitor of apoptosis; XIAP, X-linked IAP; ODN, oligonucleotide (includes oligodeoxynucleotides and oligoribonucleotides); AS, antisense; SC, scrambled (control); RP, reverse polarity; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; DOX, doxorubicin; VNB, vinorelbine; Eto, etoposide; SCID, severe combined immunodeficiency; Rag2, recombination-activating gene 2; PS, phosphothioate; PO, phosphodiester; MBO, mixed-backbone ODN; CI, combination index; NCI, National Cancer Institute; RT-PCR, reverse transcription-PCR; LFA, LipofectAMINE; DAPI, 4',6-diamidino-2-phenylindole.

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the key mitochondrial initiator caspase, caspase 9, important to the mediation of cancer chemotherapy-induced cell death (7–10). The IAPs are the only known endogenous caspase inhibitors and, thus, play a central role in the regulation of apoptosis.

**XIAP** is the most potent member of the IAP gene family in terms of caspase inhibition and apoptosis suppression (7, 9, 10). XIAP is able to bind directly to caspase-3, -7, and -9, and to block their activities (7, 11, 12) as well as to interfere with the BAX/cytochrome c cell death pathway (8, 13). Overexpression of XIAP has been shown to suppress apoptosis induced by a variety of apoptotic stimuli including tumor necrosis factor α, Fas, serum or growth factor withdrawal, ischemia, and chemoradiotherapy (14, 15). Changes in XIAP protein levels could dramatically alter a cell’s response to apoptosis. In point of fact, XIAP can be targeted for proteasome-dependent degradation on proapoptotic stimuli because of its ubiquitin protein ligase activity associated with its RING finger domain (16). This activity also promotes ubiquitin-proteasomal degradation of IAP binding partners such as caspase-3 and enhances XIAP antiapoptotic effect in Fas-induced cell death (17). Thus, XIAP regulation through the proteasome is complex.

The IAPs have been postulated to contribute to the development of some cancers, and a postulated causal chromosomal translocation involving *cIAP2/HIAP1* has been identified in MALT lymphoma (18). A recent correlation between elevated XIAP, poor prognosis, and short survival has been demonstrated in patients with acute myelogenous leukemia (19). XIAP was highly overexpressed in many tumor cell lines of the NCI (NIH) panel (20). However, there has been little direct evidence of the role XIAP plays in tumor establishment, growth, metastasis, and resistance to chemotherapy in vivo.

To demonstrate the potential of XIAP as a valid and attractive target for drug or AS therapy in H460 human NSCLC, we investigated the molecular and pharmacological effects of XIAP AS ODN treatment alone, and in combination with anticancer drugs on H460 cells in vitro and in a tumor xenograft model as a proof of principle. AS and control ODNs were evaluated for XIAP mRNA and protein down-regulation, ability to induce apoptosis alone, and sensitization of tumor cells to chemotherapy agents. These studies indicate that XIAP down-regulation represents an attractive target for improving the treatment response of NSCLC.

**MATERIALS AND METHODS**

**AS ODN Synthesis.** A library of >96 nonoverlapping chimeric ODNs, or MBOs, 19-mer AS ODNs was synthesized as 2 × 2 MBO ODNs, composed of two flanking 2′-O-methyl RNA residues at either end with PS linkages, and a central core of 15 PO DNA residues. Each final product was desalted by Sephadex G-25 chromatography (IDT Inc., Coralville, IO). This chimeric wingmer configuration, and mix of PS and PO linkages (referred to as 2 × 2 PS/PO), provided the minimum necessary stability for experimentation although reducing nonspecific toxicity associated with PS residues and associated toxic by-products of the synthesis. This configuration also provided the most cost-efficient chimeric ODN using expensive second-generation AS chemistries. Fully phosphorothioated, nonchimeric, versions of AS and control ODNs (PS-ODN) for in vivo and in vitro studies were synthesized by Trilink Biotech (San Diego, CA) and purified by RP-high-performance liquid chromatography. PS-ODNs were used in animal studies because of their reduced cost and their well-established track record and known toxicities, and based on our previous experience with PS bcl-2 AS ODNs in the same xenograft model (21).

**Tumor Cell Lines and Animal Xenograft Model.** The human NSCLC cell line (large cell type) NCI-H460 (H460) was obtained from the NCI tumor repository and was maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO₂. Cells were used in exponential growth phase, up to a maximum of 25 in vitro passages. Male SCID-RAG2 mice (7–9 weeks old, 23–26 g) were obtained from the British Columbia Cancer Agency Joint Animal Facility breeding colony and were kept in aseptic environments. A solid tumor model of H460 cells in SCID-RAG2 mice was established by s.c. implantation of 1 × 10⁶ H460 cells on the back of mice. All of the animal protocols were approved by the British Columbia Cancer Agency Animal Welfare Committee.

**Treatment of Cultured H460 Cells with AS ODNs and Anticancer Drugs.** One day before in vitro transfection, H460 cells were plated in 6- or 96-well tissue culture plates. PS or chimeric AS ODNs were delivered into cells with LFA 2000 (Life Technologies, Inc., Gaithersberg, MD) in the form of liposome-ODN complexes. On a 4.5- or 6-h transfection, the transfection medium was replaced by RPMI-10%FBS medium without transfection reagent, and cells were incubated for another 24 or 48 h before analysis.

**Real-Time Quantitative RT-PCR.** Total RNA from H460 cells treated with liposome-ODN complex for 6 h was immediately isolated using RNeasy mini-spin columns combined with DNase treatment (Qiagen), and specific XIAP mRNA was measured using a real-time quantitative RT-PCR method. XIAP forward and reverse primers (used at 600 nM) and probe (200 nm; 5′-GGTGATAAAAGTAAAGTGCTTTCACTGT-3′, 6FAM-CAACATGCTAAATGGTTCCAGGGTGCAAAT-ATC-TAMRA, and 5′-TCATGATTCTTACAGACACTCCTCAA-3′) were designed to span exon 3–4 and 4–5 junctions. One of the primers, as well as the probe, was designed to overlap an intron-exon boundary to block detection of any possible genomic DNA contamination. The RNA was reverse-transcribed and PCR was amplified using the Taqman EZ RT-PCR kit (PE/Applied Biosystems Inc.) in the ABI prism 7700 Sequence Detection System (PE/ABI). The thermal cycling conditions for the RT step were 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min, followed by 45 cycles of PCR (at 94°C for 20 s and 60°C for 1 min/cycle). The XIAP mRNA level of each sample was calculated relative to untreated control cells. XIAP mRNA levels were determined by the cycle threshold method using a threshold of 30× the baseline SD, and XIAP levels were normalized for GAPDH mRNA content, using PE/ABI supplied primers and probe.

**Western Blot Analysis.** For in vitro experiments, cells were treated with liposome-ODN complexes for 4.5 h and were incubated for another 24 h in complete medium without transfection reagent and ODN. For in vivo ODN treatments, see below (Tumor and Tissue Processing). The cells or tumor tissue were lysed with ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1%
NP40, and 0.02% sodium azide) containing protease inhibitors (Complete-Mini protease inhibitor tablets; Boehringer Mannheim GmbH, Mannheim, Germany). After incubation for 30 min on ice, samples were centrifuged at 10,000 rpm for 15 min, and were stored at −20°C. Protein content in the lysed extracts was determined using a detergent-compatible Bio-Rad assay (Bio-Rad Labs, Hercules, CA). Equal amounts of protein (40 μg/lane) were subjected to, and separated in, 12% SDS-polyacrylamide gels or 4–15% gradient SDS-polyacrylamide pre-made gels (Bio-Rad) and were transferred to nitrocellulose membranes (Mandel, Guelph, Canada). The primary antibodies against XIAP (rabbit polyclonal; Aegera Oncology Inc., Ottawa, Ontario, Canada), Bcl-2 (mouse monoclonal; DAKO, Glostrup, Denmark), Bax (rabbit polyclonal; Sigma, St. Louis, MO), caspase-3 and PARP (rabbit and mouse monoclonal, respectively; BD PharMingen, San Diego, CA) were obtained and used. The mouse antihuman β-actin monoclonal antibody was purchased from Sigma. The secondary antibody used was the appropriate horseradish-conjugated antimouse or antirabbit IgG (Promega, Madison, WI). Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) and were visualized after exposure to Kodak autoradiography film. Scanning densitometry (Molecular Dynamics, Sunnyvale, CA) was performed to quantify band intensities by volume/area integration. The amount of XIAP, caspase-3, Bcl-2, and Bax in cells was normalized to their respective lane β-actin levels.

**Measurement of Cell Viability.** Growth inhibition of H460 cells was determined by the colorimetric MTT cell viability/proliferation assay. In brief, cells were treated with liposome-ODN complex for 4.5 h, then were incubated for another 48 h at 37°C in the medium without transfection reagent or ODN in the presence or absence of anticancer drugs. MTT (25 μg/well) was added to each well and plates were incubated for 3.0 h at 37°C. The colored formazan product was then dissolved using 200 μl of DMSO. Plates were read using the microtiter plate reader (Dynex Technologies Inc., Chantilly, VA) at a wavelength of 570 nm. The percentage of dead (or growth-inhibited) cells treated with the different concentrations of ODNs was normalized to untreated controls. All of the assays were performed in triplicate.

CalcuSyn software (Biosoft, Ferguson, MO) was used to analyze data from MTT assay of cells exposed to G4 AS ODN alone or in combination with anticancer drugs to determine additive or synergistic effects. The CI equation in CalcuSyn is based on the multiple drug-effect equation of Chou and Talalay (see CalcuSyn manual) (40) and defines synergism as a more-than-expected activity effect and antagonism as a less-than-expected additive effect. Chou and Talalay defined a CI of 1 as synergism, additive, and antagonism, respectively.

**Apoptotic Flow Cytometric Assays.** Cells were treated with liposome-ODN-complex for 4.5 h and were incubated for another 48 h in the medium without transfection reagent at 37°C. Cells were harvested, washed twice with sample buffer (0.5% glucose in PBS without Ca²⁺ or Mg²⁺), and were fixed in cold 70% ethanol at 4°C for at least 18 h. Samples were centrifuged at 3000 rpm for 10 min and were resuspended in sample buffer containing 50 μg/ml PI and 400 units/ml RNase A. Samples were incubated for 30 min at room temperature and for 30 min on ice, followed by flow cytometry analysis. EXPO Software (Applied Cytometry Systems) was used to generate histograms, which were used to determine the cell cycle phase distribution after debris exclusion. The sub-G₁-G₂ cell fraction was considered as representative of apoptotic cells.

**Nuclear Morphology.** Cells were treated with liposome-ODN complexes for 4.5 h and were incubated for another 48 h at 37°C in the medium without transfection reagent or ODN. Cells were harvested and stained with 0.10 μg/ml DAPI for 30 min at room temperature. Cells were placed on a glass slide, were cytopsin, and were viewed with a Leica microscope (Germany) and ×40 objective lens under UV fluorescent illumination. Digital images were captured using ImageDatabase V. 4.01 Software (Leica, Germany).

**In Vivo Antitumor Establishment Activity.** Efficacy experiments were conducted in male SCID/RAG2 immunodeficient mice bearing H460 tumors (six mice/group). Treatments were commenced on day 3 after tumor inoculation. Saline (vehicle controls), G4 AS, or SC control ODNs were administered i.p. daily at 5 or 15 mg/kg for five doses per week over a 3-week regimen. VNB (5 or 10 mg/kg) was administered i.v. on days 3, 7, 11, and 17 after cell inoculation via a lateral tail vein, either alone or in combination with ODNs, which were administered i.p. When ODNs were administered in combination with VNB, the i.v. drug treatment was staggered 4 h after i.p. ODN treatment.

Mice were observed daily and body weight measurements and signs of stress (e.g., lethargy, ruffled coat, and ataxia) were used to detect possible toxicities. Animals with ulcerated tumors or 1 cm³ of tumor size were euthanized. Electronic caliper (Mitutoyo Corp., Kawasaki, Japan) measurements of tumors were converted into mean tumor size (cm³) using the formula: \( \frac{1}{2} \times [\text{length (cm)} \times \text{width (cm³)}] \). An average tumor size per mouse was used to calculate the group mean tumor size ± SE (n = 6 mice) from at least two independent experiments.

**Tumor and Tissue Processing.** Mouse tumors were collected on day 21 post-tumor-inoculation and treatment. One portion of tumor tissue was fixed in formalin. Paraffin-embedded tissues were sectioned and were subjected to gross histopathology using H&E staining. The other tumor portion was homogenized in lysis buffer for Western Blot analysis (see above).

**Statistical Analyses.** The Student’s t test was used to measure statistical significance between two treatment groups. Multiple comparisons were done using one-way ANOVA and post hoc test that compared different treatment groups by the Shevell test criteria (Statistica release 4.5; Soft Inc., Tulsa, OK). Data were considered significant for a P of <0.05.

**RESULTS**

**Screen Strategies for Identifying AS ODNs with Optimal Effects on XIAP Expression.** To specifically down-regulate the expression of XIAP mRNA in tumor cells, we designed a series of >100 19-mer ODN targeting various sites of XIAP mRNA. To initially test the efficacy of these ODNs in suppressing XIAP mRNA and protein levels, T24 human bladder carcinoma cells were used. This cell line was chosen for the primary screen because of its (a) ease of growth and mainte-
nance; (b) relatively high level of reproducible transfection; and (c) demonstrated reproducibility in Taqman and ELISA assays.

T24 bladder carcinoma cells, transfected with 2 × 2 MBO-ODN-Lipofectin complexes (1.0 μM) for 3 h, were used to test the ODN library for its ability to knock-down XIAP protein, as measured by ELISA after 48 h and relative to total protein. Positive hits from these results were reconfirmed for their ability to knock-down XIAP protein after 12–18 h transfection by Western analysis, and XIAP mRNA at 6–9 h of transfection by quantitative RT-PCR. Using this protocol, several candidate ODNs were identified and listed in Table 1. They do not contain CpG dinucleotides sequences, nor GGGG residues. GC content of the ODNs was between 30 and 70%. The 2 × 2 listed PS/PO chimeric ODNs showed a dose-dependent ability to decrease XIAP mRNA levels at 6–9 h in the range of 400-1200 nM ODN concentrations and demonstrated a maximum protein knock-down at 12–18 h posttransfection (data not shown).

A quick and relatively inexpensive AS screening strategy was devised, using improved second generation chemistries, consisting of 2′-O-methyl RNA residues flanking a DNA core. These chemistries offer the advantage over first-generation PS chemistries of being less nonspecifically toxic. To make the library construction economically feasible, only two flanking residues of 2′-O-methyl RNA with PS linkages were incorporated around a core of PO DNA residues, to provide the minimum stability required for in vitro experimentation. The limitation of these minimally chimeric ODNs is that these demonstrated transient knock-down post-bolus transfection, with a maximum knock-down of mRNA at 6–9 h post-transfection, and a maximum protein knock-down at 12–18 h post-transfection (data not shown). This is likely attributable to the limited stability of the chimeric ODN inside the cell. For in vivo validation studies requiring substantial amounts of materials, we reverted to inexpensive first generation fully phosphorothioated chemistry (PS-ODNs). This decision was based on our prior experience with Bcl-2 PS ODNs in our established xenograft models (21) and our understanding of the dose-limiting toxicities in vivo for first-generation chemistries, which is well documented in the literature.

Identification of Optimal XIAP AS ODNs. By using real-time quantitative PCR, chimeric ODNs targeting various sites of XIAP mRNA (see Table 1) were examined for their effect on XIAP mRNA in human H460 lung carcinoma cells. F3, G4, C5, AB6, and DE4 AS ODNs reduced the XIAP mRNA level by 50–70% of untreated controls, whereas D7 AS ODNs reduced the mRNA level by 30% (Fig. 1). In contrast, the respective ODN controls and transfectant agent alone (LFA) reduced the mRNA level only by <20%, compared with untreated controls (Fig. 1). On the basis of this finding, F3, G4, and C5 PS AS ODNs were selected for further in vitro and in vivo study.

We characterized the potency of F3, G4, and C5 ODNs on down-regulation of XIAP protein expression by Western blot analysis using actin as an internal control. Of the three ODNs tested, G4 AS ODN provided the strongest down-regulating effect on XIAP protein (Fig. 2). It reduced the XIAP protein levels by 62% at 24 h posttransfection at a concentration of 1.2 μM (Fig. 2B). F3 AS ODN at 1.2 μM reduced the XIAP protein levels by 50%, and C5 AS ODNs did not display any sequence specific effects compared with its control (Fig. 2B).

XIAP AS ODN Induced Cell Death and Chemosensitization of H460 Cells. We next tested the effects of XIAP down-regulation on H460 cell viability. As shown in Fig. 3A, G4 AS ODN effectively induced cell death in a dose-dependent manner with 55% being killed, compared with untreated control

| Table 1 Sequences of AS ODNs and their target site on XIAP mRNA

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence*</th>
<th>Position (nucleotide no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3 AS</td>
<td>ATCCTCTGTTAATGAAGGG</td>
<td>17–35</td>
</tr>
<tr>
<td>F3 RP</td>
<td>GGTATAAAGTCTCTCTCTTA</td>
<td></td>
</tr>
<tr>
<td>G4 AS</td>
<td>GCTGAGTCTCCATATTGCC</td>
<td>251–269</td>
</tr>
<tr>
<td>G4 SC</td>
<td>GCTCTTTTGGCCACTGTA</td>
<td></td>
</tr>
<tr>
<td>C5 AS</td>
<td>ACCATCTGGTACACCAGAA</td>
<td>354–372</td>
</tr>
<tr>
<td>C5 RP</td>
<td>AAGCCAATAGGCTTACCA</td>
<td></td>
</tr>
<tr>
<td>AB6 AS</td>
<td>GGTTCTCTGGTTATAGGG</td>
<td>470–488</td>
</tr>
<tr>
<td>AB6 RP</td>
<td>GGTATATGGCGTCTTGGG</td>
<td></td>
</tr>
<tr>
<td>DE4 AS</td>
<td>GGTATCTCTTTGACCAGTA</td>
<td>166–184</td>
</tr>
<tr>
<td>DE4 RP</td>
<td>ATGACCATTCTCTATAGG</td>
<td></td>
</tr>
<tr>
<td>D7 AS</td>
<td>GATTCACTCTGAAATATTAA</td>
<td>719–737</td>
</tr>
<tr>
<td>D7 RP</td>
<td>AATTAATAGGCTCATTAG</td>
<td></td>
</tr>
</tbody>
</table>

* Chimeric (2 × 2 PS/PO) and fully phosphorothioated AS ODNs (PS-ODN) have the same sequence, with the exception of the substitution of a uracil base (U) for a thymidine base (T) in the RNA flanking regions (underlined sequences). Differences in chemical structures were described in “Materials and Methods.” Corresponding control oligonucleotides are shown either as RP or as SC sequences.

The 2540-bp cDNA (partial mRNA, GenBank accession no. U45880) was used for numbering.
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Fig. 2  AS-mediated specific down-regulation of XIAP protein in H460 cells in vitro. XIAP protein levels in H460 cells, which were treated with LFA 2000 alone (LFA) or LFA plus 1.2 μM of XIAP AS ODN F3, G4, or C5 and their respective ODN controls (RP or SC) for 4.5 h and incubated for another 24 h in complete medium without transfection reagent and ODN. XIAP protein levels were analyzed by Western blotting (A), and the amount of protein was quantitated by densitometry (B). XIAP levels were normalized to cellular actin levels (n = 3), and were compared with untreated control (CNT) levels. kDa, $M_r$ in thousands.

## Antitumor Efficacy of G4 AS ODNS against H460 Tumor Xenografts.

We tested the antitumor establishment effects of G4 AS ODN in SCID/RAG2 mice bearing xenografts of H460 human non-small cell lung tumors implanted s.c. Saline-treated control tumors grew reproducibly to a size of 0.75 cm$^3$ within ~24 days (Fig. 6A). ODN treatments were initiated 3 days after tumor cell inoculation. G4 AS ODNs (5–15 mg/kg) were administered using a treatment schedule of i.p. injections given on days 3–7, 10–14, and 17–21 (once a day), based on the persistent reduction of XIAP protein levels by 62% after 24 h posttransfection in in vitro study. Treatment with G4 AS ODNs inhibited tumor growth in a dose-dependent fashion; specifically, on day 24, mean tumor sizes were 0.75, 0.45, and 0.29 cm$^3$ in the control and in the 5-mg/kg and 15-mg/kg AS ODN treatment groups, respectively (Fig. 6A). In contrast, administration of G4 SC ODN at 15 mg/kg as a control ODN provided no therapeutic activity (Fig. 6A). Additionally, none of the mice treated with ODNs displayed significant signs of toxicities, and both doses of ODNs were well tolerated. Hence, to have the best therapeutic effects, a dose of 15 mg/kg/day (5 days on, 2 days off) was selected for the future combination-treatment regimens with anticancer drugs.

### XIAP Expression in H460 Tumors Treated with G4 AS ODN.

To correlate G4 AS ODN tumor antiestablishment effects with XIAP protein expression, we examined the changes in XIAP expression in solid tumors of mice treated with 15 mg/kg of G4 AS and SC ODNs. At day 21 post-tumor-inoculation (Fig. 6A), tumors were harvested, and lysates from tumor homoge-
nates were used for Western blot analysis. XIAP and β-actin antibodies against the human protein were used, allowing for determination of human XIAP levels obtained from tumor cell specimens without contamination from XIAP derived from mouse cells. As shown in Fig. 6, B and C, XIAP protein levels in tumors treated with G4 AS ODNs were significantly reduced to ~15% of expression levels in control tumors (P < 0.005). In contrast, the XIAP protein in tumors treated with G4 SC ODN were decreased to only 76% of expression levels in control tumors, and the change was not statistically significant. XIAP protein in the group treated with G4 AS ODNs was significantly lower than in its ODN control group (P < 0.05). These results indicated that inhibition of H460 solid tumor establishment by G4 AS ODNs was correlated with the down-regulation of XIAP protein expression.

Histopathology of Tumor Specimens. To evaluate whether G4 AS ODN administration results in direct tumor cell death, we examined the morphological changes (H&E staining) of tumors after treatment (Fig. 7). Experiments were performed in parallel with the Western blot studies of tumor samples to correlate XIAP protein and tumor cell death (Fig. 6 B versus Fig. 7). At day 21 post-tumor-inoculation, tumors treated with 15 mg/kg of G4 AS, or SC ODN and saline were excised, sectioned, and stained with H&E. The results demonstrated that tumors given G4 AS treatment showed a large increase in the number of dead cells, identified morphologically by their amorphous shape and condensed nuclear material, reflecting a large percentage of cell death (Fig. 7). The enlarged insert is an amplification of the small square area, which shows morphological changes of dying tumors cells treated with G4 AS (Fig. 7). In contrast, tumors from control mice or SC ODN-treated controls were composed of densely packed live tumor cells with a very small number of dead cells (Fig. 7).

Combined Treatment of G4 AS ODN with VNB. To evaluate whether combined treatments of G4 AS ODN and a chemotherapeutic agent used for lung cancer treatment, VNB, in H460 tumors may result in any cooperative effects, we compared the therapeutic efficacy of VNB in the presence and absence of G4 AS or SC ODNs. Treatment regimens were initiated on days 3 after cell inoculations. Fig. 8A presents the in vivo efficacy results for 5-mg/kg and 10-mg/kg doses of VNB given to H460 tumor-bearing mice and compared with saline controls. The two regimens induced significant antitumor establishment effects in a dose-dependent manner (Fig. 8A) without

![Graph A](image1.png)

**Fig. 3** Dose-dependent cytotoxicity of XIAP AS ODNs alone, and combined cytotoxicity with standard chemotherapeutic agents. A, effect of XIAP G4 AS ODN treatment on the death of H460 cells. Percentage of cell death after treatment with an increasing concentration of LFA 2000 (LFA) alone or LFA-ODN complexes with G4 AS ODN or G4 SC ODN was determined by MTT assay. B, percentage of cell death after treatment with complexes of LFA and G4 AS ODN or G4 SC ODN at a 0.4-μM dose in the presence or absence of 0.5 μM DOX, 10 nm Taxol, 10 nm VNB, or 1.0 μM Etop was determined by MTT assay. Percentage of cell death in untreated controls maintained under identical experimental condition was considered as 0. All of the data represent the mean ± SD of three independent experiments.

![Graph B](image2.png)

**Fig. 4** XIAP AS-mediated effects on caspase activation and lack of effect on Bcl-2:Bax ratios. Effects of XIAP G4 AS ODN and its SC ODN controls at 1.2 μM on the expression of pro-caspase-3, PARP (full-length, Mₐ 116,000, and processed, Mₐ 85,000), Bcl-2 and Bax protein levels in transfected H460 cells compared with untreated control (CNT) and LFA 2000-treated control (LFA). Protein expression was analyzed by Western blotting. The Bcl-2 and Bax protein levels were normalized to cellular actin levels and were quantitated by densitometry. The ratio of Bcl-2:Bax is presented as the mean of two or three independent experiments, and the ratio in untreated control (CNT) cells set at 1.
showing significant signs of undesirable toxicity (i.e., body weight loss).

When G4 AS ODN (15 mg/kg) was combined with VNB (5 mg/kg) for the treatment of H460 tumors, an even more pronounced delay of H460 tumor establishment was observed compared with either treatment administered alone (Fig. 8B versus Fig. 6A and 8A). These mice did not show any significant signs of overt toxicity. Moreover, we compared the mean tumor sizes in mice treated with 5 mg/kg VNB in the presence or absence of G4 AS or SC ODNs on day 29 (Fig. 8, A and B). The tumor sizes in the group of combined VNB and G4 AS ODNs were 0.22 ± 0.03 cm³, which was significantly ($P < 0.01$) smaller than 0.59 ± 0.04 and 0.48 ± 0.05 cm³ in the treated groups of 5 mg/kg VNB alone and combination of VNB with G4 SC ODNs, respectively. However, with all of the treatments, tumor growth persisted despite concurrent G4 AS ODN and/or VNB treatment after an initial response.

**DISCUSSION**

Modulation of gene expression by AS ODNs is a promising therapeutic approach because of its target specificity and potential applicability to any sequenced gene (22, 23). Appropriately designed AS ODNs can achieve anticancer gene-targeted therapy based on reducing the level of a putative oncoprotein. Synthetic AS ODNs bind RNA molecules in a sequence-specific manner and either directly impair interaction with factors in the cytoplasm that are required for translation or recruit endogenous RNaseH to cleave the RNA backbone (24). Regardless of the exact mechanism, AS ODNs represent a potential drug therapy
In the present study, we examined a series of 19-mer AS ODNs targeting various regions of XIAP mRNA. The G4 AS ODN was identified as one of the most efficient down-regulators of XIAP mRNA and protein levels. G4 AS ODN was designed to hybridize to sequences located in the region encoding the baculovirus IAP repeat (BIR) domain. A BLASTN search of a database containing all of the sequences of GenBank was performed; this revealed a lack of similar complementarity of the AS ODN to human genes other than XIAP (birc4 gene symbol). G4 AS ODN down-regulated 60% of XIAP mRNA and protein in vitro at a concentration of 1.2 μM. At the same time, G4 AS ODN, indeed, induced a strong antiaestrogens effect in H460 human NSCLC cells in vitro and in vivo. Similar results were obtained with MDA-MB-435/LCC6 human breast cancer cells and T24 human bladder carcinoma cells (data not shown).

It is known that XIAP directly binds to, and inhibits, active caspase-3, as well as blocking the processing and activation of pro-caspase-3, recognized as the key downstream component of different major apoptotic pathways (28–30). Our data indicated that XIAP AS treatments led to the activation of caspase-3 as well as to the degradation of the caspase-3 substrate, PARP, which resulted in the morphological changes that are hallmarks of apoptosis (e.g., nuclear DNA condensation and fragmentation) and, finally, cell death. This observation is in agreement with the findings of others that, after adenoviral AS expression, XIAP down-regulation activated caspase-3 and induced apoptosis in the wild-type p53-chemoresistant cancer cells (31). Treatment of mature bone marrow-derived macrophages from mice with XIAP AS ODNs partly abolished their resistance to apoptosis (32). Using G4 AS ODN in combination with DOX, Taxol, VNB, and Etop, we provide evidence here that down-regulation of XIAP expression sensitizes cancer cells to death induction by a wide range of chemotherapeutic agents.

We did not examine, in this report, the effects of the AS ODNs on other reported activities of XIAP, such as E3 ubiquitin ligase activity or signal transduction effects on TGF-β, or JNK pathways, because the physiological relevance of these secondary activities is unclear. However, all of the reported secondary activities that are attributed to XIAP do support the antia apoptotic effects of XIAP, with the only exception being the E3 ligase activity, which can remove IAP antagonists and caspases (antia apoptotic effects) but also lead to the degradation of the IAPs themselves (proapoptotic effects) in a complex regulatory mechanism that is not fully understood.

To date, studies on biological effects of XIAP AS ODNs in vivo had not been reported. In the studies described here, we evaluated the in vivo activity of XIAP G4 AS ODNs in the xenograft solid-tumor model of H460 human NSCLC. This model was chosen to assess whether XIAP manipulation may affect tumor establishment and chemosensitivity in a human tumor cell line exhibiting high inherent XIAP expression. The data presented here are the first, to our knowledge, to demonstrate that daily i.p. G4 AS ODN treatment (5 days on, 2 days off) administered over 3 weeks was capable of achieving significant XIAP down-regulation (85% XIAP protein reduction). This was associated with antitumor establishment activity in this animal tumor model at a dose of 15 mg/kg per injection per day (5 days on, 2 days off). This effect was specific for the G4 AS ODN sequence, because there was no activity associated with
the control G4 SC ODNs. Furthermore, the histology of the AS ODN-treated tumors revealed significant induction of cell death and indicated that the decrease of tumor size was caused by an efficient tumor cell death (Figs. 6 and 7).

Although some AS ODNs containing unmethylated CpG motifs within the ODN sequence have been shown to be potent immune stimulators (33, 34), there is no CpG motif in our G4 AS ODN sequence. This strengthens the argument that the antitumor efficacy of the G4 AS ODNs observed here is a specific AS effect resulting from the sequence itself rather than an immunological effect.

XIAP is ubiquitously expressed in all normal tissues (35–37), and the function of XIAP in normal cells is presumably to suppress inadvertent activation of the caspase cascade. In addition, XIAP may provide a threshold of apoptotic resistance in the normal cell, allowing a possible recovery phase when toxic insults occur, but once this threshold is surpassed, the cell is committed to a point of no return and fully commits to death. Harlin et al. (38) developed a XIAP-deficient mouse strain and found that the XIAP-deficient mice were born at the expected Mendelian frequency and had no obvious physical or histological defects, as well as having normal life spans. This suggests the possibility of no deleterious effects in normal cells treated with G4 AS ODNs. We did not detect any overt toxicity of daily 15-mg/kg i.p. injections of G4 AS ODNs in the mouse, in terms of gross histological examination and organ and total body...
weights (data not shown). Because there is a single base difference in the 19-mer AS ODN sequence between human and murine XIAP, direct correlations between murine and human toxicity may not be appropriate.

In addition to the direct antitumor activity of G4 AS ODNs against human lung cancer xenografts, we have demonstrated that XIAP down-regulation produced by G4 AS ODNs enhanced the anticaner activity of VNB, a Vinca alkaloid, which works by interfering with microtubule assembly. VNB is approved for use as a single agent for patients with stage IV NSCLC disease and in combination with cisplatin for patients with stage III NSCLC. The combination treatment of G4 AS ODN and VNB significantly reduced the rate of tumor establishment and increased antitumor activity over that obtained with G4 AS ODN or VNB alone against human lung cancer xenografts. These results suggest that the combination of G4 AS ODN and VNB might sensitize patients with late-stage NSCLC to chemotherapy.

Planned future studies will address the efficacy of XIAP AS ODNs, alone or in combination with cytotoxic drugs, to induce regression in more clinically relevant xenograft models. The studies reported here demonstrate proof of concept that XIAP AS ODNs have antitumor establishment effects in a xenograft model, and that, like most other AS molecules targeted at cancer cells, is expected to show only true AS-mediated regression in combination with cytotoxic agents. Although data are presented here from a NSCLC xenograft model, it is not known whether NSCLC represents a valid indication for XIAP AS. This will not be known until XIAP AS is tested in the clinic. Future studies are planned with XIAP AS in other xenograft models of other histological types of cancer, because of the poor translation of results from histological type in xenograft models to the same histological type in Phase II trials (39). On the basis of these past experiences, demonstrating antitumor activity in several histological types of cancer in xenograft models increases the chances of seeing activity in human trials but not in the same histological type of cancer (39).

In summary, the studies presented here are the first to directly address the potential therapeutic role of AS ODNs targeting the XIAP gene product and the enhancement of the therapeutic efficacy of a cytotoxic agent VNB in a human NSCLC xenograft model. This information may enhance our ability to apply novel therapies targeting apoptosis regulation with conventional cytotoxic agents in a manner that will significantly improve the management of cancer with chemotherapy.

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Antisense Oligonucleotides Targeting XIAP Induce Apoptosis and Enhance Chemotherapeutic Activity against Human Lung Cancer Cells in Vitro and in Vivo

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